Survey of molecular methods for the typing of wine yeast strains

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Abstract

A survey of the genetic polymorphisms produced by distinct methods was performed in 23 commercial winery yeast strains. The microsatellite typing, using 6 different *loci*, an optimized interdelta sequence analysis and RFLP of mitochondrial DNA generated by the enzyme *Hinf* I had the same discriminatory power: among the 23 commercial yeast strains, 21 distinct patterns were obtained. Karyotype analysis originated 22 patterns, thereby allowing the discrimination of one of the three strains that were not distinguished by the other methods. Due to the equivalence of the results obtained in this survey, any of the methods can be applied at the industrial scale.

Introduction

Wine production by the use of selected *Saccharomyces cerevisiae* strains, commercially available as active dry yeast is widely accepted, being an enological practice extensively applied nowadays. The use of techniques that enable to distinguish the inoculated strain from the remaining yeast flora present in the grape must is regarded with great practical interest [1]. In the last years, several methodologies of typing based on DNA polymorphisms have been developed which allowed the discrimination among closely related yeast strains.

Chromosome separation by pulsed-field electrophoresis [2] revealed the considerable variability in the chromosomal constitution of commercial yeast strains [3], and turned to be a useful method for yeast strain identification [4,5]. As chromosome karyotyping may be too complex, laborious and time-consuming for the analysis of numerous yeast isolates, several other molecular methods of typing have been developed for this purpose.

Restriction fragment length polymorphism analysis of mitochondrial DNA (mtDNA) [5,6] was simplified [7,8] to render it a fast and easy method. Digestion of mtDNA with restriction enzymes like *Hinf*I or *Rsa*I is associated to a high polymorphism, and was also used to study the authenticity of commercial wine yeast strains [9].

The *S. cerevisiae* genome contains repetitive DNA sequences, such as the δ sequences that are frequently associated to the Ty1 transposon [10,11]. The number and the location of these elements have a certain intraspecific variability and were used as genetic fingerprint to identify *S. cerevisiae* strains [11]. PCR profile analysis of δ sequences has a good discriminating power for analyzing commercial strains [12]. On the other hand, it seems to be a minor discriminatory method when used to identify indigenous strains in a given viticultural region [13]. More recently, an extensive BLAST search allowed the optimization of the pair of primers used for interdelta analysis, resulting in highly polymorphic patterns. This

59 improved PCR-typing had a similar discriminatory power like the pulsed-field electrophoresis
60 karyotyping [14].

61 In the last few years, fingerprinting of microsatellite or SSR (Simple Sequence
62 Repeats) *loci*, that are short (1-10 nucleotides) DNA tandem repeats dispersed throughout the
63 genome and with a high degree of variability, revealed to be very useful to discriminate *S.*
64 *cerevisiae* strains [15-19]. Searching the genomic DNA database of *S. cerevisiae*, six
65 microsatellite *loci* were selected that generated 44 genotypes (with a total of 57 alleles) from
66 51 strains originating from a spontaneous fermentation [20]. This method is fast, allowing
67 multiplex PCR reactions, precise and reproducible, and therefore very powerful.

68 In the present paper four different genetic fingerprinting techniques (karyotype
69 analysis, delta sequence typing, mtDNA restriction analysis and microsatellite genotyping)
70 were used for the detailed genotyping of 23 commercial wine yeast strains. The analysis of
71 the polymorphisms produced by each of the methods allowed a detailed comparison of the
72 advantages and disadvantages of each method showing the utility and efficiency of these
73 modern approaches for fingerprinting relatively large sets of winery yeast strains.

Materials and methods

Yeast strains

23 *Saccharomyces cerevisiae* wine strains, commercially available were used in this study. Their geographic origin is indicated in Table 1.

Active dried wine yeast strains were re-hydrated and maintained in frozen stocks (glycerol, 30 %, v/v) at -80 ºC or, for short-term storage, on YPD agar medium (yeast extract, 1 %, w/v, peptone, 2%, w/v and glucose, 2 %, w/v).

DNA isolation

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 [7]. The progress of cell lysis was dependent on the strain that could last between 1 to 3 hours. DNA was quantified and used for δ sequence typing, mitochondrial RFLP and microsatellite analysis.

Delta sequence typing

Amplification reactions were performed on a BioRad iCycler thermal cycler, using the primers δ1 (5'-CAAAATTCACCTATATCT-3') and δ2 (5'-GTGGATTTTTATTCCAAC-3') (primer pair A) [7] or δ12 (5'-TCAACAATGGAATCCCAAC-3') and δ2 (primer pair B) [7]. 15 µl reaction mixture was prepared with 60 ng of DNA, 0.5 U Taq polymerase (MBI Fermentas), Taq buffer (10mM Tris-HCl, 50mM KCl, 0.08% Nonidet P40), 25 pmoles of each primer, 0.4 mM of each dNTP and 3 mM $MgCl_2$. After initial denaturation (95°C for 2 min), the reaction mixture was cycled 35 times using the following program: 95°C for 30s, 43.2°C for 1 min, 72°C for 1 min followed by a final extension at 72°C during 10 min. The amplification products were separated by electrophoresis on a 1.5 % (w/v) agarose gel containing ethidium bromide, visualized and photographed.

99

100 **Chromosomal polymorphisms**

101 Yeast chromosomal DNA was prepared in plugs as previously described [7], washed
102 in TE buffer (1 mM EDTA, 10 mMTris-HCl, pH 8.0) at 50°C for 30 min and then washed
103 again three times in the same buffer at room temperature for 30 min. The plugs were loaded in
104 a 1 % (w/v) agarose (Seakem® Gold) gel and electrophoresis was performed using a TAFE
105 (transverse alternating-field electrophoresis) system (Geneline, Beckman) under the following
106 conditions: constant voltage of 250 V for 6 hours run time with 35 s pulse time, followed by
107 20 hours at 275 V with a 55 s pulse time at constant temperature (14ºC). The electrophoresis
108 buffer consisted of 10 mM Tris Base, 0.5 mM EDTA free acid and 4 mM acetic acid. After
109 staining the gel with ethidium bromide, bands were visualized and photographed.

110

111 **Mitochondrial DNA restriction patterns**

112 The reactions were performed overnight at 37ºC and prepared for a final volume of 20
113 µl as follows: 17 µl of total DNA (60-120 µg), isolated as described, 0.5 µl of the restriction
114 endonucleases *Hinf*I or *Rsa*I (10 U/µl, MBI Fermentas), 2 µl of the appropriate 10x-buffer
115 and 0.5 µl of RNAse (10 mg/ml) (MBI Fermentas). The DNA fragments were separated on a
116 1.5 % (w/v) agarose gel containing ethidium bromide, visualized and photographed.

117

118 **Microsatellite amplification**

119 The six trinucleotide microsatellite *loci* described as ScAAT1, ScAAT2, ScAAT3,
120 ScAAT4, ScAAT5 and ScAAT6 [20] were amplified in two multiplex reactions using 20 ng
121 of template DNA, 0.5 U Taq polymerase (MBI Fermentas), the corresponding Taq buffer (10
122 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40), 0.2 mM of each dNTP and 2 mM $MgCl_2$.
123 Multiplex reaction A contained 0.05 pmol of each ScAAT1 and ScAAT6 primer pairs as well

as 0.03 pmol of ScAAT4 primer pair. The multiplex reaction B contained 0.05 pmol of ScAAT2, 0.1 pmol of ScAAT3 and 0.075 pmol of ScAAT5 primer pairs. One oligonucleotide of each pair was fluorescent-dye labeled (MWG Biotech). In both cases, the total reaction volume was 6.0 µl, and cycling was performed as described [20] in a BioRad iCycler thermal cycler. PCR reactions were diluted (1:5 for multiplex A and 1:20 for multiplex B), and 2 µl aliquots were mixed with 14 µl of formamide and 0.3 µl of a red DNA size standard (GENE-SCAN-500 ROX, Applied Biosystems). Samples were then denatured at 94 ºC for 5 min and separated by capillary electrophoresis (15 KV, 60ºC, 24 min and 27 min for multiplex reaction A and B respectively) in an ABI Prism 310 DNA sequencer (Applied Biosystems) and analyzed by using the corresponding GENESCAN software.

Reproducibility

All typings were performed at least in duplicate. The reproducibility of the described techniques was also assessed by comparing the results obtained by the analysis of DNA from two independent extractions for 5 yeast strains randomly chosen.

Results

Delta sequence typing

PCR amplification of delta sequence interspersed regions using primer pairs A or B showed a distinct degree of pattern heterogeneity as shown in Figure 1. For primer pair A, a total of 10 distinct patterns were obtained, and most of them shared three common bands around 500, 750 and 970 bp. These three bands are characteristic for pattern δ_{A3}, the pattern found in 10 of the 23 strains analyzed. Further patterns are characterized by the appearance of an additional band in close proximity to one of the three main bands (e.g., patterns δ_{A1}, δ_{A6}, δ_{A7}), by the absence of some of the three main bands (e.g., patterns δ_{A2}, δ_{A4}), or by the appearance of other extra bands (e.g., pattern δ_{A10}). For primer pair B, almost all patterns appear to have several bands in common of about 400-500 bp, and the presence of many other intense bands of different sizes produced a very high polymorphism compared to primer pair A, allowing the assignment of 21 different patterns among the 23 strains. The group of 10 strains showing the identical pattern δ_{A3} could be distinguished from each other using primer pair B that generated 10 different patterns. Interestingly, strains 1, 10 and 12 show a very characteristic pattern (δ_{B1}), with five bands sized between 300 and 500 bp. These 3 strains showed also a unique pattern (δ_{A1}) when PCR amplification was performed with primer pair A (Figure 1), indicating that they are identical or genetically very closely related.

Several faint bands, probably associated to unspecific amplification due to the low annealing temperature and to the high $MgCl_2$ concentration (3.0 mM) were not always amplified in replicates, but they were not decisive for the assignment of a pattern, as sufficient polymorphisms were obtained by the intense bands.

RFLP of mitochondrial DNA

164 The analysis of the genetic variability of 23 *S. cerevisiae* wine strains by means of
165 mtDNA restriction analysis showed a very high level of polymorphisms (Figure 2). Digestion
166 with *Rsa*I was less discriminating than *Hinf*I that generated 17 and 21 distinct patterns,
167 respectively. Strains 5, 7 and 11 shared pattern m_{R5}, while pattern m_{R7} was shared by strains 8
168 and 9. The average size of fragments obtained by *Hinf*I digestion was between 2.5 and 6 kb,
169 whereas bigger fragments (mainly between 6 and 10 kb), were obtained by *Rsa*I digestion.
170 Again, with exception of strains 1, 10 and 12, unique patterns were found with the restriction
171 enzyme *Hinf*I. Figure 4 shows the identical mtDNA restriction patterns of these 3 strains
172 using *Hinf*I or *Rsa*I.
173
174 ***Analysis of chromosomal patterns***
175 As shown in Figure 3, the pulsed field electrophoretic karyotypes of the 23 strains
176 analysed showed 22 different chromosomal patterns. In the range below 600 Kb, where the
177 resolution is better, the greatest variability was found, both in the position and in the number
178 of bands, which varied from five to ten. There was also considerable variability in the region
179 of approximately 900 Kb, where for most strains one or two bands were observed in different
180 positions.
181 The patterns of the strains 10 and 12 (K10) were again identical whereas in strain 1
182 differences in the zones of about 600 Kb (chromosomes XVI-XIII) and 900 Kb
183 (chromosomes V-VIII) were observed. A lower weak band was lost and another higher weak
184 band appeared in the zone of around 600 Kb. In addition, a band of smaller size in strain 1
185 replaced a weak band present in the region about 900 Kb. Except for these two bands, the
186 pattern of strain 1 is identical to that of strains 10 and 12, indicating that these strains are
187 genetically very closely related.
188

189 ***Microsatellite analysis***

190 The results obtained for the analysis of the 6 microsatellite *loci* AAT1-AAT6 are
191 summarized in Table 2. Unique patterns were found for 20 strains, while an identical pattern
192 was found for strains 1, 10 and 12. The number of alleles found for each *locus* varies between
193 3 and 15, being the *loci* AAT1 and AAT3 characterized by the highest polymorphism. The
194 number of genotypes varied between 4 and 18 for each *locus* separately analyzed (Table 2).

Discussion

In the present study, different methods have been applied to genetically differentiate 23 commercial wine starter yeast strains. As summarized in Table 3, depending on the technique used, distinct levels of discrimination were obtained, varying from 10 to 22 different patterns.

The power of discrimination of *S. cerevisiae* strains by PCR-based interdelta typing depended on the primer pairs used. Amplification with the initially described [11] primer pair δ1-δ2 (primer pair A) resulted in 10 different patterns, whereas the substitution of primer δ1 by primer δ12 (primer pair B), resulted in a 2-fold increase in the number of patterns obtained (Table 3). The optimized primer pair B, found by an extensive BLAST search, raised the detection of polymorphisms and allowed the unequivocal differentiation of 53 industrial, laboratory and wild-type yeast strains [14]. Delta sequence typing with the standard primer (pair A) has been reported to be very useful and easy to perform for the typing of commercial strains. However, for the delimitation of genetically close related indigenous yeast strains, this method has a low discrimination power and therefore should be combined with other typing methods like mtDNA or karyotype analysis [13,21]. In the present study, the interdelta typing of the 23 industrial strains with optimized primer pair B had almost the same level of discrimination as the pulsed-field-karyotyping. These results are consistent with the ones previously described [14].

As shown in Table 3, the 21 patterns generated by mitochondrial DNA restriction with *Hinf*I match exactly the patterns obtained by PCR-typing using primer pair B, microsatellite typing, as well as pulsed field karyoptyping (with the exception of strain 1). Additionally, in the present study, digestions with *Hinf*I allowed a much better resolution than with *Rsa*I.

Both mtDNA restriction analysis and electrophoretic karyotyping have been used in numerous studies related to the yeast ecology of spontaneous fermentations, biogeography

220 and biodiversity [22-27]. It was shown that both methods had a very similar resolving power
221 at the strain level. Nevertheless, the results obtained using the improved interdelta typing
222 methods is very promising, indicating its equivalence to mtDNA RFLP, karyotyping and
223 microsatellite analysis.

224 Using interdelta amplification, mtDNA RFLP and microsatellite typing, strains 1, 10
225 and 12 generated the same patterns (Table 3). The chromosomal patterns of strains 10 and 12
226 are identical, and were very similar to that of strain 1. Strain 1 differs from the two other
227 strains due to changes in the position of two weak bands in the zones of about 600 and 900
228 Kb. Two pairs of chromosomes, XVI / XIII and VIII / V, very close in size, are found in these
229 regions. Interestingly, a reciprocal translocation between chromosomes VIII and XVI,
230 generating two new chromosomes $VIII^{XVI}$ and XVI^{VIII} has been described as occurring
231 frequently in wine yeast strains [28]. This rearrangement, found in wine yeast strains, is
232 involved in their adaptative evolution, since the translocation results in higher expression of
233 *SSU1*, thus enabling the cells to resist higher sulfite concentrations [29]. Indeed, wine yeast
234 strains exhibit either normal chromosome VIII (of about 560 kb), chromosome $VIII^{XVI}$ (of
235 about 920 Kb), or both [29,30]. Both are actually present in strains 10 and 12 (results not
236 shown) and the opposite variations in the size of bands in strain 1 may indicate different
237 rearrangement events related these two chromosomes. All these evidences strongly suggest
238 strains 10 and 12 are genetically related to strain 1.

239 Strain "families" having the same mtDNA restriction profile and δ sequences PCR
240 product patterns, differing only by faint variations of chromosomal band position or the
241 presence of doublets have been described [23]. Differently sized chromosomes can be
242 explained by structural reorganizations, leading to structural heterozygosis [30]. Such
243 chromosomal rearrangements have been described in wine yeast genomes during vegetative
244 growth [31] or during wine fermentation [32].

The discrimination obtained by combining the allele sizes from the six microsatellite *loci* was very high. The combination of the results from *loci* AAT1 and AAT3 generated the highest polymorphism (18 and 14 genotypes), and was sufficient for the unequivocal characterization of the present population of 23 strains. However, for studies aiming at the characterization of strains that are genetically closer related, it may be necessary to include data obtained for the other four *loci*.

In summary, our results show that microsatellite typing and the optimized interdelta analysis have similar discriminatory power compared with both mtDNA restriction analysis and karyotyping. None of the typing methods was able to discriminate between two *S. cerevisiae* commercial strains (10 and 12). At least two hypotheses can be raised to explain this result: the strains are identical, although having different commercial designations, or the techniques used are not sufficiently accurate to discriminate between them. Concerning the first hypothesis, there are references reporting equivalent situations in commercial yeast strains [9]. The common geographical origin of these two strains supports this hypothesis.

The improved PCR amplification of delta sequences described by Legras *et al.* [14] is a very convenient method that does not require high equipment investment and can substitute other methods advantageously. However, some critical aspects of delta sequence typing have to be mentioned, as the PCR banding patterns depends on the quantity of template DNA [9]. Occasionally, we also found weakly amplified bands that can make the interpretation of the results difficult (not shown).

Mitochondrial DNA restriction analysis could be a good technique to differentiate yeast strains from the same ecosystem. This technique is also easy to use once the conditions have been carefully standardized and the reproducibility is better than the one of δ sequences analysis.

Karyotyping was shown to be very efficient in discriminating between strains genetically closely related as we confirmed in this study, and is still the method of choice for the detection of chromosome rearrangements. Nevertheless, this technique is time-consuming and complicated for use in industry.

The detection of microsatellite polymorphisms is a promising and powerful tool, providing accurate and unequivocal results expressed as base pair number (or as a number of repeats). This technique is the most appropriate for large-scale studies like determination of genetic proximity (phylogenetic studies) and biogeographical distribution of indigenous *Saccharomyces* strains and/or species by means of numerical analysis. It requires higher equipment investment and skilled human resources which can be seen as the only disadvantages of this technique.

In conclusion, due to the verified equivalence of the results, any of these methods could be applied for industrial applications, such as quality assurance during dry yeast production, implantation studies or tracing of contamination routes. For standard control during the fermentation process PCR amplification of δ sequences and mtDNA restriction analysis are the most appropriated methods. The choice of the most convenient technique should depend on the resources available and the objective of the work.

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392 Table 1. Commercial *Saccharomyces cerevisiae* strains used in the present study.

Strain	Origin
1	Portugal
2	Sangiovese, Italy
3	Bordelais, France
4	Rhône, France
5	Languedoc, France
6	Stellenbosch, South Africa
7	Rhône, France
8	Rhône, France
9	Valencia, Spain
10	Champagne, France
11	Loire, France
12	Champagne, France
13	Gironde, France
14	Languedoc, France
15	Gironde, France
16	Bordelais, France
17	Gironde, France
18	Portugal
19	Portugal
20	Germany
21	Not known
22	Pfalz, Germany
23	Baden-Würtenberg, Germany

393

394 Table 2. Allelic diversity of the 23 *Saccharomyces cerevisiae* commercial starter strains.

Strain	Microsatellite					
	SCAAT1	SCAAT2	SCAAT3	SCAAT4	SCAAT5	SCAAT6
1	189, 237	375	250, 346	302	219, 222	250, 256
2	201	378	247	329	216	256
3	204, 222	372, 378	259, 265	317, 329	216, 219	256, 259
4	165	384	262, 304	302, 329	216, 219	256, 259
5	246	378	262	329	216	259
6	189, 228	375, 378	250, 262	302, 329	216, 222	256
7	222	369, 384	247	302, 329	216	256
8	195	378	241	332	219	256
9	195, 216	375, 381	256	329	216	256
10	189, 237	375	250, 346	302	219, 222	250, 256
11	195	375	256	329	222	256, 259
12	189, 237	375	250, 346	302	219, 222	250, 256
13	216, 219	372, 378	247, 265	329	216, 219	256, 259
14	174	387	247	338	222	259
15	204, 219	372, 381	265	329	219, 222	256, 259
16	195	378	265	329	222	256
17	201	378	247	329	222	256
18	171, 201	375, 378	259, 268	329	219	256
19	204	369	259, 271	329	219	259
20	192	378	247, 271	329	216	256, 259
21	207	378	262	329, 332	216	256
22	219	381	259	329	219	256
23	189	381	247	290	219	256
Nº alleles	15	7	11	6	3	3
Nº genotypes	18	11	14	8	6	4

395 Table 3. Summary of the results obtained by all typing methods used. For each method a
396 different number was assigned to distinct patterns.

Strain	Pattern					
	δ sequence		mt DNA RFLP		Microsatellite SCAAT 1-6	Karyotype
	δ_A	δ_B	m_R	m_H		
1	1	1	1	1	1	1
2	2	2	2	2	2	2
3	3	3	3	3	3	3
4	3	4	4	4	4	4
5	4	5	5	5	5	5
6	5	6	6	6	6	6
7	6	7	5	7	7	7
8	3	8	7	8	8	8
9	3	9	7	9	9	9
10	1	1	1	1	1	10
11	7	10	5	10	10	11
12	1	1	1	1	1	10
13	6	11	6	11	11	12
14	8	12	8	12	12	13
15	3	13	9	13	13	14
16	9	14	10	14	14	15
17	3	15	11	15	15	16
18	10	16	12	16	16	17
19	3	17	13	17	17	18
20	9	18	14	18	18	19
21	3	19	15	19	19	20
22	3	20	16	20	20	21
23	3	21	17	21	21	22

Figure legends

Figure 1

PCR amplification fragments of delta sequence interspersed regions using primer pair A (A) or B (B). The numbers in the upper part of the figure correspond to the strains used. (δ_A) 1–10 and (δ_B) 1–21 refers to the pattern classification.

Figure 2

Mitochondrial DNA restriction patterns of the 23 commercial strains analyzed in this work. The patterns (m_H and m_R) were obtained by digestion with *Hinf*I (A) or *Rsa*I (B). The numbers in the upper part of the figure correspond to the strains used. (m_H) 1–17 and (m_R) 1–21 refers to the pattern classification.

Figure 3

Electrophoretic karyotype patterns of the 23 commercial strains analyzed. The numbers in the upper part of the figure correspond to the strains used. (K) 1-22 refers to the pattern classification. Numbers on the left give the sizes of chromosomes XVI-XIII, V-VIII and I of the reference strain S288C.

Figure 4

Analysis by delta sequence typing, mtDNA RFLP and pulse field electrophoresis of strains number 1, 10 and 12. The three strains present identical patterns, except the slight differences in strain 1 indicated by arrows.

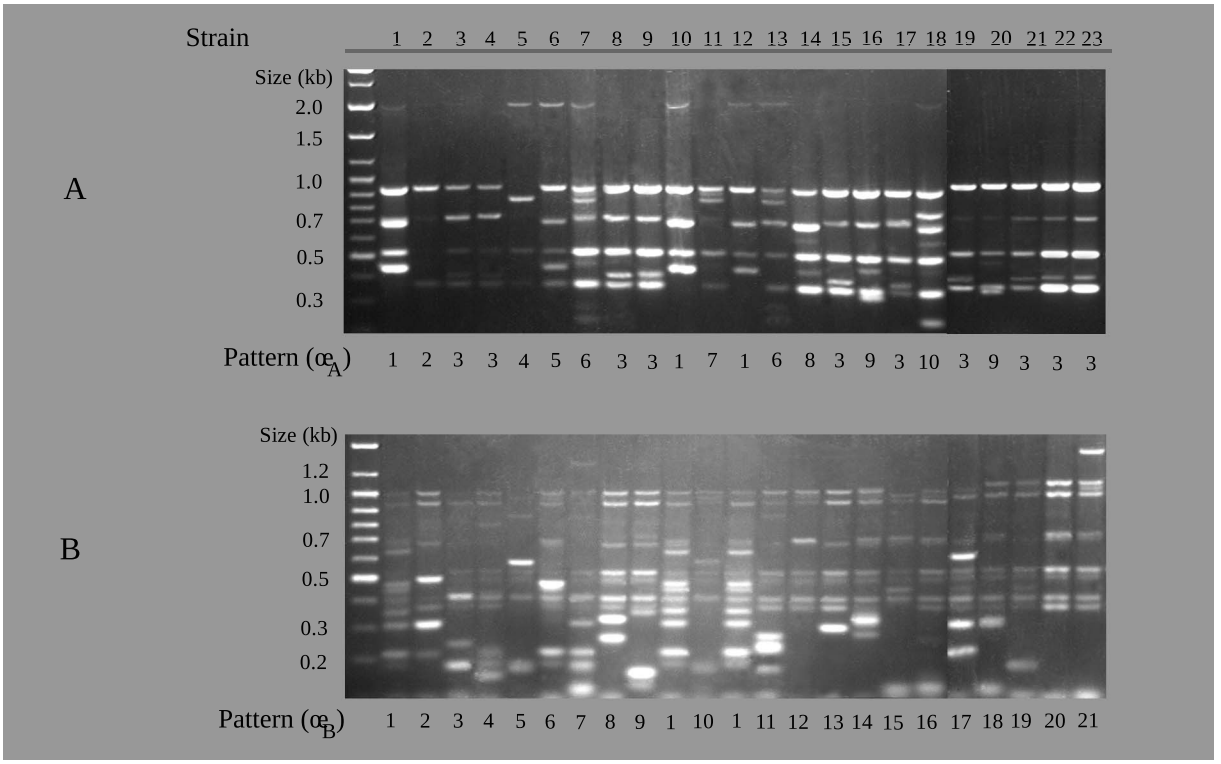
A
Strain
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Size (kb)
2.0
1.5
1.0
0.7
0.5
0.3
Pattern (œA) 1 2 3 3 4 5 6 3 3 1 7 1 6 8 3 9 3 10 3 9 3 3 3
B
Size (kb)
1.2
1.0
0.7
0.5
0.3
0.2
Pattern (œB) 1 2 3 4 5 6 7 8 9 1 10 1 11 12 13 14 15 16 17 18 19 20 21

Figure 1

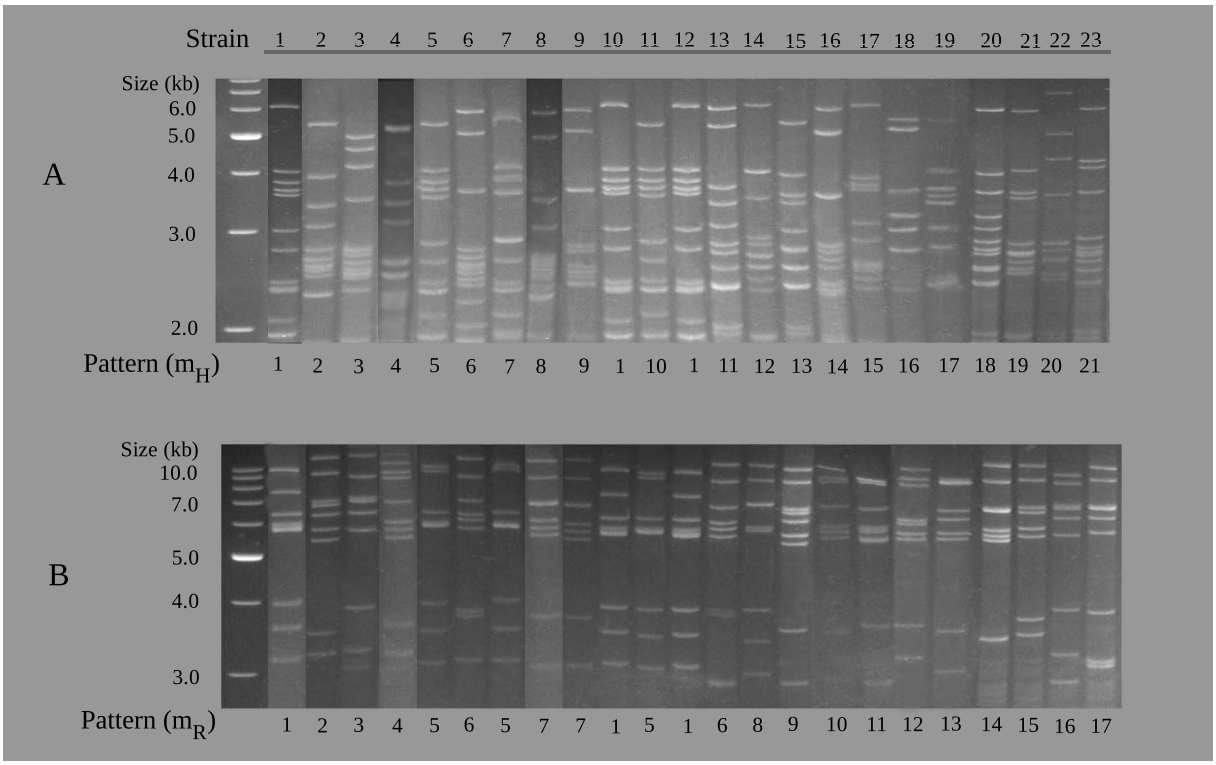
Strain 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
A
Size (kb)
6.0
5.0
4.0
3.0
2.0
Pattern (m_H) 1 2 3 4 5 6 7 8 9 1 10 1 11 12 13 14 15 16 17 18 19 20 21
B
Size (kb)
10.0
7.0
5.0
4.0
3.0
Pattern (m_R) 1 2 3 4 5 6 5 7 7 1 5 1 6 8 9 10 11 12 13 14 15 16 17

Figure 2

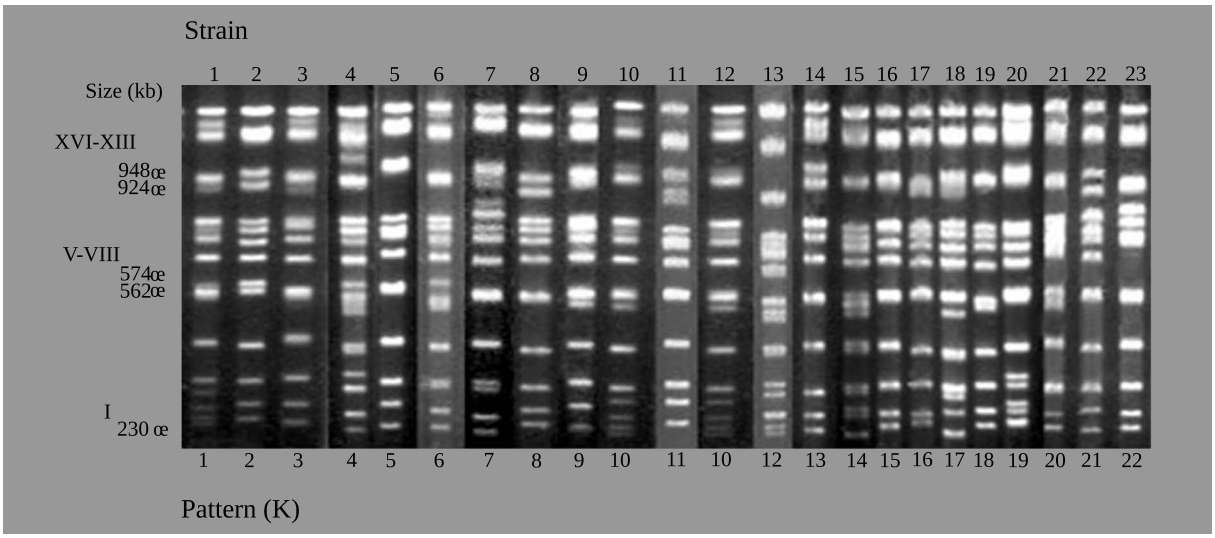
Strain
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Size (kb)
XVI-XIII
948œ
924œ
V-VIII
574œ
562œ
I
230 œ
1 2 3 4 5 6 7 8 9 10 11 10 12 13 14 15 16 17 18 19 20 21 22
Pattern (K)

Figure 3

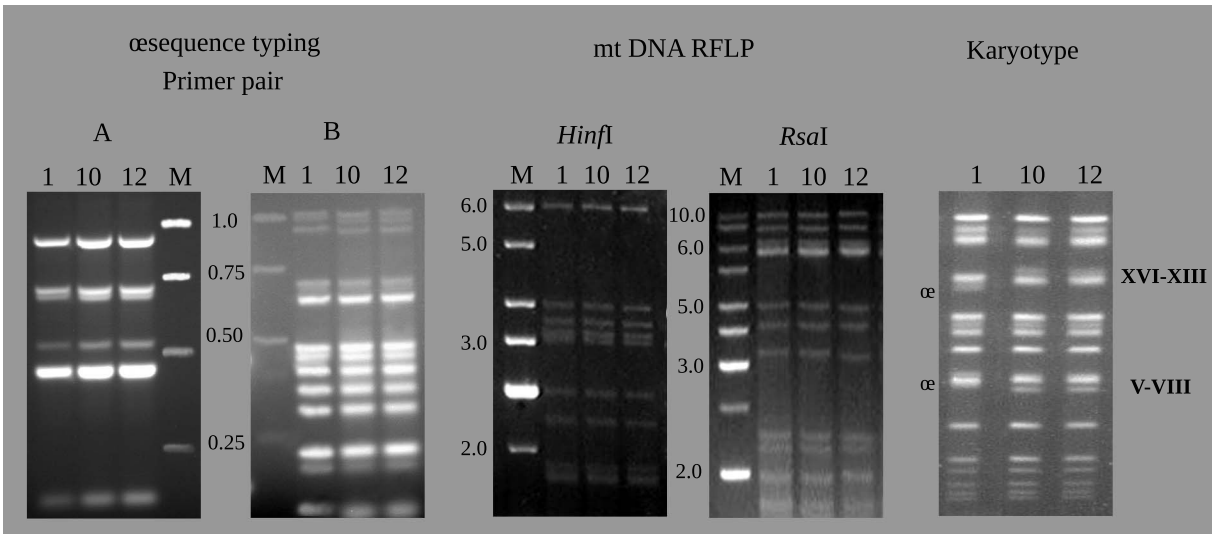
œsequence typing
Primer pair
mt DNA RFLP
Karyotype
A
B
HinfI
RsaI
1 10 12 M
M 1 10 12
1.0
0.75
0.50
0.25
6.0
5.0
3.0
2.0
10.0
6.0
5.0
3.0
2.0
1 10 12
œ
œ
XVI-XIII
V-VIII

Figure 4