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Application of the Different Electrophoresis Techniques to the Detection and Identification of Lactic Acid Bacteria in Wines

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/45759>

1. Introduction

The microorganisms play an essential role in winemaking since a mixed culture of numerous microorganisms including fungal, yeast, and bacteria species are involved in this process and are the responsible for the final quality of the wine (Bisson et al., 1993). Therefore, in order to control the fermentation processes knowing and understanding the complex microbiota involved in them is necessary.

Yeast are able to convert sugar from grapes into ethanol and many other changes that lead to wine. Lactic acid bacteria (LAB) that are often present on the surface of the grapes and can represent significant populations in musts (Lonvaud-Funel, 1999) play dual roles in wine fermentations: as wine spoilage agents and as the main effectors of malolactic fermentation (MLF). Numerous studies have been conducted on the LAB that occur on grapes, grape musts and wines and it is generally agreed that a succession of species happens during the different stages of winemaking and conservation of wines (Ribéreau-Gayon et al., 2006). Most bacterial species present in wine fermentations have been identified by traditional microbiological techniques involving cultivation. However, as it was observed with microbial ecology studies of other environments, cultivation-dependent methods often exhibit biases resulting in an incomplete representation of the true present bacterial diversity (Amann et al., 1995; Hugenholtz et al., 1998). Applications of culture-independent molecular techniques to monitor the microbial successions of various food and beverage fermentations have revealed microbial constituents and microbial interactions not witnessed by previous plating analyses (Giraffa & Neviani, 2001).

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis to separate bacterial 16S ribosomal DNA (rDNA) amplicons are common

culture independent methods employed to characterize microbial communities from specific environmental niches (Lopez et al., 2003; Muyzer & Smalla, 1998). These approaches are attractive since they enable to detect individual species as well as to get overall profiling of community structure changes with time.

Otherwise, ecology, interactions and development of the different bacterial strains during alcoholic fermentation (AF) and MLF are still a field of active research. Efficient and precise methods of strain identification and discrimination have been developed during the last years, either to prepare well-defined starters of biotechnological interest in winemaking, or to quickly assess the presence of certain strains in a wine, or to gain insight of such a complex ecosystem as wine.

Pulsed field gel electrophoresis (PFGE) has proved to be an useful tool for the identification of a wide LAB strains variety and especially for species belonging to the genus *Lactobacillus* (Charteris et al., 1997). Several restriction enzymes have been used for obtaining profiles of *Oenococcus oeni* strains: *NotI* (Kelly et al., 1993; Prevost et al., 1995; Sato et al., 2001), *ApaI* and *SfiI* (Daniel et al., 1993; Kelly et al., 1993; Lopez et al., 2007). Other used methods for LAB strain identification include ribotyping or restriction fragment length polymorphism (RFLP) of genes encoding rRNAs (Charteris et al., 1997; Zavaleta et al., 1997), random amplified polymorphic DNA (RAPD-PCR) analysis with arbitrary primers (Cocconcelli et al., 1997; Lopez et al., 2008; Spano et al., 2002), rep-polymerase chain reaction (rep-PCR) analysis (Parry et al., 2002) and multilocus sequence typing (MLST) system (de las Rivas et al., 2004; de las Rivas et al., 2006). RAPD-PCR and PFGE of macrorestriction fragments are the most frequently used (Guerrini et al., 2003; Tenreiro et al., 1994; Viti et al., 1996; Zapparoli et al., 2000; Zavaleta et al., 1997), and more recently Ruiz et al. (Ruiz et al., 2008) have obtained a better discrimination in the study of bacterial diversity by the combination of results from those two techniques.

For all these reasons, the aims of this work were: (a) applying the DGGE, PFGE and RAPD-PCR techniques to the analysis of the LAB species diversity and the intraspecific diversity of *Oenococcus oeni* in a winery of La Rioja region during three consecutive vintages, (b) getting a better knowledge of bacterial ecology throughout both AF and MLF in wine elaborated with Tempranillo, the classic red grape variety of Spain and native of Appellation of Origin Rioja, (c) evaluating the occurrence of genotypes from commercial *Oenococcus oeni* strains between the autochthonous *Oenococcus oeni* strains isolated from non inoculated fermenting wines, and (d) contributing to the maintenance of the LAB biodiversity in Rioja red wines.

2. Methodology

2.1. Wine production and wine samples

Traditional red wine fermentations from c.v. Tempranillo local grapes of 2006, 2007 and 2008 vintages at one winery of the Spanish northern region of Rioja were studied. Winemaking practices were the typical of this wine-producing area: AFs were conducted in the presence of grape skins, seeds and stalks, after the addition of sulphur dioxide and until the residual reducing sugar content was under 2 g/L. At this final point of AF, wines were

drawn off into tanks and were allowed to undergo spontaneous MLF with the endogenous microbiota. The sampled winery had never used commercial starters for MLF. One fermentation tank was sampled in each vintage. Wine samples were collected aseptically for chemical and microbiological analysis at different times: must (stage 1), tumultuous AF (density around 1,025; stage 2), at final AF (< 2 g/L glucose + fructose; stage 3), initial MLF (consumption of 10% of the initial malic acid; stage 4), tumultuous MLF (consumption of 60% of the initial malic acid; stage 5) and at final MLF (L-malic acid concentration < 0.5 g/L; stage 6).

2.2. Commercial *Oenococcus oeni* starter samples

Sixteen commercial starter cultures employed to induce MLF derived from six different companies were analyzed. These commercial cultures were selected between the most frequently used in Spain.

2.3. Chemical analysis of the musts and wines

Alcohol degree, pH, total acidity, volatile acidity, reducing sugars, free and total sulphur dioxide and L-malic and L-lactic acid content were measured according to the European Community Official Methods (European Community, 1990).

2.4. Culture dependent methods

2.4.1. Bacterial enumeration and isolation

Must or wine samples were diluted in sterile saline (0.9% NaCl) solution and plated on modified MRS agar (Scharlau Chemie S.A., Barcelona, Spain) plates supplemented with tomato juice (10% v/v), fructose (6 g/L), cysteine-HCl (0.5 g/L), D,L-malic acid (5 g/L) and pimaricine (50 mg/L) (Acofarma, S. Coop., Terrassa, Spain). Samples were incubated at 30 °C under strict anaerobic conditions (Gas Pak System, Oxoid Ltd., Basingstoke, England) for at least ten days, and viable counts were reported as the number of CFU/mL. Fifteen colonies from each wine sample were selected for reisolation and identification. Isolates were stored in 20% sterile skim milk (Difco) at -20 °C.

Every commercial lyophilized starter culture was hydrated in saline solution (0.9% NaCl) and then 100 µL aliquot from the appropriate dilution was plated at the surface of modified MRS agar without pimaricine. Because of their low viability in laboratory conditions (Maicas et al., 1999a; Maicas et al., 1999b) glycine (40 mM) and ethanol (10% v/v) were added to this medium. The plates were incubated for at least 10 days at 30 °C under anaerobic atmosphere (Gas Pak System, Oxoid Ltd.) and five colonies were isolated from each one.

2.4.2. Species identification

Species identification was carried out by previously recommended methods, which included bacteria morphology, Gram staining, and catalase (Holt et al., 1994). *Oenococcus*

oeni, *Lactobacillus plantarum* and *Lactobacillus brevis* species were confirmed by the species-specific PCR method (Beneduce et al., 2004; Zapparoli et al., 1998). In case of the identification of other species, PCR amplification of partial 16S rRNA genes was performed with WLAB1 and WLAB2 as previously described (Lopez et al., 2007). PCR products were sequenced by MacroGen Inc. (Seoul, South Korea) and sequences were used for comparison to the data in GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

2.4.3. *Oenococcus oeni* typification by PFGE

PFGE was carried out according to the method described by Birren et al. (1993) with some modifications (Lopez et al., 2007) for agarose block preparation. Because of the difficulty of typing commercial strains first of all these cells underwent to fifteen minutes of ultrasounds, moreover a higher quantity of lysozyme (100 µL/block) was added and incubated for 2 h. Macrorestriction analysis was performed with two endonucleases: *Sfi*I, following the method reported by López et al. (2007), and *Apa*I by the method reported by Larisika (2008) with the following modifications for optimal separation of fragments: 1.2% (w/v) agarose gels were submitted to 24 h with a pulse ramping between 0.5 and 20 s at 14 °C and 6 V/cm in a CHEF DRII apparatus (Bio-Rad).

2.4.4. *Oenococcus oeni* typification by RAPD-PCR

RAPD-PCR was carried out following the procedure described by Ruiz et al. (2010b) with some modifications: MgCl 100 mM, dNTP 50 mM and primer M13 100 mM. RAPD-PCR reaction was developed in a total volume of 50 µL and it was carried out with a Perkin Elmer, GeneAmp PCR System 2400 thermocycler. 20 µL of amplified products were resolved by electrophoresis in a 1.4% agarose gel in 0.5x TBE (45 mM Tris base, 89 mM, boric acid, 2.5 mM EDTA pH 8) for 3 h at 70 V.

2.4.5. Numerical analysis of PFGE and RAPD-PCR images

The conversion, normalization and further processing of images were carried out by InfoQuest™ FP software version 5.10 (Bio-Rad, USA). Comparison of the obtained PFGE patterns was performed with Pearson's product-moment correlation coefficient and the Unweighted Pair Group Method using Arithmetic averages (UPGMA). Comparison of the pulse types from the PFGE and RAPD was made by composite data set comparison with average molecular analysis by Unweighted Pair Group Method using Arithmetic averages (UPGMA) (Ruiz et al., 2008).

2.5. Culture independent methods

2.5.1. Direct DNA extraction from wines samples

A volume of 10 mL of each must or wine sample was centrifuged (30 min, 10000xg, 4 °C). The supernatant was discarded and 1.2 mL of saline solution (NaCl 0.9%) and 2.4 mL of

zirconium hydroxide (7 g/L) were added to the pellet to facilitate pelleting of the bacteria in wine (Lucore et al., 2000). After horizontal shaking during 10 min at room temperature, the suspension was again centrifuged (10 min, 500xg, 7 °C) and finally DNA was purified from the cell pellet by using a PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA USA) as per the manufacturer's instructions.

2.5.2. PCR conditions

PCR was performed using an Applied Biosystem, GeneAmp® PCR System 2700 thermocycler at a final volume of 50 µL. To amplify the region V4 to V5 of 16S rDNA gene, primers WLAB1 and WLAB2^{GC} were used as López et al. described (2003). Moreover, primers *rpoB1*, *rpoB1o*, and *rpoB2^{GC}* were employed to amplify the region of the *rpoB* gene as it was described by Renouf et al. (2006) with the next modifications: 0.5 µM of each primer, 1 mM dNTP mix and 0.5 µL of *PfuUltra* II Fusion HS DNA Polymerase (Stratagene).

2.5.3. PCR-DGGE analysis

The separation of the respective PCR products was performed with the D-CODE™ universal mutation detection system (Bio-Rad, Hercules, Calif.). PCR products obtained from WLAB1-WLAB2^{GC} primers were run on 8% (wt/V) polyacrilamide gels in a running buffer containing 2 M Tris base, 1 M Glacial acetic acid and 50 mM EDTA pH 8 (TAE), and a denaturing gradient from 35 to 55% of urea and formamide. The electrophoresis was performed at 20 V for 10 min, and 80 V for 18 h at a constant temperature of 60 °C. PCR products generated with the *rpoB1*, *rpoB1o*, and *rpoB2^{GC}* primers were separated with 8% polyacrilamide gels containing a 32 to 50% urea-formamide gradient. Electrophoresis was performed for 10 min at 20 V, and 16 h at 60 V at a constant temperature of 60 °C. The DGGE gels were stained in ethidium bromide after the electrophoresis and then were visualized with UV transillumination (GelDoc, Bio-Rad). Blocks of polyacrilamide gels which contained selected DGGE bands were excised and later incubated overnight in 20 µL of sterile and pure water at 4 °C to make DNA bands diffuse to the liquid. One microliter of this solution was used to reamplify the PCR product.

2.5.4. DNA sequencing and phylogenetic analysis

PCR products were sequenced by Macrogen Inc. (Seoul, South Korea). The quality and characteristics of the obtained sequences were analyzed with the software InfoQuest™ FP 5.10, only those ones considered as appropriate were used for comparison to the GenBank database with the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). After this preliminary study, our sequences and their homologous ones (obtained from the Nucleotide Database: <http://www.ncbi.nlm.nih.gov/nucleotide>) were assembled and submitted to phylogenetic and evolutionary analysis with MEGA version 4.0.2 (Tamura et al., 2007). The Neighbor-Joining analysis (Saitou & Nei, 1987) allowed to get information about the relations between the gotten sequences and the reliability of the identifications provided by the Nucleotide Database. The bootstrap test was based on 1000 replicates (Felsenstein, 1985).

The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2007) that allowed to calculate the equivalent units to the base substitutions per site.

3. Results and discussion

3.1. Oenological parameters of wine samples and fermentation development

Results for analytical composition of wines during three vintages are displayed in Table 1. Data were within the usual range of Tempranillo wines from this Spanish region (González-Arenzana et al., 2012b). After completion AF, alcohol content ranged between 13.0% and 14.0%, pH was between 3.32 and 3.64 and free SO₂ level was between 4.24 and 18.1 mg/L. During MLF a decrease in total acidity and a subsequent increase in pH were observed. In addition, an increase in volatile acidity was noted as it was expected. The wine from 2006 vintage showed less restrictive parameters for microbial growth, so it presented higher pH and the lowest values of alcohol content and SO₂.

Year	2006		2007		2008	
	3	6	3	6	3	6
Alcohol content (% v/v)	13.0	-	13.8	-	14.0	-
pH	3.64	3.83	3.41	3.57	3.32	3.50
Total acidity (g/L tartaric acid)	7.98	5.91	7.63	6.71	9.00	7.20
Volatile acidity (g/L acetic acid)	0.25	0.46	0.37	0.49	0.26	0.37
Total SO ₂ (mg/L)	28.4	-	38.1	-	31.6	-
Free SO ₂ (mg/L)	4.24	-	18.1	-	13.2	-
L-malic acid (g/L)	2.60	0.04	1.48	0.16	2.61	0.21
L-lactic acid (g/L)	-	1.81	-	1.10	-	1.72

-: not analyzed

Table 1. Analytical composition of wines at final AF (stage 3) and final MLF (stage 6) at each vintage.

AF completion lasted for six, sixteen and eleven days in 2006, 2007 and 2008 vintages, respectively. Viable LAB counts during AF were in the range of 10² - 10³ CFU/mL, increasing to 10⁷ - 10⁸ CFU/mL during MLF, similar to spontaneous MLF results reported by other authors (European Community, 1990; Lopez et al., 2008). The development of the MLF was related to the viable population of LAB and there was a relation between bacterial population and decrease in L-malic acid (data not shown). Important differences in MLF duration were observed between vintages and MLF completion lasted for 21, 239 and 136 days in 2006, 2007 and 2008 vintages, respectively. Different temperatures at each vintage (wine temperature below 12 °C after AF in 2007) and the lack of temperature control in the winery were the determinant factors in these differences, but factors such as pH, composition of the wine and the interaction with other microorganisms implicated in the

fermentation could also influence, as it has been reported by other authors (du Plessis et al., 2004; Lonvaud-Funel, 1999; Reguant et al., 2005a; Reguant et al., 2005b).

3.2. Species identification

3.2.1. Culture dependent microbiological analysis

Figure 1 shows the number of isolates of the viable LAB species identified at each stage and year of vinification. A total of 251 LAB isolates were recovered and identified as belonging to eight different species. The greatest diversity of LAB species was detected during the AF. *Oenococcus oeni* was present in all studied stages of the fermentation process except in 2007. It was isolated in must and tumultuous AF in 2006 and 2008 vintages, and it was the only species isolated at MLF in the three years, being therefore the predominant species, followed by *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and *Lactobacillus mali*. The other non-*Oenococcus oeni* species appeared at stages 1-2 in variable rates in the three vintages. A similar distribution of species has been also reported by other authors (Fugelsang & Edwards, 2007; Ruiz et al., 2010) and they also concluded that *Oenococcus oeni* was the main responsible species for MLF. The diversity of species found at each year was different, being the number of species isolated in 2007 almost double that in 2006 and 2008, and missing *Oenococcus oeni* until the end of the 2007 AF, a fact that could also have influence in the MLF duration, as it was indicated above.

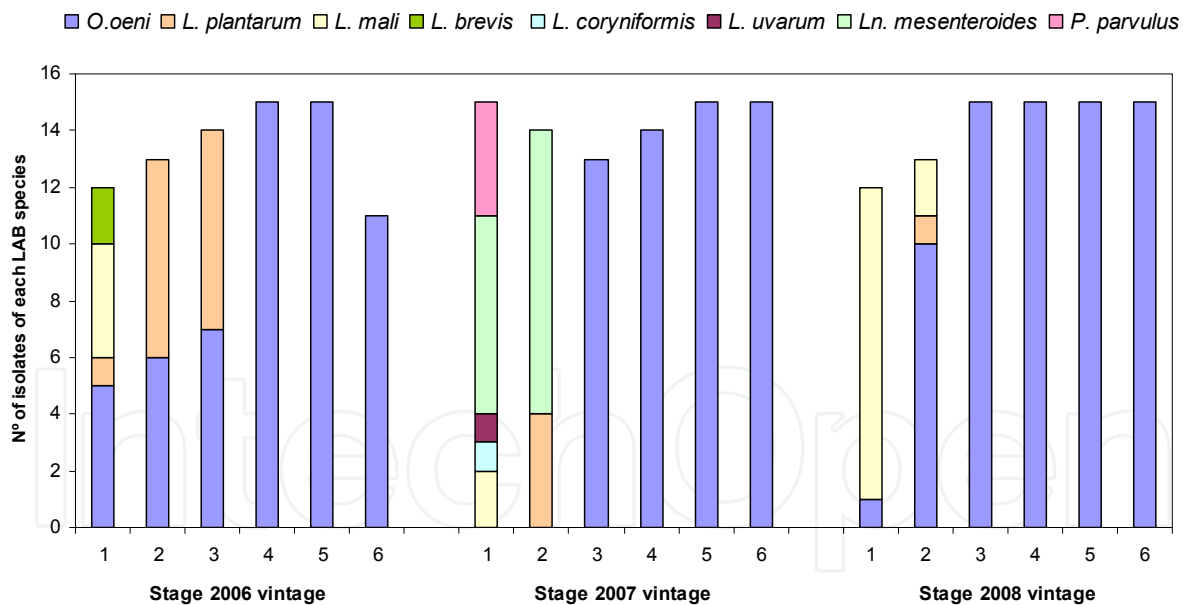


Figure 1. Number of isolates of the viable LAB species identified at each stage of vinification (1, must; 2, tumultuous AF; 3, final AF; 4, initial MLF; 5, tumultuous MLF; 6, final MLF) and in each vintage.

3.2.2. Culture independent microbiological analysis and comparison with culture dependent method

PCR-DGGE analysis of the sampled wine fermentations in the three studied vintages using primers WLAB1/2 (16S rDNA-based primer sets) and primers *rpoB1/1o/2* (β subunit of the

RNA polymerase-based primer sets) (Figure 2a and 2b, respectively) revealed different species and a profile of bacterial community structure changes during AF and MLF.

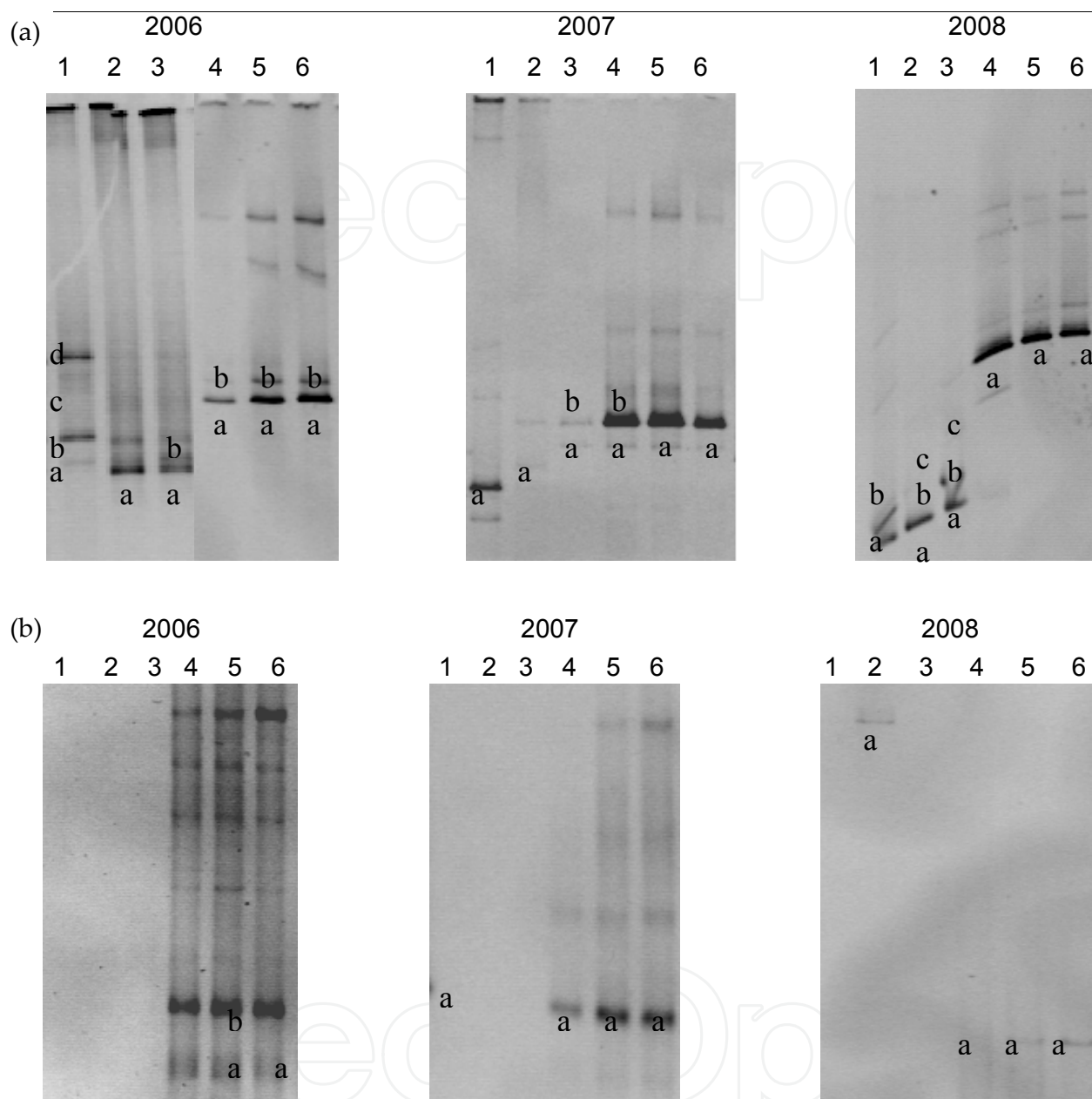
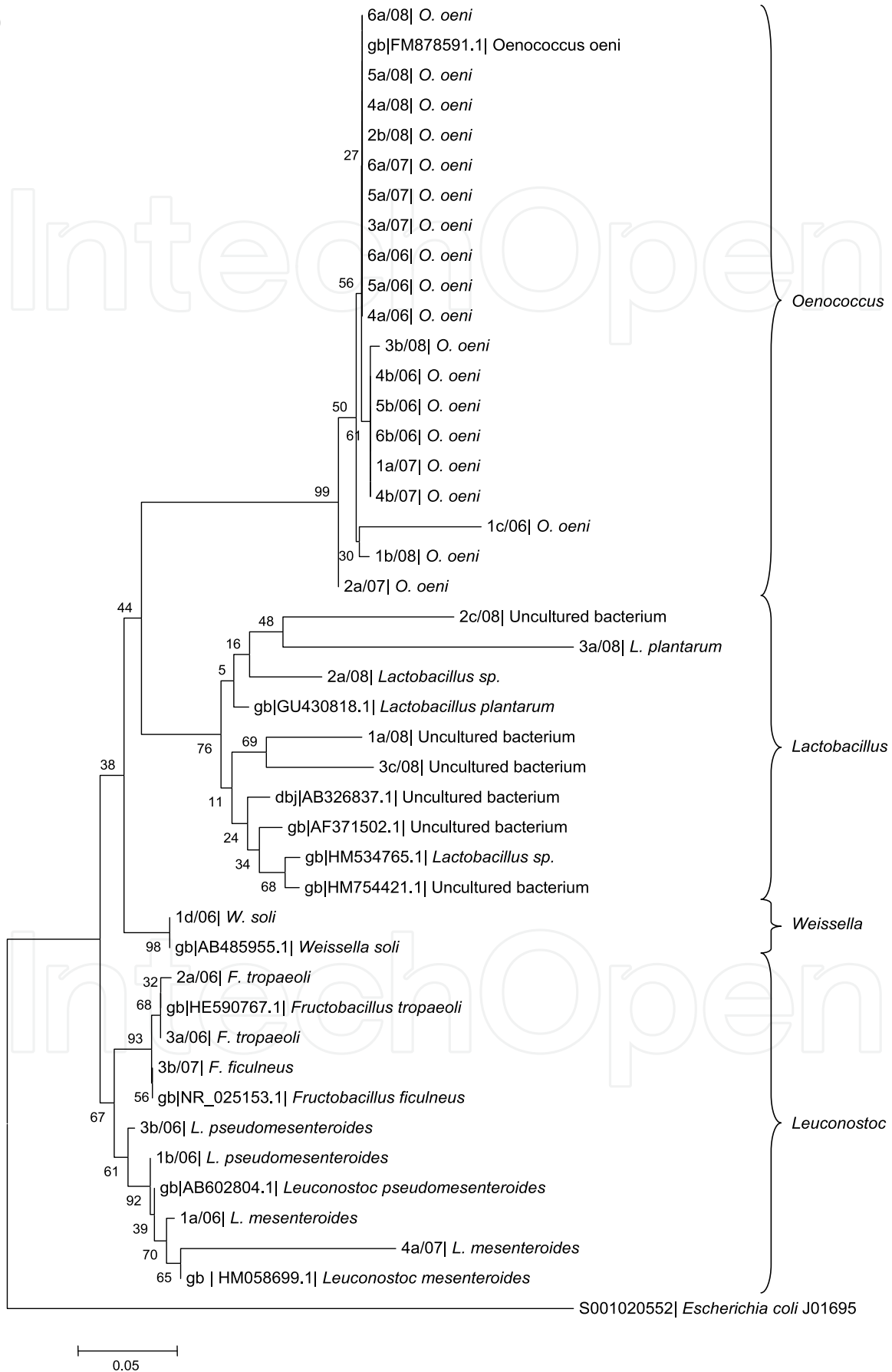


Figure 2. DGGE gels of the sampled wines at each vintage and stage of vinification (1, must; 2, tumultuous AF; 3, final AF; 4, initial MLF; 5, tumultuous MLF; 6, final MLF). Letters indicate bands excised for each gene, 16S rRNA (a) and *rpoB* (b).

The sequences obtained from the DNA excised DGGE-bands of each sample and their homologous ones from Nucleotide Database (Altschul et al., 1990) constituted a tree for each studied gene (Figure 3). Figure 3a shows a tree based on 16S rDNA gene composed by four ramifications or branches belonging to the genus *Oenococcus*, *Lactobacillus*, *Weissella* and *Leuconostoc*; and Figure 3b shows a tree based on *rpoB* gene composed by three branches belonging to *Oenococcus*, *Leuconostoc* and *Lactobacillus*.

(a)



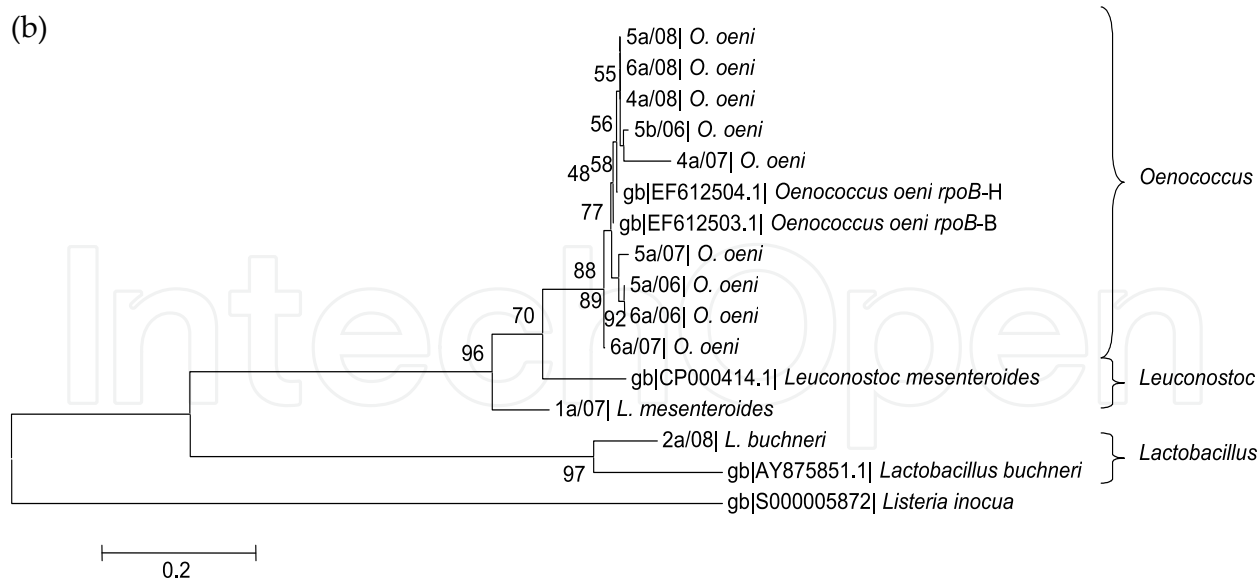


Figure 3. Phylogenetic trees generated from the method of 16S rDNA (a) and *rpoB* (b) sequences of recovered bands were inferred using the Maximum Parsimony method (Felsenstein, 1985). Numbers over the branches are bootstrap values (1000 repetitions). *Escherichia coli* and *Listeria innocua* were used as outgroups. Scale bar represents (calculated) distance. Reference strains are closely related to the sequenced bands and accession number of each gene is indicated. Band number isolated from fermenting wines are indicated by the sampled stage of vinification (1, must; 2, tumultuous AF; 3, final AF; 4, initial MLF; 5, tumultuous MLF; 6, final MLF) the letter of the position in the gel and the isolation year.

The species identification at each stage of vinification in the three studied years with culture independent techniques (16S rDNA/PCR-DGGE and *rpoB*/PCR-DGGE) and culture dependent method (plating on modified MRS) are recovered in Table 2.

A total of fourteen different LAB species were identified in the three studied vintages by traditional and culture independent methods. PCR-DGGE analysis allowed to identify nine species in comparison to the eight ones detected by culture in plate of the sampled wine. Thus, *Fructobacillus ficulneus*, *Fructobacillus tropaeoli*, *Lactobacillus buchneri*, *Leuconostoc pseudomesenteroides* and *Weissella* were not detected in the employed culture medium, while *Lactobacillus coryniformis*, *Lactobacillus brevis*, *Lactobacillus mali*, *Lactobacillus uvarum* and *Pediococcus parvulus* were not detected by PCR-DGGE. Therefore, results obtained by both methods were complementary and demonstrated the importance of using a combined analytical approach to explore microbial communities as other authors have concluded in different ecological niches (Iacumin et al., 2009). In relation with DGGE analysis, PCR-amplified bands from 16S rDNA gene gave better results than the *rpoB* bands amplification, with eight and three identified species, respectively. Nevertheless, the results obtained with the two genes were again complementary so *Lactobacillus buchneri* was only detected by *rpoB*/PCR-DGGE.

Results about diversity of LAB species found at each year and stage of vinification were very similar to those described above for culture dependent method. The greatest diversity was detected again during AF, opposite to MLF were only two species were present, *Oenococcus oeni* and *Leuconostoc mesenteroides*. The presence of *Leuconostoc mesenteroides* in

2007 at stage 4 confirmed again the possible competition of other LAB with *Oenococcus oeni* and their influence in MLF duration.

Detected LAB species	2006						2007						2008					
	1*	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
<i>Fructobacillus ficulneus</i>	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-
<i>Fructobacillus tropaeoli</i>	-	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Lactobacillus coryniformis</i>	-	-	-	-	-	-	c	-	-	-	-	-	-	-	-	-	-	-
<i>Lactobacillus brevis</i>	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Lactobacillus buchneri</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	b	-	-	-	-
<i>Lactobacillus mali</i>	c	-	-	-	-	-	c	-	-	-	-	-	c	c	-	-	-	-
<i>Lactobacillus plantarum</i>	c	c	c	-	-	-	-	c	-	-	-	-	-	c	w	-	-	-
<i>Lactobacillus uvarum</i>	-	-	-	-	-	-	c	-	-	-	-	-	-	-	-	-	-	-
<i>Lactobacillus sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-
<i>Leuconostoc mesenteroides</i>	w	-	-	-	-	-	bc	c	-	w	-	-	-	-	-	-	-	-
<i>Leuconostoc pseudomesenteroides</i>	w	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Oenococcus oeni</i>	wc	c	c	wc	◊	◊	w	w	wc	◊	◊	◊	wc	wc	wc	◊	◊	◊
<i>Pediococcus parvulus</i>	-	-	-	-	-	-	c	-	-	-	-	-	-	-	-	-	-	-
<i>Weissella sp.</i>	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UB.1	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-
UB.2	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-
UB.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-
Total species n°	7	3	4	1	1	1	6	3	2	2	1	1	3	6	3	1	1	1
Plate species n°	4	2	2	1	1	1	5	2	1	1	1	1	2	3	1	1	1	1
Total DGGE species n°	4	1	2	1	1	1	2	1	2	2	1	1	2	4	3	1	1	1
16S rDNA/DGGE species n°	4	1	2	1	1	1	1	1	2	2	1	1	2	3	3	1	1	1
<i>rpoB</i> /DGGE species n°	-	-	-	-	1	1	1	-	-	1	1	1	-	1	-	1	1	1

*stage of vinification (1, must; 2, tumultuous AF; 3, final AF; 4, initial MLF; 5, tumultuous MLF; 6, final MLF)

-; not detected

UB; uncultured bacterium

w; detected with 16S rDNA/DGGE

b; detected with *rpoB*/DGGE

c; detected in plate after culture

◊; LAB species detected with all the employed methods

Table 2. LAB species detected with culture independent techniques (16S rDNA/PCR-DGGE and *rpoB*/PCR-DGGE) and culture dependent methods at each stage of vinification (1, must; 2, tumultuous AF; 3, final AF; 4, initial MLF; 5, tumultuous MLF; 6, final MLF) during three vintages.

3.3. Strain typing of *Oenococcus oeni*

3.3.1. PFGE analysis of the strains of this study

Identification of the *Oenococcus oeni* strains of this study was successfully achieved by PFGE of DNA digested with *SfiI* and each strain presented a characteristic PFGE pattern. Digestions with *ApaI* enzyme were not more discriminating than *SfiI* restriction (data not

shown). Cluster analysis and visual inspection of the PFGE patterns from the 187 *Oenococcus oeni* isolates recovered from wine fermentations in the three years gave a total number of 37 distinct genotypes (Figure 4). Twenty-four of them were detected in 2006, five in 2007 and fifteen in 2008 vintages (Figure 5). The lower clonal diversity found in 2007 vintage would be related with the low temperatures during MLF (below 12 °C) what could make few genotypes be able to get adapted to the difficult conditions.

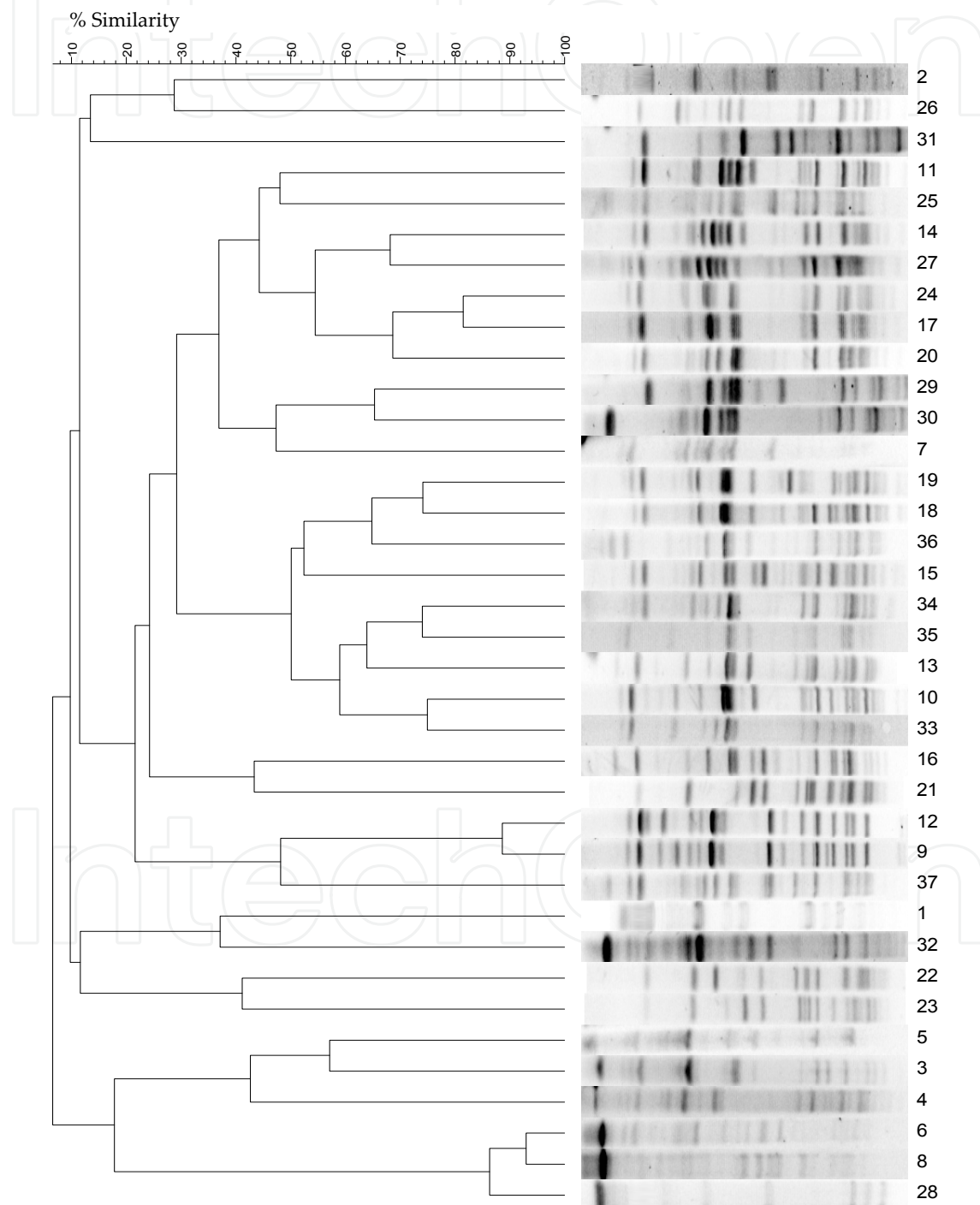


Figure 4. UPGMA dendrogram based on the *Sfi*I PFGE patterns of the 37 *Oenococcus oeni* genotypes. Figure from González-Arenzana et al. (2012a).

Comparing coincident genotypes for the three vintages, it was observed that between the genotypes isolated in 2006 vintage two were found in 2007 (genotypes 18 and 20) and four in 2008 (genotypes 3, 13, 17 and 18). Moreover, two genotypes isolated in 2007 (genotypes 18 and 25) were also detected in 2008 vintage. Only one genotype (18) was identified in the three studied years. The frequency of participation of each genotype varied from year to year, thus dominant genotypes one year were minority or not present at other one which suggested the adaptation of *Oenococcus oeni* strains to the winery conditions every year. Similar results were reported by other authors in studies about bacteria and yeast populations (Gutierrez et al., 1999; Reguant et al., 2005a; Ruiz et al., 2010; Santamaría, 2009).

Interestingly, no genotype was isolated in all fermentation stages so fourteen genotypes appeared only at AF (stages 1-3), six were present at all MLF stages (4, 5 and 6) and three of them were also detected at the end of AF. Most fermentation stages showed mixed *Oenococcus oeni* strains populations, which confirmed that several *Oenococcus oeni* strains occurred in a single spontaneous MLF (Lopez et al., 2007; Renouf et al., 2009; Ruiz et al., 2010). The number of different genotypes identified at each stage ranged from 0 to 5, and from 3 to 9, at stages 1-3 and 4-6, respectively. Genotypes 9 and 18 in 2006 vintage, 20 and 26 in 2007, and 13 in 2008 were the predominant ones during MLF. Three out of these genotypes (13, 18 and 20) could be considered as interesting *Oenococcus oeni* strains for the selection of new malolactic starter cultures as individual or mixed strains, because in addition to be dominant in most of the MLF stages at each vintage, they were isolated at more than one year in quality wines.

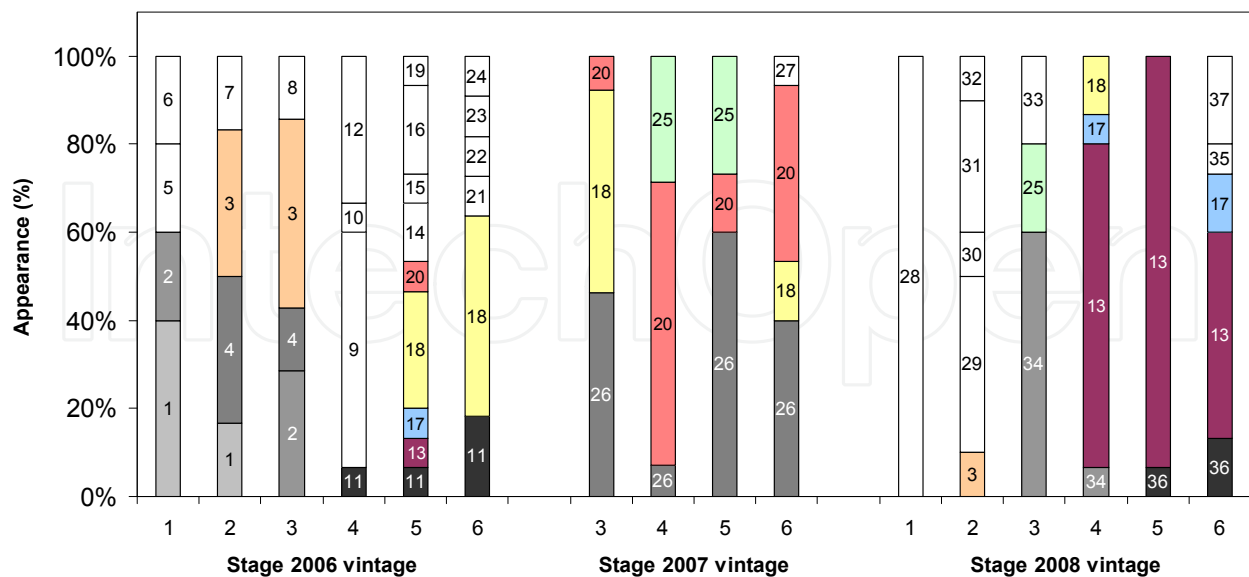


Figure 5. Frequency of appearance (%) of *Oenococcus oeni* genotypes at each stage (1, must; 2, tumultuous AF; 3, final AF; 4, initial MLF; 5, tumultuous MLF; 6, final MLF) and vintage. Genotypes without color only appeared once, genotypes with grey color appeared only in one year at more than one stage and genotypes with color appeared in more than one vintage.

3.3.2. Comparison of the PFGE and RAPD-PCR profiles from the wine fermentation strains and commercial strains

The thirty-seven genotypes of the indigenous *Oenococcus oeni* strains from wine fermentations and fourteen PFGE and RAPD-PCR patterns obtained of strains from several commercial cultures were submitted to comparison by bioinformatics and visual analysis (data not shown). Figure 6 shows that genotypes I, II, III and IV from commercial starter cultures resulted indistinguishable from four indigenous genotypes (23, 13, 21 and 18, respectively), despite commercial malolactic cultures had never been employed in the sampled cellar.

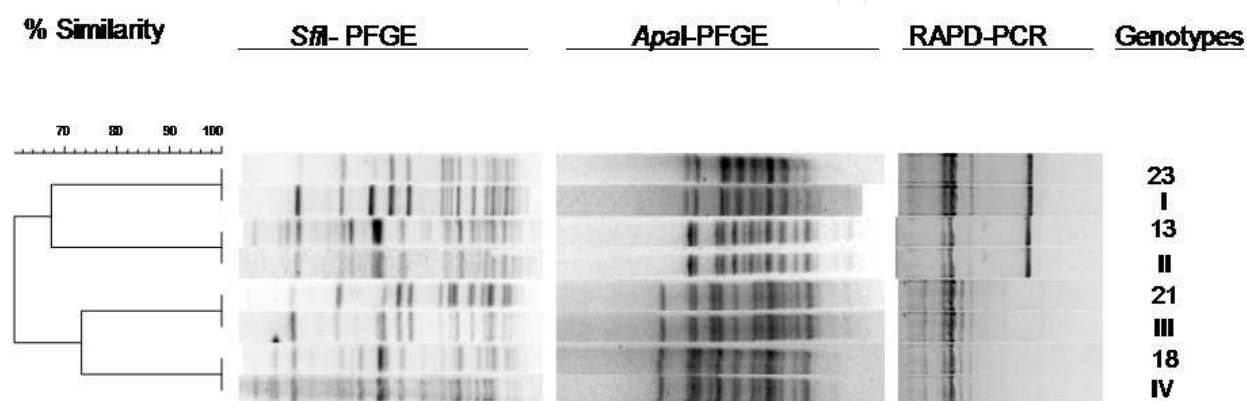


Figure 6. Consensus dendrogram obtained by combination *Sfi*I-PFGE, *Apa*I-PFGE and M13 RAPD-PCR patterns corresponding to the commercial strains (I to IV) and their respective indistinguishable genotypes from indigenous *Oenococcus oeni* strains from this winery.

These all four autochthonous genotypes were detected in 2006 vintage, year which showed the greatest strain diversity, two of them occurred in 2008 and one in 2007, with an important frequency of appearance in some cases (Table 3).

Genotype	Isolation stage	Frequency of appearance at each vintage (%)		
		2006	2007	2008
13	4-5-6	2	-	45
18	3-4-5-6	15	14	3
21	6	2	-	-
23	6	2	-	-

Table 3. *Oenococcus oeni* genotypes indistinguishable to commercial patterns; isolation stage (1, must; 2, tumultuous AF; 3, final AF; 4, initial MLF; 5, tumultuous MLF; 6, final MLF) and frequency (%) of their appearance (frequency of appearance (%) = n° of isolates that presented a specific PFGE pattern \times 100/total n° of isolates per vintage) at each vintage.

Therefore, despite two of these strains had been previously considered as interesting for the selection of new malolactic starter cultures, the possible identical strain identification with already marketed strains suggested reject these two indigenous *Oenococcus oeni* isolates from

a future selection process regardless of their oenological properties as malolactic starter cultures.

4. Conclusion

This study has been a contribution to a better description of the LAB ecology along the process of Tempranillo wines winemaking.

The study about the microbial diversity of viable LAB populations showed that the species diversity was higher at the AF stage where eight different species were identified. *Oenococcus oeni* was detected during AF in variable proportions and it became the majority species during spontaneous MLF.

This work allowed to increase the endogenous strain collection of LAB isolated from fermenting wines of the Appellation of Origin Rioja what meant a contribution to the preservation of biodiversity and wine peculiarity of this region and a starting point for future research.

The analysis of the total LAB populations by culture independent techniques (PCR-DGGE) showed that the species diversity detected along the winemaking process was higher than the one found by the study of viable LAB, identifying up to nine different LAB species. The LAB species variability was also higher at the previous stages to the MLF. Once spontaneous MLF started this variability was greatly reduced, with *Oenococcus oeni* and *Leuconostoc mesenteroides* as the only detected species.

The results obtained with culture dependent and independent techniques were complementary so in studies conducted in microbial ecology they both should be used to achieve a broader view of the studied ecosystem.

PFGE has shown to be a suitable method for strain differentiation, for monitoring individual strains and determining which strains actually survive and carry out MLF. The results of *Oenococcus oeni* typification indicated the high diversity of indigenous *Oenococcus oeni* strains responsible for MLF of the wines of this study and the complexity of the ecology involved in a fermentating wine. The frequency of participation of each genotype varied from year to year, thus dominant genotypes one year were minority or not present at other one, which suggested the adaptation of *Oenococcus oeni* strains to the winery conditions every year.

Several genotypes could be considered as interesting *Oenococcus oeni* strains for the selection of new malolactic starter cultures as individual or mixed strains because, in addition to be isolated at more than one year in quality wines, they were dominant in most of the MLF stages at each vintage. The comparison of the patterns from commercial cultures and the genotypes from indigenous *Oenococcus oeni* strains showed four indistinguishable genotypes. The presence of these four genotypes for one to three years, and in some cases with a high frequency of appearance, demonstrated the significance of this study in order to exclude these genotypes from a future selection process.

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Acknowledgement

This work was supported by funding and predoctoral grant (B.O.R. 6th March, 2009) of the Government of La Rioja, the I.N.I.A. project RTA2007-00104-00-00 and FEDER of the European Community and was made possible by the collaborating winery.

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