Systematic and Applied Microbiology 40 (2017) 1–10

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Exploring the biodiversity of two groups of *Oenococcus oeni* isolated from grape musts and wines: Are they equally diverse?

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ARTICLE INFO

Article history:
Received 23 May 2016
Received in revised form 4 November 2016
Accepted 5 November 2016

Keywords:
Oenococcus oeni
Wine
Grape must
Diversity
Carbohydrate fermentation
DNA fingerprints

ABSTRACT

One hundred and four *Oenococcus oeni* isolates were characterised by the carbohydrate fermentation (CH) profile and DNA fingerprinting. Forty-four isolates came from grape must, and 60 from wines sampled at the end of alcoholic fermentation or during malolactic fermentation. The grape must isolates fermented more CH than the wine isolates. In genotypical terms, no clear boundary between grape must and wine isolates was found. Diversities were deduced by considering the isolates of grape must and of wine separately and jointly. By considering only CH fermentation abilities, the group of grape must isolates gave higher diversity index (DI_{CH}) values than those isolated from wine; i.e., these isolates were metabolically more diverse. The contrary occurred when the DNA fingerprints were used to calculate $DI_{RAPD\text{-}VNTR}$: wine isolates were genotypically more diverse than grape must ones. With a polyphasic approach, which considered metabolic and genotypic data, the diversity index of both isolate groups (from grape must and wine) was the same, 0.993, which was slightly lower than that calculated from all the isolates (0.997).

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Introduction

Two main groups of organisms involved in wine production are yeasts and lactic acid bacteria (LAB), which are responsible for alcoholic fermentation (AF) and malolactic fermentation (MLF), respectively. AF consists in transforming sugars into ethanol and carbon dioxide. During the MLF process, the L-malic acid present in wine is converted into L-lactic acid and carbon dioxide. MLF is a crucial step in winemaking as it enhances the organoleptic characteristics of wine and lowers the risk of microbial alteration [17,37,38]. Among the lactic acid bacteria associated with the winemaking process, *Oenococcus oeni* is the species mainly responsible for MLF because it is the best adapted bacterium to overcome wine stressing conditions, which is why it is often used as a malolactic starter culture [27,56,62]. Although this species is generally isolated from wine, it has been occasionally described in grape musts [33].

Growth of LAB in wine depends largely on sugars, organic acids, amino acids and vitamins, which are found in grape must or wine [23,38,43]. Like most heterofermentative LAB, *O. oeni* is able to degrade hexoses, pentoses and other carbohydrates from must [59]. Glucose and fructose are major residual sugars in wine after completing AF, whose concentration may vary from 10 g/L to below 0.5 g/L, depending on wine style [2]. Fructose is generally found at higher concentrations than glucose. Other carbohydrates, like arabinose, xylose, ribose and trehalose, may be present in wine, but at lower levels than glucose and fructose [7]. Several authors have studied the ability to ferment carbohydrates of *O. oeni* to (a) characterise this species; (b) realise how it influences its growth; and (c) know how it impacts the final characteristics of the wines in which this species develops [7,25,32,34,35,44].

Many other authors have characterised *O. oeni* strains at molecular level by different techniques to fulfil various goals. Random Amplified Polymorphism DNA (RAPD) [1,9,26,36,42,46,47,56,64], Pulse Field Gel Electrophoresis (PFGE) [35,40,45,63], Amplified Fragment Length Polymorphism (AFLP) [11–13], Variable Number of Tandem Repeats (VNTR) [15,16,22] and Multilocus Sequence

Abbreviations: GM, genotypic profiles (combined RAPD and VNTR fingerprints) derived from comparing the grape must *O. oeni* isolates; GW, genotypic profiles (combined RAPD and VNTR fingerprints) derived from comparing the wine *O. oeni* isolates; GT, genotypic profiles (combined RAPD and VNTR fingerprints) derived from comparing the totality of *O. oeni* isolates; PM, polyphasic profiles (combined CH fermentation and RAPD and VNTR fingerprints) derived from comparing the grape must *O. oeni* isolates; PW, polyphasic profiles (combined CH fermentation and RAPD and VNTR fingerprints) derived from comparing the wine *O. oeni* isolates; PT, polyphasic profiles (combined CH fermentation and RAPD and VNTR fingerprints) derived from comparing all the *O. oeni* isolates.

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http://dx.doi.org/10.1016/j.syapm.2016.11.003
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Typing (MLST) [3,6,18,22] have been the most commonly used techniques. The objectives pursued by these authors were diverse: strain typing, study the population dynamics of *O. oeni* during winemaking [1,5,11,13,15,31,39,46], evaluate intraspecific biodiversity [3,4,6], investigate the temporal and biogeographical distribution of strains [1,5,25,26,35,42], determine relationships between phenotypical properties and genomic characteristics [11–13,26,63]. Although several researchers have attempted to find relationships between phenotypic and genomic features, the majority have found no correlation among the clusterings obtained by the two types of characteristics [13,26,63]. Very few authors have attempted to assess diversity with a polyphasic approach by combining phenotypic and genotypic characteristics [5].

As far as we know, the majority of works conducted on the characterisation of *O. oeni* strains have been performed on strains isolated from the final moments of alcoholic fermentation or during malolactic fermentation. This is the first time that a similar number of grape must and wine isolates has been compared. From a basic and applied point of view, it is challenging to know if evidence exists as to the *O. oeni* isolates from different fermentation times being separated into distinct metabolic or genetic groups.

The aims of this work were to determine if the *O. oeni* isolates from grape must and wine were metabolically and genetically distinguishable, to know if the diversities of both groups were similar or not, and finally, whether these diversities reflected that of all the isolates, regardless of their origin. To this end, single and combined analyses using different characterisation techniques were run to study both groups separately and jointly.

Material and methods

Origin of the Oenococcus oeni *isolates*

One hundred and four isolates were obtained from different types of grape musts, wines and wineries in various regions of Spain and Portugal. Wine isolates were taken from late alcoholic fermentation or malolactic fermentation (Table 1). Isolates were lyophilised and deposited in the private culture collection of the ENOLAB Laboratory (Universitat de València, Spain).

Isolation of bacterial isolates

The samples from grape musts and wines, after being appropriately diluted, were spread on solidified *Leuconostoc oenos* medium (MLO) [66] added with 0.15 mg/L of Actistab (0.075 mg/L natamycin) (Gist-Brocades) to prevent yeast growth. Plates were incubated at 28 °C for 7 days. The colonies grown on surfaces were isolated and lyophilised.

Identification of isolates at the species level

Isolates were presumptively identified as *O. oeni* by colony and cell morphologies, cell arrangement, Gram-positive character, absence of catalase, heterofermentative catabolism glucose, and lactic acid production from glucose, according to classic keys for LAB identification [14,28,51,60]. Gram-positive character and catalase activity were determined as described by Cappuccino and Sherman [14]. Production of lactic acid from hexoses was analysed by HPLC, as Frayne described [21]. Each isolate was examined microscopically to determine cellular morphology and arrangement. We determined the type of glucose fermentation following the procedure described by Zúñiga et al. [66].

In order to confirm the phenotypical identification of the *O. oeni* isolates, a couple of primers specific for *O. oeni*, I_2B (CCCTACTGCTGCCTCCCGTAGGAGT) and I_6B (TTCGGGTGAAGTGAGGCAATGACTA), were used (Ferrer, personal communication). These primers amplify an *O. oeni* specific region of the rRNA 16S gene. The expected amplicon size was approximately 300 base pairs (bp). Each specific-PCR reaction was performed in a total volume of 50 μL with the following components: 50 mM of I_2 and I_6 primers, 50 *p*mol of each dNTP (Roche), 0.5 μL of Taq polymerase (DyNAzyme II DNA Polymerase; Thermo Scientific), 5 μL of 10X Taq buffer (Thermo Scientific), 0.5 μL of 50 mM $MgCl_2$ (Thermo Scientific) and 40 μL of MilliU water (Millipore). A DNA template was obtained from a single colony suspended in 10 μL of sterile MilliU water (Millipore) and 1 μL of this cell suspension was added to the *O. oeni* specific-PCR reaction, as described by Rodas et al. [51]. Each set of reactions included a negative control. PCR reactions were carried out in a PTC-100TM thermal cycler (MJ Research, Watertown, USA) using an initial denaturation time of 5 min at 95 °C, followed by 34 amplification cycles, which comprised a denaturation step at 95 °C for 30 s, an annealing step at 73 °C for 1 min and 30 s, and an extension step at 72 °C for 30 s. Reactions were completed with a 7-min elongation step at 71 °C, followed by cooling to 10 °C.

Amplified products were resolved by electrophoresis on 1.2% (w/v) type D-1 Low-EEO agarose (Pronadisa, Madrid, Spain) in 0.5X of TBE buffer (45 mmol/L Trisbase, 89 mmol/L boric acid, 2.5 mmol/L EDTA; pH 8). Ladder 1Kb Plus (Invitrogen) was used as a DNA molecular weight marker. The electrophoretic conditions were 80 V for 45 min (200/2.0 power supply; Bio-Rad Laboratories, Richmond, CA, USA). Gels were stained with ethidium bromide (0.5 μg/mL; MO BIO) and images were digitalised with GelPrinter Plus by TDI (Madrid, Spain).

Phenotypic characterisation: carbohydrate fermentation

Isolates were metabolically characterised by studying their abilities to ferment carbohydrates. Fermentation tests on different carbohydrates were performed using the Carbohydrate Fermentation Basal semisolid Medium (CFBM) described by Garvie [24], which contained 2% (v/v) of the following carbon sources: amylose, D-arabinose, L-arabinose, D-arbutin, D-cellobiose, dextrin, D-fructose, D-galactose, D-glucose, D-lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-ribose, D-sucrose, D-salicin, D-trehalose, D-xylose and L-xylose. Sugars were purchased from Sigma–Aldrich.

Cells from 0.8 $O.D._{600nm}$ MLO cultures were harvested by centrifugation at 6842 × *g* for 15 min (Heraeus Multifuge 1 SR), washed twice, re-suspended in the same volume of sterile saline solution and inoculated at 4% (v/v) in CFBM. Tubes were incubated at 28 °C for 30 days. The CH fermentation results were recorded as positive if the medium's colour changed from blue–green to yellow.

Genotypic characterisation

A Random Amplified Polymorphic DNA (RAPD) analysis was carried out using primer M13, according to the method described by Zapparoli et al. [64]. Each RAPD-PCR reaction was performed in a total volume of 50 μL with the following components: 50 mM of primer M13, 50 *p*mol of each dNTP (Roche), 1 μL of Taq polymerase (DyNAzyme II DNA Polymerase; Thermo Scientific), 5 μL of 10X Taq buffer (Thermo Scientific), 0.5 μL of $MgCl_2$ at 50 mM (Thermo Scientific) and 41 μL of MilliU water (Millipore). A DNA template was obtained as mentioned above and 1 μL of this cell suspension was added to the RAPD-PCR reaction. PCR reactions were carried out in a Techne TC-312 Thermal cycler (Durviz S.L., Valencia, Spain) using the amplification conditions described by Rodas et al. [52]. Each set of reactions included a negative control. Amplified products were resolved as previously mentioned.

A multiple-locus variable number of tandem repeat (VNTR) analysis was carried out according to the method described by Claisse and Lonvaud-Funel [15], with one modification; all pairs of

Table 1
Origin of the *Oenococcus oeni* isolates.

Geographical origin	Grape variety	Wine type	Fermentation stage	Isolates
Los Isidros[a]	Bobal	Rose	Must	**75**
Venta del Moro[a]	Garnacha	Red	Wine	118
Venta del Moro[a]	Macabeo	White	Wine	120
Requena[a]	Macabeo	White	Wine	122
Los Ruices[a]	Bobal	Red	Wine	123, 124, 125
Requena[a]	Red grape mixture	Red	Wine	128, 138 M, 171, 196
Los Ruices[a]	Bobal	Rose	Wine	129, 130
Los Ruices[a]	Macabeo	White	Wine	133
Venta del Moro[a]	Bobal	Red	Wine	134, 242
El Derramador[a]	Macabeo	White	Wine	142, 234
Sinarcas[a]	Bobal	Rose	Wine	149, 205A, 206, 213
Sinarcas[a]	Tempranillo	Rose	Wine	158
Fuenterrobles[a]	Bobal	Red	Wine	160B, 160C, 237
Casas de Prada[a]	Bobal	Rose	Wine	168, 169, 194, 197, 225, 228A, 251
Cuevas de Utiel[a]	Bobal	Rose	Wine	176A
Casas de Prada[a]	Tempranillo	Red	Wine	182, 188, 202, 229
Sinarcas[a]	Tempranillo	Red	Wine	217B, 218A, 219, 222M, 223A, 224
Barbastro[b]	Tempranillo	Red	Wine	226SM
Utiel[a]	Bobal	Red	Wine	235, 236
Venta del Moro[a]	Bobal	Rose	Wine	238, 239, 240
Camporrobles[a]	Bobal	Rose	Wine	246
Corrales de Utiel[a]	Bobal	Red	Wine	248
Corrales de Utiel[a]	Tardana	Red	Wine	254
Los Isidros[a]	Bobal	Rose	Wine	255
Fuente La Higuera[a]	Tempranillo	Red	Wine	504
Peñafiel[c]	Tempranillo	Red	Wine	B19
Fuensalida[d]	Tempranillo	Red	Wine	Z1, Z2, Z3, Z6, Z8
Oporto[e]	Syrah	Red	Must	**Po2, Po4, Po5, Po6, Po8, Po9, Po10, Po14, Po15, Po16**
Requena[a]	Merseguera	White	Must	**Me1, Me2, Me3, Me6, Me8**
La Rioja[f]	Tempranillo	Red	Must	**Ix1, Ix5, Ix6, Ix9, Ix13, Ix14, Ix15, Ix19, Ix21**
Camporrobles[a]	Bobal	Red	Must	**FS2, FS10, FS13, FS14, FS16, FS17, FS18, FS19, FS20, FS21, FS24, FS26, FS29, FS30, FS31, FS34, FS35, FS36, FS37**

In the normal type: isolates from wine, and in bold type: isolates from grape must.
[a] Valencia.
[b] Huesca.
[c] Valladolid.
[d] Toledo.
[e] Portugal.
[f] La Rioja.

primers were used together in a multiplex amplification reaction and not in five different reactions, as done originally. The sequences of these primers have been reported by Claisse and Lonvaud-Funel [15]. Amplifications were performed in a 50-μL reaction volume that contained: 1 μL of the cell suspension obtained as mentioned earlier, 0.5 μL of 50 mM of each primer, 1 μL of 50 pmol of each dNTP (Roche), 0.5 μL of Taq DNA polymerase (Invitrogen), 5 μL of 10X Taq buffer (Invitrogen), 2 μL of 50 mM $MgCl_2$ (Invitrogen) and 29.5 μL of MilliU water (Millipore). Reactions were run in a PTC-100TM thermal cycler (MJ Research, Watertown, USA). Each cycle consisted of an initial denaturation time of 5 min at 95 °C, followed by 40 amplification cycles, which comprised a denaturation step at 95 °C for 30 s, an annealing step at 58 °C for 30 s, and an extension step at 72 °C for 30 s. Reactions were completed with a 7-min elongation step at 72 °C, followed by cooling to 10 °C. Each set of reactions included a negative control. Amplified products were gel-resolved under the same conditions as those described above and digitalised to obtain a densitometric curve for each isolate.

Data analysis

The carbohydrate fermentation results and digitalised images of the DNA fingerprinting patterns were analysed by the BioNumerics software (version 6.6; Applied Maths, Kortrijk, Belgium). Similarity matrices and clustering analyses were performed by the Unweighted-Pair Group Method with Arithmetic mean (UPGMA) using the simple matching similarity coefficient for the carbohydrate fermentation results, and the Pearson correlation coefficient for the densitometric curves of the RAPD and VNTR patterns. To obtain a measure of reproducibility of each technique, 10 isolates were randomly selected and analysed in duplicate. The lowest value obtained in the reproducibility study was defined as the threshold to determine if isolates belonged to the same cluster or not.

Grouping analyses were run by taking into account single techniques and combinations of them. The composite analyses of the RAPD and VNTR fingerprints on the one hand, and of the combined fingerprints and CH fermentation patterns on the other hand, were done using UPGMA and the above-mentioned similarity coefficients. The last grouping type resulted in polyphasic consensus dendrograms, which were built for the grape must and wine isolates analysed separately and together.

Calculating diversity indices

Diversity indices (*DI*) were calculated by using the formula proposed by Hunter and Gaston [29] and the index was obtained from the following mathematical expression:

$$\boldsymbol{DI} = 1 - \frac{1}{\boldsymbol{N}(\boldsymbol{N}-1)}\sum_{\boldsymbol{j}=1}^{\boldsymbol{S}} \boldsymbol{n_j}\left(\boldsymbol{n_j}-1\right)$$

where N is the total number of isolates studied, S is the total number of groups described, and n_j is the number of isolates that belong

Table 2
Fermentative profiles deduced from carbohydrates fermentative abilities of 104 isolates of *Oenococcus oeni.*

Profile	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
No. isolates per profile[a]	3	21/**1**	1	4	1	1/**1**	4	18	2/**5**	1/**2**	1	2/**5**	**9**	**4**	**3**	**2**	**3**	**2**	**7**	1
Total number of CH fermented	3	3	4	4	7	8	3	2	4	6	3	4	3	4	7	8	10	3	5	4
Amylose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-Arabinose	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L-Arabinose	–	+	+	+	+	–	–	–	–	+	–	+	–	+	+	–	+	+	+	–
Arbutin	+	–	–	+	+	+	–	–	–	–	–	+	+	–	–	+	+	–	–	+
Cellobiose	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+	+	+	–	–	–
Dextrin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fructose	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	–	–	–
Galactose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glucose	–	–	–	–	+[d]	+[d]	–	–	+	+	+[d]	+	+	+	+	+	+	+	+	+
Lactose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Maltose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mannose	–	–	–	–	–	+	–	–	–	+	–	–	–	–	+	+	+	–	–	–
Melibiose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	+	–
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Salicin	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–
Trehalose	–	–	–	–	+	+	+	–	+	+	–	–	–	+	+	+	+	–	+	+
D-xylose	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	–	–	–	–	–	–
L-xylose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

+: positive reaction; –: negative reaction.
[a] In bold: number of isolates from grape must.
[d] Delayed reaction; ND: not determined.

to the jth group. This index measures the probability of two unrelated isolates, taken randomly from a population, belonging to two different groups. It is based on the number of groups and the number of isolates in each group. Index values can vary from 0 to 1. Diversity increases as the *DI* approach 1.

The *DI* were calculated from the grouping obtained from single techniques and from combining two or three of them. Several *DI* were obtained when considering all the *O. oeni* isolates on the one hand, and the sets of isolates from grape must and wine separately on the other hand.

Results

Isolation and identification of bacteria

White convex colonies (1 mm diameter) were isolated from the MLO plates. This type of colony is typical of the *O. oeni* grown on this medium. Cell shape (spherical to lenticular), cell arrangement (chains of variable length), the Gram + character, absence of catalase, the heterofermentative utilisation of glucose and the production of lactic acid from hexoses led us to presumptively identify isolates as *O. oeni.* One hundred and four isolates exhibited these characteristics: 44 came from grape musts and 60 from wines. Identity was confirmed by the fact that a single PCR product of approximately 300 bp was obtained by the specific PCR of *O. oeni*. Sequencing the fragment showed the expected sequence that matched the corresponding amplified fragment of the *O. oeni* rRNA 16S gene.

Although sampling was done from the beginning to the end of each fermentation process, *O. oeni* isolation was very rare in the grape must stage, whereas isolation from wines was much more frequent.

Carbohydrate fermentation patterns of the O. oeni *isolates*

When the fermentative abilities of the 104 isolates were considered, some general traits were observed, which were all able to ferment ribose, but were unable to ferment amylose, dextrin, galactose, lactose, maltose, mannitol, sucrose and D- and L-xylose (Table 2). However, they displayed a variable reaction for D- and L-arabinose, arbutin, cellobiose, fructose, glucose, mannose, melibiose, salicin and trehalose. According to the abilities to ferment these carbohydrates, the *O. oeni* isolates were grouped into 20 different fermentative profiles (Table 2). Profiles B and H were the commonest, whereas the least frequent were C, E, K and T, all of which comprised a single isolate (Table 3). Profile H consisted of isolates with fewer fermentative abilities, which fermented only fructose and ribose. The other big group of isolates clustered in profile B and fermented L-arabinose, ribose and fructose. Profile Q grouped the isolates able to ferment the largest number of CH (10).

When we separately considered isolates from wine and from grape must, we observed that 79% of the wine isolates fermented only 2–3 CH, whereas the 75% of the must ones fermented more than three of them. Thus the majority of the grape must isolates

Table 3
Fermentative profiles and the strains included in them. Letters of the profiles shown in bold mean that the profiles contained exclusively or mainly grape must isolates. The isolate names in bold mean that isolates came from grape must, and the rest came from wine.

Profile	Isolate
A	129, Z6, Z8
B	120,122, 124, 125, 128, 130, 133, 138M, 142, 171, 196, 197, 217B, 223A, 224, 234, 238, 239, 246, 251, Z1, **FS35**
C	205A
D	218A, 240, 248, 254
E	118
F	**75**, 255
G	158, 169, 182, 222M
H	134, 149, 168, 176A, 188, 194, 202, 206, 213, 219, 225, 228A, 235, 236, 237, 242, Z2, Z3
I	123, 160B, **Po14**, **Me3**, **Me6**, **FS17**, **FS30**
J	160C, **Me8**, **FS36**
K	229
L	226SM, 504, **Po9**, **Po16**, **FS2**, **FS20**, **FS29**
M	**Po2**, **Po4**, **FS10**, **FS14**, **FS18**, **FS19**, **FS26**, **FS34**, **FS37**
N	**Po8**, **Ix5**, **Ix14**, **FS24**
O	**FS13**, **FS16**, **FS21**
P	**Po10**, **FS31**
Q	**Po5**, **Po6**, **Po15**
R	**Me1**, **Me2**
S	**Ix1**, **Ix6**, **Ix9**, **Ix13**, **Ix15**, **Ix19**, **Ix21**
T	B19

grouped in profiles I, J, L, M, N, O, P, Q, R and S showed the largest number of fermented carbohydrates, whereas the wine isolates clustered in A, B, D, G and H were the profiles with fewer fermented CH. We also observed some differences between these two groups in terms of their ability to ferment certain CH; all the isolates from wine, except for two, fermented fructose, while this ability was a variable character for the grape must isolates. The opposite occurred with glucose, which was fermented by all the grape must isolates except for one, but by only 23% of the wine isolates. Twenty percent of the grape must isolates were able to ferment cellobiose, mannose and melibiose, but no wine isolate was able to.

Genotypic analysis

In order to determine whether *O. oeni* isolates differed from a genotypic point of view, molecular techniques were applied. By considering all 104 *O. oeni* isolates, a RAPD analysis discriminated 62 genotypic profiles defined at a minimum similarity level of 96.6%, the lowest value determined in the reproducibility study. Nineteen genomic groups comprised two isolates or more, while the remaining 43 consisted of a single isolate. The cophenetic correlation was 0.74. The VNTR analysis grouped the 104 *O. oeni* isolates into 36 different genotypic profiles (15 contained two isolates or more, and 21 included only a single isolate; data not shown). The reproducibility of the VNTR patterns was 95.5% ± 2.3% and the cophenetic correlation was 0.83. When the results of the RAPD and VNTR fingerprints were combined to build a composite dendrogram, 88 GT profiles were defined at a cut-off similarity value of 97.5%. Ten profiles consisted of two isolates or more, and the remaining 78 profiles contained a single isolate (Supplementary Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.syapm.2016.11.003). The cophenetic correlation was 0.76. Hence the 104 *O. oeni* isolates constituted 88 genetically different strains.

When the isolates from must and wine were considered in two separate groups, the RAPD analysis discriminated 29 and 42 genotypic profiles in the must and wine groups, respectively (at a similarity level of 96.6%), whereas the VNTR analysis respectively discriminated 24 and 15 genotypic profiles (at a similarity level of 95.7%) in the same groups (data not shown). The composite dendrograms from the RAPD and VNTR fingerprints showed 37 GM profiles in the group of 44 grape must isolates, and 53 GW profiles in the 60 isolates of the wine group (Supplementary Figs. S2 and S3 in the online version at DOI: http://dx.doi.org/10.1016/j.syapm.2016.11.003, respectively). No clear separation of the wine and must isolates fingerprints was observed. Although 50% of the grape must isolates clustered together in groups A, B and C (Supplementary Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.syapm.2016.11.003), a few wine isolates were also placed in these groups. They did not separate at any cut-off level.

Combined analysis of the metabolic and genotypic characteristics (polyphasic analysis)

After taking into account the combined RAPD-VNTR fingerprints and the CH profiles of the 104 isolates, 94 PT profiles were defined. Seven different profiles were represented by two isolates or more (PT37, PT39, PT65, PT70, PT74, PT78 and PT87), and 88 were single members (Fig. 1). The calculated global cophenetic correlation value was 0.78. Profiles PT37 and PT39 comprised four and two isolates, respectively, all with the same origin. Profile PT74 contained three isolates (120, 128 and 130) and profiles PT65, PT70, PT78 and PT87 consisted of two isolates each. None of the isolates that grouped in these clusters came from the same wine, except for the couple 223A and 224. No relationship was found between the clustering in Fig. 1 and the geographical origin of the isolates (Table 1), except for isolates 223A and 224, which came from the same winery and wine.

When considering the two groups of isolates separately (from grape must and wine), the polyphasic analysis showed that for the 98.2% similarity value, the grape must isolates clustered in 40 PM profiles, of which profiles PM19 and PM21 were represented by two isolates or more, and the remaining 38 were single members (Fig. 2). At the same similarity level value, the wine isolates grouped in 54 combined PW profiles. Five different profiles (PW18, PW21, PW24, PW36 and PW50) were represented by two isolates or more, and 49 were single members (Fig. 3). The cophenetic correlation values for these two analyses were 0.78 and 0.80, respectively.

Diversity analysis by different characterisation techniques

The *DI* values were deduced to estimate the metabolic, genotypic and global *DI* of all the isolates, or of those that pertained to the separately analysed grape must and wine. The *DI* values were deduced from Table 3, Figs. 1–3, and Supplementary Figs. S1–S3 in the online version at DOI: http://dx.doi.org/10.1016/j.syapm.2016.11.003, and also from the dendrograms built with the RAPD and VNTR fingerprints separately (data not shown). The calculated *DI* are described in Table 4, where we can see that higher metabolic diversity was found mainly in the group of the grape must isolates (0.903), whereas genotypic diversity ($DI_{\text{RAPD-VNTR}}$) was higher in the wine isolates (0.995). When considering the genotypic data, the *DI* obtained from RAPD clustering were always higher than those obtained from the VNTR fingerprint. The RAPD analysis showed higher diversity in the wine group than in that of the grape must isolates. However, the contrary occurred with the VNTR analysis. The combined genotypic analysis solved this apparent inconsistency and reasserted the existence of a higher *DI* in the group of wine isolates.

The polyphasic analysis (metabolic and genomic data) gave higher *DI* values than the metabolic or genotypic analyses for all 104 isolates (DI_{PT}) and the grape must group, but were slightly lower for the group of wine isolates. This was the result of the low DI_{VNTR} of the latter group, which instead diminished increased diversity. For this reason, the polyphasic analysis provided a more accurate vision of real diversity.

Discussion

O. oeni belongs to a heterogeneous group from a metabolic point of view, as evidenced by Davis et al. [17], Pardo et al. [43], Lafon-Lafourcade et al. [34], Edwards et al. [19], Cappello [13] and Bravo-Ferrada et al. [5].

When we compared our data with those obtained by other authors, we found that all our isolates fermented ribose. Davis et al. [17] found that only 55% of the tested isolates were able to ferment this sugar. The genes that codify the conversion of ribose-5P into ribulose-5P are all present in the isolates used to build the *O. oeni* pan-genome [57], which could explain the universal use of ribose by *O. oeni*. The percentage of *O. oeni* isolates that fermented both glucose and fructose was similar to that reported by Lafon-Lafourcade et al. [34], whereas, Davis et al. [17] indicated that 100% of the isolates of *O. oeni* were able to metabolise them. The inability of some strains of this species to ferment glucose is related to NADH/NADPH re-oxidation problems, as formerly described by Maicas et al. [41]. If glucose was the only carbon source present, its catabolism would not assure the proper re-oxidation of these cofactors and glycolysis would be blocked. However, if a glucose-fructose mixture was used, fructose would be reduced to mannitol and this reaction would permit the re-oxidation of NADH/NADPH. Hence glucose would be metabolised quite easily [41]. The different

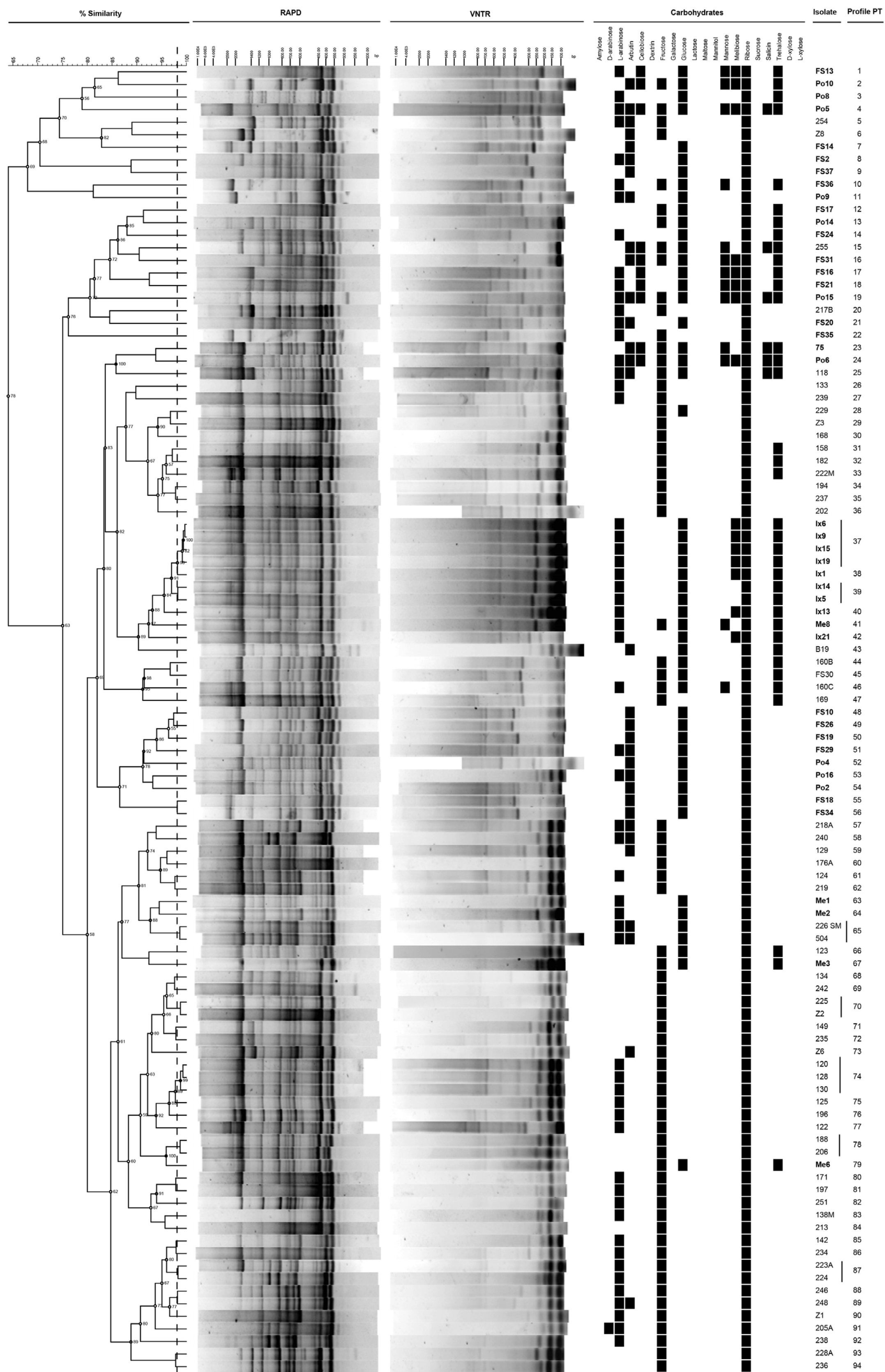

Fig. 1. Dendrogram derived from comparison of all techniques combined (RAPD, VNTR and carbohydrate fermentation profiles) of 104 *O. oeni* isolates. Clustering is based on the UPGMA method, using Pearson coefficient of correlation with 1% optimization and 2.5% of tolerance values. The dashed line indicates the cut-off level (98.2%) at which isolates are or not grouped in the same PT profile. Names in bold type indicate isolates from grape must and in normal type from wine. ■ Positive reaction on CH fermentation.

Table 4
Diversity indices calculated from the totality of isolates and from grape must and wine isolates separately.

Characteristics considered	Grape must isolates	Wine isolates	Total isolates
Metabolic (DI_{CH})	0.903	0.785	0.904
RAPD fingerprints (DI_{RAPD})	0.962	0.985	0.980
VNTR fingerprints (DI_{VNTR})	0.926	0.749	0.900
Combined fingerprints ($DI_{RAPD-VNTR}$)	0.985	0.995	0.995
Polyphasic analysis (DI_{P})	0.993	0.993	0.997

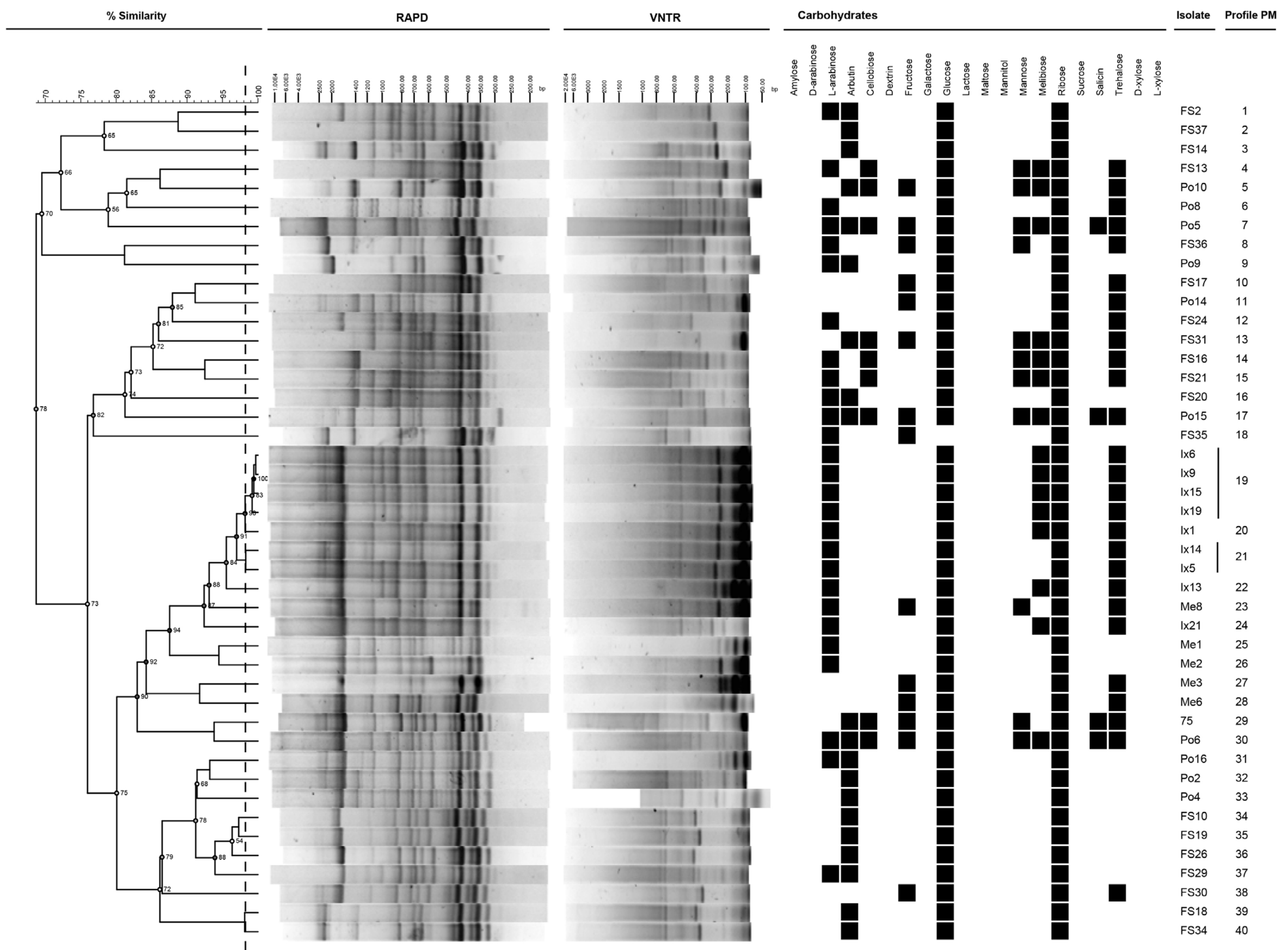

Fig. 2. Dendrogram derived from comparison of all techniques combined (RAPD, VNTR and carbohydrate fermentation profiles) of forty-four *O. oeni* isolates from grape must. Clustering is based on the UPGMA method, using Pearson coefficient of correlation with 1% optimization and 2.5% of tolerance values. The dashed line indicates the cut-off level (98.2%) at which isolates are considered or not grouped in the same PM profile. ■ Positive reaction on CH fermentation.

behaviour displayed by the *O. oeni* strains to glucose fermentation can be explained by the fact that strains differ in lactate dehydrogenase effectiveness terms or in the alternative re-oxidation systems of these cofactors [20,61]. According to our data, L-arabinose was fermented by 48% of the wine isolates. Sternes and Borneman [57] found that the three enzymes needed to catabolise L-arabinose (L-arabinose isomerase, EC 5.3.1.4, L-ribulokinase EC.7.1.16 and ribulose-phosphate 4-epimerase EC 5.1.3.1) were present in the core-genome assembly, which indicates that they were present in at least 75% of the strains. The lower percentage of our isolates that efficiently ferment this pentose could be the result of sporadic mutations that lead to non-functional enzymes, which prevent the catabolism of this sugar. The ability to ferment pentoses was a criterion adopted by Peynaud and Domerq [44] to differentiate two species of *Leuconostoc* found in wine (*Leuconostoc gracile* unable to ferment pentoses and *Leuconostoc oenos* (syn. *O. oeni*) able to ferment L-arabinose, L-xylose, or both). Nonetheless, this proposal was not considered to be sufficiently relevant to separate *L. oenos* into two different species [58].

The fermentation of arbutin, cellobiose, mannose, salicin and trehalose was a variable character among our isolates, unlike that observed by Davis et al. [17], who reported that the ability to ferment these carbohydrates was a general trait of their isolates. These authors, and also Bravo-Ferrada et al. [5], determined that several of their isolates fermented sucrose and D-arabinose. In our case, no isolate metabolised sucrose, and only one catabolised D-arabinose. None of our isolates was able to ferment melibiose, mannitol, D- and L-xylose. Davis et al. [17] found isolates capable of using these carbohydrates. As deduced from the CH fermentation profiles, the metabolic diversity among the isolates that came from different

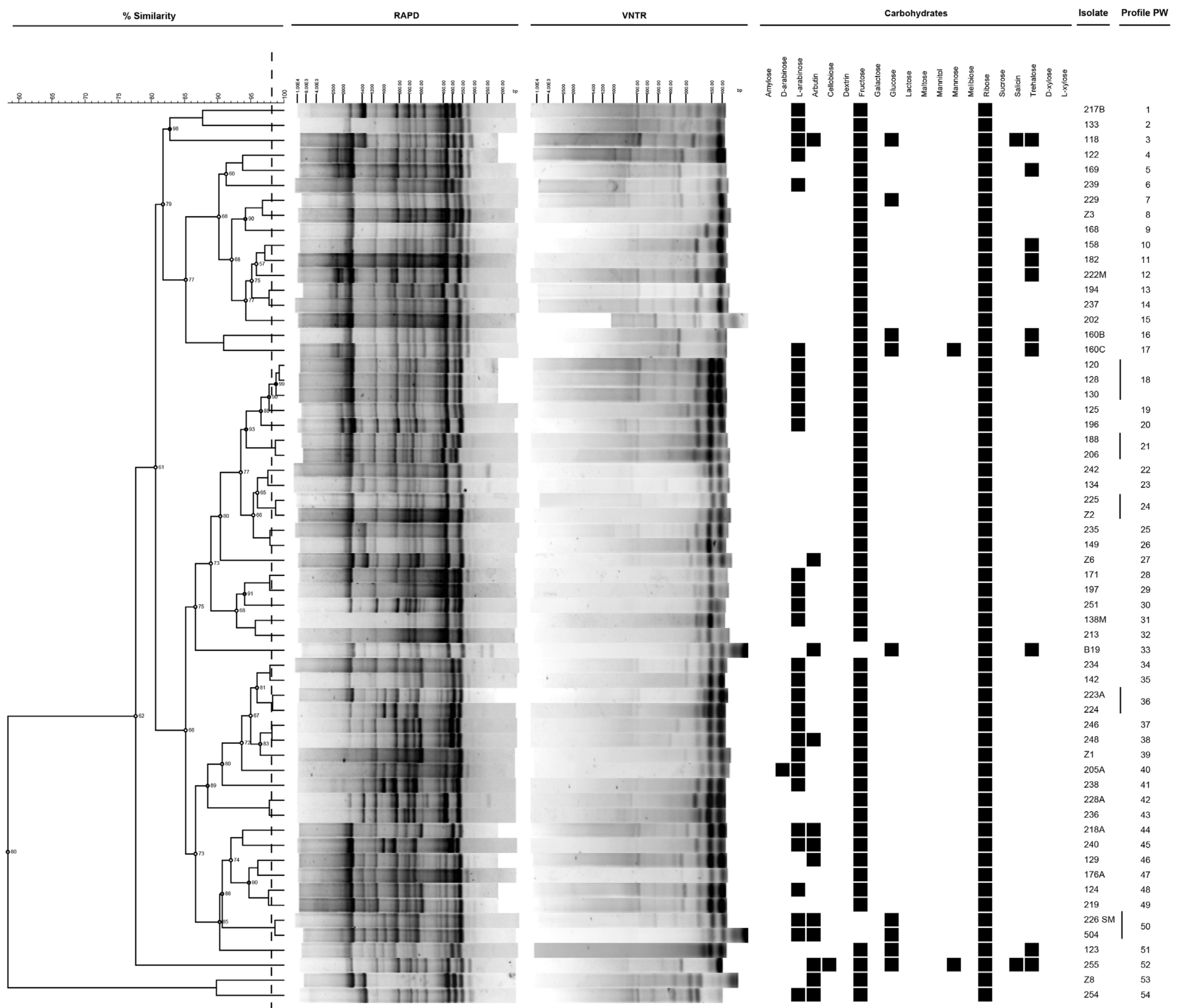

Fig. 3. Dendrogram derived from comparison of all techniques combined (RAPD, VNTR and carbohydrate fermentation profiles) of sixty *O. oeni* isolates from wine. Clustering is based on the UPGMA method, the coefficient of correlation corresponding to each characterization technique, with 1% optimization and 2.5% of tolerance values. The dashed line indicates the cut-off level (98.2%) at which isolates are considered or not grouped in the same PW profile. ■ Positive reaction on CH fermentation.

geographical regions and wines was vast. In any case, we need to be cautious when making comparisons because studies of metabolic abilities were not performed using the same preculture and culture media, nor incubation conditions, then the results could vary, as previously demonstrated by Jensen and Edwards [32] and by Pardo et al. [43]. Currently, knowledge about the transport of sugars in *O. oeni* is scarce. Neither the biochemistry nor the energetics of sugar transport has been characterised, and no experimental correlation of sugar transport to specific carriers of genes exists [65]. This fact avoids having to compare if the differences in sugar fermentation between strains are related to transport genes or to fermentative pathways genes. Several sugar catabolism-related genes have been characterised in the *O. oeni* genome. Although the genes of the catabolic pathways of hexoses are well-identified, the genes of the main pathways of pentose catabolism are not. Recently, Sternes and Borneman [57] proposed that D-Xylose could be fermented by the pentose phosphate pathway, which they described for the first time in *O. oeni*.

Some metabolic differences between isolates from grape must and wine have been observed. Grape must isolates are able to ferment more carbohydrates than wine isolates. This could be because the habitat where they come from contains more carbohydrates, mainly sugars, than wine. Sugars are present in wine in very low concentrations (generally less than 2 g/L) [7] and the remaining ones are the less preferred by *Saccharomyces cerevisiae*, e.g., pentoses and fructose. *S. cerevisiae* is unable to ferment pentoses [50] and, although this yeast can ferment fructose, this sugar is metabolised more slowly than glucose [53]. Campbell-Sills et al. [8] suggested that ancestral *O. oeni* strains were adapted to low ethanol-containing environments, such as overripe fruits or fruit juices, and that they are domesticated to cider and wine that contained a few sugars and high alcohol. Perhaps grape must isolates are representative of the more ancient *O. oeni* specimens. The fact that the phylogenetic study of Campbell-Sills et al. [8] did not include strains from unfermented juices avoids having to check this hypothesis.

RAPD and VNTR techniques have been used by several authors for different aims: to estimate the diversity of *O. oeni* strains [1,10,12,22,31,36,39,40,42,46,47,56], to discover if any biogeographical relationships exist between strains and place of provenance [4,6,35,42,45], to study the evolution of *O. oeni* populations during winemaking, and to determine if the inoculated *O. oeni* conducts the MLF [30,39,40,55].

We used these two techniques to characterise the *O. oeni* isolates from quite separate wine fermentation stages, to estimate if there were genotypic patterns that supported their separation into two groups (grape must or wine isolates), and to quantify the diversity of grape must and wine isolates, considered both separately and jointly. Studies into genotypic variability and *DI* vary in terms of not only the number and types of geographical origin of the samples, vintages, types and number of primers used, but also the number of fingerprints used in the analysis. Hence it is difficult to make comparisons. Accordingly, Bartowsky et al. [1] analysed genomic variability in a set of 17 *O. Oeni* isolates by using four different RAPD primers. These authors found that variability depended on the RAPD primer used, and they described that the most marked discrimination was achieved by analysing all the patterns (fingerprints) obtained with the different primers. Similar results were obtained by Zapparoli et al. [63] after they analysed 60 isolates, and high genomic diversity was reported in both cases. Nevertheless, Reguant and Bordons [46], Capozzi et al. [10], Solieri et al. [56] and Marques et al. [42] were unable to demonstrate that high diversity existed in the set of isolates that they analysed with M13 [42,56] and primers Coc and On2 [10,46]. In the present work, we found higher genomic diversity in our *O. oeni* group (0.980) than those found by other authors, who used primer M13 to type *O. oeni*.

Like Claisse and Lonvaud-Funel [15], we also followed the VNTR technique to genetically characterise the *O. oeni* isolates, but we did not obtain the same results despite employing the same pairs of primers (TR1–TR5). They reported *DI* values of 0.994 [15], while our *DI* value was much lower (0.900). One explanation for this disagreement could lie in the fact that the above-cited authors obtained five different VNTR fingerprintings per strain, and each resulted from the amplification with five different pairs of primers, whereas we had only one single fingerprinting, which resulted from combining the five VNTR primers in a single PCR reaction. The first strategy provided much more variability that the second one. Another difference to the analysis performed by Claisse and Lonvaud-Funel [15] was that they analysed number of repeats and number of alleles for the five tandem repeats (TR1–TR5) by capillary electrophoresis, whereas we used densitometric curves, as previously described. However, Garofalo et al. [22], who used the same original methodology as Claisse and Lonvaud-Funel [15], did not find as high a discriminating power of VNTR as the French researchers did: from 50 isolates, they were able to discriminate only 27 VNTR profiles. Claisse and Lonvaud-Funel [16] also attempted to simplify the technique in a later paper, and they performed only two multiplex PCR reactions instead of five single PCRs using combinations of the original VNTR 1–5 primers.

In order to analyse intraspecific diversity in *O. oeni*, some researchers [22,25,54] have combined the data they obtained from two different molecular typing techniques. Ruiz et al. [54] and González-Arenzana et al. [25] noticed that intraspecific diversity increased if the RAPD and PFGE fingerprints were analysed together instead of separately. Our result agrees with their findings.

Others authors have combined not only genetic typing techniques, but also phenotypic ones in a polyphasic approach to explore *O. oeni* diversity. Guerrini et al. [26] grouped 84 *O. oeni* isolates into eight profiles by combining phenotypic and genotypic data. Compared to their results, a much wider variability was found in this work as 94 groups were detected from 104 *O. Oeni* isolates after combining CH fermentation profiles and genomic fingerprints.

Notwithstanding, the use of molecular fingerprints is in many cases was considered enough to discriminate *O. oeni* strains [48,49] and to quantify diversity. However, our work demonstrated that the isolates which shared the same genomic profiles could have different fermentative profiles, and *vice versa.* Consequently, the polyphasic approach provided higher diversity.

Conclusions

Our results demonstrated that the *O. Oeni* isolates from grape must fermented more carbohydrates and were metabolically more diverse than the isolates isolated from wine. On the contrary, higher genomic diversity was found in the group of wine isolates. The RAPD analysis gave higher *DI* values than VNTR, but the highest *DI* values were obtained when combining the RAPD and VNTR profiles. The combination of metabolic and genomic data (polyphasic approach) gave the highest diversity values. The diversity found in each *O. oeni* group was similar and slightly lower than that of the set with all the strains. Our results demonstrate a high metabolic and genetic intraspecific diversity in *O. oeni*.

Acknowledgments

We acknowledge the Generalitat Valenciana, European Regional Development Funds (ERDF), and the Spanish Ministry of Science and Innovation for supporting this research. The Generalitat Valenciana awarded a “Santiago Grisolia” grant to Liz Erika Cruz-Pio to do her PhD thesis. ERDF and the Spanish Ministry of Science and Innovation partially financed this work through Project RM2010-00001-00-00.

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