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# Genomic diversity of *Oenococcus oeni* from different winemaking regions of Portugal

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**Summary.** *Oenococcus oeni* is an alcohol-tolerant, acidophilic lactic acid bacterium that plays an important role in the elaboration of wine. It is often added as a starter culture to carry out malolactic conversion. Given the economic importance of this reaction, the taxonomic structure of this species has been studied in detail. In the present work, phenotypic and molecular approaches were used to identify 121 lactic acid bacteria strains isolated from the wines of three winemaking regions of Portugal. The strains were differentiated at the genomic level by M13-PCR fingerprinting. Twenty-seven genomic clusters represented by two or more isolates and 21 single-member clusters, based on an 85% similarity level, were recognized by hierarchic numerical analysis. M13-PCR fingerprinting patterns revealed a high level of intraspecific genomic diversity in *O. oeni*. Moreover, this diversity could be partitioned according to the geographical origin of the isolates. Thus, M13-PCR fingerprint analysis may be an appropriate methodology to study the *O. oeni* ecology of wine during malolactic fermentation as well as to trace new malolactic starter cultures and evaluate their dominance over the native microbiota. [**Int Microbiol** 2011; 14(3):155-162]

**Keywords:** *Oenococcus oeni* · lactic acid bacteria (LAB) · Portuguese winemaking regions · genomic diversity · M13-PCR · fingerprinting

## Introduction

In the mid 1960s, Ellen Garvie [13] isolated, characterized, and named *Leuconostoc oenos* as the bacterial agent of malolactic fermentation (MLF). This species is a Gram-positive, catalase negative, microaerophilic and heterofermentative coccus [14]. With the introduction of molecular techniques, however, a new genus, *Oenococcus*, was described, and *Leuconostoc oenos* was reclassified as *Oenococcus oeni* [10]. Due to its resistance to high ethanol concentrations (<15% v/v) and tolerance of low pH (as low as 2.9), *Oenococcus oeni* is the species of lactic acid bacteria (LAB) most frequently associated with MLF in wine. In this reaction, L-malate is converted to L-lactate and carbon dioxide. MLF promotes the deacidification and microbial stability of wines [16,20,27,40]. However, it can either positively or negatively influence the sensorial profiles of wines, with the overall effects largely dependent on the particular strain involved and on the type of wine being produced [4].

In the last 20 years, molecular biology techniques have provided new information on microbial biodiversity. Yet, it is

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difficult to identify strains within species, especially when microorganisms belonging to a genomically homogeneous species are analyzed. Strains belonging to *O. oeni* are clearly distinguishable from *Leuconostoc* species by chromosomal DNA-DNA hybridization [9,25,26,36,42], 16S and 23S rRNA sequence analysis [11,32,33,36,42], 16S-23S rDNA intergenic spacer region sequencing (ITS-PCR) [23,52] and ribotyping [6,45,46,50,53]. Several studies on genotyping diversity among *O. oeni* strains, carried out using molecular techniques including DNA fingerprinting, restriction endonucleases analysis–pulsed field gel electrophoresis (REA-PFGE) [19,21,22,28,29,36,41,42,45,50], randomly amplified polymorphic DNA-PCR (RAPD-PCR) [2,6,22,24,36–38, 41,50], and differential display PCR (DD-PCR) [22,36,41] suggest that this species is phylogenetically homogeneous, although physiologically diverse. Delaherche et al. [8], based on sequence analyses of nine genes, claimed that *O. oeni* is a single bacterial species displaying genomic variation, which may be correlated to malolactic activity. However, recent studies [39] using multilocus sequence typing (MLST) and physiological characterization have again raised the hypothesis of subspecific divisions within this taxon. Given the taxonomic structure of *O. oeni*, the availability of reliable methods for strain differentiation is crucial for monitoring the survival and contribution of inoculated and autochthonous bacteria and to select individual *O. oeni* strains with desirable organoleptic properties. Since the wine dynamics of *O.oeni* populations are also conditioned [37,38] by the available species and strain diversity (from spontaneous and controlled inoculation) as well as the winemaking conditions (e.g. temperature, wine chemical profile), the identification and typing of MLF-promoting isolates is a reliable approach to assess their ability to dominate the native microbiota and to correlate their dominance/performance with distinct winemaking conditions.

In the present work, 121 *O. oeni* strains were isolated from wines of different winemaking regions of Portugal and identified using a phenotypic and molecular approach. M13-PCR fingerprinting analysis was carried out to evaluate the genetic diversity of this collection of *O. oeni* strains and to search for underlying patterns of regional/geographical strain diversity.

## Materials and methods

**Bacterial strains.** The 121 bacterial isolates of *Oenococcus oeni* used in this work are listed in Table 1. Among them, 100 were isolated from wines, at the end of spontaneous MLF, recovered from four wineries of Dão (Carregal do Sal, Viseu, Mangualde and Mealhada), two wineries of Ribatejo (Dois Portos and Arruda dos Vinhos) and one winery of Alentejo (Reguengos). Additionally, 20 *O. oeni* isolates from Nelas (Dão) and one *O. oeni* isolate from Ourém (Ribatejo), previously isolated and identified [PhD thesis, R. Tenreiro, Univeristy of Lisbon, 1995], were obtained from the

**Table 1.** *Oenococcus oeni* strains used in this study

| Region/Sub-region wine | | *Oenococcus oeni* strains |
|---|---|---|
| Dão | Nelas | bOg18, bOg20, bOg22, bOg23, bOg27, bOg29, bOg30, bOg31, bOg32, bOg33, bOg34, bOg35, bOg36, bOg39, bOg40, bOg41, bOg42, bOg43, bOg44, bOg45 |
| | Carregal do Sal | DS5 |
| | Silgueiros | ID4, ID5 |
| | Mangualde | ID6, ID38, ID39, ID40, ID42, ID43, ID44, ID45, ID46, ID47, ID48, ID53, ID55, ID56, ID57, ID58, ID62, ID65, ID70 |
| | Mealhada | ID41 |
| Ribatejo | Dois Portos | EVN1, EVN2, ENV7, E169, IO1, IO2, IO24, IO25, IO27, IO30, IO58, IO59, IO60, IO61, IO62, IO63, IO64, IO66, IO75, Agro1, Agro2, Agro3, Agro4, Agro5, Agro6, Agro7, Agro8, Agro9, Agro10, EVN19, EVN22, EVN26 |
| | Ourém | bOg38 |
| | Arruda dos Vinhos | IER1, IER2, IER3 |
| Alentejo | Reguengos | IAL7, IAL8, IAL9, IAL10, IAL11, IAL12, IAL13, IAL14, IAL15, IAL16, IAL17, IAL18, IAL19, IAL20, IAL21, IAL22, IAL23, IAL24, IAL25, IAL26, IAL27, IAL28, IAL29, IAL30, IAL31, IAL33, IAL34, IAL35, IAL36, IAL37, IAL49, IAL50, IAL51, IAL52, IAL54, IAL59, IAL60, IAL61, IAL63, IAL64, IAL66, IAL71 |

*Oenococcus oeni* culture collection of the Center of Biodiversity, Functional and Integrative Genomics (BioFIG/FCUL, Lisboa, Portugal). In this study, the type strain *O. oeni* DSMZ 20252$^T$ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was also included as were seven commercial malolactic starters (Viniflora oenos and Viniflora CH35 from Christian Hansen, Hørsholm, Denmark; GM from Microlife Technics, Sarasota, Florida, USA; Alpha, Beta and VP41 from Proenol, Vila Nova de Gaia, Portugal; PSU-1 from Pennsylvania University, Philadelphia, USA).

**Bacteria isolation.** The bacteria were isolated by spreading 100 ml of wine samples onto plates with medium promoting the growth of *Leuconostoc oenos* [5] (MLO, tryptone 1%, yeast extract 0.5%, glucose 1%, fructose 0.5%, magnesium sulfate 0.02%, manganese sulphate 0.005%, ammonium citrate 0.35%, Tween 80 0.1%, tomato juice 10% and cysteine 0.05%), adjusted to pH 4.8. Cycloheximide (100 mg /l, Sigma-Aldrich, St. Louis, USA) was added to inhibit the growth of yeasts and molds. The plates were incubated anoxically inside jars containing an Anaerocult C system (Merck, Darmstadt, Germany) at 30ºC for 12 days. Colonies were then selected and further isolated as pure cultures by repeated streaking onto plates containing MTJ medium (70% MRS medium, Merck, Darmstadt, Germany; 30% tomato juice broth, Difco & BD, Franklin Lakes, NJ USA). Bacterial strains were maintained as frozen stocks at –80ºC in MTJ broth media and 20% (v/v) glycerol as cryoprotective agent. Working cultures were cultivated at 30ºC in MTJ broth, until stationary phase. Purity was checked by plating on corresponding agar media and microscopic examination.

**Identification of the bacterial strains.** Bacterial isolates were first selected on the basis of their genus-specific *Oenococcus* characteristics. Catalase-negative and Gram-positive cocci were screened for the release of $CO_2$ from glucose based on the production of gas in inverted Durham tubes containing MRS broth [15]. Since this property is shared by other LAB genera, the API 50 CHL system (bioMérieux, Craponne, France) was also used for species identification, according to manufacturer's instructions.

For DNA isolation, the strains were grown in MTJ broth until stationary phase at 30ºC. Cells were recovered by centrifugation and total DNA was obtained using an UltraClean Microbial DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA). The DNA concentration was determined spectrophotometrically at 260 nm. Ethidium bromide staining was used to visualize the DNA after electrophoresis through a 1% (w/v) agarose gel (Seakem, Cambrex Bio Science, Rockland Maine, USA). Molecular identification of *O. oeni* strains was performed by 16S rRNA gene amplification and restriction analysis with the enzyme *Fse*I as described by Marques et al. [31]. The results were confirmed by partial sequencing of the 16S rRNA genes of several randomly selected isolates and of the type strain *O. oeni* DSMZ20252$^T$. 16S rDNA was amplified with the universal primers pA and pH [46] and the amplified fragments were purified using a Concert Rapid PCR purification system (Gibco BRL, Carlsbad, CA, USA). The sequencing reactions were performed using the internal primer 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) at the MWG Biotech sequencing service (Ebersberg, Germany). The BLAST algorithm was used to compare the sequences with those of the U.S National Center for Biotechnology Information GenBank entries [1], and an identification at species level was assumed when at least 97% homology with the 16S rDNA sequence of a known species was determined [43].

**M13-PCR fingerprinting.** Genomic DNA from all *O. oeni* strains was used as template for PCR fingerprinting using as a primer the M13 minisatellite core sequence (csM13) [17] with the sequence 5′-GAGGGTGGCGGTTCT-3′. Approximately 50 ng of total DNA was subjected to PCR amplification in a reaction mixture containing 1× PCR buffer, 2.5 mM $MgCl_2$, 200 mM of each deoxyribonucleotide (Invitrogen, Carlsbad, CA, USA), 50 pmol of primer (Invitrogen), and 1 U of Taq DNA polymerase (Invitrogen) in a final volume of 25 ml. The reaction mixtures were subjected to amplification in a thermocycler (Biometra, Goettingen, Germany). PCR cycling conditions consisted of: 94ºC for 5 min followed by 40 cycles of 94ºC for 1 min, 40ºC for 2 min and 72ºC for 2 min, plus one additional cycle at 72ºC for 7 min for chain elongation. PCR profiles were resolved by agarose (1.2% w/v) gel electrophoresis in 0.5× TBE buffer (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA; Invitrogen), at 90 V for 3 h. DNA was visualized under UV light after ethidium bromide staining and the results photographed with Kodak 1D software (Kodak, USA).

**Data analysis.** The images of the gels were captured using the Kodak electrophoresis documentation software 1D. The images were then saved as TIFF files and exported into the pattern analysis software package BioNumerics version 4.61 (Applied Maths, Kortrijk, Belgium) for processing. To obtain a measure of reproducibility, 12 isolates were randomly selected and analyzed in duplicate. The similarity between each duplicate pair was determined from an analysis based on a dendrogram computed with the Pearson correlation coefficient and the unweighted pair group method with arithmetic average (UPGMA) as the agglomerative clustering [47]. The reproducibility value was determined as the average value for all pairs of duplicates. Strain relationships, based on the molecular characters as determined from the fingerprints, were analyzed by hierarchical numerical methods with Pearson correlation similarity and UPGMA clustering. A cut-off value of 85% similarity was used to distinguish the clusters. The intra-regional genomic diversity of *O. oeni* was evaluated with the indexes of Simpson [18] and Shannon [51]. The Simpson index (*D*) measures the probability of two non-related strains, taken from the tested population, belonging to two different genomic types and is based on the number of types and isolates for each type. The Shannon index (*J'*) is an evenness measure, expressing the observed diversity as the proportion of the possible maximum diversity and reflecting the homogeneity/heterogeneity of the distribution of isolates among the genomic types.

## Results and Discussion

**Isolation and identification of the strains.** From 81 wines (23 Dão wines, 24 Ribatejo wines and 34 Alentejo wines), a culture collection of 100 bacterial isolates (23 from Dão, 35 from Ribatejo and 42 from Alentejo) was obtained. A primary classification was performed based on cell morphology and cellular arrangement, Gram staining, catalase activity, and $CO_2$ production from glucose. All isolates were Gram-positive, catalase negative, had similar cell arrangements (single, pairs and long chains), and were heterofermentative. The isolates showed the same fermentation pattern in API 50 CHL galleries, producing acid only from arabinose, esculin, fructose, galactose, glucose, and xylose. As six non-matching tests with the most closely related taxon (*Lactobacillus brevis*) were obtained, no acceptable phenotypic identification was possible using the API database. These results further reinforce the low reliability of this system as an identification tool for wine LAB, especially *O. oeni*, as described by others [34, and PhD thesis, R. Tenreiro 1995].

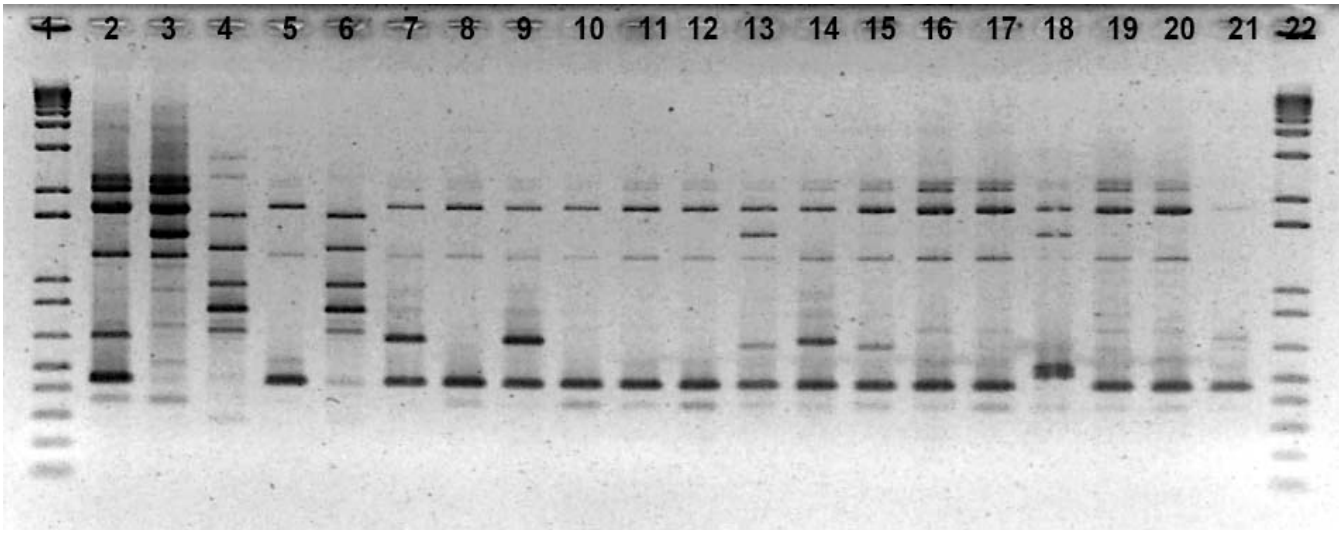


**Fig. 1.** Representative M13-PCR profiles of several strains of *Oenococcus oeni*. Lanes 1 and 22: molecular ladder 1 kb plus (Invitrogen); lanes 2–21: *O. oeni* isolates from wines of different winemaking regions in Portugal.

However, the assays used for the primary classification offer a practical screening strategy and allowed us to conclude that the bacterial isolates belonged to a group of heterofermentative cocci LAB.

The bacterial isolates were identified as *O. oeni* using the molecular methodology described by Marques et al. [31]. This method is based on 16S rRNA gene amplification with universal primers followed by restriction enzyme analysis with the endonuclease *Fse*I, generating two fragments of 326 and 1233 bp. These results were confirmed based on the partial 16S rDNA sequence of some isolates (10%), randomly selected, and that of the type strain *O. oeni* DSMZ20252$^T$. The DNA sequences were analyzed and compared using the BLAST network service (NCBI). The resulting fragments were approximately 98% similar to the 16S rRNA gene isolated from an *O. oeni* strain (GenBank accession number X95980), confirming that the isolated strains belonged to *O. oeni* species (data not shown).

**M13-PCR fingerprinting.** The intraspecific diversity of our culture collection of 121 *O. oeni* strains obtained from three winemaking regions throughout Portugal was evaluated by M13-PCR fingerprinting analysis. The primer csM13 provided suitable fingerprints, with well defined amplification patterns (Fig. 1).

The reproducibility of the fingerprints with primer csM13, estimated by the similarity average value for all pairs of duplicates, was 96 ± 0.4%. The DNA fingerprinting patterns were analyzed on BioNumerics software (v4.61, Applied Maths) and the genetic similarity between the 121 *O. oeni* strains was displayed in the form of a dendrogram, depicted in Fig. 2.

The cophenetic correlation coefficient was 0.93, which demonstrates the faithfulness of a dendrogram in preserving the pairwise distances between the original unmodeled data points. Although a value of 1.0 means that the concordance (as a linear relation) between the input data and the tree is theoretically perfect, in practice the relationship is unlikely to be totally linear. Romesburg [Cluster Analysis for Researchers. Wadsworth, Inc., USA, 1984] suggested that a cophenetic correlation of 0.8 or above indicates that the dendrogram does not greatly distort the original structure in the input data. However, the cophenetic correlation coefficient is not always a very reliable measure of the distortion due to a hierarchical model [12].

At a similarity level of 85%, the M13-PCR fingerprinting analysis organized the *O. oeni* strains in 49 genomic groups (27 different genomic clusters, represented by two or more isolates and 22 single-member genomic clusters). Six major genomic clusters (I–VI) were also defined, based on the overall hierarchical relationships, with distinctive composition in terms of the regional origin of the isolates. *O. oeni* strains from the Dão region were distributed into 19 genomic groups, including seven unique profiles as single-member clusters. Strains from the Ribatejo region were grouped in 22 genomic groups, with ten of them as single-member clusters, while those from the Alentejo region belonged to 14 genomic clusters, with five single-member clusters. Although nine out

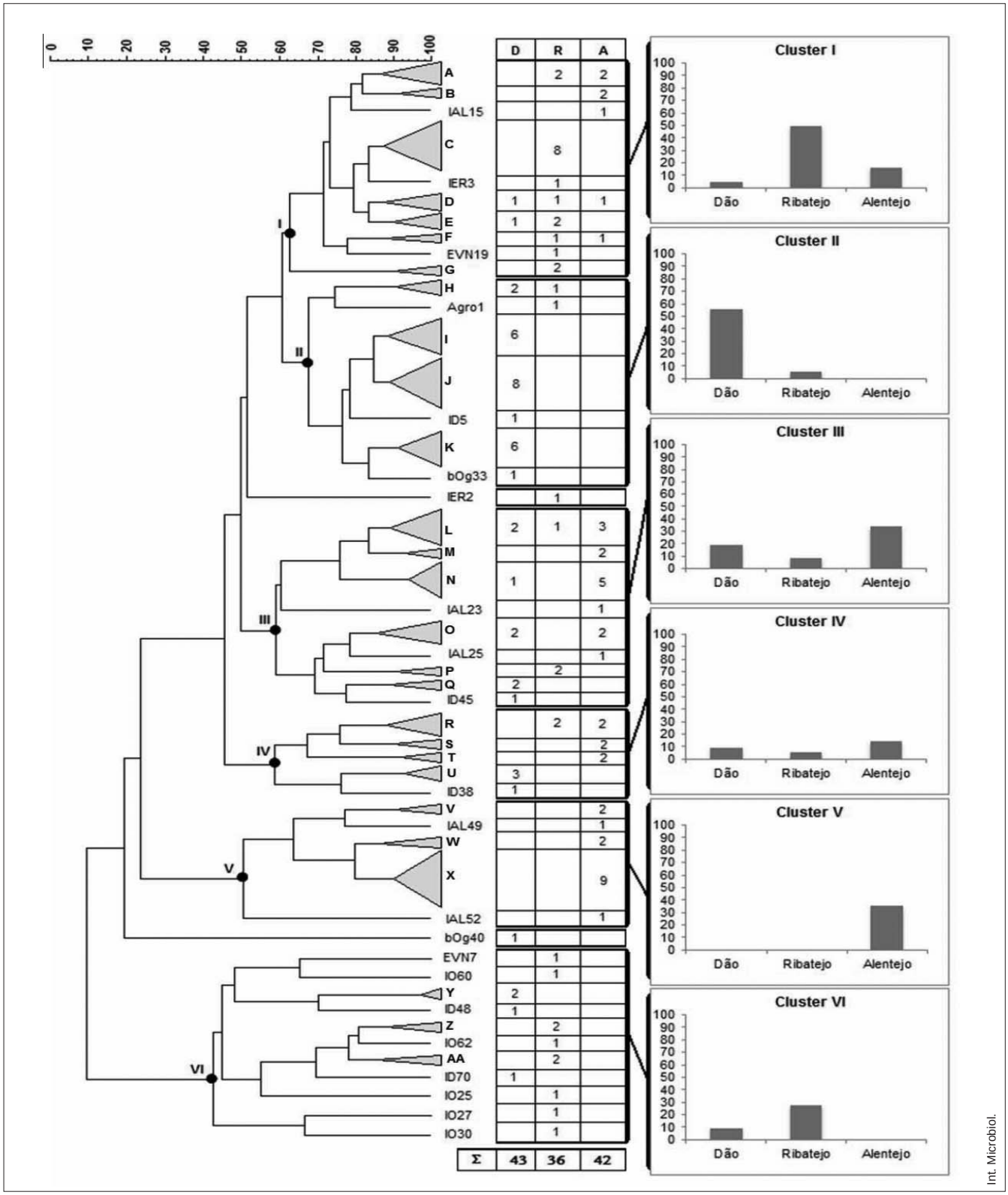


**Fig. 2.** Dendrogram of the 121 *Oenococcus oeni* isolates from different winemaking regions in Portugal based on the M13-PCR fingerprint analysis (Pearson correlation coefficient and UPGMA clustering). Alphabetic letters indicate the genomic groups of strains defined at an 85% similarity. The number of isolates from each region is displayed. (D: Dão; R: Ribatejo; A: Alentejo), as is the relative distribution of strains in each major cluster I-VI (as a percentage of the total number per region).

**Table 2.** *Oenococcus oeni* diversity indexes for the isolates in winemaking regions

| | | Diversity index* | |
|---|---|---|---|
| Winemaking region | % Types | *D* | *J'* |
| Dão | 47 (20/43) | 0.93 | 0.89 |
| Ribatejo | 50 (18/36) | 0.92 | 0.91 |
| Alentejo | 43 (18/42) | 0.93 | 0.92 |

**D*: Simpson diversity index; *J'*: Shannon diversity index; % Types: (number of types/number of isolates)×100, in each winemaking region.

of the 27 genomic clusters (A, D, E, F, H, L, N, O, and R) comprised a mixture of *O. oeni* isolates from more than one region (9 isolates from Dão, 10 from Ribatejo, and 16 from Alentejo), the remaining 18 genomic clusters were formed only by isolates from the same region (6 from Dão, 5 from Ribatejo, and 7 from Alentejo), pointing to a regional partitioning of the genomic diversity in this species. *O. oeni* isolates from the same wine were distributed by different clusters, which indicated the presence of different types of *O. oeni* strains in the same wine.

Seven commercial malolactic starters (VP41, Alpha, Beta, Viniflora oenos, Viniflora CH35, GM, and PSU-1) and the *O. oeni* type strain (DSMZ 20252$^T$) were also submitted to fingerprint analysis. For each starter, a unique and discriminative DNA fingerprint was obtained, with the exception of the starters Viniflora oenos and Viniflora CH35, which were grouped in the same genomic cluster (data not shown). Each of these commercial *O. oeni* strains has different winemaking origins.

Shannon-Weaver and Simpson diversity indexes were applied to assess the intra-regional genomic diversity of *O. oeni* strains from the different winemaking regions of Portugal (Table 2). Both the percentage of types and the values of the Simpson and Shannon-Weaver diversity indexes, obtained with M13-PCR fingerprinting, were closely similar and high enough for each winemaking region so as to confirm the high genomic diversity of *O. oeni*, as previously determined by MLST, macrorestriction, and physiological characterization [35,39,45].

**Evaluation of regional distribution of *Oenococcus oeni* genomic groups.** Among the 49 genomic groups defined by M13-PCR fingerprinting analysis (Fig. 2), 40 were unique to a particular winemaking region. Seven genomic groups (A, E, F, H, N, O, and R) were shared by two regions each, while the remaining two (D and L) were

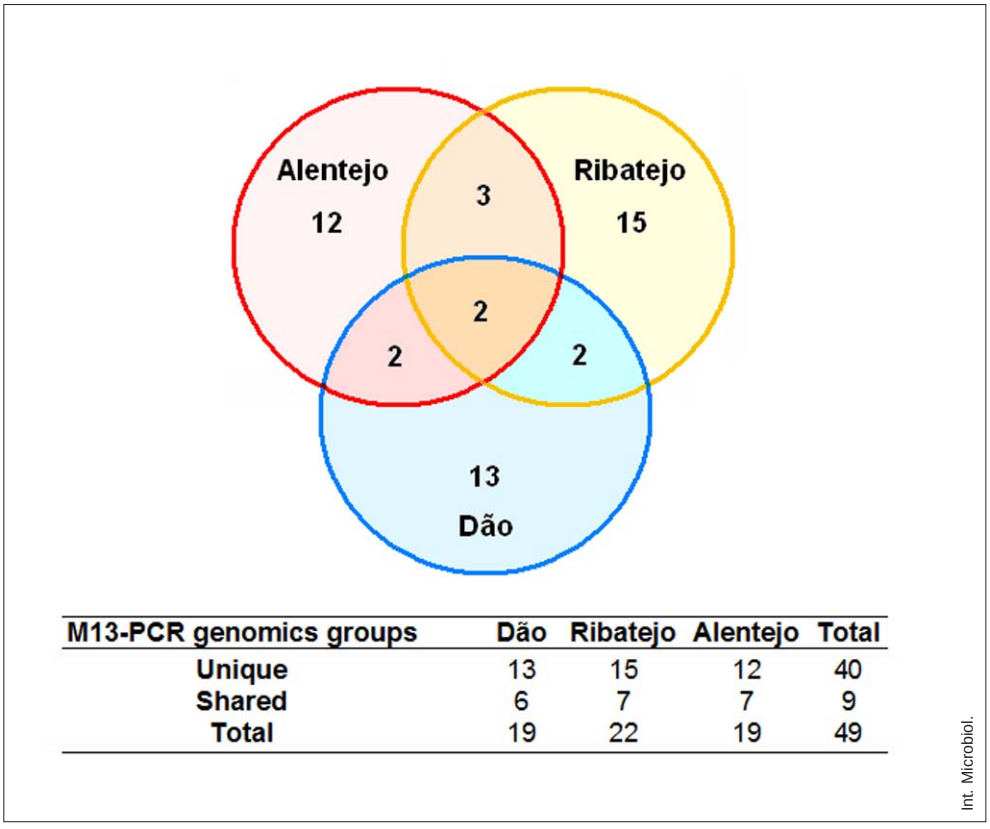


**Fig. 3.** Regional distribution of the 49 M13-PCR genomic groups of *Oenococcus oeni* isolates from Portuguese wines of different winemaking regions.

the only ones that included isolates from the three regions. When the uniqueness/commonness ratio of genomic groups was analyzed for each winemaking region (Fig. 3), a 2:1 proportion was found between specific genomic profiles unique to that region and genomic profiles shared with at least another region. This pattern of geographically associated diversity is also obvious from the composition of the six major genomic clusters (I–VI; Fig. 2) in terms of the regional origin of the isolates. Overall, these data point to a global partitioning of the genomic diversity of *O. oeni* according to the geographical origin of the isolates and to the occurrence either of an alopatric or ecological speciation process in this wine species. Similar conclusions have been reached in other bacterial groups subjected to highly selective or heterogeneous environments [49].

During the last several years, the diversity of *O. oeni* strains within and around wineries has been extensively examined. The results obtained from the application of different techniques, such as studies of the patterns of total soluble cell proteins [9], 16S and 23S sequence analyses [32], RAPD-PCR [53] and DD-PCR [22], suggest that *O. oeni* is a homogeneous species. More recently, de las Rivas et al. [7] submitted five genes (*gyr*B, *ddl*, *mle*A, *pgm*, and *rec*P) to MLST in order to evaluate the allelic diversity and population structures of various oenococcal isolates. This analysis was able to completely differentiate 18 strains, suggesting a higher level of genetic heterogeneity among oenococcal isolates. These authors argued that the high level of diversity in *O. oeni* is an example of a panmictic genetic population, in which the high frequency of recombination among constituents results in the randomization of sequences and the generation of linkage equilibrium. Marcobal et al. [30] showed that high mutation rates in *O. oeni* explain some of the discordant observations reported for this species. They suggested that the lack of *mut*S and *mut*L in *O. oeni*, combined with the high mutation rate, accounts for the high allelic diversity among strains, as seen from the MLST data.

In oenology, biodiversity is strictly correlated to habitat. Consequently, it is conditioned by selective factors that inhibit or favor the presence not only of one species over the other but also of a strain or biotype. The present study aimed to differentiate *O. oeni* isolates from different winemaking regions of Portugal and to reveal the underlying patterns of regional/geographical strain diversity. Our results confirm the predominance of *O. oeni* species in the hostile conditions prevailing in wine and the high adaptation capacity of the various strains in the winery environment [53]. M13-PCR fingerprinting allowed the genomic discrimination of *O. oeni* while a cluster analysis of M13-PCR patterns revealed a correlation between strain distribution and geographical area of origin. This approach can be useful in following the evolution of *O. oeni* populations during malolactic fermentation in wine and in assessments of the *O. oeni* ecology in wine.

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**Competing interests**. None declared.

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