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# Microsatellite typing of *Lachancea thermotolerans* for wine fermentation monitoring

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ABSTRACT

Climate change is causing a lack of acidity during winemaking and oenologists use several solutions to cope with such a problem. *Lachancea thermotolerans*, which has the potential to tolerate the harsh physicochemical conditions of wine, has emerged as a promising alternative for pH management during winemaking and, currently, it is the most valuable yeast used for acidity control in wine. In this work a manageable method for *L. thermotolerans* genotyping based on a multiplexed microsatellite amplification in 6 different loci was developed. The proposed method was used to distinguish between 103 collection strains obtained from different geographical and isolation sources, and then challenged against a 429 *L. thermotolerans* isolates from several wineries and harvests. The procedure was also tested for fermentation monitoring and strain implantation. This approach was conceived to simplify the methodology available for *L. thermotolerans* genotyping, making it easy for applying in wine-related laboratories. This method can be applied to distinguish between *L. thermotolerans* strains in selection programs and to follow implantation of inoculated strains during winemaking with optimal results.

## 1. Introduction

The new climatic conditions in the different wine-growing areas due to global climate change are altering the composition of the wine grape, triggering consequences in the process of wine production (Santillán et al., 2019). Rising temperatures bring changes in vine phenology causing modifications in the chemical composition and microbiological quality of the grapes that arrives at the winery. In this regard, the main grape changes are related to higher sugars concentration (implying higher probable alcoholic degrees) and a lower acidity. Microbial populations developed during the winemaking process and sensory quality of the wine are affected by these changes (De Orduña, 2010; Volschenk et al., 2006).

There are several tools for acidity control in wines based on acid or base addition or the use of different microorganisms (Vicente et al., 2022). There are some mechanisms to control the acidity of must and wine authorized by the International Wine Organization (OIV, 2012; Volschenk et al., 2006). Nevertheless, numerous studies show the interest of using selected yeast strains for wine pH regulation (Pacheco et al., 2012; Vicente et al., 2022). *Lachancea thermotolerans* stands out for its ability to acidify wine through the production of lactic acid (up to 9 g/L) without significant increments in acetic acid under oenological conditions (Porter et al., 2019; Vicente et al., 2022; Vilela, 2019). For that reason, it is one the most valuable yeasts used for acidity control, with some commercially available strains. Furthermore, *L. thermotolerans* improves wine aromatic complexity through the production of different aromas, *e.g.*, 2-phenylethanol (Vicente et al., 2021a).

For the isolation and selection of yeasts with these characteristics, the use of a simple, precise, and reproducible genotyping method is very convenient. Several attempts to study the *L. thermotolerans* intraspecific diversity have been accomplished. The first one described, analysed the mitochondrial DNA of *L. thermotolerans* using restriction analysis approaches. This study showed a high homology among this species, without influence of the geographic or niche origin of strains (Belloch et al., 1997). Later, applying NGS techniques, the highly conserved mitochondrial DNA structure was confirmed (Freel et al., 2014; Friedrich et al., 2012). Other approaches analyzing different microsatellites have been described to study the intraspecific diversity of this species, being valuable for phylo-ecological studies (Banilas et al., 2016;

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Hranilovic et al., 2017). Nevertheless, these techniques are hardly implementable in winery-related strain selection procedures. The fluorescent labelled-multiplexed SSR analysis followed by a capillary electrophoresis revealed the influence of the geographical source of isolation in the population architecture in strains coming from several vineyards, a fact that was not supported applying other typing techniques based on tandem-repeat tRNA (Banilas et al., 2016) but later confirmed by deeper studies (Hranilovic et al., 2017).

With the specific objective of developing a comprehensive tool to search new *L. thermotolerans*, in this work a genotyping procedure for this species has been developed. This method can be applied to distinguish between *L. thermotolerans* strains in selection programs and to follow implantation of inoculated strains during winemaking or dry yeast production procedures. New primers have been designed to select those that allow a good resolution using agarose gel electrophoresis. Here we have verified the technique against several collections to prove its specificity and sensibility, both in collection and natural isolates. We tested the usefulness of the technique by following-up the implantation of an *in vitro* co-culture of *L. thermotolerans* strains under different complexity levels.

## 2. Materials and methods

### 2.1. Yeast strains and molecular identification of isolates

The yeast strains used as controls in this study, and coming from different environments and substrates, are listed in Table S1. Briefly, 103 *Lachancea thermotolerans* strains that were provided from different laboratories, culture collections and yeast producing companies were employed for technique verification. The specificity was assayed using several strains from other *Lachancea* species as well as other yeast genera. As well, a collection of *L. thermotolerans* autochthonous isolates coming from different fermentative stages (must, sulphited must and wines at 1040 and 999 densities) and from several vineyards of different Spanish wine appellations (Ribera de Duero, Rioja, and Manzanilla – Sanlúcar de Barrameda) in two consecutive vintages (2020 and 2021) is listed in Table S2. These autochthonous yeasts cultures were isolated using lysine agar for the selective isolation of non-*Saccharomyces* yeast. Each isolated was re-streaked twice in lysine agar to obtain pure cultures and then cryopreserved in 25 % glycerol at −80 °C. For yeast propagation, YMA agar plates were used (0.5 % proteose peptone, 0.3 % yeast extract, 0.3 % malt extract, 1.0 % glucose, 1.5 % agar) and incubated at 28 °C. Total genomic DNA was purified using the isopropanol method as elsewhere described (Querol et al., 1992) and stored at −20 °C for further analysis. During the 2020 vintage, the taxonomic identification of the isolates was based in the 26S large subunit of rRNA gene sequencing using NL-1/NL-4 primers (Kurtzman and Robnett, 1998). Sequences obtained by Sanger sequencing were compared by BLAST-search and confirmed using the sequence of the type strain at the MYCOBANK database. During the 2021 vintage, as the technique had already been verified, we employed the proposed technique to simultaneously identify and genotype the *L. thermotolerans* isolates.

### 2.2. Microsatellites identification and primers design

For microsatellite identification and primer design, the complete genome (including mitochondrial DNA) of the type strain *L. thermotolerans* CBS 6340 was downloaded from NCBI (Accession number: ASM14280v1) and used as template. Tandem repeated sequences were identified using the Tandem Repeats Finder described by Benson (1999) and studied as possible microsatellites candidates. Different loci by chromosome were manually selected according to the repeat length (between 50 and 400 bp) and the number of repeats (between 2 and 80 repetitions). Loci with repetitions lower than 50 bp were discarded due to the resolution limit of agarose electrophoresis, while those with higher number of repeats were preferred as they allow a better discrimination. Primers were designed using Primer3 (https://primer3.ut.ee/) at the flanking regions of each microsatellite candidate. For specificity, primers were firstly tested using BLAST search (in standard databases) and then *in vitro* using several strains (see Primer verification and selection for microsatellites fingerprinting amplification section).

### 2.3. Primer verification and selection for microsatellites fingerprinting amplification

Ten strains, representing a wide diversity in terms of geographic origin of the isolates, of *L. thermotolerans* (10-1488, CBS 10520, CBS 2907, CECT 1951, CONCERTO, DBVPG 3418, DMKU-RK 361, PYCC 4135, PYCC 6986 and UWOPS 85-312.1) were used in a first selection stage for the analysis of the most variable microsatellites. To select the primers that allowed the maximum discrimination between strains individual PCRs for each primer pair were performed in triplicate in a final volume of 25 μL containing 100 ng of genomic DNA, 2 μM of each primer (Integrated DNA Technologies, USA) and DreamTaq Green DNA polymerase 2× (ThermoFisher, USA). PCR was performed in a ProFlex PCR system (Applied Biosystems, USA) with an initial denaturation cycle at 95 °C for 5 min, 25 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min and a final extension step at 72 °C for 10 min. DNA electrophoresis was carried out using 15 μL of the PCR product in a 1.6 % (*w/v*) agarose gel and resolved in 1× TAE at 70 V for 110 min. After that, DNA was stained using a 1× GelRed® solution (Biotium, USA) in 0.1 M NaCl. A 100 to 3000 base pairs DNA weight marker (VWR, USA) was employed for band sizing. Gel images were captured employing a Gel Analyzer System (Axygen Scientific, USA).

### 2.4. Microsatellites fingerprinting and strain classification

The selected primers were multiplexed in a single PCR as described above with some modifications. The final concentration of B and F chromosome loci primers was reduced to 0.5 μM each. The rest of PCR conditions were maintained.

For strain classification, gel band analysis was performed using GelAnalyzer v.19.1 (www.gelanalyzer.com). Band fingerprinting of every gel was translated into binary (0 and 1) matrices, where the y-axis shows the name of every single strain and the x-axis shows the size of all the amplicons present in all the different electrophoresis. Then, the isolates were clustered according to the Sørensen-Dice coefficient and represented by hierarchical clustering using the Ward calculation methods employing *ade4* package from R studio (Dray and Dufour, 2007; R Core Team, 2013). Correlation between genotypic and geographical distance matrices was calculated based on Mantel's statistic according to Pearson's product-moment correlation implemented in R studio, using *geosphere* and *vegan* packages.

### 2.5. Co-cultures for strain monitorization

To assess the adequacy of the technique to monitor strain's implantation during wine fermentation, different co-culture assays were developed. Four strains (ROD21-99, A11-606, A11-612 and UWOPS 79-116) were selected to perform the *in-vitro* co-cultures at different complexity levels: two-strain, three-strain, and four-strain communities. All the cultures were inoculated in 2.5 mL of SGM (Synthetic Grape Must) at an initial cellular density of $10^6$ cells/mL (Vicente et al., 2023a) using 12-well microtiter plates. 100 μL samples were taken at 0, 24 and 96 h, serially diluted and spread on YMA plates. From each sample, 20 colonies per community were randomly taken, genotyped according to the hereto described technique and compared to the fingerprint of the strains in pure culture. Then, implantation percentages were calculated for each strain in each community and time.

## 3. Results and discussion

### 3.1. Microsatellites description and primers design and selection

Here we present an accurate genotyping method for *L. thermotolerans* selection and strain monitorization. The genome, including mitochondrial DNA, of the type strain of *L. thermotolerans* CBS 6340 was analysed for the detection of microsatellites. In total, 1038 Simple Sequence Repeats (SSR) were present in this reference genome, 23 of them located in the mitochondrial chromosome. SSR were filtered according to the repetition length and the number of repetitions, maintaining only the longest and those presenting a high repetition number to achieve a better discrimination using agarose gel electrophoresis (which resolution is lower if compared to other techniques but easier to implement in cellar laboratories). Finally, the most adequate microsatellite candidates were selected for *in vitro* testing. The selected microsatellites and their primers for PCR are described in Table 1. The first approach revealed the unsuitability of the mitochondrial microsatellites since all the ten strains that were initially used presented the same amplification products according to that previously reported (Belloch et al., 1997; Friedrich et al., 2012; Freel et al., 2014). The low mutation rate together with the selection process that the species has suffered have led to a low rate of nonsynonymous substitutions and a great homogeneity in its mitochondrial genome (Friedrich et al., 2012; Freel et al., 2014). The genomic-located loci showed a better performance, nevertheless, some of them indicated a great homogeneity among the studied strains. In some cases, no amplification was detected for different primers in several strains. Despite this fact, those primers were not discarded since they allow a differentiation among strains in a presence/absence criterion. At the end, six of them, located in different chromosomes (A, B, F, G, and H) were selected by two reasons: they were those that presented the higher divergence among all the strains tested and, the combination of twelve different primers is suitable enough for a multiplexed PCR.

### 3.2. Multiplexed-PCR validation

The analysis, that was performed using five different *Lachancea* non-*thermotolerans* species and thirty-four yeasts isolates belonging to genera different from *Lachancea* (Table S1), showed a great specificity. No amplification products were obtained in any case, indicating that this is a species-specific tool. This fact allows a direct isolate-fingerprinting analysis without requiring a previously molecular (*e.g.*, 26S rDNA sequencing) identification of the isolate as occur with the interdelta genotyping method (Legras and Karst, 2003). This approach was followed to identify all *L. thermotolerans* isolates from 2021 vintage used in this work (Fig. S1). The application of this technique in more than one hundred collection and reliable strains allowed the discrimination up to 30 different amplicons in the whole population. Employing the resulting presence/absence matrix and the following clustering we were able to discriminate up to 99 out of the 103 analysed strains, which means a 96 % of sensibility (Fig. 1) (Table S3). The genotypic clustering obtained by employing these microsatellites lacked geographical significance since the genotypic differences of the strains were not related with the geographical origin of the isolate, as Mantel correlation test showed ($p = 0.658$) (Fig. 2-A). This fact may be linked to the non-clonal reproduction of the species, since if cells mate, different recombinants showing admixed microsatellite markers can be produced during meiosis (sporulation). This fact can distort any geographical or heritability pattern by producing different combinations for each marker. This fact has been previously observed in *Candida albicans*, where some clades have lost the typical clonal evolutionary pattern that is commonly expected in a species that presents asexual reproduction (Taylor et al., 2015).

### 3.3. Protocol application in a yeast selection process

The application of the procedure in a collection of autochthonous isolates coming from several vineyards and harvests allowed the differentiation of 190 different fingerprints among the 428 studied isolates

**Table 1**
Designed primers of the study for multiplex-PCR. Chromosome location, primer name, sequence, melting temperature, length and number of repetitions in *L. thermotolerans* CBS 6340, and GeneID from NCBI. In bold, those employed for *L. thermotolerans* typing.

| Chromosome | Primer name | Sequence (5′ → 3′) | Tm (°C) | Amplified region and length | Gene ID |
|---|---|---|---|---|---|
| A | LTA-f/r | GTAAGAACCGCTGTAAGC | 55.0 | 49,746–51,360 | – |
| | | TACTGGATCCACCTCC | 53.4 | | |
| | **LTA2-f/r** | **GAGAAGAAGATGGAGTTTGGG** | **61.0** | **49,697–51,421 (1724 bp)** | **8290277** |
| | | **CTCCAGTTTCCTCGGTTCC** | **64.0** | | |
| B | **LTB-f/r** | **AGAAACGGGGCTTCACAGG** | **66.4** | **588,507–589,963 (1500 bp)** | **8290945** |
| | | **GTTTTGGCTAGTCCGCTTTGGG** | **64.2** | | |
| C | LTC-f/r | GGATTGGAGT GCGATTTGCC | 64.5 | 298,710–299,639 (930 bp) | – |
| | | CGCTGTAGCGATGTTTCC | 56.3 | | |
| D | LTD-f/r | CATCAACAGTTGTTGATGG | 58.0 | 240–1329 (1089 bp) | 8294947 |
| | | TGTGGAGGTAGATTGAGC | 56.0 | | |
| E | LTE-f/r | TGAGAAAACACTGTTATGCG | 59.4 | 1,050,934–1,051,789 (855 bp) | 8292157 |
| | | TGTGACCAGTACGAAGGCC | 56.4 | | |
| F | **LTF-f/r** | **GCTCTGTCTCCACGGTGTCTGC** | **67.9** | **440,838–441,463 (625 bp)** | **8292622** |
| | | **GGAGTTAGTGGTGGTAGAGG** | **60.5** | | |
| G | **LTG-f/r** | **CTAGTACTCAACCTACAACTCG** | **60.1** | **286,981–288,075 (1094 bp)** | **8293462** |
| | | **GGGACAAAGGGTAAGATTCG** | **58.40** | | |
| | **LTG2-f/r** | **GATAGGAAACGCTAGGAGACTCG** | **64.6** | **1,025,921–1,028,099 (2178 bp)** | **8,93842** |
| | | **CTTAAGAGAACAGTCGAACTGC** | **60.1** | | |
| H | **LTH-f/r** | **CGAGTTTGCGGAAGACAGTGG** | **63.2** | **10,105–10,897 (792 bp)** | **–** |
| | | **GATGCTAGCGCTATGACTAGC** | **61.2** | | |
| Mitochondrial | LTmt1-f/r | GACCCAGTTACTTATTAGGATG | 58.4 | 746–904 (158 bp) | 3238975 |
| | | CCATAATATTTATTATGGTAGC | 52.7 | | |
| | LTmt2-f/r | CATTTATAATTTATATCAAGCAG | 52.3 | 5900–6100 (200 bp) | – |
| | | CTCATTTATTAAAGGAACCC | 52.3 | | |
| | LTmt3-f/r | CTTCTTCTTATTTAAAAGATGC | 55.5 | 16,100–17,350 (1117 bp) | 3238973, 3239002, 3238980, 3238971 |
| | | CAGTTTACTGCTTTACCACTAAGC | 62.0 | | |
| | LTmt4-f/r | GTTTAATGGTTAAACTGTTAGATTGC | 60.1 | 18,401–19,080 (679 bp) | 3238992, 3238983, 3238977 |
| | | CTAATCATACTAAATTTAAATCACC | 55.9 | | |
| | LTmt5-f/r | GTATTAAAGGACAATATTCACG | 54.7 | 22,176–22,524 (348 bp) | 3238984 |
| | | CCTCATAAATATTTTTTATTACGG | 55.0 | | |

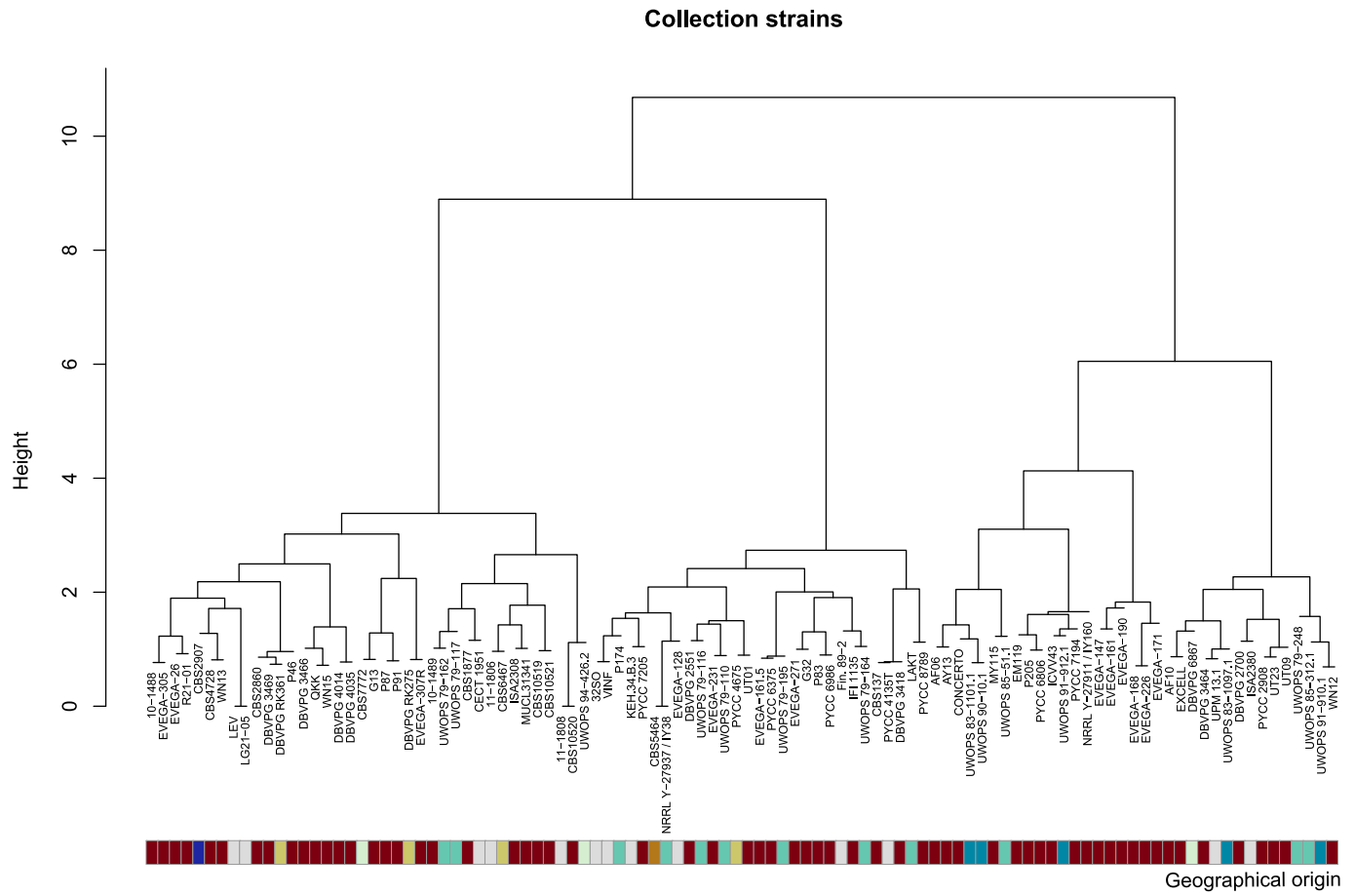


**Fig. 1.** Dendrogram showing the different clusters based on Sørensen-Dice coefficient constructed using Ward's methods for *L. thermotolerans* collection strains. The colour bar indicates the geographical origin of each strain: Europe, purple; Africa, navy blue; Asia, yellow; Australia, orange; South America, light green; North America, light blue; no data, white.

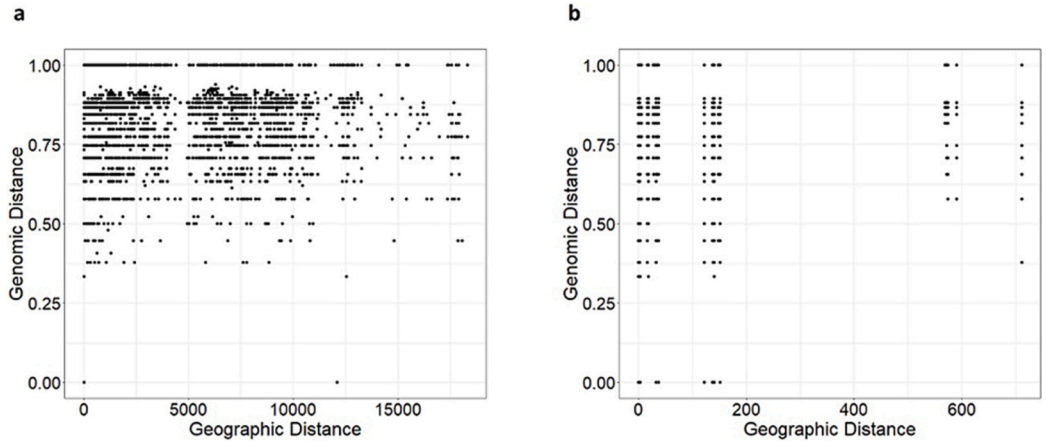


**Fig. 2.** Correlation analysis between geographical and genotypic distance. A) Collection strains. B) Isolated strains.

(based in 30 different amplicons), which means that around the 45 % of the isolates presented a unique fingerprint (Fig. 3, Table S4). The diversity present among the *L. thermotolerans* isolates was extremely high as showed by others studying the intraspecific diversity of *L. thermotolerans* in Greek isolates (Banilas et al., 2016). Nevertheless, this data did not show any kind of population structure since any geographical clustering significance was observed ($p = 0.03$) (Fig. 2-B).

In our study, it was not possible to discriminate the strains by their geographical distribution, unlike the results obtained in Banilas' study, where the strains were isolated from two winemaking regions separated by about 500 km and the sea in between, that conforms an important geographical barrier for allopatric differentiation. The impact of the intraspecific diversity of *S. cerevisiae* in wine fermentation is still partially unknown as well as for other yeast species. Some non-*Saccharomyces* species, such as *Hanseniaspora uvarum* and *Starmerella bacillaris,* show a great diversity (Masneuf-Pomarede et al., 2016) as *L. thermotolerans.* This diversity has been probably driven by the selective pressure in wine-related environments (Hranilovic et al., 2017). The

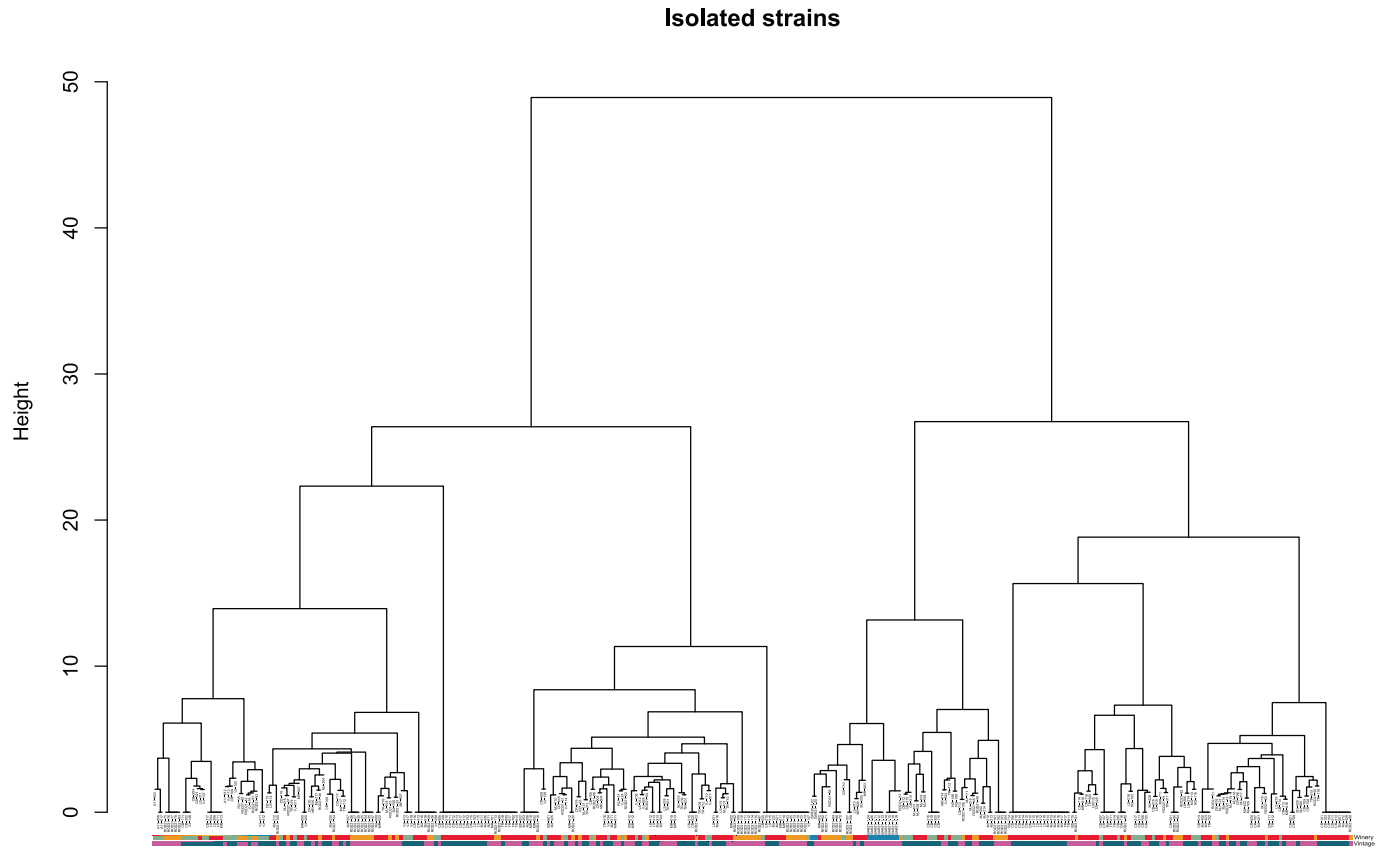


**Fig. 3.** Dendrogram showing the different clusters based on Sørensen-Dice coefficient constructed using Ward's methods for *L. thermotolerans* natural isolates. The colour bar indicates geographical origin (light green: Winery P (Ribera de Duero); orange: Winery R (Rioja); red: Winery H (Ribera de Duero); blue: Winery B (Manzanilla – Sanlúcar de Barrameda)) and vintage (blue: 2020; purple: 2021).

results concerning the *terroir* designation (regarding geographical influence) in non-*Saccharomyces* species is unclear. Some genetic patterns regarding *S. cerevisiae* have been described, confirming the singularity of some stains in a certain geographical location (de Celis et al., 2019), fact that has not been confirmed in other wine-related species (Banilas et al., 2016). Despite this fact, the genetic profile is not the unique condition that confirms the uniqueness of a strain, the oenological phenotypes are essential for *terroir* confirmation.

### 3.4. Protocol verification for its application in wine-fermentation monitoring

Finally, we carried out an additional verification of the PCR technique to test the suitability for strain monitoring studies, both in wine fermentation and dry yeast biomass production. With this purpose we performed several co-cultures containing different strains of *L. thermotolerans* mixed up at different complexity levels and different times, from 0, to check the initial inoculation ratio, to 96 h, when the lactic acid production peaked, and *S. cerevisiae* is usually inoculated in sequential fermentations involving both species (Vicente et al., 2021b; Vicente et al., 2023b). The application of the technique for wine-monitoring purposes showed the accuracy of the PCR multiplex-based genotyping method hereto presented. The evolution of the strains expressed as implantation percentages are shown in Table 2. We were able to track every strain along the fermentation since each strain showed its characteristic band profile in all the analysis. Huge differences were observed among the implantation capacity of the strains. Some strains (*e.g.*, UWOPS 79-116) were able to grow faster in the conditions tested, displacing others, and becoming dominant at the end of the fermentation trials. This fact is of great importance since, in rational strain selection procedures, one of the most valuable characteristics is the rapid growth and dominance of the selected strain over the autochthonous microbiota. These strain selection procedures will be essential for building synthetic yeast starter-culture consortia with microbial *terroir* effects (Pretorius, 2020).

**Table 2**
Implantation percentages of each strain used in the synthetic *L. thermotolerans* communities at different sampling times.

| Community | Sampling time (h) | Incidence (%) | | | |
|---|---|---|---|---|---|
| | | A11-606 | ROD21-99 | UWOPS 79-116 | A11-612 |
| A | 0 | 55 | 45 | – | – |
| | 24 | 35 | 65 | – | – |
| | 96 | 5 | 95 | – | – |
| B | 0 | 50 | 30 | 20 | – |
| | 24 | 40 | 45 | 15 | – |
| | 96 | 0 | 40 | 60 | – |
| C | 0 | 25 | 25 | 25 | 25 |
| | 24 | 0 | 25 | 20 | 55 |
| | 96 | 0 | 35 | 40 | 25 |

## 4. Conclusions

The simplicity, reproducibility, and valuable results that this technique achieves are extremely applicable in wine-related strain selection procedures. The method has been tested in different groups of strains, both from collection and natural origin. The first trial allowed us to determine the discrimination capacity of our method; the second one tested the real performance of it in a strain selection procedure. The final validation showed valuable results since every strain can be followed-up

along the time even in high-complexity *L. thermotolerans* communities. So, the method described here is a simple procedure to implement that allows, not only the strains classification, but also the fermentation monitoring as well as the competition capacity of the strains that defines their performance in different industrial applications.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110186.

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