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Development of microsatellite markers for *Lachancea thermotolerans* typing and population structure of wine-associated isolates

Georgios Banilas[a], Georgios Sgouros[b,c], Aspasia Nisiotou[b,*]

[a] Department of Enology and Beverage Technology, Technological Educational Institute of Athens, Ag. Spyridonos Street, 12210, Greece
[b] Institute of Technology of Agricultural Products, ELGO DEMETER, 1 S. Venizelou Str., Lykovrysi, 14123, Greece
[c] Department of Molecular Biology and Genetics, Democritus University of Thrace Dragana, Alexandroupolis, 68100, Greece

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ABSTRACT

Lachancea (*Kluyveromyces*) *thermotolerans* is an important member of the grape/wine yeast community with great technological potential for the wine industry. Although several molecular marker techniques have been developed for typing different yeast species, no one has been designed so far for *L. thermotolerans*. Here we present a simple and efficient method based on a multilocus SSR analysis for molecular typing and genetic diversity assessment of *L. thermotolerans* isolates. Following whole genome screening, five polymorphic microsatellite markers were selected and tested on a panel of grape isolates from different vineyards of two geographically separated viticultural zones, Nemea and Peza, in Greece. The SSR method proved quite discriminatory as compared to tandem repeat-tRNA-PCR, a fingerprinting method for typing non-*Saccharomyces* yeasts. Genetic analysis based on SSR data revealed a clear structure between the populations of the two zones. Furthermore, significant differences were also detected in a number of phenotypic characters of enological interest. A positive correlation was observed between phenotypic and genotypic diversity. Taking together, present results support the microbial *terroir* concept in the case of *L. thermotolerans* in Greece, which is an important prerequisite for the exploitation of selected genotypes as fermentation starters with region-specific characters.

1. Introduction

Lachancea thermotolerans (formerly *Kluyveromyces thermotolerans*) is an ascomycetous yeast species associated with fruits, *Drosphila* species and other plant-feeding insects (Ganter, 2006). It is considered a regular inhabitant of the grape/wine ecosystem, as it has been frequently encountered in grapes and fermenting musts in several viticultural regions worldwide (Jolly et al., 2003; Mills et al., 2002; Nisiotou et al., 2007; Torija et al., 2001). *L. thermotolerans* belongs to the group of the so-called non-*Saccharomyces* or 'wild' yeasts, originally derived from grapes, which due to their numerical supremacy in fresh must they commence the alcoholic fermentation. Although they are later replaced by the most alcohol tolerant *S. cerevisiae*, *L. thermotolerans* may exhibit further persistence and survive the elevated ethanol concentrations at the end of the fermentation course (Mills et al., 2002; Nisiotou et al., 2007).

For long, the presence of non-*Saccharomyces* yeasts in winemaking has been viewed under skepticism, as they have been associated with stuck or sluggish fermentations and wines with unacceptable organoleptic characteristics (Ciani et al., 2010). However, their role has been recently revisited, since it has been shown that their activity may be also highly beneficial (Capozzi et al., 2015; Jolly et al., 2014). Therefore, different grape-related yeast species of various genera, like *Candida*, *Metschnikowia*, *Pichia*, *Torulaspora*, and *Lachancea*, have been evaluated as potential wine yeast starters in mixed cultures with *S. cerevisiae* (Benito et al., 2015; Comitini et al., 2011; Englezos et al., 2015). Selected strains are currently used in commercially produced mixed inocula for wine production. Among the non-*Saccharomyces* wine yeasts, *L. thermotolerans* has attracted much attention for the aroma compounds it may confer to wines, including significant amounts of 4-methyl-4-sulfanylpentan-2-one (box-tree odor) and 3-sulfanylhexan-1-ol (grapefruit and passion fruit nuances) and for increased production of lactic acid and glycerol (Ciani and Ferraro, 1998; Comitini et al., 2011; Gobbi et al., 2013; Kapsopoulou et al., 2007; Soden et al., 2000; Zott et al., 2011). These properties have led to the development of a commercial *L. thermotolerans* dry yeast product, which according to the manufacturer produces lactic acid giving roundness and balanced acidity and confers increased flavor

* Corresponding author.
E-mail address: anisiotou.wi@nagref.gr (A. Nisiotou).

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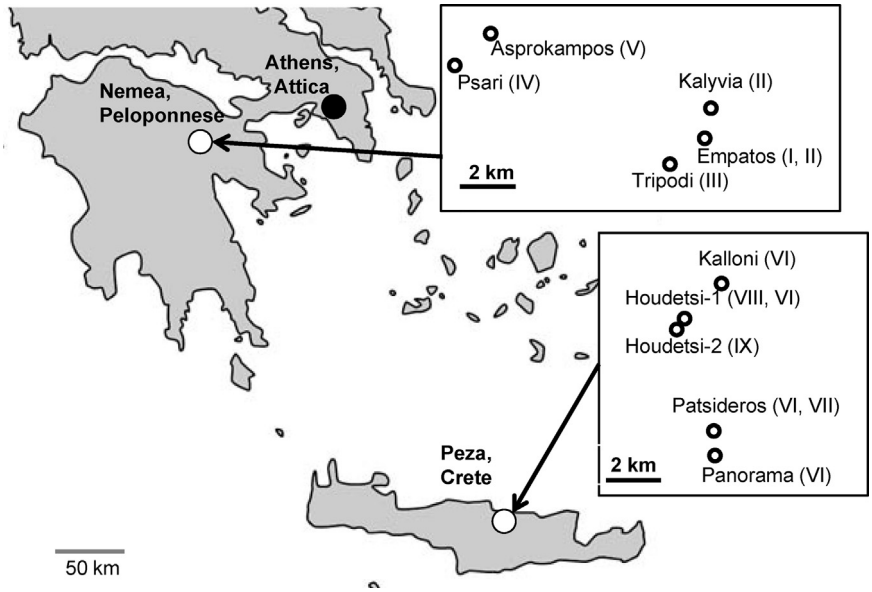

Fig. 1. Map of the vineyards in the viticultural zones of Nemea and Peza in Southern Greece. Genotypes (I–IX) detected in each vineyard are indicated in parentheses.

impact to wines (http://www.chr-hansen.com/food-cultures-and-enzymes/wine/cards/collection-cards/speciality-yeast).

Despite the importance of *L. thermotolerans* for the wine and food industry, our current knowledge on the ecology, distribution and population genetics of this species is limited. Recently, the genome diversity and evolution of *L. thermotolerans* was investigated by analyzing the mitochondrial (mt) genomes of 50 isolates from diverse geographical regions and ecological niches and a relatively low mt genetic diversity was found (Freel et al., 2014). Currently, much information at the genomic level is available, given that the genome of the type strain CBS 6340 has been completely sequenced (Talla et al., 2005). However, in order to facilitate strain discrimination and identification of wine-important non-*Saccharomyces* yeasts, there is an increasing need to develop methods for molecular marker-assisted genotyping. Such methods should ideally be highly discriminatory, affordable, simple and rapid, in order to track and monitor selected strains in complex microbial communities during the fermentation course. The need to develop such methods is further underlined by the increasing interest on ecological aspects of wine yeasts, mainly due to possible association of microbial mosaic to wine "*terroir*". The recently emerging concept of 'microbial *terroir*', i.e. the association of native yeast populations with distinctive geographical wine phenotypes, may strengthen the originality and typicity of wines and deliver added value to the product.

Although different molecular methods have been developed for typing wine yeasts, most of them have been applied to *S. cerevisiae*. To our knowledge, not any molecular marker method has been developed so far for *L. thermotolerans* typing. Microsatellites or simple sequence repeats (SSRs) are tandem repeats of a short DNA sequence motif, usually up to 6 bp long. They are characterized by a relatively high mutation rate, presenting high variability in the repeat number. Alleles are easy-to-score by means of PCR amplification and fragment-size analysis. SSRs provide several advantages over most other size-based molecular markers, among which are the high reproducibility within and among laboratories and the unambiguous scoring, while the analysis can be semi-automated. More importantly, unlike other fingerprinting methods such as RAPDs and AFLPs, SSRs are codominant markers, allowing extracting evolutionary relationships among individuals. They are thus widespread in the genome of most eukaryotes and have proved particularly suitable markers for molecular fingerprinting, population genetics, and phylogenetic analysis in various taxa, including wine-related yeasts (Albertin et al., 2014; Goddard et al., 2010; Richards et al., 2009; Tofalo et al., 2013). With respect to the grape/wine ecosystem, SSRs have been mainly applied to *S. cerevisiae* (Legras et al., 2007; Richards et al., 2009; Schuller and Casal, 2007). Only recently they are beginning to expand to few non-*Sacharomyces* species, like *Brettanomyces bruxellensis* (Albertin et al., 2014), *Candida zemplinina* (Masneuf-Pomarede et al., 2015), and *Hanseniaspora uvarum* (Albertin et al., 2016). This is probably due to the lack of genome sequencing data available in most of the wild yeast species, a major limitation to a wider exploitation of SSRs. Here, taking advantage from the complete genome sequence of *L. thermotolerans*, a set of SSR markers was developed able to differentiate isolates of grape origin and to describe the genetic structure of populations from two geographically separated viticultural zones in Greece. A thorough analysis of enologically important characteristics of strains from different geographical regions was also conducted that showed important phenotypic to genotypic relatedness.

2. Materials and methods

2.1. Yeast isolation and species identification

L. thermotolerans yeasts were isolated from fermented grape must originating from various vineyards of Nemea (Northern Peloponnese) or Peza (Crete) region, representing two major viticultural zones in Greece (Fig. 1, Table 1). In Nemea all vineyards have been cultivated with the red grapevine variety 'Agiorgitiko', whereas in Peza three varieties were sampled, the red ones 'Mandilari' and 'Kotsifali' and the white variety 'Vilana'. After harvest, grapes were placed into sterile plastic bags and transferred at 4 °C to the laboratory, where crushed with a stomacher and let to ferment spontaneously in sterile bottles. Yeasts were isolated at the end of the fermentation course after culture plating in Lysine agar medium (Oxoid, Unipath Ltd., Hampshire, UK). The type strain *L. thermotolerans* CBS6340 was also included in the analysis. All isolates were identified at the species level after PCR amplification using the universal primers ITS1 and ITS4 and sequencing analysis of the 5.8S-ITS