International Journal of Food Microbiology 233 (2016) 1–10

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Multilocus analysis reveals large genetic diversity in *Kluyveromyces marxianus* strains isolated from Parmigiano Reggiano and Pecorino di Farindola cheeses

Giuseppe Fasoli [a], Eladio Barrio [b,c], Rosanna Tofalo [a], Giovanna Suzzi [a], Carmela Belloch [b,*]

[a] Faculty of BioSciences and Technology for Food, Agriculture and Environment, University of Teramo, Via C.R. Lerici 1, 64023 Mosciano Sant' Angelo, Italy
[b] Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC), Ave. Agustín Escardino 7, 46980 Paterna, Valencia, Spain
[c] Department of Genetics, Universitat de València, C/Dr. Moliner, 56100 Burjassot, Valencia, Spain

ARTICLE INFO

Article history:
Received 20 January 2016
Received in revised form 16 May 2016
Accepted 30 May 2016
Available online 6 June 2016

Keywords:
Kluyveromyces marxianus
Italian dairy
Genetic diversity
Inter-RTL
mtDNA RFLPs
IGS KmSSB1-KmRIO2 sequence
Mating type

ABSTRACT

In the present study, we have analysed the genetic diversity in *Kluyveromyces marxianus* isolated from Parmigiano Reggiano and Pecorino di Farindola cheesemaking environment. Molecular typing methods inter-RTL fingerprint and mtDNA RFLPs, as well as, sequence diversity and heterozygosity in the intergenic region between KmSSB1 and KmRIO2 genes and analysis of the mating locus were applied to 54 *K. marxianus* strains. Inter-RTL fingerprint revealed a large degree of genetic heterogeneity and clustering allowed differentiation of *K. marxianus* strains from different geographical origins. In general, inter-LTR profiles were more discriminating than RFLPs of mtDNA; however our results also indicate that both techniques could be complementary unveiling different degrees of genetic diversity. Sequence analysis of the intergenic region between KmSSB1 and KmRIO2 genes revealed 26 variable positions in which a double peak could be observed in the sequence chromatogram. Further analysis revealed the presence of heterozygous strains in the *K. marxianus* population isolated from Parmigiano Reggiano. On the other hand, all strains isolated from Pecorino di Farindola were homozygous. Two very different groups of haplotypes could be observed as well as mixtures between them. Phylogenetic reconstruction divided *K. marxianus* dairy strains into two separate populations. A few heterozygous strains in an intermediate position between them could also be observed. Mating type locus analysis revealed a large population of diploid strains containing both *MATa* and *MATα* alleles and few haploid strains, most of them presenting the *MATα* allele. Different scenarios explaining the presence and maintaining of homozygous and heterozygous diploids as well as hybrids between them in the Parmigiano Reggiano *K. marxianus* population are proposed. A principal component analysis supported the large differences between *K. marxianus* isolated from Parmigiano Reggiano and Pecorino di Farindola.

1. Introduction

The yeast *Kluyveromyces marxianus* and its sister species *Kluyveromyces lactis* are characterized by their unique ability to ferment lactose (Lachance, 2011). The study of lactose metabolism, among other reasons, has highlighted *K. lactis* as a model for non-conventional yeasts (Fukuhara, 2006; Dujon et al., 2004; Schaffrath and Breunig, 2000). On the other hand, *K. marxianus* has been found especially suitable for biotechnological applications, based on its broad substrate spectrum, high growth rates and thermotolerance (Fonseca et al., 2008; Lane and Morrissey, 2010). Among these, production of enzymes, bioingredients and aroma compounds, removal of lactose from food or bioremediation stand out for their industrial utilization (Fonseca et al., 2008). Additionally, its GRAS (Generally Regarded As Safe) and QPS (Qualified Presumption of Safety) status favours the interest of the dairy and beverage industry on *K. marxianus* (Lane and Morrissey, 2010).

K. marxianus is generally described as a lactose fermentative homothallic yeast, commonly isolated from dairy environments, though it can also be found in a variety of different habitats. Very limited knowledge is available on *K. marxianus* when compared with other yeasts of biotechnological interest such as *Saccharomyces cerevisiae*. Several studies have pointed out the genetic heterogeneity of *K. marxianus*. Karyotyping of *K. marxianus* strains from different isolation sources and geographical origins revealed a rich intraspecific polymorphism (Belloch et al., 1998; Fasoli et al., 2015) which was in agreement with the wide haplotype diversity observed through RFLPs of mitochondrial DNA (mtDNA) (Belloch et al., 1997; Suzzi et al., 2000). Restriction analysis of the non-transcribed spacer (NTS) region of rDNA allowed reproducible

* Corresponding author at: Institute of Agrochemistry and Food Technology, Ave. Agustín Escardino 7, 46980 Paterna, Valencia, Spain.
E-mail address: belloch@iata.csic.es (C. Belloch).

http://dx.doi.org/10.1016/j.ijfoodmicro.2016.05.028
0168-1605/

discrimination at the intraspecific level on *K. marxianus* (Pérez-Brito et al., 2007). Additional RAPD PCR fingerprinting studies were found useful to differentiate at strain level (Tofalo et al., 2014; Lopandic et al., 2006; Suzzi et al., 2000); and the variability of the insertion of the long terminal repeat (LTR) retrotransposons Tkm1 was used for assessing genetic diversity within *K. marxianus* from diverse origins (Sohier et al., 2009). Analysis of the mating type (*MAT* locus) supports the emerging view that stable haploid and diploid *K. marxianus* strains occur in natural environments (Lane et al., 2011). The MAT locus in *K. marxianus* seems to be organized as in *K. lactis* (Butler et al., 2004), and it is constituted by MATa1 and MATa2, and MATα1, MATα2 and MATα3 alleles. Lane et al. (2011) analysed the mating type in *K. marxianus* by PCR amplification of the *MAT* locus. They concluded that strains containing bands consistent with MATa and MATα genes were diploid and strains producing a single band consistent with MATa or MATα genes were haploid. In recent years, the complete genome sequences of several *K. marxianus* strains have been obtained unveiling gene encoding enzymes for several metabolic traits (Llorente et al., 2000; Jeong et al., 2012; Suzuki et al., 2014).

In this study, we have analysed genetic diversity, heterozygosity and mating type in *K. marxianus* strains isolated from Italian dairy sources. For this purpose, strains isolated from natural starters in the production of Parmigiano Reggiano cheese, cow whey, fermented milk as well as some strains isolated during the production and ripening of Pecorino di Farindola cheese, were analysed by inter-LTR PCR fingerprinting and mtDNA restriction analysis. The level of heterozygosity was evaluated by sequencing the intergenic spacer region between genes KmSSB1 and KmRIO2, and mating type was determined by PCR amplification of *MATa* and *MAT*α alleles.

2. Materials and methods

2.1. Yeast strains and growth conditions

Fifty four *K. marxianus* isolates from Italian dairy origin were examined in this work. Parmigiano Reggiano strains (LM) were isolated from natural whey starter cultures belonging to 35 different dairies located in geographically contiguous areas of Northern Italy (Emilia Romagna region, Italy). Pecorino di Farindola strains (M) were isolated along the cheese making process, strain FM09 was isolated from fermented milk and strain VG4 was isolated from cow whey. All yeast strains were conserved as culture stocks at −80 °C in 15% (v/v) glycerol.

2.2. DNA isolation and inter-LTR PCR fingerprinting

Yeast cells were cultured overnight on GPY medium (2% w/v glucose, 0.5% w/v peptone and 0.5% w/v yeast extract) at 25 °C. DNA was extracted as reported by Querol et al. (1992) and dilutions containing about 10 ng/μl prepared using a Nanodrop spectrophotomether ND-1000 (Thermo Fisher Scientific, USA). Inter-LTR PCR was carried out as described by Sohier et al. (2009) using KM1 (5′-GTTGGTATAATATCTGG-3′) and KM2 (5′-TTCTAAGGTCCCTACTAC-3′) primers. PCR products were separated on 2% (w/v) agarose gel in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8) at 60 V for 3 h, stained with RedSafe (INtRON Biotech., Korea) and visualized under UV light. DNA fragment sizes were determined using a 100 bp DNA ladder (Life Technologies, USA). Reproducibility of the technique was verified using internal controls of few strains which were included in all PCR DNA amplifications and electrophoresis. *K. marxianus* CBS 834 type of *Saccharomyces kefir* was included as reference strain of dairy origin.

2.3. Mitochondrial DNA restriction analyses (mtDNA RFLPs)

MtDNA restriction analysis was performed as reported in Belloch et al. (1997). For this purpose, a total of 300 ng of DNA was digested with restriction endonuclease *Hin*fI (Roche, Switzerland) according to the manufacturer's instructions. Restriction fragments were separated on 2% (w/v) agarose gel in 1 × TAE buffer at 25 V for 16 h, stained with GelRed™ (Biotium, USA) and visualized under UV light. DNA fragment sizes were estimated against a DNA marker consisting of lambda phage DNA digested with *Pst*I.

2.4. Sequencing of the intergenic spacer region (IGS) between genes KmSSB1 and KmRIO2

The IGS region was amplified using primers KmSSB1 (5′-CAAATACCGCATATGAGATGTRTCTAAYTTCAT-3′) and KmRIO2 (5′-GAACAAATGGTCAACCARGCYGARGA-3′). An additional internal primer (5′-CGCTTTTACATGTCTACGTTAT-3′) was designed to obtain the whole sequence. PCR reactions were performed in 50 μl final volume containing 0.25 μl rTaq (5 U) DNA polymerase, 4 μl dNTP mix (2.5 mM), 5 μl of 10X buffer (Takara Bio Inc., Japan), 3 μl $MgCl_2$ (1.5 mM) (Sigma, USA), 1 μl primer (50 pmol/μl) (Isogen Life Science, The Netherlands) and 7.5 μl solution containing around 100 ng of genomic DNA. Amplification was performed on a Mastercycler Pro (Eppendorf, Germany) with an initial denaturation at 95 °C for 5 min followed by 45 cycles consisting of 45 s at 94 °C, 35 s at 57, 1 min at 72 °C and a final extension of 10 min at 72 °C. Bands of PCR products were cut from the agarose gel and purified with High Pure PCR Product Purification Kit (Roche, Switzerland) following the manufacturer's instructions. Sequencing reactions were carried out using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) in an Applied Biosystems (Model 310) automatic DNA sequencer. *K. marxianus* CECT 10585^T (CBS 712^T), type strain of the species, and CECT 1043 (CBS 608) were included as reference strains.

2.5. Mating type analysis

Primer design for determining the mating type in *K. marxianus* was carried out taking into account previous results by Butler et al. (2004) and Lane et al. (2011) as well as sequences deposited in GenBank. Primer pairs for amplification were SLA2 (5′-TATACATGGGATCATAAATC-3′) (Lane et al., 2011) and MATa1D (5′-GGTTTGGCAGGAGTACAACTA-3′) and MATα1D (5′-TGAAATCCAAAGCACCAACT-3′). PCR reactions were performed in 25 μl final volume containing 0.75 μl (5 U) Expand Long Template PCR System (Roche, Switzerland), 5 μl 10X buffer 1 containing 1.75 mM $MgCl_2$, 3.5 μl dNTP mix (2.5 mM) (Takara Bio Inc., Japan), 0.5 μl each primer (50 pmol/μl) (Isogen Life Science, The Netherlands) and 2.5 μl solution containing around 200 ng of genomic DNA. Amplification was performed on a Mastercycler Pro (Eppendorf, Germany) with an initial denaturation at 94 °C for 2 min followed by 10 cycles consisting of 10 s at 94 °C, 30 s at 46 °C and 3 min at 68 °C and 35 cycles consisting of 10 s at 94 °C, 30 s at 50 °C and 3 min at 68 °C and a final extension of 10 min at 68 °C. The type strain of the closest relative *K. lactis* CECT 1961^T (CBS 683^T), was used as a negative control and, *K. marxianus* CECT 1043 (CBS 608) was used as a positive control (Lane et al., 2011). PCR products were separated on 1% (w/v) agarose gel in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8) at 60 V for 3 h, stained with RedSafe (INtRON Biotech., Korea) and visualized under UV light. DNA fragments size was estimated against a DNA marker consisting of lambda phage DNA digested with *Pst*I.

2.6. Data analysis: dendrograms, heterozygosity analysis and phylogenetic reconstruction

A similarity matrix was generated based on presence (1) and absence (0) of bands in the electrophoretic patterns of inter-LTRs and RFLPs of mtDNA (see DiB1). Bands ranging between 600 bp to over 2.0 Kb and 1.98 Kb to 11.5 Kb were considered in the case of LTRs and *Hin*fI RFLPs of mtDNA, respectively. Clustering by the UPGMA (unweighted pair-group method using arithmetic averages) method