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ORIGINAL ARTICLE

Comparison of RAPD-PCR and PFGE analysis for the typing of *Streptococcus thermophilus* strains isolated from traditional Turkish yogurts

Fatima Ghazi[1] · Mebrouk Kihal[1] · Neslihan Altay[2] · Güzin Candan Gürakan[3]

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Abstract The dairy industry is constantly developing new starters with specific traits. Therefore, strains from natural sources have huge potential for exploration and characterization. The aim of this work was to identify the most efficient, useful, and reliable option for typing 55 *Streptococcus thermophilus* isolates from traditional Turkish yogurts and four industrial strains. Then, we statistically evaluated whether our conclusions could be extended to the true population. For this purpose, the results of the rapid amplified polymorphic DNA polymerase chain reaction (RAPD-PCR; using the OPI-02 MOD, M13, and XD9 primers) and pulsed-field gel electrophoresis (PFGE; using the *Sma*I and *Apa*I enzymes) typing methods were compared. The discriminatory power, typeability, and reproducibility were analyzed. Additionally, the congruence between typing methods was quantified using the adjusted Rand index (AR), Wallace index (W), and expected Wallace coefficient. Both methods revealed high genetic diversity of the *S. thermophilus* strains, even in the same yogurt sample. The numerical combination of results for these primers or restriction enzymes increased the congruence between the methods and provided more complete information on the strains. The comparison of these two options showed that using *Sma*I with *Apa*I was more advisable and useful than using OPI-02 MOD with M13. Additionally, the first combination represented the best tool to discriminate *S. thermophilus* strains (Simpson's index of diversity [DI] of 0.999 [0.997–1.000]). This finding was statistically supported. The RAPD (OPI-02 MOD) typing result showed an ability to confidently predict the PFGE (*Sma*I, *Apa*I) type (AR = 0.782 [0.618–0.949], W = 0.946 [0.865–1.000]). This result had some statistical support and might represent an important application in the dairy industry for screening strains.

Keywords *Streptococcus thermophilus* · Typing · PFGE · RAPD-PCR · Comparison

✉ Fatima Ghazi
ghazifati@yahoo.fr

[1] Laboratory of Applied Microbiology, Department of Biology, Faculty of Nature and Life Sciences, Oran University 1, Ahmed Ben Bella, BP 16, Es-Sénia 31100, Oran, Algeria

[2] Department of Food Engineering, Selçuk University, 42250 Konya, Turkey

[3] Department of Food Engineering, Middle East Technical University, 06531 Ankara, Turkey

Introduction

Streptococcus thermophilus is an essential component of natural or commercial starters and many types of fermented dairy products, such as yogurts and cheeses. It is considered to be the second most important species of industrial lactic acid bacteria after *Lactococcus lactis*. *Streptococcus thermophilus* is largely ingested by the human population and has a market value of approximately 40 billion US$ annually (Iyer et al. 2010).

The technological, probiotic, and sensorial characteristics of lactic acid bacteria are strain-specific. Therefore, strain typing is important due to the increasing interest in strain-related properties for applications in the dairy industry (Sánchez et al. 2004; Ruiz et al. 2008; Picozzi et al. 2010). The differentiation of *S. thermophilus* at the strain level is also a source of interest for monitoring patented technologies in yogurt production. Furthermore, strain typing could provide traceability and standardization of the product.

Rapid amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) have been applied to evaluate

the genetic diversity and typing of *S. thermophilus* strains. However, each method has advantages and disadvantages.

RAPD-polymerase chain reaction (PCR) is considered to be a useful approach to obtain genetic data (Ramos et al. 2008; Singh et al. 2009). It is a rapid, simple, easy, and less expensive technique that is universally applicable to any genome. Prior knowledge of the target sequence is not needed. However, RAPD-PCR has a tendency for a reproducibility problem in the band patterns (Singh et al. 2009). For this reason, the RAPD reaction conditions should be highly standardized (Ramos et al. 2008; Singh et al. 2009). In contrast, PFGE has a superior discriminatory capacity and high reproducibility. It is called the "gold standard" and has been universally recognized as the appropriate method to determine strain-specific diversity (Picozzi et al. 2010). However, PFGE is labor-intensive, requires equipment with a high cost that is rare in most microbiology and molecular biology laboratories, and can run only a limited number of samples at one time (Singh et al. 2009).

In previous studies, the restriction enzymes *Apa*I and *Sma*I and the primers M13, OPI-02 MOD, and XD9 were most frequently used to evaluate the genetic diversity and typing of *S. thermophilus* strains (Boutrou et al. 1995; Moschetti et al. 1998; O'Sullivan and Fitzgerald 1998; Giraffa et al. 2001; Andrighetto et al. 2002; Mora et al. 2002; Jenkins et al. 2002; Sánchez et al. 2004; Girard and Moineau 2007; Michaylova et al. 2007; Tosi et al. 2007; Rizzotti et al. 2009; Lazzi et al. 2009; Morandi and Brasca 2012). Therefore, we used these primers and restriction enzymes in this study. Then, we performed combined numerical analysis of RAPD-PCR patterns with two primers (OPI-02 MOD and M13) and combined numerical analysis of PFGE patterns with the two restriction enzymes.

This work consisted of an analysis and comparison of the results obtained using the PFGE and RAPD-PCR methods. The aim was to identify the best option for typing 59 *S. thermophilus* strains originating from homemade yogurts from different areas in Turkey. Then, our conclusions were statistically evaluated to determine whether they could be extended to the true population.

The analysis and comparison were based on two approaches. First, the parameters used to evaluate the successful implementation of the bacterial genotyping methods, typeability, discriminatory power, execution time, feasibility, and reproducibility (preferably after a period of a few months) were analyzed (Coenye et al. 2002; Carriço et al. 2006; Behringer et al. 2011) Then, a quantitative evaluation of the congruence between the typing methods was performed to support our decision as advocated by other authors (Severiano et al. 2011). This approach allowed us to determine whether these typing methods identified the same relationship between strains.

As previously proposed by other authors (Carriço et al. 2006; Pinto et al. 2008; Severiano et al. 2011), the following measures were computed to evaluate the congruence, correspondence, or agreement between the results of the typing methods: adjusted Rand's index, Wallace's index (and their confidence intervals), and the expected Wallace value if the classifications were independent

Materials and methods

Bacterial strains and growth conditions

A total of 55 *S. thermophilus* strains were isolated from traditional Turkish yogurts (Altay Dede 2010); an additional four industrial strains were also studied. The bacteria were grown at 42 °C in M17 broth (Merck) at pH 6.8 and stored at −80 °C in M17 glycerol.

The Turkish strains originated from different regions, as indicated in Table 1.

PFGE genotyping

The restriction enzymes *Sma*I (Fermentas) and *Apa*I (Fermentas) were used separately for PFGE analysis. Genomic DNA preparations of *S. thermophilus* strains and DNA restrictions for PFGE were performed as described by O'Sullivan and Fitzgerald (1998), with the following modifications: after lysis using lysozyme (Applichem), the cells immobilized in agarose blocks (low melting point Prona agarose) were incubated for 30 min at room temperature in a solution containing 1 % (w/v) SDS, 10 mM Tris–HCl, and 50 mM EDTA (pH 8.5); then, the blocks were incubated in the same solution supplemented with 2 mg/ml proteinase K (Novagen, Merck) for 36 h at 50 °C. The agarose blocks were subsequently washed nine times in 5 ml of 50 mM EDTA (pH 8.5) for 30 min at 4 °C and stored in the same buffer.

Endonuclease restriction of *S. thermophilus* genomic DNA embedded in agarose was performed with 3U of *Sma*I (Fermentas) and 20 U of *Apa*I (Fermentas) at 37 °C in 200 μl of restriction buffer for both restriction enzymes.

The fragments obtained by the restriction endonucleases were electrophoresed on 1 % pulsed-certified agarose (Bio-Rad) in the CHEF System (Amersham Gene Navigator) at 200 V for 23 h, with the following switch times; 7 s (12 h), 10 s (6 h), and 15 s (5 h) for the *Sma*I enzyme and 2 s (6 h), 4 s (6 h), 8 s (6 h), and 12 s (5 h) for the *Apa*I enzyme. A total of 50 μM of thiourea was added to the 0.5X TBE running buffer.

The gels were stained with 0.5 mg of ethidium bromide in 500 ml of distilled water for 30 min in a covered container, then destained in distilled water for 60 min. The destained gels were placed on a UV box, and images were captured as TIFF files using the Gel Doc XR Digital Imaging System (Bio-

Table 1 *Streptococcus thermophilus* strains included in this study

Strain source	Strain code	Geographical origin
K1 yogurt	K1-15, K1-12, K1-1, K1-27, K1-28, K1-2, K1-19, K1-11, K1-9, K1-21, K1-22, K1-26, K1-31, K1-13, K1-14, K1-30, K1-23, K1-24, K1-29, K1-16, K1-7, K1-20, K1-18	Erzincan
S1 yogurt	S1-3, S1-1	Mersin
N8 yogurt	N8-2	Mersin
N1 yogurt	N1-1	Mersin
N2 yogurt	N2-3, N2-4, N2-1	Antalya
N3 yogurt	N3-1, N3-3, N3-7, N3-6, N3-2, N3-4	Antalya
N4 yogurt	N4-2,N4-3, N4-1	Antalya
N5 yogurt	N5-4, N5-5, N5-7, N5-2, N5-3, N5-1, N5-6	Antalya
N6 yogurt	N6-5, N6-1,N6-2, N6-6, N6-3, N6-4	Mersin
N9 yogurt	N9-4, N9-2, N9-1	Mersin
Commercial starter culture	Ta040-1, Ta040-2	Danisco
Commercial starter culture	Yo-mix 410-3	Danisco
Culture collection	LMG18311	BCCM/LMG Bacteria Collection, Gent, Belgium

Rad). The λ ladder DNA (concatemers of λ cl857 Sam7) from Bio-Rad (ranging from 48.5 to 1000 kb) was used as the DNA size standard.

RAPD-PCR genotyping

RAPD-PCR analysis was performed with the OPI-02 MOD primer (5′-GCTCGGAGGAGAGG-3′), M13 primer (5′-GAGGGTGGCGGTTCT-3′), and XD9 primer (5′-GAAGTCGTCC-3′), as previously described by Ghazi et al. (2013).

The GeneJET™ Genomic DNA Purification Kit (Fermentas) was used for DNA extraction according to the manufacturer's instructions. All DNA solutions were stored at −20 °C. The 100 bp Plus DNA ladder (Fermentas) was used as the molecular size standard. Gels were stained with 0.5 mg of ethidium bromide in 500 ml of distilled water for 20 min in a covered container, then destained in distilled water for 5 min. The gel images were taken using the Gel Doc XR Digital Imaging System (Bio-Rad).

Reproducibility study for PFGE and RAPD-PCR

DNA extraction was repeated twice on different days for ten test strains and four times for the reference strain (*S. thermophilus* LMG 18311) to test the reproducibility of the PFGE method. The DNA restriction and the running assay were applied for all 59 strains in different gels at least two and three times for the *Apa*I and *Sma*I enzymes, respectively. The reference strain *S. thermophilus* LMG 18311 was included in almost all PFGE gels with the *Sma*I enzyme. The RAPD-PCR reproducibility study was performed as described by Ghazi et al. (2013).

Analysis and comparison of PFGE and RAPD-PCR typing results

RAPD-PCR and PFGE gels were visualized by UV transillumination. The patterns were converted, normalized, and further processed using GelCompar II software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). After automatic band detection, band assignment was manually curated for all gel images.

Strains were grouped using the Dice correlation coefficient via the software. Then, a dendrogram was generated for each typing result from the matrix of similarity of Dice coefficients by the unweighted pair group method using arithmetic averages (UPGMA) clustering algorithm. Composite datasets of PFGE gels using the *Apa*I and *Sma*I enzymes and RAPD gels using the M13 and OPI-02 MOD primers were created to obtain combined PFGE (*Sma*I, *Apa*I) and RAPD-PCR (M13, OPI-02 MOD) dendrograms. UPGMA analysis of the composite dataset was performed using the "average from experiments" setting of the Applied Maths software.

In this study, the definition of clusters (or types) relied on the partitioning of the resulting dendrogram based on similarity values for the analysis and comparison of the two sets of results between these typing methods. Three cutoff thresholds were defined: the first at the value of 100 % similarity (first similarity threshold); the second similarity threshold was determined according to the criteria of Tenover et al. (1995), as previously described (Struelens

et al. 1992; Murchan et al. 2003); and the third similarity threshold cutoff was defined on the basis of the geographical origin of the strains.

Multidimensional scaling is another method for clustering strains that is different from UPGMA. It was applied to the *S. thermophilus* strains and lactic acid bacteria to visualize the diversity revealed by RAPD-PCR as described in previous studies (Giraffa et al. 2001; Oguntoyinbo and Narbad 2012). In our case, the method was only performed to help define the similarity threshold cutoff for the resulting dendrograms. For this purpose, the previous matrices of similarities were introduced into the XLSTAT 2012 (Addinsof) software and analyzed.

To estimate the congruence between the results of the typing methods at the three similarity thresholds, an online tool (http://www.comparingpartitions.info) was used to calculate the following measures: adjusted Rand index (AR) (Hubert and Arabie 1985), Wallace index (W) (Carriço et al. 2006), and their confidence intervals (CIs) (Pinto et al. 2008; Severiano et al. 2011), and the expected Wallace coefficient (Wi). The Simpson's index of diversity (DI) and its CI were also computed (Hunter and Gaston 1988).

The adjusted Rand index provides an estimation of the overall congruence between two typing methods. The Wallace index provides information about the directional correlation between typing methods; for given data, the value of $W_{A \rightarrow B}$ expresses the probability that two individuals are classified together using method B if they have been classified together using method A (Carriço et al. 2006; Severiano et al. 2011).

The expected Wallace coefficient under independence is a measure to assess whether the results of two typing methods could agree by chance alone (Pinto et al. 2008).

Results

The yielded dendrograms are shown in Figs. 2, 3, 4, 5, 6, and 7.

In this section, the results of the PFGE and RAPD-PCR patterns and reproducibility study are presented. Then, descriptive (or qualitative) and quantitative comparisons were performed between the assigned clusters of the PFGE and RAPD PCR typing methods for three cases to study the similarity thresholds. The DI, AR, and W values are dependent on the specific sample of *S. thermophilus* strains taken from the true population. To evaluate the population parameters, the statistical CIs were calculated to indicate the reliability of our evaluation and to validate whether our conclusions had statistical support.

PFGE patterns

Excellent reproducibility was detected for the PFGE method. The repeated experiments performed using *Sma*I or *Apa*I yielded identical patterns for each culture, even over periods of months and up to one year.

The PFGE analysis was performed for the 59 strains using the *Sma*I and *Apa*I enzymes. Five of these strains (N3-3, N3-6, N3-2, N5-4, and N2-4) were nontypeable, even though thiourea was added to solve the problem of high DNA degradation (Okatani et al. 2001; Goering 2010). Therefore, they were excluded from further analysis.

PFGE with the *Sma*I enzyme yielded patterns with 10 to 14 bands for most of the strains; however, 6, 8, and 9 bands were produced for three strains, N9-4, N9-1, and N2-1, respectively. The bands ranged from approximately 48.5 kb to 339.5 kb in size (Fig. 1b).

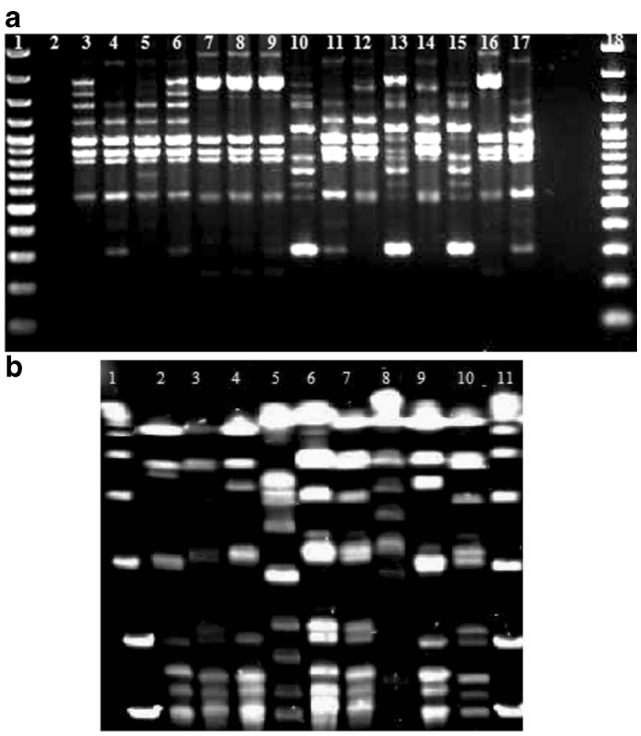

Fig. 1 **a** RAPD-PCR patterns of *S. thermophilus* strains obtained with OPI-02 MOD primer. Lanes 1 and 17, molecular size marker; lane 2, negative control; lane 3, N9-4; lane 4, N5-3; lane 5, N9-2; lane 6, N9-1; lane 7, K1-29; lane 8, K1-16; lane 9, K1-7; lane 10, N3-2; lane 11, N5-1; lane 12, N3-4; lane 13, K1-20; lane 14, N6-4; lane 15, Ta040-02; lane 16, K1-18; lane 17, N5-6. **b** PFGE patterns of *S. thermophilus* strains obtained with *Sma*I restriction enzyme. Lanes 1 and 11, molecular size marker; lane 2, N5-7; lane 3, K1-31; lane 4, S1-3, lane 5, Ta040-1; lane 6, K1-21; lane 7, K1-14; lane 8, N6-5; lane 9, N6-6; lane 10, K1-15

PFGE with the *Apa*I enzyme yielded patterns of 10 to 18 bands. Bands within a range of molecular weight values between 14.5 kb to 242.5 kb were taken into consideration.

RAPD-PCR patterns

The optimization of the PCR reaction mixtures and running conditions allowed us to obtain identical repeatable patterns with the M13 and OPI-02 MOD primers on different days (Ghazi et al. 2013).

Five months later, we repeated the experiment. Six strains produced identical patterns with the OPI-02 MOD primer, while four out of the six strains produced identical patterns with M13. Extra faint bands appeared for the two remaining strains. Therefore, OPI-02 MOD seemed to have better reproducibility (Ghazi et al. 2013).

Despite its excellent discriminatory power, the XD9 primer was omitted from the study due to its poor reproducibility and congruence results.

Amplification using OPI-02 MOD yielded 5 to 11 bands ranging from approximately 300 to 3000 bp in size, while the M13 primer generated 1 to 6 fragments ranging from approximately 600 to 3000 bp in size (Fig. 1a).

Types generated at the first similarity threshold (100 % similarity) and comparison between the PFGE and RAPD-PCR results

The only isolates (K1-19 and K1-2) sharing the same type in the combined PFGE (*Sma*I, *Apa*I) dendrogram also presented the same type in the RAPD-PCR (M13, OPI-02 MOD) dendrogram (Figs. 4 and 7). These isolates yielded identical patterns in all typing results from PFGE (*Sma*I), PFGE (*Apa*I), RAPD-PCR (OPI-02 MOD), and RAPD-PCR (M13) (Figs. 2, 3, 5, and 6). Because they came from the same sample, they probably represented the same strain isolated two times. A higher number of types (exactly eight) was revealed by the RAPD-PCR (M13, OPI-02 MOD) dendrogram (Fig. 7).

Combining the results of both restriction endonucleases increased the discriminatory power, yielding 53 PFGE genotypes from 54 strains, with a DI of 0.999 [0.997–1.000]. This finding had some statistical support because the 95 % CIs of the DI did not overlap for PFGE (*Sma*I, *Apa*I) and PFGE (*Apa*I). Likewise, the combined results using the two primers enhanced the number of RAPD genotypes to 38, with a DI of 0.976 [0.956–0.996] (Figs. 4 and 7; Table 2). In this case, the fact that the discriminatory power of the PFGE (*Sma*I, *Apa*I) was higher than the RAPD-PCR (M13, OPI-02 MOD) had statistical support because their DI 95 % CIs did not overlap.

The adjusted Rand and Wallace indices obtained at the 100 % similarity threshold were very low (data not shown). However, the adjusted Rand index between RAPD (OPI-02 MOD) and RAPD (M13) was 0.201 [0.060–0.348], while the index was 0.073 [0.000–0.307] between PFGE (*Sma*I) and PFGE (*Apa*I). These values allowed us to exclude the possibility that the two chosen primers (or the two restriction enzymes) targeted the same part of the DNA.

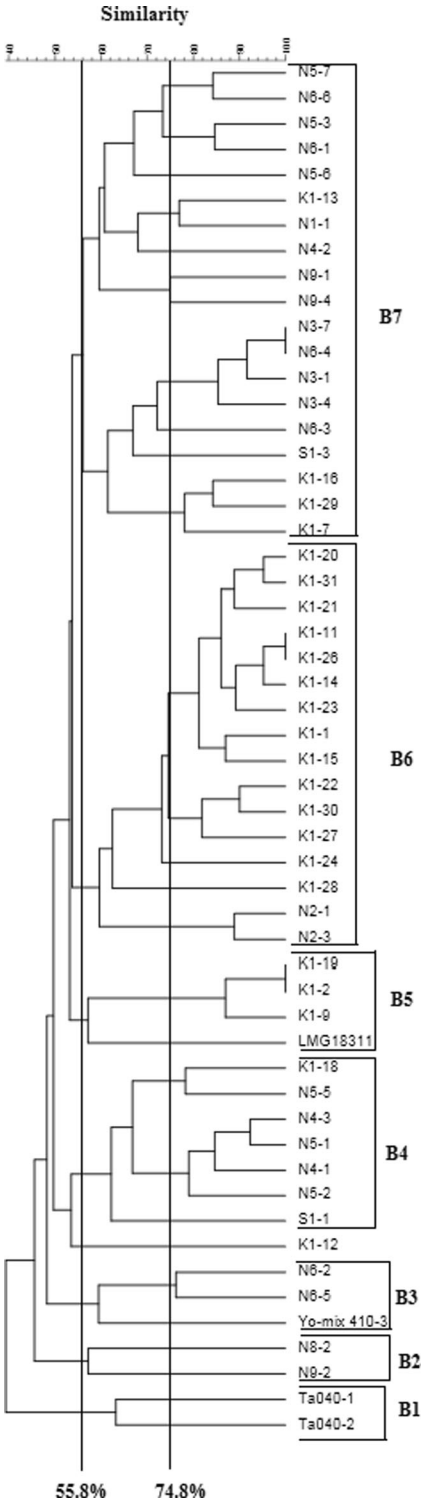

Fig. 2 PFGE (*Sma*I) dendrogram. B1, B2, B3, B4, B5, B6, and B7 clusters delineated at 55.8 % similarity

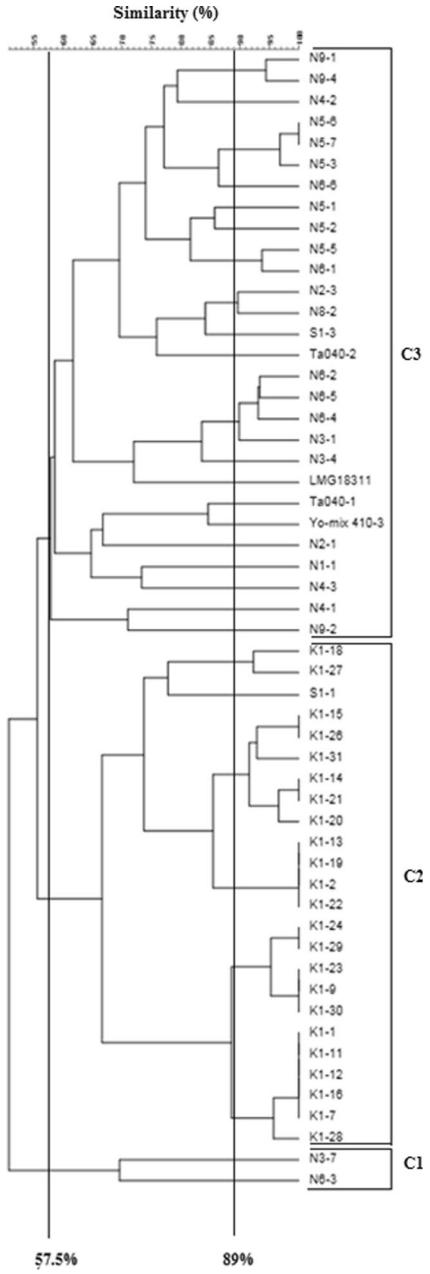

Fig. 3 PFGE (*Apa*I) dendrogram. C1, C2, and C3 clusters delineated at 57.5 % similarity

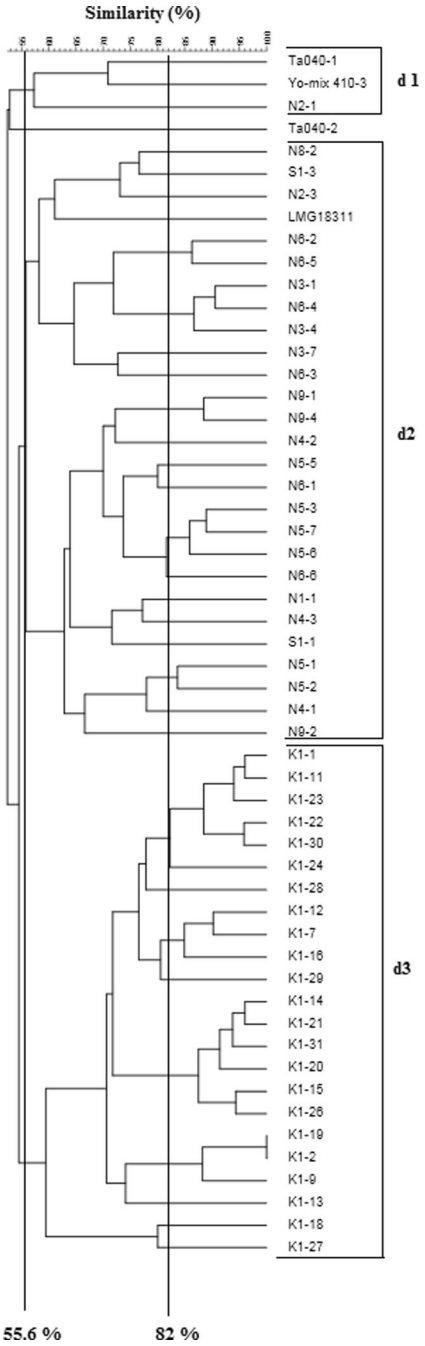

Fig. 4 Combined PFGE (*Sma*I, *Apa*I) dendrogram. d1, d2, and d3 clusters delineated at 55.6 % similarity

Types generated at the second similarity threshold (according to the criteria of Tenover et al.) and comparison between the PFGE and RAPD-PCR results

The criteria of Tenover et al. (1995) have been widely applied in epidemiological studies for the interpretation of PFGE patterns (Coenye et al. 2002; Murchan et al. 2003; Carriço et al. 2006). According to the authors, profiles varying from each other in the positions of up to three bands should be considered closely related, while profiles differentiated by up to six bands should be considered possibly related. The reasoning behind this recommendation is that differences in one to three bands would be the result of a single genetic event (a point mutation in a restriction site, a deletion, or an insertion), and a six-band difference would be the result of two genetic events (Tenover et al. 1995; Barrett et al. 2006). Therefore, the 74.8 % Dice similarity coefficient for the PFGE (*Sma*I) dendrogram (Fig. 2) and the 89 % Dice similarity coefficient for the PFGE (*Apa*I) dendrogram (Fig. 3) were designed to express similarity threshold values above which all clusters included strains differing from each other by a maximum of six

Table 2 Number of types and index of diversity (with 95 % CI) of each typing method at the three similarity thresholds for the 54 strains

	First similarity threshold cutoff studied		Second similarity threshold cutoff studied		Third similarity threshold cutoff studied	
	Number of types (clusters) found	DI	Number of types (clusters) found	DI	Number of types (clusters) found	DI
PFGE (*Sma*I)	51	**0.998** [0.995–1.000]	27	**0.955** [0.928–0.983]	8	**0.774** [0.708–0.841]
PFGE (*Apa*I)	41	**0.984** [0.970–0.997]	27	**0.957** [0.938–0.977]	3	**0.542** [0.492–0.592]
PFGE (*Sma*I, *Apa*I)	53	**0.999** [0.997–1.000]	34	**0.972** [0.954–0.990]	4	**0.576** [0.513–0.639]
RAPD (OPI-02 MOD)	36	**0.973** [0.953–0.994]	23	**0.955** [0.936–0.973]	6	**0.641** [0.567–0.714]
RAPD (M13)	19	**0.827** [0.745–0.908]	19	**0.827** [0.745–0.908]	5	**0.627** [0.555–0.698]
RAPD (OPI-02 MOD, M13)	38	**0.976** [0.956–0.996]	26	**0.939** [0.908–0.969]	3	**0.589** [0.530–0.648]

bands (less than or equal to six bands). The average value of 82 % was used for the PFGE (*Apa*I, *Sma*I) dendrogram (Fig. 4).

Criteria similar to those of Tenover et al. (1995) for RAPD-PCR patterns do not exist in the literature. The similarity cutoff values of 100 %, 81.2 %, and 85.6 % were used for the RAPD-PCR (M13), RAPD-PCR (OPI-02 MOD), and RAPD-PCR (M13, OPI-02 MOD) dendrograms, respectively (Figs. 5, 6, and 7) for comparison with the PFGE clusters. These values were designed on the basis of RAPD-PCR clusters including strains that were grouped together in the PFGE clusters (defined according to the criteria of Tenover et al.). The delineated clusters are illustrated in Table 3.

The results of PFGE with *Sma*I and *Apa*I and their numerical combination showed that the commercial *S. thermophilus* strains LMG 18311, Yo-mix 410-3, Ta040-1, and Ta040-2 were not closely or possibly related to any of our strains according to the criteria of Tenover et al. (1995). This fact was clearly observed in the combined RAPD-PCR (M13, OPI-02 MOD) dendrogram, but was not necessarily apparent in the RAPD-PCR (OPI-02 MOD) or RAPD-PCR (M13) dendrograms. As result, the *S. thermophilus* strains from traditional Turkish yogurts seem to be genetically unrelated to the commercial strains.

In the combined PFGE (*Sma*I, *Apa*I) dendrogram, all of the following clusters included strains from the same yogurt sample: AS1, AS2, AS3, AS4, AS5, AS7, AS8, and AS9 (Table 3). However, the N3-1 and N3-4 strains from the N3 yogurt sample and the N6-4 strain from the N6 yogurt sample (Table 1) were grouped within the AS6 cluster that was identical to the R6 cluster in the combined RAPD-PCR (M13, OPI-02 MOD) dendrogram. Moreover, the N3-1 and N6-4 strains revealed patterns that were distinguishable by one band and three bands in the PFGE results with the *Sma*I and *Apa*I restriction enzymes, respectively. This result showed that they were genetically closely related and possibly from the same lineage, although they originated from different samples and different regions (Antalya and Mersin).

Four clusters (AS1, AS2, AS3, and AS8) were delineated for the K yogurt strains: two clusters (AS4, AS9) for the N5 yogurt, one cluster (AS5) for the N6 yogurt, and one cluster (AS7) for the N9 yogurt (Table 3). Other strains from the S1, K1, N2, N3, N4, N5, N6, N8, and N9 yogurts and the commercial starters remained single (clusters of a single strain). Therefore, the combination of the results of both restriction enzymes revealed that one yogurt sample could include strains from several clusters of closely or possibly related strains. None of the resulting dendrograms included all strains of the K yogurt sample in one cluster at similarity threshold values calculated according to the criteria of Tenover et al. (1995) and the similarity threshold defined for the RAPD-PCR results. The R4, R5, and R6 clusters from the RAPD-PCR (M13, OPI-02 MOD) dendrogram were composed of a mixture of strains isolated from the N3, N4, N5, N6, and N9 yogurts. In summary, the genetic diversity within strains from the same yogurt sample was illustrated by the RAPD-PCR and PFGE results, but this observation was clearer with the combined results of PFGE with the *Sma*I and *Apa*I enzymes. Furthermore, strains from the S1, N1, N2, N3, N4, N5, N6, and N8 yogurts were genetically closer to one another and genetically farther from the strains of the K yogurt.

At this similarity threshold, higher adjusted Rand and Wallace indices were obtained compared to those yielded at the 100 % similarity threshold (data not shown). However, these values were low. The maximum value of the adjusted Rand of 0.269 [0.077–0.463] was found between the PFGE (*Sma*I, *Apa*I) and RAPD (OPI-02 MOD, M13) typing results.

Types generated at the third similarity threshold and comparison between the PFGE and RAPD-PCR results

It was clear that the clusters of the strains were correlated with their geographical origin in the case of the combined PFGE (*Sma*I, *Apa*I) dendrogram. The isolates from the Kemah (Erzincan) yogurt sample were grouped together in the d3

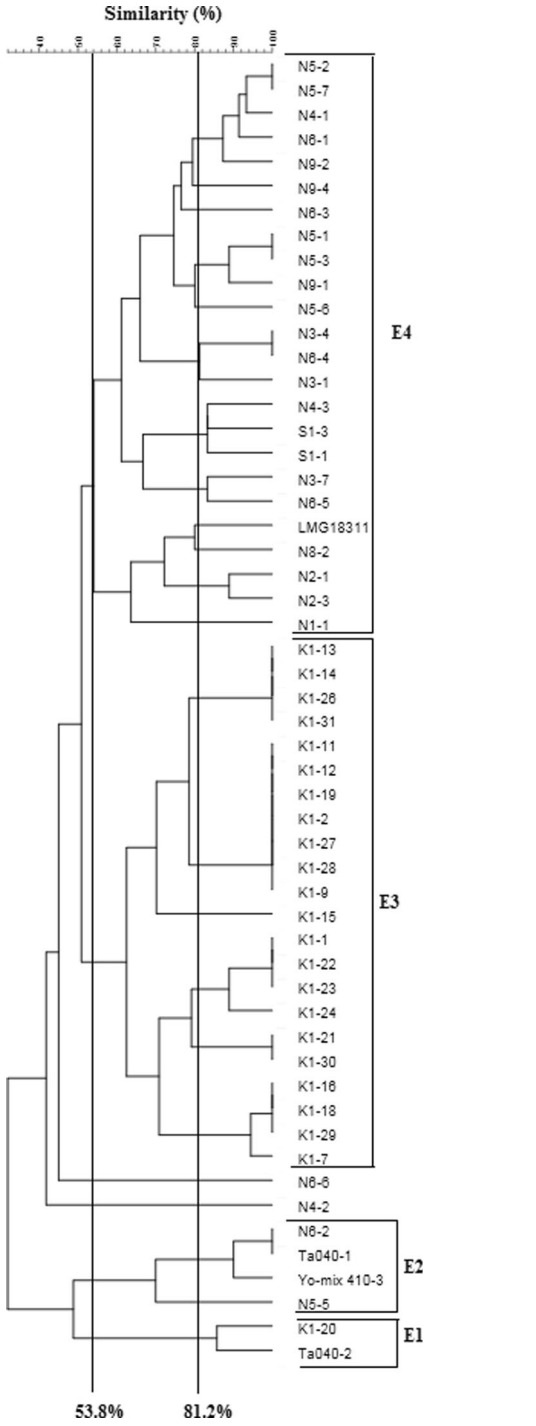

Fig. 5 RAPD-PCR (OPI-02 MOD) dendrogram. E1, E2, E3, and E4 clusters delineated at 53.8 % similarity

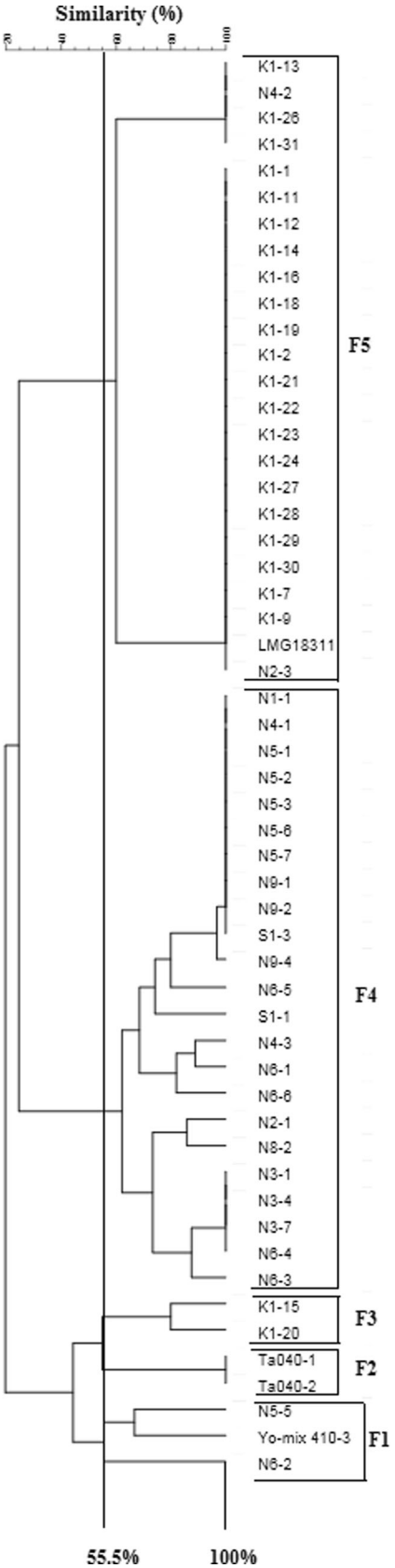

Fig. 6 RAPD-PCR (M13) dendrogram. F1, F2, F3, F4, and F5 clusters delineated at 55.5 % similarity

cluster, while the strains from different samples from the Antalya and Mersin regions were grouped together in the d2 cluster (Fig. 4). The Antalya and Mersin regions are located closer to each other and far from the Kemah (Erzincan) region. Moreover, the combined PFGE (*Sma*I, *Apa*I) dendrogram showed that the commercial strains Ta040-1, Ta040-2, and Yo-mix 410-3 were clearly outgroups for the Turkish yogurt

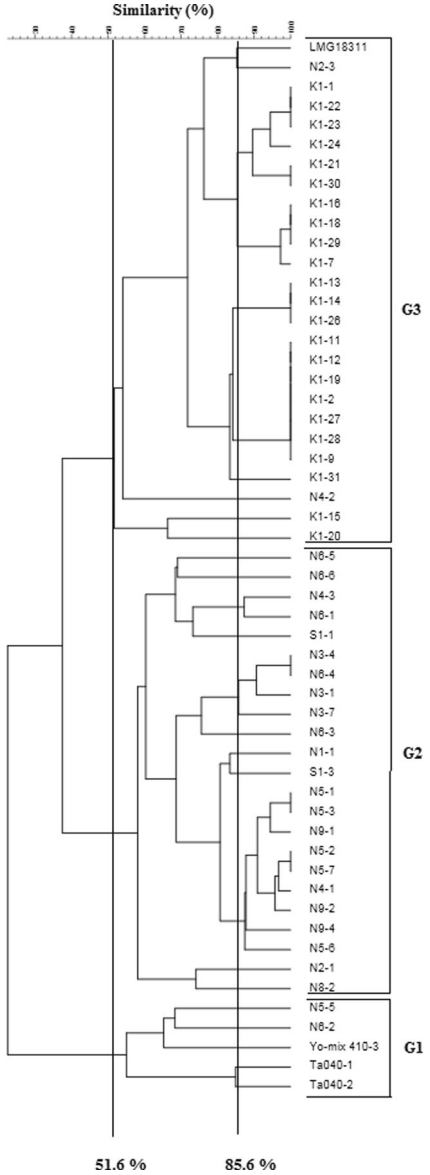

Fig. 7 Combined RAPD-PCR (OPI-02 MOD, M13) dendrogram. G1, G2, and G3 clusters delineated at 51.6 % similarity

strains. The exception was the *S. thermophilus* LMG18311 strain, which was isolated from yogurt produced in the United Kingdom in 1974. This strain was grouped with strains of the Antalya-Mersin yogurts.

Multidimensional scaling scatter plot (MDS) analysis produces a two-dimensional plot in which the strains are spread according to their relatedness. MDS tended to group the strains into two separated areas that were in good agreement with the PFGE (*Sma*I, *Apa*I) dendrogram clusters (data not shown).

Although the MDS plot clustered the *S. thermophilus* strains in accordance with their geographical origin, it did not group them, for all PFGE and RAPD-PCR typing results, according to their sample of isolation source (data not shown). Therefore, the corresponding clusters in the remaining dendrograms were mostly correlated to the geographical origin of the isolates to obtain an acceptable comparison. The definition of the PFGE and RAPD-PCR clusters was based on the similarity threshold cutoff of 55.8 % for the PFGE (*Sma*I), 57.5 % for the PFGE (*Apa*I), 55.6 % for the PFGE (*Sma*I, *Apa*I), 53.8 % for the RAPD-PCR (OPI-02 MOD), 55.5 % for the RAPD-PCR (M13), and 51.6 % for the RAPD-PCR (M13, OPI-02 MOD) dendrograms (From Figs. 2, 3, 4,5, 6, and 7).

The highest adjusted Rand and Wallace indices were found at this threshold level (Tables 4 and 5). The best congruence was found between the results of RAPD (OPI-02 MOD) and PFGE (*Sma*I, *Apa*I) (AR of 0.782 [0.618–0.949], W of 0.946 [0.865–1.000]), but this outcome had no statistical support based on the 95 % CIs of AR or W.

Only three of the calculated 95 % CIs for W included the respective Wi ($W_{ApaI \rightarrow SmaI}$, $W_{SmaI \rightarrow ApaI}$, $W_{OPI\text{-}02\ MOD \rightarrow SmaI}$). In all other comparisons, the congruence between typing methods could not be attributed to chance alone, because there was dependence among these typing methods (Table 5).

The numerical combination of the results increased the adjusted Rand index to 0.688 [0.453–0.925] using the value obtained between the RAPD-PCR (M13, OPI-02 MOD) and PFGE (*Sma*I, *Apa*I) compared with the values obtained between the RAPD (OPI-02 MOD) or RAPD (M13) with PFGE (*Apa*I) or PFGE (*Sma*I). Similarly, higher values were obtained between the results of PFGE (*Sma*I, *Apa*I) and either RAPD (OPI-02 MOD) or RAPD (M13) (Table 4). The same trend was observed for the Wallace indices (Table 5). However, these differences were not statistically significant based on the 95 % CIs.

Interestingly, there was a good, bidirectional correlation between the PFGE (*Sma*I, *Apa*I) and RAPD-PCR (M13, OPI-02 MOD) types based on the Wallace indices. In contrast, a good correspondence was only found in the direction of the PFGE (*Sma*I, *Apa*I) type for both RAPD (OPI-02 MOD) and RAPD (M13) types, but not in the reverse direction (Table 5).

Discussion

In this study, the PFGE and RAPD-PCR typing results for 59 *S. thermophilus* strains were analyzed and compared. Among them, 55 strains were isolated from traditional yogurts collected from different Turkish regions.

We aimed to determine which method was more suitable for typing these 59 *S. thermophilus* strains based on the data obtained from our PFGE and RAPD-PCR typing experiments. Moreover, we also investigated whether we could extend this result to the true population.

Both the PFGE and RAPD-PCR typing method results revealed a large degree of genomic diversity within the studied *S. thermophilus* strains, which was in agreement with previous

Table 3 Clusters obtained in the PFGE and RAPD-PCR dendrograms at similarity coefficients selected according to the criteria of Tenover et al.

Strains designation	PFGE *Sma*I, 74.8 %	PFGE *Apa*I, 89 %	PFGE *Sma*I + *Apa*I, 82 %	RAPD M13, 100 %	RAPD OPI-02 MOD, 81.2 %	RAPD OPI-02 MOD + M13, 85.6 %
K1-14	S1	A1	AS1	M2	O1	R1
K1-15	S1	A1	AS1	*	*	*
K1-26	S1	A1	AS1	M1	O1	R1
K1-21	S1	A1	AS1	M2	O13	R2
K1-31	S1	A1	AS1	M1	O1	*
K1-20	S1	A1	AS1	*	O12	*
K1-1	S1	A2	AS2	M2	O2	R2
K1-11	S1	A2	AS2	M2	O3	R3
K1-23	S1	A8	AS2	M2	O2	R2
N5-7	S2	A6	AS4	M3	O9	R5
N6-6	S2	*	*	*	*	*
K1-19	S3	A3	AS3	M2	O3	R3
K1-2	S3	A3	AS3	M2	O3	R3
K1-9	S3	A8	AS3	M2	O3	R3
N5-3	S4	A6	AS4	M3	O7	R5
N6-1	S4	A10	*	*	O9	R4
N6-2	S5	A5	AS5	*	O11	*
N6-5	S5	A5	AS5	*	O5	*
N6-4	S6	A5	AS6	M4	O6	R6
N3-1	S6	A5	AS6	M4	O6	R6
N3-4	S6	*	AS6	M4	O6	R6
N3-7	S6	*	*	M4	O5	R6
K1-13	S13	A3	*	M1	O1	R1
N1-1	S13	*	*	M3	*	*
K1-16	S8	A2	AS8	M2	O8	R7
K1-29	S8	A8	*	M2	O8	R7
K1-7	S8	A2	AS8	M2	O8	R7
K1-22	S9	A3	AS2	M2	O2	R2
K1-30	S9	A8	AS2	M2	O13	R2
K1-27	S9	A4	*	M2	O3	R3
N2-3	S10	A9	*	M2	O4	*
N2-1	S10	*	*	*	O4	*
K1-18	S11	A4	*	M2	O8	R7
N5-5	S11	A10	*	*	*	*
N4-3	S12	*	*	*	O10	R4
N5-1	S12	A4	AS9	M3	O7	R5
N4-1	S12	*	*	M3	O9	R5
N5-2	S12	*	AS9	M3	O9	R5
K1-12	*	A2	AS8	M2	O3	R3
K1-28	*	A2	*	M2	O3	R3
N8-2	*	A9	*	*	*	*
N5-6	*	A6	AS4	M3	*	R5
N9-4	S7	A7	AS7	*	*	R5
N9-1	S7	A7	AS7	M3	O7	R5
K1-24	*	A8	*	M2	O2	R2
N4-2	*	*	*	M1	*	*
N6-3	*	*	*	*	*	*
S1-3	*	*	*	M3	O10	*
LMG18311	*	*	*	M2	*	*
S1-1	*	*	*	*	O10	*
N9-2	*	*	*	M3	O9	R5
Yo-mix 410-3	*	*	*	*	O11	*
Ta040-1	*	*	*	M5	O11	*
Ta040-2	*	*	*	M5	O12	*

S, A: Clusters generated from PFGE; M, O: clusters generated from RAPD-PCR; AS: cluster from combined analysis of PFGE (*Sma*I, *Apa*I); R: clusters generated from combined analysis of RAPD-PCR (M13, OPI-02 MOD)

*Single strains

Table 4 Adjusted Rand indices and respective 95 % CIs (Jackknife pseudovalues) for the PFGE and RAPD-PCR typing methods at the third similarity threshold

	PFGE (*Sma*I)	PFGE (*Apa*I)	PFGE (*Sma*I, *Apa*I)	RAPD (M13)	RAPD (OPI-02 MOD
PFGE (*Apa*I)	**0.141** [0.000–0.304]				
PFGE (*Sma*I, *Apa*I)	**0.259** [0.085–0.436]	**0.723** [0.523–0.923]			
RAPD (M13)	**0.255** [0.087–0.425]	**0.470** [0.232–0.709]	**0.638** [0.414–0.865]		
RAPD (OPI-02 MOD)	**0.205** [0.029–0.384]	**0.609** [0.400–0.819]	**0.782** [0.618–0.949]	**0.762** [0.565–0.963]	
RAPD (OPI-02 MOD, M13)	**0.286** [0.107–0.467]	**0.533** [0.283–0.783]	**0.688** [0.453–0.925]	**0.921** [0.823–1.000]	**0.759** [0.554–0.965]

Table 5 Wallace indices and respective analytical 95 % CIs for the PFGE and RAPD-PCR typing methods at the third similarity threshold

	PFGE (*Sma*I)	PFGE (*Apa*I)	PFGE (*Sma*I, *Apa*I)	RAPD (M13)	RAPD (OPI-02 MOD)	RAPD (OPI-02 MOD, M13)
Wi	0.226	0.458	0.424	0.373	0.359	0.411
PFGE (*Sma*I)		**0.607** [0.453–0.760]	**0.687** [0.530–0.845]	**0.616** [0.465–0.767]	**0.551** [0.386–0.716]	**0.697** [0.561–0.832]
PFGE (*Apa*I)	**0.299** [0.185–0.414]		**0.814** [0.680–0.948]	**0.624** [0.471–0.778]	**0.684** [0.534–0.834]	**0.698** [0.568–0.827]
PFGE (*Sma*I, *Apa*I)	**0.366** [0.237–0.494]	**0.878** [0.762–0.994]		**0.735** [0.585–0.884]	**0.801** [0.658–0.943]	**0.806** [0.680–0.931]
RAPD (M13)	**0.373** [0.246–0.499]	**0.766** [0.616–0.916]	**0.835** [0.706–0.964]		**0.833** [0.698–0.968]	**1.000** [1.000–1.000]
RAPD (OPI-02 MOD)	**0.346** [0.216–0.477]	**0.872** [0.754–0.989]	**0.946** [0.865–1.000]	**0.866** [0.741–0.991]		**0.912** [0.811–1.000]
RAPD (OPI-02 MOD, M13)	**0.383** [0.255–0.511]	**0.777** [0.633–0.922]	**0.832** [0.705–0.958]	**0.908** [0.810–1.000]	**0.798** [0.656–0.939]	

authors' reports (Giraffa et al. 2001; Andrighetto et al. 2002; Jenkins et al. 2002; Mora et al. 2002; Michaylova et al. 2007; Tosi et al. 2007; Blaiotta et al. 2011; Morandi and Brasca 2012).Various RAPD or pulsotypes of *S. thermophilus* (even different clusters of closely or possibly related strains according to the criteria of Tenover et al.) seemed to be involved in the fermentation process in the same sample of traditional Turkish yogurt. Indeed, continuous evolution and adaptation of indigenous strains to their environment makes traditional products a rich source for the isolation of strains with phenotypes that are valuable for technological applications. The dairy industry is constantly improving starter cultures by including strains with better performances. These new strains can be obtained using different technologies, such as recombinant DNA method (this method is currently not an option) or screening of wild-type strains from natural sources (Derkx et al. 2014). Consequently, there is interest in preserving and exploring the huge diversity of these natural yogurts. Therefore, the identification of a valuable, efficient, easy, and quick typing method that is able to characterize a large number of strains will be very useful.

The good degree of reproducibility observed with PFGE using the *Sma*I and *Apa*I enzymes and RAPD-PCR using the OPI-02 MOD primer could allow the establishment of reference patterns for the potential tracking of *S. thermophilus* strains. Obviously, interlaboratory reproducibility is an issue, particularly for the RAPD-PCR (OPI-02 MOD) typing method. Thus, the numerical combination of PFGE with the *Sma*I and *Apa*I enzymes seemed to be more appropriate for such purpose, because it was more discriminative (53 different profiles out of 54) and had statistical support (Table 2). However, PFGE is a more laborious and time-consuming technique, while the RAPD-PCR typing method is easier, faster, and less expensive to perform. Additionally, PFGE has a problem of nontypeability of some strains. Therefore, it might be possible to use reproducible primers in combination with the OPI-02 MOD primer to increase the discriminatory index.

The lowest discriminatory power was obtained with the M13 primer at the first studied similarity threshold cutoff. This result had statistical support with the true population because its DI 95 % CI did not overlap with those of the other primer, restriction enzymes, or the combinations (Table 2). At this cutoff value, the discriminatory indices for the PFGE (*Apa*I), PFGE (*Sma*I), RAPD-PCR (OPI-02 MOD), PFGE (*Sma*I, *Apa*I), and RAPD-PCR (M13, OPI-02 MOD) experiments were greater than 0.90. These values are preferred for the reliable interpretation of the results for the genotyping method (Hunter and Gaston 1988; Coenye et al. 2002; van Belkum et al. 2007).

At the third similarity threshold, RAPD-PCR (M13) and RAPD-PCR (OPI-02 MOD) had almost the same discriminatory power (Table 2) and were in high agreement (AR = 0.762 [0.565–0.963]). However, there was no congruence between the PFGE (*Sma*I) and PFGE (*Apa*I) results (AR = 0.141 [0.000–0.304]), and the information provided by one method was independent or unrelated to the information provided by the other method (as revealed by the Wi values in Table 5). Therefore, it seems more advisable to combine the results obtained with the *Sma*I and *Apa*I enzymes than to combine the results obtained with the OPI-02 MOD and M13 primers. PFGE with *Apa*I performed well in identifying strains related by the RAPD-PCR results based on the AR and W values. This finding had statistical support based on the result obtained with OPI-02 MOD primer (Table 4) and the strong predictive power of the RAPD-PCR typing results (using OPI-02 MOD, M13, and their numerical combination) in the direction of the PFGE (*Apa*I) result (Table 5).

Several conclusions emerged from our limited dataset (59 studied strains) when evaluating whether the same relationship between strains was identified by the PFGE and RAPD-PCR typing methods. There was no congruence at the first and second similarity thresholds. However, the information that was in good agreement at both levels was more reliable than the results from each typing method alone.

Combining the results offered more complete information and well-clarified findings compared to the results obtained by the application of one primer or one restriction enzyme. Moreover, the performance of PFGE (*Sma*I, *Apa*I) was higher than RAPD-PCR (M13, OPI-02 MOD). The same observations were made at the third similarity threshold, where good congruence was detected. Combining the results increased the discriminatory power at the first similarity threshold and the congruence at the third similarity threshold. Thus, the comparison and numerical combination of the results obtained with different primers or restriction enzymes was able to provide a broader view, as previously suggested by other authors (Sánchez et al. 2004; Ruiz et al. 2008).

The results of the third similarity threshold suggested that, if two strains belonged to the same cluster according to the RAPD (OPI-02 MOD) typing result, one could confidently predict that they would also share the same PFGE (*Sma*I, *Apa*I) type (W = 0.946 [0.865–1.000]). The reverse conclusion could not be made with the same certainty, because only four out of five pairs of strains sharing the PFGE (*Sma*I, *Apa*I) cluster were also grouped in the same RAPD-PCR (OPI-02 MOD) type. This outcome needs to be verified statistically for the true population; then, the use of the RAPD-PCR (OPI-02 MOD) method will be sufficient to predict the PFGE (*Sma*I, *Apa*I) types. Obviously, complete agreement in terms of the AR value is preferable. PFGE has the advantage of having been previously used to create online databases that enable the comparison of strain profiles. Because RAPD-PCR is quicker and easier, it might be possible for us to apply it to a large number of strains to predict PFGE types. For instance, PFGE types correlated to the geographical origins

or industrial traits of strains (O'Sullivan and Fitzgerald 1998; Jenkins et al. 2002). Moreover, some strains that are not typeable by PFGE are typeable by RAPD-PCR. Therefore, RAPD-PCR with OPI-02 MOD could be a first step for screening strains of interest.

Predicting the PFGE (*Sma*I, *Apa*I) type from the RAPD-PCR (OPI-02 MOD) type had some statistical support, because it led to much lower error rates (from 0 to 13.5 %) compared to any other correspondence (as shown by the 95 % CIs in Table 5). Specifically, the reverse correspondence would lead to error rates ranging from 5.7 to 34.2 %. Moreover, the adjusted Wallace index that avoids the overestimation of unidirectional congruence (Severiano et al. 2011) was 0.905 [0.765–1.000], indicating the strength of this analysis between the RAPD-PCR (OPI-02 MOD) and PFGE (*Sma*I, *Apa*I) types. In summary, the OPI-02 MOD primer showed interesting features in this study.

In light of our findings, comparing the results of typing methods appears to be important, despite the limited number of studied strains.

Generally, several techniques have been used to characterize the same collection of *S. thermophilus* strains. An important consideration is how much information is added from the use of another method (or combining results) in terms of discriminatory power, type assignment, or even phylogenetic information about the strains. The comparison allows the user to keep a useful typing method and exclude a useless one. However, the ability to predict PFGE results from RAPD results might have an important application as the first step in screening strains of interest.

Five of the cited points had statistical support, while three conclusions lacked statistical support and required further scrutiny. It is possible to improve this work by decreasing the confidence intervals as much as possible in order to obtain more reliable results regarding the true population. This purpose could be achieved by using a larger number of strains and/or random sampling. Regardless, *Sma*I, *Apa*I, and OPI-02 MOD are of value in typing *S. thermophilus* strains.

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