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Does fingerprinting truly represent the diversity of wine yeasts? A case study with interdelta genotyping of *S. cerevisiae* strains.

Walter P. Pfliegler[1*] and Matthias Sipiczki[1]

[1] Department of Genetics and Applied Microbiology, University of Debrecen, Debrecen, Hungary.

Abbreviated running headline: Fingerprinting *vs.* true diversity.

Correspondence*

Walter P. Pfliegler, Department of Genetics and Applied Microbiology, University of Debrecen, Egyetem tér 1., H4032 Debrecen, Hungary. E-mail: walterpfliegler@gmail.com

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Significance and Impact of the Study:

Genotyping is routinely used for assessing the diversity of a large number of isolates/strains of a single species, e.g. a collection of wine yeasts. We tested the efficiency of interdelta genotyping on a collection of *Saccharomyces* wine yeasts from four wine regions of Hungary that was previously characterized physiologically. Interdelta fingerprinting recovered neither physiological nor geographical similarities and additionally, the two different primer pairs widely used for this method showed conflicting and barely comparable results. Thus this method does not necessarily represent the true diversity of a strain collection, but detailed clustering may be achieved by the combined use of primer sets.

Abstract

Simple and efficient genotyping methods are widely used to assess the diversity of a large number of microbial strains, e.g. wine yeasts isolated from a specific geographic area or a vintage, etc. Such methods are often also the first to be applied, to decrease the number of strains deemed interesting for a more time-consuming physiological characterization. Here, we aimed to use a physiologically characterized strain collection of 69 *S. cerevisiae* strains from Hungarian wine regions to determine whether geographic origin or physiological similarity can be recovered by clustering the strains with one or two simultaneously used variations of interdelta genotyping. Our results indicate that although a detailed clustering with high resolution can be achieved with this method, the clustering of strains is largely contrasting when different primer sets are used and it does not recover geographic or physiological groups.

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Keywords

wine, genotyping, diversity, yeast physiology, fermentation

Introduction

An ever-increasing number of genotyping methods have been proposed in recent years aiming at the efficient and quick characterization of microbial strains, including RAPD (Random Amplified Polymorphic DNA) or micro- and minisatellite PCR, AFLP (Amplified Fragment Length Polymorphism), TRFLP (Terminal Restriction Fragment Length Polymorphism), mtDNA-RFLP (Mitochondrial DNA Restriction Length Polymorphism), MLST (Multilocus Sequence Typing), or interdelta PCR, e.g. (de Barros Lopes *et al.* 1999; Corich *et al.* 2005; Muñoz *et al.* 2009; Maqueda *et al.* 2010; Blättel *et al.* 2013; Pfliegler *et al.* 2014; Ramírez-Castrillón *et al.* 2014).

The question of effectiveness (e.g. resolving different strains) and comparability of different fingerprinting methods is important. Different methods have different levels of strain resolution and the grouping of strains may depend on the chosen method. For example, Ayoub *et al.* (2006) found that MLST performed better in resolving geographic origin and interdelta PCR was more useful in differentiating closely related strains. By using ~50 *Saccharomyces* spp. yeasts (commercial strains and vineyard or winery isolates) it has been shown that interdelta polymorphism and *COX1* intron polymorphism fingerprinting produced a very similar number of genotype groups, however, the clustering of strains was very different when the two methods were compared (Liu *et al.* 2014). Similarly, Pfliegler *et al.* (2014) used RAPD and micro/minsatellite fingerprinting with altogether 5 different primers to characterize ~50 *Starmerella bacillaris* (*Candida zemplinina*) isolates and found that the

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use of different primers greatly affected the clustering of strains. Identical or almost identical strains with one method often showed surprisingly less similarity when another one was applied, despite that the overall diversity of different strains from a wide range of geographic locations was relatively low. Subsequently, isolates from the same species have been compared with microsatellite typing specifically designed for the species' microsatellite loci after it's genome was sequenced. That method proved to produce a higher level of diversity and more detailed clustering (Masneuf-Pomarede *et al.* 2015). The same method was also successfully used in the case of the species *Torulaspora delbrueckii* (Albertin et al. 2014).

Based on these observations the use of fingerprinting methods may have limitations when used to assess microbial diversity. For example, in the field of food microbiology, the important physiological characteristics and the genetic diversity of strains may not be correlated correctly by one or another fingerprinting method, especially when different methods yield conflicting results in the grouping of strains. Being the most important microorganisms in the production of fermented beverages, the physiological traits of *S. cerevisiae* and other wine yeasts are crucial in understanding the whole process of fermentation. *S. cerevisiae* is arguably the best-known and most intensively researched among these and several methods are routinely used by different laboratories for assessing its genetic diversity. One such method is the interdelta genotyping, a PCR-based method used for amplifying genomic regions located between the more-or-less randomly distributed delta sequences (Legras & Karst 2003; Tristezza *et al.* 2009). The interdelta-PCR (also δ-PCR) results in a varying number of fragments of different size, these are visualized by gel electrophoresis or analyzed by capillary electrophoresis and the yeasts are clustered based on the number and size of their fragments, much like with the methods of RAPD or ALFP. In this work, we aimed to determine whether the physiological diversity and geographic origin

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of a group of *S. cerevisiae* wine yeasts is consistent with the clustering obtained with interdelta genotyping, and, whether different primer pairs for interdelta analysis produce comparable results.

To test the interdelta genotyping method from these aspects, we chose to run a fingerprinting analysis of the *S. cerevisiae* strains described in Csoma *et al.* (2010) who identified and characterized altogether 86 *S. cerevisiae* and *S. uvarum* strains with a combination of molecular genetic and physiological analysis. The strains were isolated from spontaneously fermenting wines in four wine regions of Hungary (Badacsony, Kunság, Mecsekalja and Szekszárd regions). A very high diversity of the examined features of *S. cerevisiae* isolates was recorded with almost every isolate possessing a unique combination of properties. This diverse set of wine yeasts is ideal for testing whether the variations of the interdelta-genotyping method yield a consistent clustering of the strains.

Results and discussion

PCR conditions and reproducibility.

We used two primer pairs for interdelta analysis, delta1-2 and delta12-2 and tested all strains with two separately isolated stocks of genomic DNA to evaluate reproducibility. In the first set of experiments, different PCR conditions were also tested. Various authors have used a great variety of PCR conditions and especially Tm temperatures for interdelta genotyping [e.g. 42 and 46°C by Legras & Karst (2003), 52°C by Siesto *et al.* (2012)]. We tested two Tm temperatures, 45°C and 55°C using 4 randomly chosen *S. cerevisiae* and additionally, 5 different *S. uvarum* strains (strains 3, 12, 29, 68 and 71) identified and described by Csoma *et al.* (2010). Interdelta genotyping is known to produce no bands for *S. uvarum* (Legras &

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Karst 2003) and thus its strains were used as negative control. At the lower Tm, several weak bands in the range of 1000-8000 bp were obtained from *S. uvarum* (much larger bands than normally expected with interdelta genotyping), while these were completely absent or almost unrecognizably faint when using the higher Tm. *S. cerevisiae* strains, however, displayed almost identical patterns with the two different conditions and furthermore, both stocks of gDNA produced the same band patterns for each strain. Thus we concluded that the higher Tm is favorable for the genotyping experiments. This observation held true for both primer pairs.

Comparison of different primer sets.

In their work dedicated to the interdelta optimization, Legras and Karst (2003) concluded that the delta12-2 primer combination allows to produce more diverse sets of bands from wine yeasts than the traditionally used delta1-2 pair and recommended using the former pair or the delta12-21 pair for genotyping. In that study, 3 primer pairs and 53 laboratory and commercial strains were tested and the differences in clustering of the strains with the traditional and the newly designed primer pairs was not discussed in detail. We analyzed the patterns obtained from our collection of wine strains using delta1-2 and delta12-2 combinations. In contrast to the mentioned analysis, the strains in this study were all wine yeasts obtained from naturally fermenting must from different wine regions of Hungary, and from 2 subsequent vintage seasons. This means that our set of strains includes yeasts of less diverse origin (as they were all isolated from similar environments from locations not more than ~300 km apart from each other).

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In our study, well-reproducible electrophoretic band patterns with both primer pairs were obtained. Most sets of bands localized between ~250-2500 base pairs (bp) with delta1-2 primers (4 to 12 different bands were obtained for each strain) and between ~50-600 bp with delta12-2 (4 to 13 different bands were obtained for each strain) (Figure 1a-b). These results are broadly similar to those of Legras and Karst (2003) who obtained strong bands of ~280-1200 bp for the former primer pair and bands of ~100-870 bp with the latter primer pair. The latter primer pair produced a somewhat more diverse set of bands and the average similarity of the strains was lower with this primer pair (mean similarity was 0.600 with delta1-2 vs. 0.456 with delta12-2 when similarities of all strain pairs were evaluated). Supplementary Figure S1a-b. shows the two different dendograms obtained with the two primer sets. In some cases, delta1-2 PCR produced different band patterns for strains that were identical with delta12-2 PCR reaction (strains 11-372 and 11-375; strains 11-381 and 11-384).

Surprisingly, the two dendograms generated for the two primer sets in this study both contained numerous clades, but were highly different in terms of overall topology and strains that were located on the same branch of the UPGMA dendogram with one primer pair were placed on highly diverging branches when the other primer pair was used (Figure S1). This means that the two primer pairs amplified different genomic interdelta regions and thus produced practically uncomparable band patterns. For example, 22 pairs of strains were obtained with delta1-2 that differed from each other by only one band. Of these, only 8 pairs were grouped together in the same way when delta12-2 was used. The strains of the remaining pairs were placed on different branches of the delta12-2 dendogram. These results highlight the fact that different fingerprinting primers, even if designed for the same target (the delta sequences), often produce highly different and incomparable clusterings of strains and this may also be the case when interdelta genotyping is compared with other methods. Our results concerning the two different primer sets are summarized in Figure 2., where the

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similarity indices (obtained using delta1-2 or delta12-2) for all strain pairs are compared and plotted against each other. Similarity indices obtained with the two primer sets showed very low correlation (r^2=0.1139).

Comparison of phenotype, geographical origin and interdelta results

We could not detect any geographical clustering of the strains regardless of which primer pair was used for genotyping. Similar strains often had different geographical origins and strains located on distant branches were often collected from the same location and the same vintage year. The high number of strains collected in Badacsony wine region resulted in clusters with a majority of strains originating from this region, but nevertheless, these clusters never consisted of purely Badacsony strains (Figure S2). These observations indicate that these wine regions do not have isolated *Saccharomyces* populations and there is a considerable exchange of strain between them, or the accidental presence of commercial starter yeasts obscure the differences between the spontaneously fermenting musts from which the trains originated from. Or the interdelta analysis is not necessarily applicable as a geographic population analysis of these yeasts. As the two primer pairs gave conflicting clusterings of the strains, the latter possibility seems more likely.

Similarly, strains that had similar phenotypes proved to have highly dissimilar interdelta patterns, and genotypically similar strains (e.g. 11-621 vs. 11-742, 11-745 vs. 11-773, 11-771 vs. 11-793, and 11-946 vs. 11-956 on the delta1-2 dendogram; 11-771 vs. 11-793, and 11-844 vs. 11-928 on the delta12-2 dendogram; 11-771 vs. 11-793, and 11-844 vs. 11-928 on the combined dendogram) had different physiological profiles. We compared the positions of strains on the dendograms and the characteristic features of the strains [outstandingly high osmotic, ethanol or copper tolerance as described in Csoma *et al.* (2010)]

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and the clustering of the strains based on metabolite profiling after microfermentations conducted at 14°C and 26°C, as described in the supplementary files of Csoma *et al.* (2010). The latter data was only available from the source article for 29 strains. These comparisons are visualized on Figure S2. Similarly, in a recent analysis by Aponte and Blaiotta (2016), yeasts of the Taurasi DOCG region in Italy were characterized with a detailed physiological evaluation and determination of interdelta genotypes. The authors noted that isolates with the same interdelta pattern showed different technological traits and their physiological clustering did not correlate with interdelta grouping.

From our results two conclusions can be drawn. (1) The use of interdelta genotyping for wine yeasts yields a large number of PCR bands that can be used to differentiate strains with high resolution, especially when the results obtained by the two primer sets are combined, in one dendogram, as shown in Figure S1c. Importantly, the use of a single primer set is not enough to estimate genetic relatedness between strains. (2) The clustering topology and the similarity indices yield little or no information on the geographic and physiological relatedness of the isolates, even when results of two primer sets are combined, as clusterings with two different primer sets produce such contradictory results.

In the field of the microbiology of fermented beverages, strain genotyping is often the first choice for a preliminary characterization of a large number of strains isolated from a single source, e.g. a winery (Liu *et al.* 2015). This preliminary characterization is useful to reduce the number of strains that are subsequently characterized by more time-consuming methods (e.g. physiological characterization and trial fermentation). In this way strains with identical genotypes may be treated as identical or near-identical and genotype categories can be generated - one strain of each category can subsequently be used for further experiments (e.g. Liu *et al.* 2015). However, based on our results and previous findings (e.g. Pfliegler *et al.* 2014), the use of genotyping for reducing the number of strains or isolates, especially

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when only a single RAPD or interdelta primer/primer combination is used, could lead to the loss of physiologically diverse yeasts and thus to misleading results regarding the diversity of a strain collection.

PCR-fingerprinting methods probably have an inherent limitation regarding the comparability of band pattern similarities and strain relatedness. Similarity of band patterns obtained with a single primer or primer combination does not necessarily indicate genetic relatedness, nor physiological conformity. Nevertheless, the use of different primers or primer combinations may reliably identify genetically closely related strains, especially if band patterns of two strains are identical. Combined methods are in use [e.g. RAPD combined with interdelta genotyping with two primer sets were used by Šuranská *et al.* (2016)], but in these cases the individual methods and physiological characterization are rarely compared. Whether similar and closely related strains are physiologically close to each other is unpredictable. In an era when whole-genome sequencing enables the species-specific development of new strain typing primer sets (e.g. Masneuf-Pomerade et al. 2015), it will be crucial that the development of new methods is followed by surveying their limitations in (1) strain resolution, (2) recovering geographic origin, and, most importantly in food microbiology, (3) physiological similarity.

Materials and methods

Strains and genomic DNA

The altogether 69 *S. cerevisiae* strains used in this study are listed in Table S1. and correspond to the strains and numbering used by Csoma *et al.* (2010). Strains were kept in the strain collection of the Dept. of Genetics and Applied Microbiology (University of Debrecen) in 30% glycerol stocks at -70°C.

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PCR primers used for interdelta genotyping.

The primers used in this study were described by (Ness *et al.* 1993; Lavallée *et al.* 1994; Legras & Karst 2003). The primer sequences are as follows: delta1 (5’-CAAAATTCACCTATWCTCA-3’), delta2 (5’-GTGGATTTTTATTCCAACA-3’) and delta12 (5'-TCAACAATGGAATCCCAAC-3'). PCR reactions were carried out with the following primer combinations: delta1-2 and delta 12-2 (Legras & Karst 2003).

Conditions for interdelta-PCR.

DNA was isolated and purified according to the method described in Hanna and Xiao (2006). Concentration of the genomic DNA for RAPD-PCR was measured with an UVS-99 Micro-Volume UV/Vis Spectrophotometer (ATCGene) and DNA-concentrations were set to 100ng/µl subsequently. For each reaction, 50 ng was used. PCR reactions were performed with the following programs: 94°C for 5 min, 30x(94°C 50 sec, 55°C 50 sec, 72°C 50 sec), 72°C 5 min. For amplification, GoTaq® DNA Polymerase was used with GoTaq Green Buffer (Promega) according to the manufacturer's instructions. 25 pmols of primers were used per reaction. PCR reactions were conducted using an Applied Biosystems 2720 thermal cycler in a final volume of 25 µl.

Gel electrophoresis and analysis

PCR-products were loaded onto 2% w/v agarose gels stained with ethidium-bromide and electrophoresis was carried out with 90V for 60 minutes in 1xTBE buffer, visualization was performed under UV light. A 1 kb DNA ladder marker and a PhiX174/HinfI marker served as size standards (Thermo Scientific). Negative controls were used for each round of

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experiments. PCR patterns were acquired using a MiniBIS Pro transilluminator (DNR Bio-Imaging Systems Ltd.) and the GelCapture version 5.8 software. PCR reactions were carried out at least twice in separate reactions with separately isolated DNA stocks to test reproducibility and only unambiguous bands were used in the analysis. Patterns were analyzed using the GelAnalyzer 2010 software (http://www.gelanalyzer.com/) with manual adjusting. Similarity indices were calculated and dendograms were generated using the unweighted pair group method with arithmetic averages (UPGMA) algorithm available at http://genomes.urv.es/UPGMA/ (Garcia-Vallvé *et al.*, 1999) with Dice coefficient. Trees were created with FigTree v1.4.2.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Figure legends:

Figure 1.a. Examples of delta-PCR patterns. a: with primer combination delta1-delta2. b. with primer combination delta12-delta2. M1kb: 1 kb size marker. MPhi: PhiX174/HinfI size marker.

Figure 2. Comparison of the two different dendograms. Similarity indices obtained with the two primer sets for all strain pairs are plotted against each other.

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Supporting Information

Table S1. List of *S. cerevisiae* strains used in this study.

Figure S1. Dendogram of the strains based on the patterns obtained with primers delta1-2 (S1a), delta12-2 (S1b) and dendogram based on combined patterns (S1c).

Figure S2. Geographic origin and physiological characteristics of the strains as described by Csoma *et al.* (2010), depicted on the dendograms generated with the two primer pairs and the combined dendogram.

Legend:

† High osmotolerance (15-25x increase in optical density in 60% glucose, 96 hours after inoculation); ‡ Very high osmotolerance (>25x increase in optical density in 60% glucose, 96 hours after inoculation); ○ High ethanol tolerance (minimal inhibotory concentration >20% v/v); ◊ High copper tolerance (minimal inhibotory concentration >2 mM); **B** wines produced in microfermentation at 14°C have high levels of acetaldehyde and acetic acid and variable levels of ethyl-acetate, n-propanol, acetoin, amyl alcohols and isobutanol (cluster B in Csoma et al.); **C** wines produced in microfermentation at 14°C have low-to-medium levels of acetaldehyde, acetic acid, ethylacetate, n-propanol, acetoin, amyl alcohols and isobutanol (cluster C in Csoma et al.); **D** wines produced in microfermentation at 26°C have low levels of n-propanol, acetoin and acetaldehyde and medium-to-high levels of amyl alcohols (cluster D in Csoma et al.); **E** wines produced in microfermentation at 26°C have high levels of ethyl-acetate and acetic acid and variable content of acetaldehyde, n-propanol, acetoin, amyl alcohols and isobutanol (cluster E in Csoma et al.); **G** wines produced in microfermentation at 26°C have low levels of acetaldehyde, acetic acid, ethyl-acetate, n-propanol, acetoin, amyl alcohols and isobutanol (cluster G in Csoma et al.); **Badacsony: Németh 2006**

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Spontaneously fermenting 'Kéknyelű' must, Németh Winery, Badacsony wine region (2006); **Badacsony: Németh 2007 O** Spontaneously fermenting 'Olaszrizling' must, Németh Winery, Badacsony wine region (2007); **Badacsony: Németh 2007 K** Spontaneously fermenting 'Kéknyelű' must, Németh Winery, Badacsony wine region (2007); **Mecsekalja: Res. Inst. 2006** Spontaneously fermenting 'Cirfandli' must, Research Institute of Viticulture and Enology, Pécs, Mecsekalja wine region (2006); **Mecsekalja: Res. Inst. 2007** Spontaneously fermenting 'Cirfandli' must, Research Institute of Viticulture and Enology, Pécs, Mecsekalja wine region (2007); **Szekszárd: Pálos 2006** Spontaneously fermenting 'Kadarka' must, Pálos Miklós Winery, Szekszárd wine region (2006); **Kunság: Frittmann 2006** Spontaneously fermenting 'Ezerjó' must, Frittman Brother Ltd., Soltvadkert, Kunság wine region (2006); **Kunság: Frittmann 2007** Spontaneously fermenting 'Ezerjó' must, Frittman Brothers Ltd., Soltvadkert, Kunság

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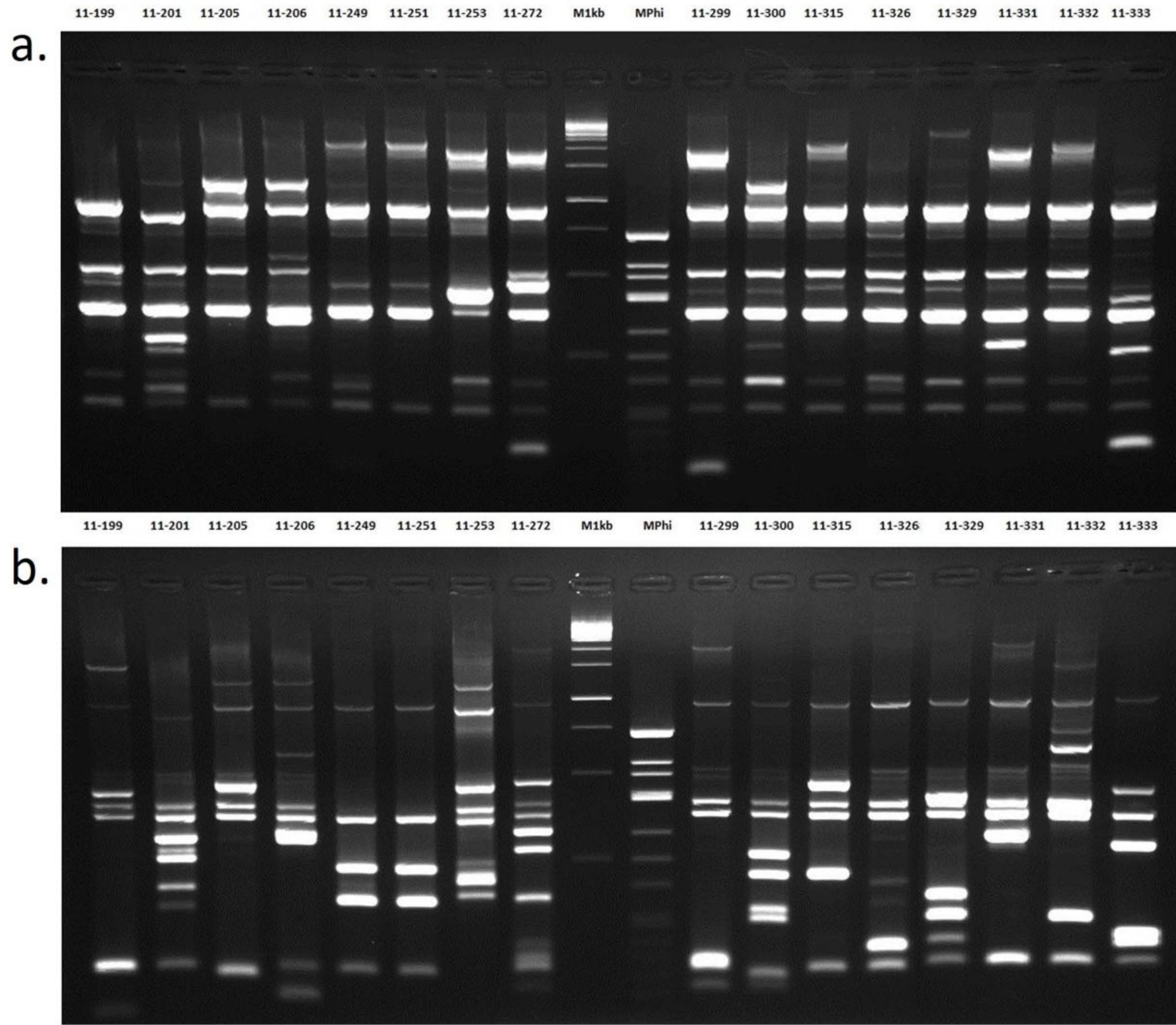

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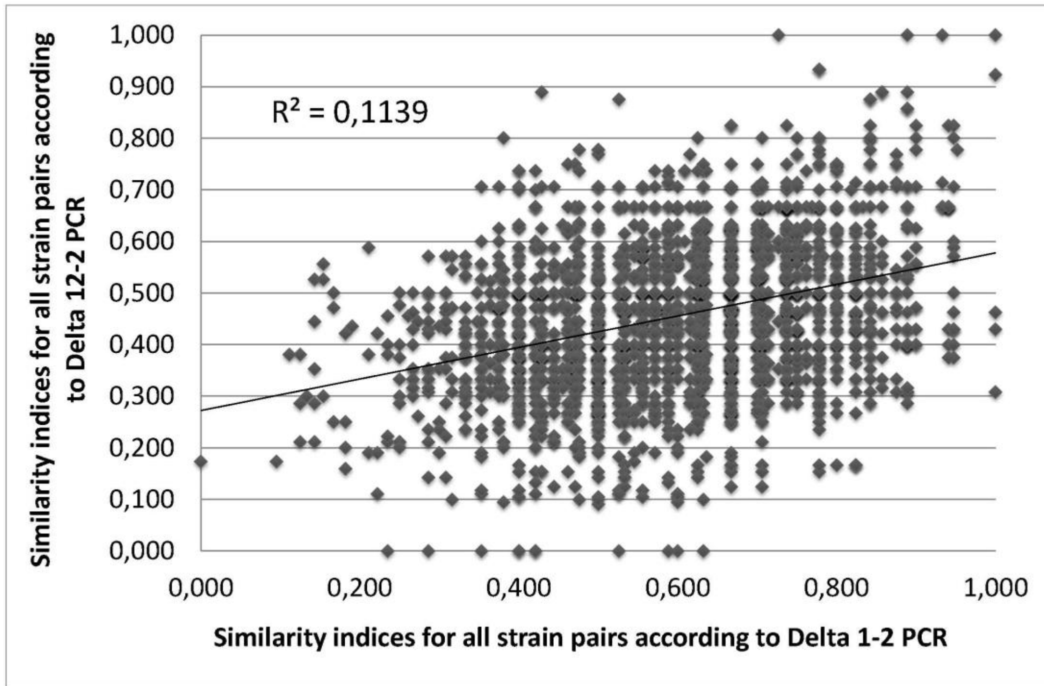

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