Randomly amplified polymorphic DNA (RAPD) for discrimination of *Pediococcus pentosaceus* and *Ped. acidilactici* and rapid grouping of *Pediococcus* isolates

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A. NIGATU, S. AHRNÉ, B.A. GASHE AND G. MOLIN. 1998. In a taxonomic study of the known *Pediococcus* species, together with 116 isolates from fermenting *tef* dough and fermented *kocho* using a PCR-based RAPD procedure, all the different species developed well differentiated agarose gel electrophoresis profiles. Analyses of the images with the Pearson product moment correlation coefficient (r) and UPGMA clustering algorithm in the GelCompar version 4·0 software gave a distinct identification scheme within and between type strains and food isolates of *Pediococcus* species. The procedure is simple, rapid for grouping of isolates, applicable to all species of pediococci and particularly useful for differentiating between strains of *Ped. pentosaceus* and *Ped. acidilactici*.

INTRODUCTION

Pediococcus species are major lactic acid microflora in fermenting plant materials (Garvie 1986). As a result of their widespread habitats and physiological adaptations, pediococci are frequently isolated from beverages, fermenting foods and dairy products. They commonly develop with other plant associated lactic acid bacteria (LAB) (Garvie 1986; Tanasupawat *et al*. 1993).

Pediococci are often isolated from Ethiopian traditional plant derived lactic acid fermented foods such as *tef* dough and *kocho* (Gashe 1985, 1987; Nigatu and Gashe 1994a,b). However, as available phenotypic procedures are limited, it is difficult to assign isolates to known species as it is hard to differentiate easily and clearly between species of pediococci. This is particularly true of *Pediococcus pentosaceus* and *Ped. acidilactici* (Tanasupawat *et al*. 1993; Judicial Commission 1996). The phenotypic procedures in use are time consuming, laborious and imprecise and this has hampered distinction of field isolates for taxonomic purposes. Therefore, although detailed taxonomic studies on pediococci date back to 1884 (Garvie 1986), there is still a lack of precise and rapid procedures for clearly identifying an isolate belonging to this genus to the species level.

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The use of DNA base composition and DNA–DNA hybridization results as applied to bacterial taxonomy has been useful and accurate (Garvie 1986; Ezaki *et al*. 1989). Nevertheless, as these are tedious and expensive procedures requiring sophisticated laboratory equipment, it is still not feasible to use them for routine identification. To the authors' knowledge, there is no rapid and suitable molecular genetic taxonomic procedure in common use for identification of pediococci.

Randomly amplified polymorphic DNA (RAPD) is an accurate technique, easily adoptable in most laboratories, which has proved useful for strain typing in *Lactobacillus plantarum* (Johansson *et al*. 1995) and in differentiating *Lactobacillus* species not easily discriminated using phenotypic tests (Van Reenen and Dicks 1996). In this paper, the application of a PCR-based RAPD procedure is described for clear distinction of the species of *Pediococcus* and rapid identification and grouping of field strains from Ethiopian fermented foods. In particular, the clear separation of *Ped. pentosaceus* and *Ped. acidilactici* employing this method is emphasized.

MATERIALS AND METHODS

Strains

Serial dilutions of fermented *tef* dough and *kocho* samples were seeded on Rogosa agar and incubated in a BBL GasPak

system, (BBL, Becton Dickinson Microbiological Systems, Cockeysville, MD, USA) at 30 °C. Two hundred Gram-positive, catalase-negative cocci occurring in pairs or tetrads were randomly picked from grown colonies and sub-cultured in modified *Lactobacillus*-carrying medium, LCM (Efthymiou and Hansen 1962) containing 2% glucose. They were further purified, alternately on solid or in broth LCM, six to nine times and purity was checked microscopically. Seven type strains of *Pediococcus* (obtained from Culture Collection University of Gothenburg, CCUG, Sweden), namely *Ped. urinaeequi* CCUG 28094^T, *Ped. parvulus* CCUG 28439^T, *Ped. dextrinicus* CCUG 18834^T, *Ped. acidilactici* CCUG 32235^T, *Ped. pentosaceus* CCUG 32205^T, *Ped. inopinatus* CCUG 38496^T and *Ped. damnosus* CCUG 32251^T were also sub-cultured twice on LCM agar. The type strains and isolates were subjected to PCR and agarose gel electrophoresis. All strains were stored at −80 °C in a freezing buffer (containing 3·6 mmol l^{-1} K_2HPO_4, 1·3 mmol l^{-1} KH_2PO_4, 2·0 mmol l^{-1} Na-citrate, 1·0 mmol l^{-1} $MgSO_4$ and 12% glycerol) (Ahrné *et al*. 1989) until used.

Preparation of crude cell extract

Overnight cultures (1 ml) grown in LCM at 30 °C in Eppendorf tubes were centrifuged at 14 000 rev min^{-1} for 5 min and the cells washed with 1 ml sterile double distilled water (SAQ) twice. To each pellet was added 0·25 ml SAQ and about eight sterile glass beads (0·2 mm diameter). Cells were disintegrated by thorough cold shaking for 30–45 min using an Eppendorf Mixer (Model 5432, Eppendorf, Hamburg, Germany) at 4 °C. The tubes were then centrifuged for 5 min and clear supernatant fluid was transferred into sterile Eppendorf tubes and kept at −20 °C until further use.

PCR processing

Into a reaction tube was added 34·5 μl SAQ, 5 μl of 10 × PCR reaction buffer (Boehringer Mannheim Scandinavia, Bromma, Sweden), 1 μl each from dATP, dCTP, dGTP and dTTP (Perkin Elemr, Branchburg, NJ, USA) 5 μl Primer 73 (a 9-mer with a base sequence of 5′-ACGCGCCCT-3′, Symbicon AB, Umeå, Sweden), 1 μl of crude cell extract and 0·5 μl of Taq polymerase (Boehringer Mannheim Scandinavia). The buffer contained 1·5 mmol l^{-1} $MgCl_2$. The reaction mixture was overlaid with mineral oil and the amplification was done in a Perkin Elmer Cetus DNA thermal cycler (Model 480, Perkin Elmer, Norwalk, USA). The cycles performed had the following temperature profile: 94 °C, 45 s; 30 °C, 120 s; 72 °C, 60 s for four cycles followed by 94 °C, 5 s; 36 °C, 30 s; 72 °C, 30 s for 26 cycles; the extension step was increased by 1 s for every new cycle. The PCR amplification reaction was terminated at 75 °C for 10 min, after which samples were cooled to 4 °C.

Gel electrophoresis and photography

Conventional gel electrophoresis was carried out as follows. Submerged horizontal slab gels with 1·5% agarose (Type III:High EEO, Sigma, St Louis, MO, USA) in single strength TB buffer (89 mmol l^{-1} boric acid, 23 mmol l^{-1} H_3PO_4, 2·5 mmol l^{-1} EDTA, pH 8·3) as described by Johansson *et al*. (1995), were prepared and run at 100 V for 2·5 h in a TB electrophoresis buffer without cooling. Aliquots (20 μl) from each sample were mixed with 5 μl of dye; 3 μl DNA molecular weight marker VI (Boehringer Mannheim Scandinavia) mixed with 5 μl dye and 17 μl distilled water served as a standard (in all cases, fresh buffer was used for every gel electrophoresis run). Gels were rinsed with distilled water and then stained in ethidium bromide (0·2 μg ml^{-1}) for 5 min. Following this, they were kept in distilled water in a tray for another 5 min. De-wetted gels were visualized and then photographed at 302 nm on a u.v. transilluminator board (UVP Inc., San Gabriel, CA, USA) with a polaroid camera loaded with polaroid film (Polaroid, Number 665, Polaroid Corporation, Cambridge, MA, USA) and developed as recommended by the manufacturer.

Image preparation, comparison and reproducibility

Developed bands on the photo positive and negative were scanned with a flatbed Scanner (UMAX UC630 Max Colour) at a resolution of 200 dots per inch.

The length of each lane finally tracked corresponded to the length of the lane for the DNA molecular weight markers. All scanning, tracking, normalizing of data and analyses of the normalized gels were done using GelCompar Version 4·0 program (Applied Maths, Kortrijk, Belgium). The percentage similarity was based on the compared zones 8–386 (i.e. the length corresponding to that of the DNA molecular markers) in all cases representing the best lane zone for the DNA molecular weight marker as found satisfactory on the reference gel. Similarity matrix and cluster analysis of the matrix of similarity values were calculated using the Pearson product moment correlation coefficient (r) with UPGMA (unweighted pair group method using arithmetic averages) and Ward's clustering algorithms. Dendrograms were produced, and major clusters with a cut-off point of 33% or above in the UPGMA and 85% in the Ward's clustering analyses similarity levels (relatedness) were taken as representing a single cluster.

In order to check the reproducibility of the RAPD procedure, duplicate fresh cultures were run in different experiments from all of the seven type strains and five field isolates and the resultant gels analysed.

RESULTS

Agarose gel electrophoresis band profiles of the crude DNA extracts from the type strains of *Pediococcus* are shown in Fig. 1.

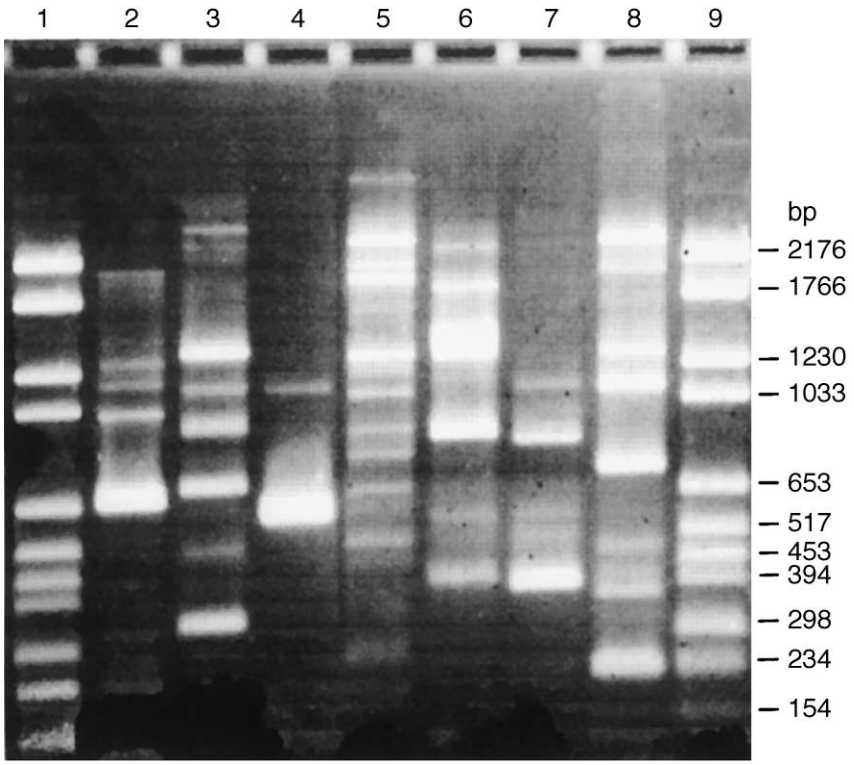

Fig. 1 RAPD agarose gel electrophoresis band profiles with ethidium bromide of type strains of *Pediococcus* species. Lanes 1 and 9 represent the reference DNA molecular weight (bp) marker VI and lanes 2–8 are bands for *Ped. acidilactici* CCUG 32235^T, *Ped. pentosaceus* CCUG 32205^T, *Ped. inopinatus* CCUG 38496^T, *Ped. parvulus* CCUG 28439^T, *Ped. damnosus* CCUG 32251^T, *Ped. dextrinicus* CCUG 18834^T and *Ped. urinaeequi* CCUG 28094^T, respectively

Band patterns developed for a type or a field strain used in duplicate experiments were stable and consistently the same. Each of the seven species showed distinct bands of differing molecular weights, revealing a specific DNA fingerprint for each species, and each one of the type strains had band patterns which related them to some field strains. For both the type and field strains, most of the bands developed lay within the length of the molecular weight marker (i.e. between 0·1 and 3·0 kilobases, kb). The number of bands per species varied between two and 12 (Fig. 1). As a result, 116 of the 204 food isolates fell into one or the other type strain as follows: *Pediococcus parvulus*, 33 isolates; *Ped. inopinatus*, eight isolates; *Ped. dextrinicus* and *Ped. damnosus*, one isolate each; *Ped. acidilactici*, 21 isolates; *Ped. pentosaceus*, 50 isolates; and *Ped. urinaeequi*, two isolates. The rest of the isolates were not closely related to any one of the seven type strains. Figure 2 shows this grouping pattern of the type strains with the 116 food isolates. As shown in the figure, the association and relatedness within and between the named species and isolates gave a high degree of similarity. The Pearson product moment correlation coefficient (r) with UPGMA clustering algorithm produced the best association and enabled the relatedness at a higher percentage to be defined by comparison with the Ward's method of cluster analysis (data not shown). As a result, clear differences were obtained between the different *Pediococcus* species, and clustering levels for the field isolates with the known strains were well defined. The degree of relatedness between the seven type strains of pediococci was above 89% S_J and individual values of similarities have been calculated in a matrix (data not shown). This similarity was very high and maximum relatedness was observed between the pairs *Ped. acidilactici* and *Ped. inopinatus*, and *Ped. damnosus* and *Ped. dextrinicus*, the similarity being 96% in each case.

Another observation was the advantage of using a photo negative over the positive. The clarity of the bands also enabled a visual comparison of lane differences.

DISCUSSION

In this study, the use of crude DNA extract for amplification using the PCR procedure described gave satisfactory results. The use of RAPD together with a G-C-rich primer and the specific DNA molecular weight marker VI easily developed characteristic agarose gel electrophoresis band profiles which gave genetic fingerprints for each of the type strains of *Pediococcus* species. It was found that the field strains from the two Ethiopian lactic acid fermented foods were grouped around the type strains and hence, easily identified. The amplification of a unique band pattern for each species also suggests the conservation of DNA.

Previous studies on DNA–DNA hybridization among

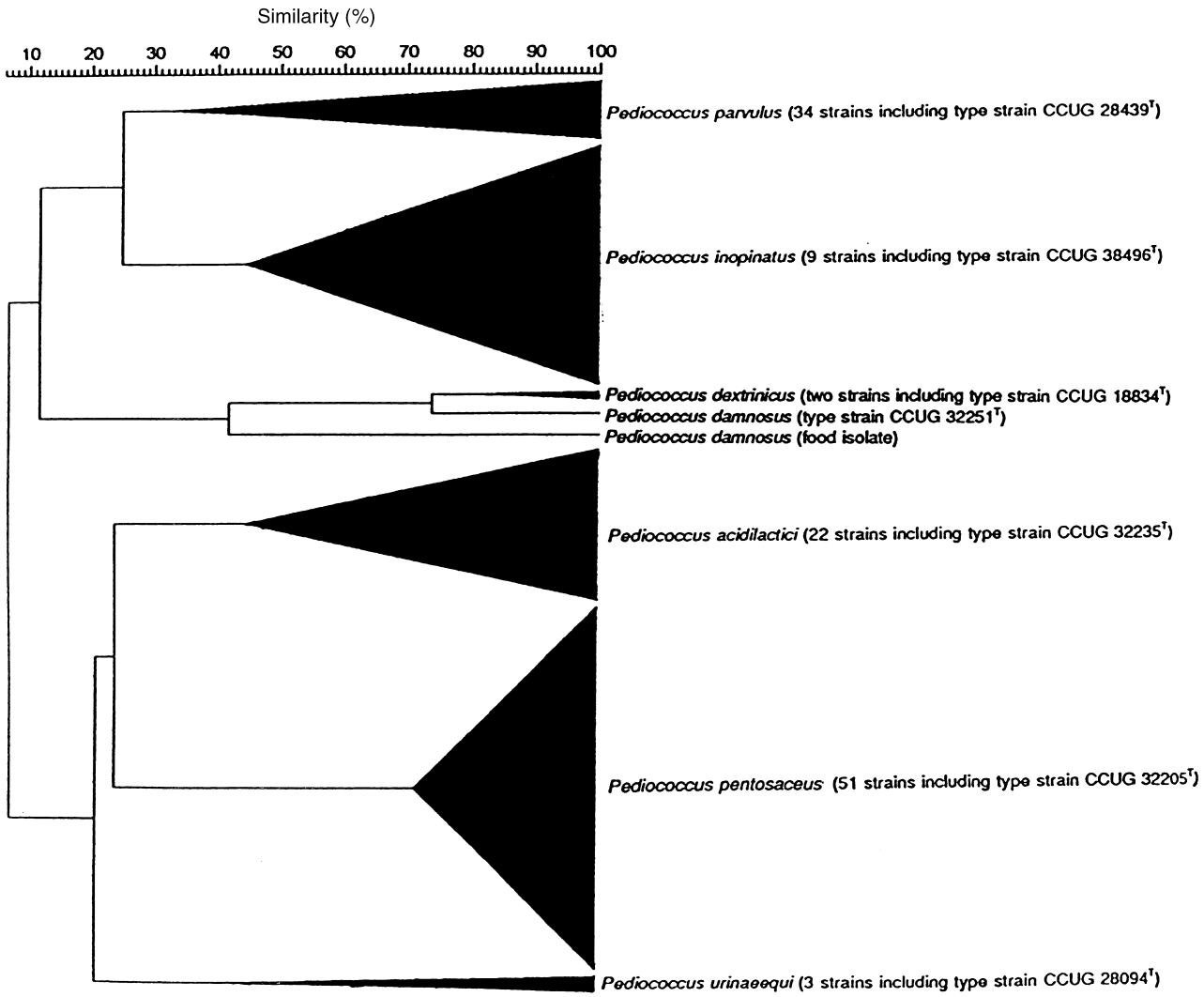

Fig. 2 Dendrogram, showing clustering profiles of type strains of *Pediococcus* species and food isolates, produced using the Pearson product moment correlation coefficient (r) and UPGMA clustering algorithm with the GelCompar version 4·0 program

species of the genus *Pediococcus* (Garvie 1986) have shown the occurrence of the highest degrees of homology between *Ped. damnosus* and *Ped. inopinatus* (40–50%).

In this study, the genetic relatedness of *Pediococcus* species indicated that *Ped. acidilactici* and *Ped. inopinatus* form one pair and *Ped. damnosus* and *Ped. dextrinicus* another. Both pairs revealed the maximum similarity value in the Ward's clustering algorithm and 67% in the UPGMA analysis (data not shown). The relatedness amongst these seven species was also high, as the minimum value was 89·6% for *Ped. damnosus* or *Ped. dextrinicus* and the rest of five species. *Pediococcus pentosaceus* and *Ped. acidilactici* were clearly differentiated when all the type strains were used for assigning isolates as shown in the dendrogram (Fig. 2) and also, when the type strains were compared individually with one another (Fig. 1). In this study, *Ped. pentosaceus* has been found to be much closer to *Ped. parvulus* than to *Ped. acidilactici*. A higher percentage of relatedness was also observed between the two species in this study as opposed to the very low values from DNA:DNA hybridization studies ($< 10\%$) reported previously (Garvie 1986). A similar pattern of discrepancy has also been found between the two methods in the similarity values for *Ped. acidilactici* with *Ped. inopinatus*, or *Ped. damnosus* with *Ped. dextrinicus*, which were very high in this study for both relationships (data not shown), while they were $< 10\%$ in the DNA:DNA hybridization studies (Garvie 1986). This difference might be ascribed to the differences in the two methods of study. Nevertheless, there was agreement

between the two studies regarding the degree of relatedness between *Ped. dextrinicus* and the other species which in both methods, had lower values.

From these results, RAPD as a genetic tool was found to be very efficient for discriminating as well revealing the degree of relatedness of isolates, provided that the procedure was properly followed. In comparison to hybridization procedures, RAPD is cheaper, more rapid and requires less labour. Its reproducibility is high, as observed when using the same procedure consistently for the same strains in different experiments. It is also possible to study a very large number of field isolates or populations in a very short time.

The statistical software used has also proved its wider application as a tool for pediococci taxonomy as supplemented with the UPGMA and Ward's clustering algorithms. The UPGMA clustering analysis has been used to set cut-off points at definite percentages of relatedness between the field and type strains, at the same time thereby clearly separating the different named species at lower percentages (Fig. 2). The Ward's method of analysis was helpful for determining the relationships at higher similarity levels.

Finally, it is possible to reuse the database and the gels produced whenever they are needed, rather than one time physiological and phenotypic observations and records which are subjective and prone to various drawbacks.

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