

Polyphasic study of wine *Lactobacillus* strains: taxonomic implications

A. M. Rodas, S. Ferrer and I. Pardo

Correspondence

I. Pardo

Isabel.Pardo@uv.es

ENOLAB – Laboratori de Microbiologia Enològica, Departament de Microbiologia i Ecologia, Facultat de Ciències Biològiques, Universitat de València, Dr Moliner 50, 46100 Burjassot, València, Spain

One hundred and seventy-eight lactobacilli isolated from wine were characterized by a polyphasic approach. Strains were phenotypically identified at genus and species level by classical tests including the analysis of cell morphology, homo/heterofermentative character, sugar fermentation patterns, growth at different temperatures and the optical nature of the isomer of lactic acid produced from glucose. Molecular techniques such as random amplification of polymorphic DNA (RAPD), amplified 16S rDNA restriction analysis (16S-ARDRA), PFGE-RFLP and ribotyping were used to characterize strains, and their potential for identification and/or typing was evaluated. The information obtained with these techniques was processed with the BioNumerics software in order to analyse relationships existing between isolated strains and various reference species of the genus. Then, taxonomic dendrograms were obtained, and this information allowed the proposal of molecular procedures suitable for the identification and typing of these wine micro-organisms. The techniques useful for both identification and typing were RAPD and ribotyping, while 16S-ARDRA was only useful for identification and PFGE-RFLP only for typing purposes. The wine strains were identified as *Lactobacillus brevis* (19 strains), *Lactobacillus collinoides* (2 strains), *Lactobacillus hilgardii* (71 strains), *Lactobacillus paracasei* (13 strains), *Lactobacillus pentosus* (2 strains), *Lactobacillus plantarum* (34 strains) and *Lactobacillus mali* (10 strains).

INTRODUCTION

Lactobacillus is a genus of lactic acid bacteria (LAB) described as a heterogeneous group of 'regular non-sporing Gram-positive rods' (Kandler & Weiss, 1986). Lactobacilli are found in a great variety of habitats (Hammes *et al.*, 1991). The genus *Lactobacillus* consists of more than 110 species with validly published names (Euzéby, 1997). They are genetically quite diverse; their G+C content ranges from 32 to 54 mol%. This shows that *Lactobacillus* is not a well-defined genus (Vandamme *et al.*, 1996), as has been demonstrated by the transfer of some *Lactobacillus* species to newly created genera: *Carnobacterium* (Collins *et al.*, 1987), *Atopobium* (Collins & Wallbanks, 1992), *Weissella* (Collins *et al.*, 1993) and *Paralactobacillus* (Leisner *et al.*, 2000). These reorganizations are mainly supported by

DNA–DNA reassociation studies and 16S rRNA gene sequence data (Collins *et al.*, 1991; Schleifer & Ludwig, 1995).

The classical division of the lactobacilli is based on their fermentative characteristics: (i) obligately homofermentative, (ii) facultatively heterofermentative and (iii) obligately heterofermentative. Identification to the species level by simple phenotypic tests may sometimes be difficult because most of the bacteria have very similar nutritional requirements and grow under similar environmental conditions (Vandamme *et al.*, 1996).

The development of molecular biological methods such as cellular fatty acids analysis (Rizzo *et al.*, 1987), whole-cell protein analysis (Dicks & van Vuuren, 1988; Patarata *et al.*, 1994), DNA–DNA hybridization (Schillinger *et al.*, 1989; Lonvaud-Funel *et al.*, 1991; Sohier & Lonvaud-Funel, 1998), electrophoretic patterns of peptidoglycan hydrolases (Lortal *et al.*, 1997) and ribotyping (Zhong *et al.*, 1998; Motoyama *et al.*, 2000) have proved useful for identification of some species of lactobacilli. Among PCR-based methods, species-specific primers (Sohier *et al.*, 1999; Chagnaud *et al.*, 2001), internal *rrn* spacer region primers (Berthier & Ehrlich, 1998; Nour, 1998; Dubernet *et al.*, 2002), random amplification of polymorphic DNA (RAPD) (Cocconcelli *et al.*, 1995;

Abbreviations: 16S-ARDRA, restriction analysis of the amplified 16S rRNA gene; LAB, lactic acid bacteria; RAPD, random amplification of polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences of strains 8, 59b, 68, 71, 88, 116, 154, 166, 203 and 459 determined in this study are AY681126–AY681135, respectively.

Additional dendrograms, examples of RAPD profiles and the 16S rRNA gene sequence similarity matrix are available as supplementary material in IJSEM Online.

Daud Khaled *et al.*, 1997; Nigatu *et al.*, 2001), analysis of repetitive bacterial DNA elements (rep-PCR) (Gevers *et al.*, 2001), tDNA-PCR (Baele *et al.*, 2002) and amplified 16S rDNA restriction analysis (16S-ARDRA) (Ventura *et al.*, 2000; Rodas *et al.*, 2003) have been employed.

In the present work, we have applied a set of molecular and phenotypic techniques to characterize wine lactobacilli, comparing them with reference strains in a polyphasic approach. The species found in wine are *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus collinoides*, *Lactobacillus coryniformis* subsp. *coryniformis*, *Lactobacillus fermentum* (= *Lactobacillus cellobiosus*; Dellaglio *et al.*, 2004), *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus fructivorans* (= *Lactobacillus trichodes*; Weiss *et al.*, 1983; Vandamme *et al.*, 1996), *Lactobacillus hilgardii*, *Lactobacillus kunkeei*, *Lactobacillus mali*, *Lactobacillus nagelii*, *Lactobacillus plantarum* and *Lactobacillus paracasei* subsp. *paracasei* (Edwards *et al.*, 1998, 2000; Rodas *et al.*, 2003). Reference strains of these species and other closely related species (Schleifer & Ludwig, 1995) were included in the analysis. Taxonomic dendrograms were deduced from the comparisons and taxonomic relationships were established. We also evaluated the discriminatory power of each individual method (RFLP-PFGE, RAPD, 16S-ARDRA, ribotyping) for identification and typing of 178 lactobacilli isolated from grape must and wine.

METHODS

Bacterial strains, growth conditions and storage. The 178 bacterial strains used in this study were obtained from a microbiological survey of 32 grape musts and wine samples, belonging to the Utiel-Requena and Jumilla Origin Denominations in Spain. *Lactobacillus* reference strains included in this study are listed in Table 1. All strains were grown in MRS broth (Scharlab) supplemented with L-cysteine hydrochloride (0.5 g l⁻¹) at 28 °C. Cultures were maintained frozen at -20 °C in 20 % v/v glycerol and lyophilized.

Isolation and phenotypic and biochemical characterization of strains. The grape must and wine samples were diluted and inoculated on MRS agar plates supplemented with cycloheximide (0.1 mg l⁻¹) to inhibit yeasts and L-cysteine hydrochloride (0.5 mg l⁻¹). The plates were incubated at 28 °C from 48 to 72 h.

Wine strains were purified on MRS agar and characterized as strains of *Lactobacillus* on the basis of their Gram-positive character, absence of catalase activity and production of lactic acid from hexose fermentation (Cappuccino & Sherman, 1987). Analysis of the final products of sugar catabolism was done by HPLC as described by Frayne (1986). Each strain was examined microscopically for cellular morphology and arrangement and for motility. We determined the type of glucose fermentation following the procedure described by Zúñiga *et al.* (1993).

Phenotypic identification was performed according to Hammes *et al.* (1991), who propose an range of different tests for identifying homofermentative or heterofermentative lactobacilli. The phenotypic traits studied were: (i) growth at 15 and 45 °C in MRS broth, (ii) production of acid from different carbohydrates in MRS fermentation broth (Scharlab) and (iii) the optical nature of the isomer of lactate formed, determined by HPLC and L-lactic kit (cat. no. 139084; Roche).

Table 1. Reference strains and 16S rRNA gene sequence accession numbers

Sources of strains are abbreviated as: CECT, Colección Española de Cultivos Tipo, Universidad de Valencia, Burjassot, Spain; ATCC, American Type Culture Collection, Manassas, VA, USA; NCFB, National Collection of Food Bacteria, c/o NCIMB Ltd, Aberdeen, UK; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. NA, No sequence available in the databases.

Taxon	Strain	Accession no.
<i>L. brevis</i>	CECT 216	M58810
<i>L. buchneri</i>	CECT 4111 ^T	M58811
<i>L. casei</i>	CECT 475 ^T	D16551
<i>L. casei</i>	ATCC 334	D86517
<i>L. cellobiosus</i>	CECT 562 ^T	AJ575812
<i>L. collinoides</i>	CECT 922 ^T	AB005893
<i>L. coryniformis</i> subsp. <i>coryniformis</i>	CECT 982 ^T	M58813
<i>L. curvatus</i>	CECT 904 ^T	AJ270951
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	CECT 4005 ^T	AB007908
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i>	CECT 286 ^T	M58814
<i>L. delbrueckii</i> subsp. <i>lactis</i>	CECT 282	AY050173
<i>L. farciminis</i>	CECT 571 ^T	M58817
<i>L. fermentum</i>	CECT 4007 ^T	M58819
<i>L. fructivorans</i>	NCFB 2167 ^T	M58818
<i>L. graminis</i>	CECT 4017 ^T	NA
<i>L. helveticus</i>	CECT 4305 ^T	X61141
<i>L. hilgardii</i>	NCFB 264 ^T	M58821
<i>L. mali</i>	CECT 4149	M58824
<i>L. nagelii</i>	DSM 13675 ^T	Y17500
<i>L. oris</i>	CECT 4021 ^T	X94229
<i>L. paracasei</i> subsp. <i>paracasei</i>	CECT 4022 ^T	D79212
<i>L. pentosus</i>	CECT 4023 ^T	D79211
<i>L. plantarum</i>	CECT 748 ^T	D79210
<i>L. reuteri</i>	CECT 925 ^T	X76328
<i>L. rhamnosus</i>	CECT 278 ^T	D16552
<i>L. sakei</i>	CECT 906 ^T	M58829
<i>L. zeae</i>	ATCC 15820 ^T	D86516

RFLP-PFGE. To perform this technique, DNA must be extracted in an agarose matrix to prevent mechanical DNA breakage. Genomic DNA in agarose plugs was prepared by a modification of the method of Matushek *et al.* (1996). Bacterial strains were grown in MRS to an OD₆₀₀ of 0.5, cells were harvested from 2.5 ml culture by centrifugation and resuspended in 0.5 ml 2× lysis buffer [12 mM Tris/HCl (pH 7.6), 2 M NaCl, 200 mM EDTA (pH 8.0), 1 % w/v Brij 58, 0.4 % w/v deoxycholate, 1 % w/v sodium lauroyl sarcosine] containing 1 mg lysozyme ml⁻¹ (Sigma), 60 µg RNase A ml⁻¹ (DNase-free; Sigma) and 40 U mutanolysin ml⁻¹ (Sigma). Cell suspensions were mixed with 0.5 ml of 1.5 % w/v low-melting-point agarose (SeaPlaque GTG agarose; FMC) and pipetted into a plug mould (syringe of 1 ml). Solidified plugs were cut with a sharp

blade forming slices of 1 mm thickness, which were incubated for 12 h at 37 °C in 6 ml of 1 × lysis buffer, containing 5 mg lysozyme ml⁻¹, 30 µg RNase A ml⁻¹ and 10 U mutanolysin ml⁻¹. After incubation, the lysis solution was replaced with 6 ml ES [0.5 M EDTA (pH 9.0), 1% w/v sodium lauroyl sarcosine] containing 100 µg proteinase K ml⁻¹ (Boehringer Mannheim) and 1% SDS, and incubated 6 h at 50 °C. The slices were washed three times with 10 ml TE 10/0.1 buffer [10 mM Tris/HCl (pH 8.0), 0.1 mM EDTA (pH 8.0)] and incubated with 10 ml TE 10/0.1 buffer for 1 h at 50 °C. The solution was replaced with 10 ml fresh buffer and then incubated at room temperature for 1 h. Finally, the slices were immersed in 10 ml TE 10/100 buffer [10 mM Tris/HCl (pH 8.0), 100 mM EDTA (pH 8.0)] for storage at 4 °C. Under these conditions, discs were stable for several months, without DNA degradation or apparent changes in quality. Before digestion, the slices were washed twice in TE 10/0.1 buffer for 1 h and equilibrated for 1 h in the appropriate restriction enzyme buffer. Single digestions with *Sfi*I, *Not*I and *Sma*I were performed for 27 h at 50, 37 and 25 °C, respectively. Electrophoresis was carried out in a CHEF DR II apparatus (Bio-Rad) in 1% w/v SeaKem GTG agarose (FMC) with 0.5 × TBE buffer (45 mM Tris/HCl, 45 mM boric acid and 1 mM EDTA, pH 8.0) at 15 °C. A constant voltage (200 V) was applied to the system and fragment separation was performed using a two-phase program. To separate the *Sfi*I and *Not*I fragments of obligately heterofermentative lactobacilli, a pulse time was applied from 0.5 to 20 s for 12 h and then another from 30 to 60 s for 8 h. For homofermentative and facultatively heterofermentative lactobacilli digested with *Not*I, a pulse time was used from 0.5 to 5 s for 12 h and then another from 5 to 10 s for 8 h. Electrophoretic conditions for separating the *Sma*I fragments in all lactobacilli were a pulse time from 0.5 to 5 s for 10 h and then another from 0.5 to 10 s for 4 h. MidRange PFG marker I or LowRange PFG marker (New England BioLabs) were used as molecular size standards. Agarose gels were stained with ethidium bromide (0.5 µg ml⁻¹) and images were digitized with a GelPrinter Plus system (TDI).

16S-ARDRA. This technique was applied following the protocol described by Rodas *et al.* (2003). Template DNA was prepared from cell suspensions in water; primers pA and pH were used to amplify 16S rRNA genes as already described (Edwards *et al.*, 1989). Amplified fragments were quantified and then separately digested with three enzymes (*Bfa*I, *Mse*I and *Alu*I). Digestion products were separated by conventional gel electrophoresis using a 1 Kb plus ladder (Invitrogen, BRL) as a molecular size standard. Electrophoresis conditions were previously described by Rodas *et al.* (2003). Staining and digitization of images were done as described in the RFLP-PFGE method.

RAPD. DNAs used for random amplifications were obtained from agarose slices for RFLP-PFGE purposes. One slice was cut into four pieces with a sterile sharp blade, a quarter was suspended in 20 µl of distilled water and then heated at 94 °C for 5 min to melt the agarose and 1 µl of this solution was used in the PCR mixture.

Three random primers were used separately in this study, 16R and 17R described by Tompkins *et al.* (1996), and COC described by Cocconcelli *et al.* (1995). DNA amplification was carried out in a 50 µl PCR mixture containing 200 µM dNTPs, 1 µM selected random primer, 10 mM Tris/HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 2 mM MgCl₂, 1.5 U DyNzyme II DNA polymerase (Finnzymes) and 1 µl template DNA prepared as described above. PCR was performed in a Techne Thermal cycler (Techne). The amplification conditions for all primers used were those described by Tompkins *et al.* (1996) but we included a ramp rate of 20 °C min⁻¹.

RAPD products were resolved by electrophoresis in 1.2% (w/v)

SeaKem LE agarose (FMC) in 0.5 × TBE gels and a 1 Kb plus ladder was used as a molecular size standard. Staining and digitization of images were done as described in the RFLP-PFGE method.

Ribotyping. Ribotyping was performed using the RiboPrinter microbial characterization system (Qualicon). DNA was obtained from colonies grown on MRS agar at 28 °C for 2–4 days. Ribotypes were obtained as reported in the manufacturer's protocol and *Eco*RI was used to restrict the DNA.

Data analysis. Digitized images of all molecular techniques and phenotypic traits were introduced on BioNumerics software version 2.5 (Applied Maths), which carried out conversion, normalization and further analysis of the patterns. Phenotypic profiles were analysed using the simple matching similarity coefficient and UPGMA. PFGE patterns with *Sfi*I and *Not*I and ARDRA patterns were grouped with the UPMGA clustering method using the band-based Dice similarity coefficient. *Sma*I PFGE patterns, RAPD and ribotypes were analysed using Pearson's product moment correlation coefficient and the UPMGA clustering method. The level of reproducibility of each experiment was calculated from duplicate experimental profiles of 10 randomly selected strains. The overall data for each strain obtained from each technique were combined on the BioNumerics software, maintaining the same coefficients used for single pattern analysis and clustered by using the UPMGA method.

Diversity indexes were calculated using the DIVERS online informatic program (F. J. Pérez-López and F. M. Sola-Fernández; available at <http://perso.wanadoo.es/jp-l/descargas.htm>).

Amplification and sequencing of the complete 16S rRNA gene, and sequence data analysis. Complete 16S rRNA genes were amplified as described for the 16S-ARDRA technique. The primers used for complete sequencing of the 16S rRNA gene were: pA, pH (Edwards *et al.*, 1989), I2B (5'-CCCTACTGCTGCCTCCC-GTAGGAGT-3'), S1i (5'-GGACTACCAGGTATCTA-3') and S2d (5'-AGTGGAACTCCATGTGT-3'). Amplification products were purified using the UltraClean PCR clean-up kit (Mobio) and sequenced using an ABI-PRISM 377 (PE-Applied Biosystems) automated sequencer and the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (PE-Applied Biosystems).

A total of 11 strains (8, 59b, 68, 71, 88, 116, 154, 166, 203, 449 and 459) were sequenced. 16S rRNA gene sequences of about 1500 bp were compared with the full dataset of about 22 000 reference sequences of the ARB database (<http://www.arb-home.de/>) using the program package ARB (Ludwig *et al.*, 2004). This database contains 250 almost full-length sequences corresponding to LAB. The respective ARB tools were used for automated sequence alignment. The alignment was checked visually and corrected manually using the sequence editor ARB_EDIT. Sequence similarity values were calculated by comparing nucleotides at the corresponding positions. A tree was derived from the corrected distance values by using the neighbour-joining method (Saitou & Nei, 1987). Maximum-parsimony- and maximum-likelihood-based trees, respectively using the full dataset or a selection of about 28 sequences, were obtained and compared in order to make an estimate of the confidence. The effects on the results of a base-frequency filter in the sequence comparison analysis were also evaluated.

RESULTS AND DISCUSSION

Phenotypic identification

One hundred and seventy-eight wine strains were classified as belonging to the genus *Lactobacillus* from their morphology, Gram-positive character, absence of catalase activity

and motility. Ninety-two strains showed obligately heterofermentative metabolism and 86 were homofermentative or facultatively heterofermentative strains.

The dendrogram constructed by numerical analysis of the phenotypic profiles of the heterofermentative lactobacilli is available as Supplementary Fig. A (dendrogram I) in IJSEM Online. Above 93% similarity (S), 10 clusters could be delineated, named He1–He10, six of which were formed only by one reference strain (He5–He10). Cluster He1 comprised 20 wine strains, *L. brevis* CECT 216 and *L. collinoides* CECT 922^T. Cluster He2 comprised 48 wine strains and *L. hilgardii* NCFB 264^T. Cluster He3 and He4 comprised wild strains without any reference strain. The reproducibility level was 100%S. The calculated cophenetic correlation value for the phenotypic profiles was 0.94, indicating an excellent level of reliability.

The dendrogram that was obtained after numerical analysis of the phenotypic profiles of homofermentative and facultatively heterofermentative lactobacilli is available as Supplementary Fig. A (dendrogram II) in IJSEM Online. Above 86%S, 15 clusters could be delineated, named Ho1–Ho15, seven of which were formed only by one reference strain. Cluster Ho1 comprised one wine strain and *L. coryniformis* subsp. *coryniformis* CECT 982^T. Cluster Ho3 comprised 25 wine strains and *L. mali* CECT 4149. Cluster Ho4 comprised *Lactobacillus delbrueckii* subsp. *bulgaricus* CECT 4005^T and *Lactobacillus helveticus* CECT 4305^T. Cluster Ho7 comprised nine wine strains and *L. casei* ATCC 334. Cluster Ho8 comprised six wine strains and two reference strains, *Lactobacillus zeae* ATCC 15820^T and *L. casei* CECT 475^T. Cluster Ho9 comprised 10 wine strains without reference strains. Cluster Ho12 comprised 32 wine strains and four reference strains: *L. plantarum* CECT 748^T, *Lactobacillus rhamnosus* CECT 278^T, *L. paracasei* subsp. *paracasei* CECT 4022^T and *L. nagelii* DSM 13675^T. Cluster Ho14 comprised only two wine strains. The reproducibility level was 100%S. The calculated cophenetic correlation value for the phenotypic profiles was 0.83, indicating a good level of reliability.

All the wine strains that grouped without or with more than one reference strain were named as *Lactobacillus* spp. The two reference strains of *L. casei* were placed in different clusters: the type strain CECT 475^T was in the same group as the type strain of *L. zeae*, ATCC 15820^T. This incongruence in the results prevents the identification of wild strains grouped in clusters Ho7 and Ho8. These phenotypic traits allowed us to identify only three species of wine lactobacilli: *L. hilgardii* (48 strains), *L. coryniformis* subsp. *coryniformis* (1 strain) and *L. mali* (25 strains).

Numerical analysis of RFLP-PFGE patterns

Single digestions of DNA were performed with *Sfi*I, *Not*I and *Sma*I, respectively yielding 1–30 fragments of 15–1000 kb, 1–35 fragments of 15–800 kb and more than 35 fragments of 4–100 kb. The level of reproducibility was 100%S for

digestion with *Sfi*I and *Not*I and 92.07%S for *Sma*I. As the *Sfi*I and *Not*I fragments of *L. delbrueckii* subsp. *bulgaricus* CECT 4005^T, *L. delbrueckii* subsp. *delbrueckii* CECT 286^T, *L. delbrueckii* subsp. *lactis* CECT 282, *L. fermentum* CECT 4007^T and *L. cellobiosus* CECT 562 were of approximately 4–97 kb, the electrophoretic conditions used to separate them were the same as for *Sma*I fragments.

Based on a computerized numerical analysis of combined RFLP-PFGE patterns, we grouped the strains into 22 clusters at a similarity level of 50%S (Supplementary Fig. B in IJSEM Online). This technique did not enable the assignment of isolated strains to species because the majority of clusters contained none or more than one reference strain; only cluster M5 included 23 wine strains and *L. hilgardii* NCFB 264^T. The global cophenetic correlation value for the PFGE was 0.89, indicating a good level of reliability. Analyses of the single PFGE-RFLP patterns with *Sfi*I, *Not*I and *Sma*I are not shown, but the results were concordant with those obtained using the combined analysis.

The typing power of RFLP-PFGE was high; analysis of digestion bands obtained with *Sfi*I, *Not*I and *Sma*I revealed 130, 118 and 130 distinct patterns, respectively, from 178 isolated *Lactobacillus* strains. One hundred and forty-three distinct patterns were found after combined numerical analysis; 122 strains showed a specific restriction pattern, whereas 56 strains were grouped in 21 different patterns. Strains sharing the same pattern came from the same sample, leading us to think that they represented the same strain isolated several times. Ferrero *et al.* (1996), Zapparoli *et al.* (1998) and Ventura & Zink (2002) reported discriminatory, clear and reproducible RFLP-PFGE patterns in various *Lactobacillus* species, which allowed its use for typing studies. Our results agree with their observations.

16S-ARDRA fingerprinting analysis

Based on a computerized numerical analysis of combined *Bfa*I and *Mse*I 16S-ARDRA patterns, we grouped the strains into 26 clusters at a similarity level of 98% (Supplementary Fig. C in IJSEM Online). Cluster A1 comprised *L. zeae* ATCC 15820^T and *L. casei* CECT 475^T. Cluster A2 contained 13 wine strains, *L. paracasei* subsp. *paracasei* CECT 4022^T and *L. casei* ATCC 334. Cluster A4 comprised *Lactobacillus reuteri* CECT 925^T and *Lactobacillus oris* CECT 4021^T. Cluster A5 comprised two wine strains and *L. collinoides* CECT 922^T. Cluster A8 comprised 10 wine strains and *L. mali* CECT 4149. Cluster A12 comprised 71 wine strains and *L. hilgardii* NCFB 264^T. Cluster A14 comprised 36 wine strains, *L. plantarum* CECT 748^T and *Lactobacillus pentosus* CECT 4023^T. Cluster A17 comprised one wine strain and *L. coryniformis* subsp. *coryniformis* CECT 982^T. Cluster A18 comprised 19 wine strains and *L. brevis* CECT 216. Cluster A25 contained *L. fermentum* CECT 4007^T and *L. cellobiosus* CECT 562^T and cluster A26 grouped the three subspecies of *L. delbrueckii*. Clusters A6, A7, A9–A11 and A21 comprised one or more wine strains without a reference strain and were named *Lactobacillus* spp. The remaining clusters

contained only reference strains. The reproducibility of the method was 98.52 %S for each enzyme. The calculated global cophenetic correlation value for the 16S-ARDRA patterns was 0.96, indicating an excellent level of reliability.

The 16S-ARDRA patterns with *Bfa*I and *Mse*I permitted us to distinguish between almost all reference strains and to identify six species of wine lactobacilli: *L. paracasei* subsp. *paracasei* (13 strains), *L. collinoides* (2 strains), *L. mali* (10 strains), *L. hilgardii* (71 strains), *L. coryniformis* subsp. *coryniformis* (1 strain) and *L. brevis* (19 strains).

The pairs of strains *L. zae*e ATCC 15820^T–*L. casei* CECT 475^T, *L. paracasei* subsp. *paracasei* CECT 4022^T–*L. casei* ATCC 334, *L. reuteri* CECT 925^T–*L. oris* CECT 4021^T, *L. plantarum* CECT 748^T–*L. pentosus* CECT 4023^T and *L. fermentum* CECT 4007^T–*L. cellobiosus* CECT 562^T and the subspecies of *L. delbrueckii* could not be differentiated by *Bfa*I and/or *Mse*I 16S-ARDRA. However, we achieved a good discrimination of *L. reuteri* and *L. oris* from the different profiles obtained with *Alu*I (Rodas *et al.*, 2003). Giraffa *et al.* (1998) were able to discriminate *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* by 16S-ARDRA with *Eco*RI. We confirmed their results with our reference strains. The discrimination of single species in the other strain pairs was impossible with *Alu*I or other enzymes tested.

Our results show that *L. plantarum* and *L. pentosus* grouped together in the same cluster. These species share more 99 % 16S rRNA gene sequence similarity (Collins *et al.*, 1991; Quere *et al.*, 1997) and therefore it is impossible to discriminate them by 16S-ARDRA. Despite their high 16S rRNA gene sequence similarity, Zanoni *et al.* (1987) demonstrated that they are separate species on the basis of DNA–DNA hybridization studies. The 36 wine strains grouped together with these species could be assigned to either of these species.

We could also not discriminate between *L. fermentum* and *L. cellobiosus*. Chenoll *et al.* (2003) published the 16S rRNA gene sequence of the former species and showed that this sequence shared more than 99 % similarity with that of *L. fermentum*. Other data supporting their taxonomic proximity are the high phenotypic similarities, the complete DNA–DNA relatedness found by Kandler & Weiss (1986) and their identity when they were analysed by the tDNA-PCR technique (Baele *et al.*, 2002). Recently, Dellaglio *et al.* (2004) proposed the reclassification of *L. cellobiosus* as a later heterotypic synonym of *L. fermentum*.

The *L. casei* group comprises three species: *L. casei*, *L. paracasei* and *L. rhamnosus* (Collins *et al.*, 1989). However, the taxonomic position of the *L. casei* group remains unclear. Dellaglio *et al.* (2002) requested an Opinion of the Judicial Commission of the International Committee on Systematics of Prokaryotes to replace the type strain of *L. casei*, ATCC 393^T, with ATCC 334, to reject the name *L. paracasei*, to include *L. paracasei* strains in *L. casei* and to

reclassify strain ATCC 393^T as a member of *L. zae*e. Thirteen of our wine strains clustered together with the type strain of *L. paracasei* subsp. *paracasei* and *L. casei* ATCC 334, whereas type strains of *L. casei* CECT 475^T and *L. zae*e ATCC 15820^T clustered at 100 %S in another cluster (Supplementary Fig. C in IJSEM Online). These results support the request of Dellaglio *et al.* (2002). Our wine strains will remain named *L. paracasei* while the Judicial Commission considers the request of Dellaglio *et al.* (2002).

16S-ARDRA generates species-specific patterns in the majority of the studied species, but it is not useful for typing purposes because the 16S rRNA gene sequence is highly conserved at the species level.

RAPD fingerprinting analysis

Reproducibility of RAPD fingerprinting was evaluated in preliminary experiments using a subset of strains. Primer 16R gave poor reproducibility with homofermentative and facultatively heterofermentative lactobacilli, but produced reproducible species-specific profiles with heterofermentative lactobacilli. Thus, we decided to use this primer to analyse the latter group. Primer 17R showed clear and reproducible specific patterns for all reference species. Primer COC (Cocconcelli *et al.*, 1995) was chosen to analyse homofermentative lactobacilli, as it generated species-specific and highly reproducible patterns. The reproducibility of the method ranged between 93.10 and 98.53 %S for these three primers. An example of the RAPD patterns obtained from some reference strains and some isolates is available as Supplementary Fig. D in IJSEM Online. Amplification with primer 16R generated 2–18 fragments of 200–4000 bp, primer 17R generated 5–20 fragments of 100–5000 bp and primer COC generated 10–30 fragments of 100–4000 bp.

On the basis of a computerized numerical analysis of combined RAPD patterns, we grouped strains into 34 clusters at a similarity level of 50 % (Supplementary Fig. E in IJSEM Online), named R1–R34. Cluster R4 was formed by *L. cellobiosus* CECT 562^T and *L. fermentum* CECT 4007^T. Cluster R6 comprised 10 wine strains and *L. mali* CECT 4149. Cluster R11 comprised 28 wine strains and *L. plantarum* CECT 748^T. Cluster R17 comprised 16 wine strains and *L. brevis* CECT 216. Cluster R24 consisted of 71 wine strains and *L. hilgardii* NCFB 264^T. Cluster R28 contained 13 wine strains and two reference strains: *L. paracasei* subsp. *paracasei* CECT 4022^T and *L. casei* ATCC 334. Cluster R31 comprised two wine strains and *L. collinoides* CECT 922^T. Cluster R34 comprised two wine strains and *L. pentosus* CECT 4023^T. Clusters R5, R7, R9, R12, R14, R18, R19, R23 and R30 grouped one or more wine strains without a reference strain and were named as *Lactobacillus* spp. The remainder of the clusters comprised only single reference strains. The calculated cophenetic correlation value for the RAPD patterns was 0.94, indicating an excellent level of reliability.

The RAPD patterns allowed us to identify six species of wine lactobacilli: *L. mali* (10 strains), *L. plantarum* (28 strains), *L. brevis* (16 strains), *L. hilgardii* (71 strains), *L. paracasei* subsp. *paracasei* (13 strains), *L. collinoides* (2 strains) and *L. pentosus* (2 strains).

The technique permitted discrimination between all reference strains except the pair *L. cellobiosus* and *L. fermentum*; this result is concordant with the results obtained by 16S-ARDRA. The pair *L. plantarum*–*L. pentosus*, which could not be separated with 16S-ARDRA, were differentiated with RAPDs, and 30 of the 36 strains grouped with these two species were identified; two strains belonged to *L. pentosus* and 28 strains belonged to *L. plantarum*. This is the first time that *L. pentosus* has been reported in wine. The six strains clustered in R12 were adjacent to *L. plantarum* (44.74 %S) but did not show the species-specific pattern of this species. The group *L. casei* could be differentiated with the RAPD technique, except *L. paracasei* subsp. *paracasei* CECT 4022^T and *L. casei* ATCC 334, supporting our previous results and the request of Dellaglio *et al.* (2002).

Analysis of the results with primer 17R revealed 123 distinct patterns from 178 wine *Lactobacillus* strains. The analysis of 92 wine heterofermentative lactobacilli with primer 16R revealed 17 patterns, whereas primer COC gave 63 distinct patterns from 86 wine homofermentative lactobacilli. The combined analysis of amplification profiles obtained with the three primers showed 131 distinct patterns from the 178 isolated strains.

The RAPD technique has been described as a useful technique for both identification and typing (Cocconcelli *et al.*, 1995; Daud Khaled *et al.*, 1997; Nigatu *et al.*, 2001). In our study, the three selected primers show both characteristics, although primer 17R is of wider application than the other two, because it can be used for all wine lactobacilli and yielded more complex patterns, exhibiting a high typing ability. The discriminatory power of RAPD is lower than that of PFGE.

Ribotyping analysis

Ribotyping was applied to two different sets of wine strains. One set consisted of *L. plantarum* CECT 748^T, *L. pentosus* CECT 4023^T and 10 selected wine strains from cluster A14 in Supplementary Fig. C in IJSEM Online and clusters R11, R12 and R34 in Supplementary Fig. E. The second set consisted of *L. paracasei* subsp. *paracasei* CECT 4022^T, *L. casei* ATCC 334 and 10 selected wine strains from cluster A2 in Supplementary Fig. C and cluster R28 in Supplementary Fig. E. *L. casei* CECT 475^T, *L. rhamnosus*

CECT 278^T and *L. zeae* ATCC 15820^T were also analysed in order to clarify the species assignment of the wine strains.

The dendrogram obtained from the numerical analysis of ribotypes grouped the strains into six clusters at a similarity level of 49 %S (Supplementary Fig. F in IJSEM Online), named E1 – E6. Cluster E1 comprised 10 wine strains and two reference strains: *L. paracasei* subsp. *paracasei* CECT 4022^T and *L. casei* ATCC 334. Cluster E2 comprised nine wine strains and *L. plantarum* CECT 748^T. Cluster E4 comprised one wine strain and *L. pentosus* CECT 4023^T. The reproducibility level was 97 %S. The calculated cophenetic correlation value for the ribotypes profiles was 0.93, indicating an excellent level of reliability.

Ribotyping technique enabled us to confirm the identification of 20 studied strains; 10 strains belonged to *L. paracasei* subsp. *paracasei*, nine to *L. plantarum* and one to *L. pentosus*. We observed a specific-species band pattern for *L. plantarum*; however, within the cluster we could also differentiate two subclusters, as also observed with RAPD (clusters R11 and R12 in Supplementary Fig. E). Differences were evident among the patterns of the reference species.

Ribotyping results enabled the assignment to species of those strains grouped in the same cluster with *L. pentosus* and *L. plantarum* by 16S-ARDRA. Ribotyping confirmed the results obtained with RAPD and clarified the taxonomic status of some strains not grouped by this last technique (cluster R12 in Supplementary Fig. E). As in previous dendrograms, some wine strains were grouped with the type strain of *L. paracasei* subsp. *paracasei* and also with *L. casei* ATCC 334. The grouping of these latter reference strains was observed in RAPD, 16S-ARDRA and ribotyping, supporting the proposal of Dellaglio *et al.* (2002).

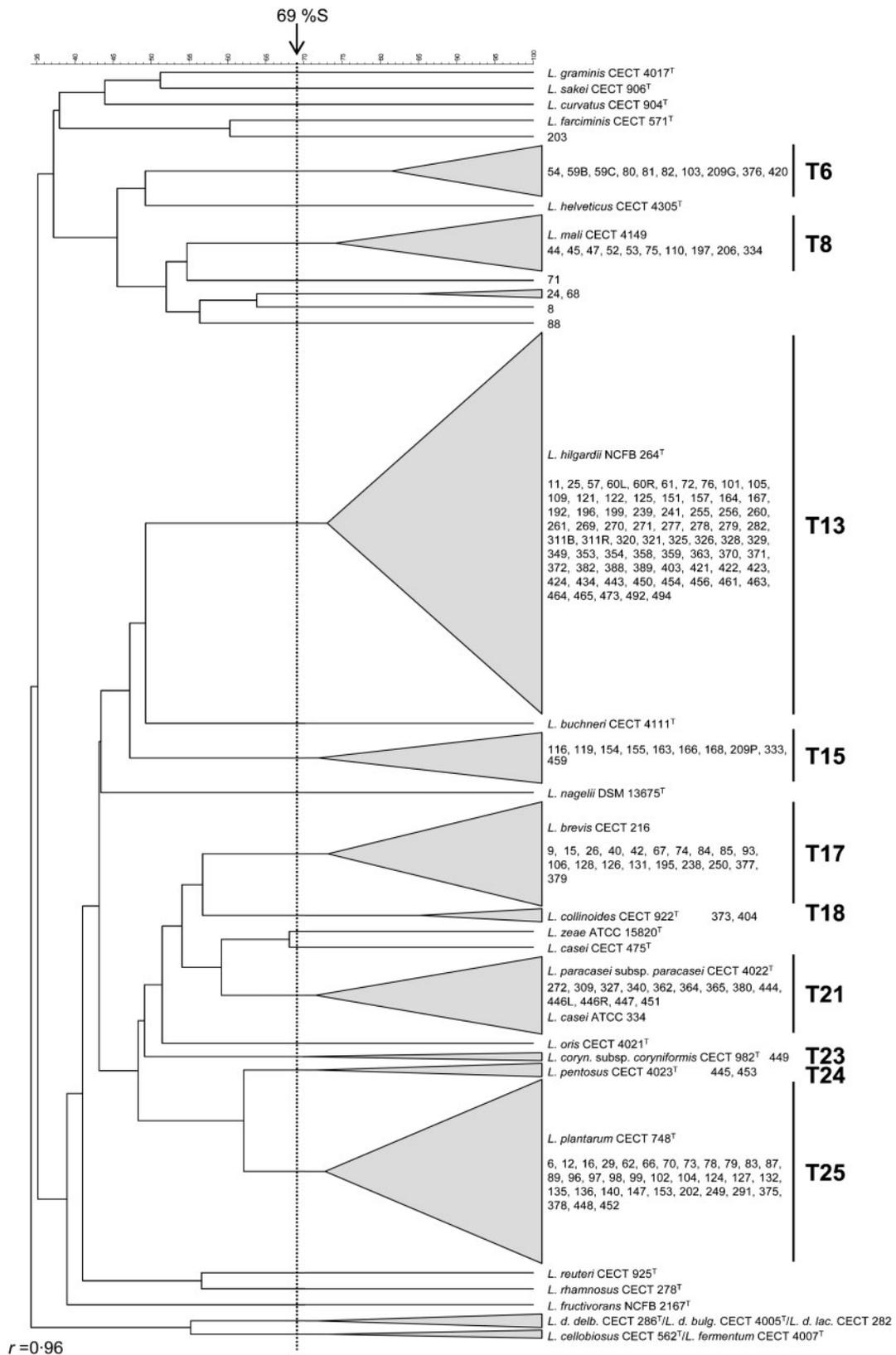
Global analysis

On the basis of a computerized numerical analysis of all combined patterns, a dendrogram was constructed (Fig. 1). Above 69 %S, 30 clusters could be delineated. Cluster T8 comprised 10 wine strains and *L. mali* CECT 4149. Cluster T13 contained 71 wine strains and *L. hilgardii* NCFB 264^T. Cluster T17 comprised 19 wine strains and *L. brevis* CECT 216. Cluster T18 contained two wine strains and *L. collinoides* CECT 922^T. Cluster T21 consisted of 13 wine strains, *L. paracasei* subsp. *paracasei* CECT 4022^T and *L. casei* ATCC 334. Cluster T23 contained one wine strain and *L. coryniformis* subsp. *coryniformis* CECT 982^T. Cluster T24 contained two wine strains and *L. pentosus* CECT 4023^T. Cluster T25 comprised 34 wine strains and *L.*

Fig. 1. Dendrogram derived from comparison of the combined 'all techniques' patterns obtained from wine LAB and reference strains. Levels of similarity between patterns were calculated by using the similarity coefficient of each technique and the clustering is based on the UPGMA method. The vertical dotted line indicates the 69% similarity value for delineating clusters. The cophenetic correlation for this dendrogram was 0.96. Subspecies of *L. delbrueckii* are abbreviated as *L. d. delb.* (subsp. *delbrueckii*), *L. d. bulg.* (subsp. *bulgaricus*) and *L. d. lac.* (subsp. *lactis*); *L. coryn.*, *L. coryniformis*.

plantarum CECT 748^T. Clusters T5, T6, T9, T10, T11, T12 and T15 comprised one or more wine strains without any reference strain and were named as *Lactobacillus* spp. The

remaining clusters consisted of reference strains. The calculated global cophenetic correlation value was 0.96, indicating an excellent level of reliability.



By means of the polyphasic study, 152 wine lactobacilli were identified as *L. brevis* (19 strains), *L. collinoides* (2 strains), *L. coryniformis* subsp. *coryniformis* (1 strain), *L. hilgardii* (71 strains), *L. paracasei* subsp. *paracasei* (13 strains), *L. pentosus* (2 strains), *L. plantarum* (34 strains) and *L. mali* (10 strains) and the remaining 26 strains were unidentified.

Different authors have described difficulties with LAB phenotypic identification schemes (Vandamme *et al.*, 1996; Björkroth *et al.*, 2002). In our work, phenotypic identification allowed us to identify only three species. Moreover, of the 25 wine strains identified phenotypically as *L. mali*, only 10 grouped with this reference strain in the global analysis: 10 strains were close to *L. helveticus* CECT 4305^T and five strains were close to *L. mali* CECT 4149 (Fig. 1; clusters T8, T6 and T9–T12, respectively). All 48 wine strains identified phenotypically as *L. hilgardii* were assigned to this species by the global analysis, but this analysis also included 22 additional strains previously unidentified by phenotypic traits. Members of this last group ferment arabinose, while *L. hilgardii* is unable to ferment this carbohydrate according to Kandler & Weiss (1986) and Hammes *et al.* (1991). In agreement with our results, Sohler *et al.* (1999) found *L. hilgardii* wine strains that could ferment this sugar. Strain 449 was identified as *L. coryniformis* subsp. *coryniformis* by phenotypic traits, 16S-ARDRA and global analysis; however, RAPD clustering did not assign it to this species, though it was placed close to it. To verify the relationship between *L. coryniformis* subsp. *coryniformis* and strain 449, we sequenced the 16S rRNA gene from this strain. Phylogenetic analysis showed that the sequences share more than 99% similarity (data not shown), confirming the results obtained by other techniques that classify strain 449 as *L. coryniformis* subsp. *coryniformis*.

The global analysis reflects the information from each experiment; however, the more congruent techniques with global analysis are RAPD and 16S-ARDRA. Amplification with the 17R primer shows a specific-species band pattern, and numerical analysis (data not shown) exhibits a similar clustering and identifies the same number of species from wine as global analysis. The 16S-ARDRA technique permits the identification of the reference strains and the assignment to species of our wine lactobacilli using the sequential analysis described by Rodas *et al.* (2003). A numerical confirmation of this statement was obtained by calculating the diversity indices to species level of the global analysis: values of 0.782 and 1.895 were obtained for the Simpson ($1-\lambda$) and Shannon–Weaver (H') indices. When these calculations were applied to those individual tests that provided a correct identification of the majority of strains (RAPD with primer 17R and 16S-ARDRA with *MseI*), similar values of diversity were obtained to those of the global analysis [for RAPD-17R, the Simpson ($1-\lambda$) and Shannon–Weaver (H') indices were 0.800 and 2.082, respectively; for ARDRA-*MseI* they were 0.778 and 1.844]. For these reasons we propose that these techniques are useful to simplify and

clarify the identification of lactobacilli. 16S-ARDRA has advantages over RAPD: it is less dependent on reaction conditions and the interpretation of results is easier.

Twenty-six wine strains remained unidentified after the global analysis. They are grouped in seven clusters: T5, T6, T9, T10–T12 and T15 (Fig. 1). We selected one representative strain of each cluster, except for cluster T15, from which four strains were chosen because preliminary results indicated that this group was more distant from reference strains (<95% sequence similarity). The selected strains were 8, 59b, 68, 71, 88, 116, 154, 166, 203 and 459: their almost complete 16S rRNA gene sequences (approx. 1500 bp long) were subjected to similarity searches with the ARB database. The results obtained from the application of the three alternative treeing methods were congruent. All strains were assigned to the genus *Lactobacillus*. The analysis sited them specifically within the heterogeneous *L. casei* group as defined by Collins *et al.* (1991) and redefined into four groups by Schleifer & Ludwig (1995). The corresponding overall 16S rRNA gene sequence similarity matrix is available as Supplementary Table A in IJSEM Online. Fig. 2 displays a phylogenetic tree of *Lactobacillus* species, based on the neighbour-joining method, which included the 16S rRNA gene sequence data of these 10 strains. In the analysis, we have included *Oenococcus oeni* as the outgroup.

Strain 203 was found within the *L. plantarum* group as defined by Schleifer & Ludwig (1995). This group represents a cluster of related species consisting of *Lactobacillus alimentarius*, *Lactobacillus farciminis* and the recently described *Lactobacillus paralimentarius* (Cai *et al.*, 1999), *Lactobacillus kimchii* (Yoon *et al.*, 2000), *Lactobacillus mindensis* (Ehrmann *et al.*, 2003) and *Lactobacillus versmoldensis* (Kröckel *et al.*, 2003). With the exception of *L. kimchii*, all these species have been isolated from sourdoughs, and not from wine. The closest relatives of strain 203 were *L. kimchii* (99.3%), *L. alimentarius* (98.2%) and *L. paralimentarius* (97.8%) (Supplementary Table A). These three species were not included in this study because their habitats do not include wine (kimchi and sourdough). In further studies, we will compare strain 203 in depth with *L. kimchii*, *L. paralimentarius* and *L. alimentarius* by a polyphasic approach that will include DNA–DNA hybridization. Then, we will ascertain whether strain 203 could constitute a novel species or a new description from wine of one of these three species *L. kimchii*, *L. paralimentarius* and *L. alimentarius*.

Phylogenetic analysis placed the other nine wine strains within the *L. salivarius* group as defined by Schleifer & Ludwig (1995). This group represents a cluster of related species consisting of *Lactobacillus agilis*, *Lactobacillus animalis*, *Lactobacillus aviarius*, *L. mali*, *Lactobacillus murinus*, *L. ruminis*, *Lactobacillus salivarius* and the recently described *L. nagelii* (Edwards *et al.*, 2000), *Lactobacillus acidipiscis* (Tanasupawat *et al.*, 2000) and *Lactobacillus equi* (Morotomi *et al.*, 2002). The majority of these species have

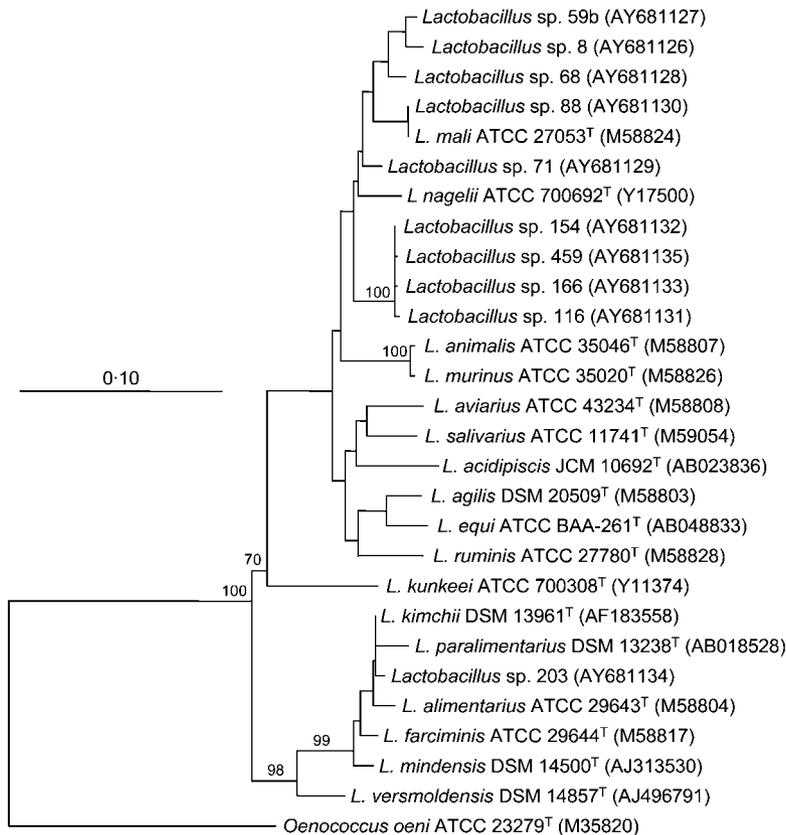


Fig. 2. Phylogenetic tree showing the positions of strains 8, 59b, 68, 71, 88, 116, 154, 166, 203 and 459, and some *Lactobacillus* species based on 16S rRNA gene sequences. Clustering is based on neighbour-joining. Bootstrap values (expressed as percentages of 1000 replicates) of 70% or greater are shown at branch points. GenBank accession numbers are given in parentheses. Bar, 10% nucleotide substitutions.

been isolated from human or animal sources, while *L. mali* and *L. nagelii* have been isolated from wine. The closest relative of strains 8, 59b, 68 and 71 was *L. mali*, with 96.8, 97.8, 97.8 and 96.7% sequence similarity, respectively; *L. nagelii* had approximately 95% sequence similarity to these strains except strain 71, which exhibited 96.1% similarity. Strain 88 shared 100% 16S rRNA gene sequence similarity with *L. mali* (Supplementary Table A). Although this result and the phenotypic traits indicate that strain 88 should belong to *L. mali*, the rest of the tests and the global analysis do not allow assignment to this species. 16S rRNA gene sequences of strains 116, 154, 166 and 459 were almost identical (99.9% similarity). The closest relatives of strains 116, 154, 166 and 459 were *L. mali* (approx. 95%) and *L. nagelii* (approx. 95%) (Supplementary Table A). DNA–DNA hybridizations must be done to reveal whether these nine strains belong to *L. mali* or *L. nagelii* or represent novel species.

After the global analysis, the number of different strains retained is 146 from 178 wine lactobacilli, PFGE being the most discriminatory technique (143 different patterns). One hundred and twenty-seven strains could be differentiated by global analysis and 51 strains were grouped in 19 groups. As has been stated before, some of these 178 strains could have been isolated several times from the same sample.

This vast study reveals that the majority of lactobacilli in wine belong to *L. hilgardii* (40%), *L. plantarum* (19%), *L.*

brevis (10.7%), *L. paracasei* subsp. *paracasei* (7.3%) and *L. mali* (5.6%), the first three being the most typical in wine. The species *L. collinoides*, *L. coryniformis* subsp. *coryniformis* and *L. pentosus* appear much less frequently and were always associated with spoiled wine. The latter three species and *L. paracasei* subsp. *paracasei* were described in wine for the first time by Rodas *et al.* (2003) and their presence is confirmed in the present work. Furthermore, the application of this polyphasic approach has allowed the detection of seven putative novel species and contributes to expanding our knowledge on the confusing genus *Lactobacillus*. Moreover, this approach allows evaluation of the taxonomic value of different techniques currently used, and clarifies controversial results obtained by the application of a single technique. The inclusion of a good set of reference strains is necessary to obtain consistent results. In addition to the taxonomic knowledge obtained in this work, we have demonstrated a simple identification scheme for wine lactobacilli: 16S-ARDRA with *MseI* and RAPD with primer 17R in cases of ambiguity.

ACKNOWLEDGEMENTS

This work was supported by the Spanish CYCYT ALI97-1077-C02-01 and AGL2000-0827-C02-01. A. M. R. was supported by a grant from the Conselleria d'Educació i Ciència, of Generalitat Valenciana, FPI98-AG03-179. We wish to thank Dr M^a Carmen Macián Rovira for kindly helping with the phylogenetic analysis.

REFERENCES

- Baele, M., Vanechoute, M., Verhelst, R., Vancanneyt, M., Devriese, L. A. & Haesebrouck, F. (2002). Identification of *Lactobacillus* species using tDNA-PCR. *J Microbiol Methods* **50**, 263–271.
- Berthier, F. & Ehrlich, S. D. (1998). Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA spacer region. *FEMS Microbiol Lett* **161**, 97–106.
- Björkroth, K. J., Schillinger, U., Geisen, R., Weiss, N., Holzapfel, W. H., Korkeala, H. & Vandamme, P. (2002). Taxonomic study of *Weissella confusa* and description of *Weissella cibaria* sp. nov., detected in food and clinical samples. *Int J Syst Evol Microbiol* **52**, 141–148.
- Cai, Y., Okada, H., Mori, H., Benno, Y. & Nakase, T. (1999). *Lactobacillus paralimentarius* sp. nov., isolated from sourdough. *Int J Syst Bacteriol* **49**, 1451–1455.
- Cappuccino, J. G. & Sherman, N. (1987). *Microbiology: a Laboratory Manual*. Menlo Park, CA: Benjamin/Cummings.
- Chagnaud, P., Machinis, K., Coutte, L. A., Marecat, A. & Mercenier, A. (2001). Rapid PCR-based procedure to identify lactic acid bacteria: application to six common *Lactobacillus* species. *J Microbiol Methods* **44**, 139–148.
- Chenoll, E., Macián, M. C. & Aznar, R. (2003). Identification of *Carnobacterium*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* by rDNA-based techniques. *Syst Appl Microbiol* **26**, 546–556.
- Cocconcelli, P. S., Porro, D., Galandini, S. & Senini, L. (1995). Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci. *Lett Appl Microbiol* **21**, 376–379.
- Collins, M. D. & Wallbanks, S. (1992). Comparative sequence analyses of the 16S rRNA genes of *Lactobacillus minutus*, *Lactobacillus rimaie* and *Streptococcus parvulus*: proposal for the creation of a new genus *Atopobium*. *FEMS Microbiol Lett* **92**, 235–240.
- Collins, M. D., Farrow, J. A. E., Phillips, B. A., Ferusu, S. & Jones, D. (1987). Classification of *Lactobacillus divergens*, *Lactobacillus piscicola* and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus, *Carnobacterium*. *Int J Syst Bacteriol* **37**, 310–316.
- Collins, M. D., Phillips, B. A. & Zannoni, P. (1989). Deoxyribonucleic acid homology studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., com. nov. *Int J Syst Bacteriol* **39**, 105–108.
- Collins, M. D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J. A. E., Martínez-Murcia, A., Phillips, B. A., Williams, A. M. & Wallbanks, S. (1991). Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiol Lett* **77**, 5–12.
- Collins, M. D., Samelis, J., Metaxopoulos, J. & Wallbanks, S. (1993). Taxonomic studies on some *Leuconostoc*-like organisms from fermented sausages: description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. *J Appl Bacteriol* **75**, 595–603.
- Daud Khaled, A. K., Neilan, B. A., Henriksson, A. & Conway, P. L. (1997). Identification and phylogenetic analysis of *Lactobacillus* using multiplex RAPD-PCR. *FEMS Microbiol Lett* **153**, 191–197.
- Dellaglio, F., Felis, G. E. & Torriani, S. (2002). The status of the species *Lactobacillus casei* (Orla-Jensen 1926) Hansen and Lessel 1972 and *Lactobacillus paracasei* Collins et al. 1989. Request for an Opinion. *Int J Syst Evol Microbiol* **52**, 285–287.
- Dellaglio, F., Torriani, S. & Felis, G. E. (2004). Reclassification of *Lactobacillus cellobiosus* Rogosa et al. 1953 as *Lactobacillus fermentum* Beijerinck 1901. *Int J Syst Evol Microbiol* **54**, 809–812.
- Dicks, L. M. T. & van Vuuren, H. J. J. (1988). Identification and physiological characteristics of heterofermentative strains of *Lactobacillus* from South African red wines. *J Appl Bacteriol* **64**, 505–513.
- Dubernet, S., Desmasures, N. & Guéguen, M. (2002). A PCR-based method for identification of lactobacilli at the genus level. *FEMS Microbiol Lett* **214**, 271–275.
- Edwards, U., Rogall, T., Blocker, H., Emde, M. & Bottger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* **17**, 7843–7853.
- Edwards, C. G., Haag, K. M., Collins, M. D., Hutson, R. A. & Huang, Y. C. (1998). *Lactobacillus kunkeei* sp. nov., a spoilage organism associated with grape juice fermentations. *J Appl Microbiol* **84**, 698–702.
- Edwards, C. G., Collins, M. D., Lawson, P. A. & Rodriguez, A. V. (2000). *Lactobacillus nagelii* sp. nov., an organism isolated from a partially fermented wine. *Int J Syst Evol Microbiol* **50**, 699–702.
- Ehrmann, M. A., Martin, R. A. & Vogel, R. F. (2003). Molecular analysis of sourdough reveals *Lactobacillus mindensis* sp. nov. *Int J Syst Evol Microbiol* **53**, 7–13.
- Euzéby, J. P. (1997). List of bacterial names with standing in nomenclature: a folder available on the Internet (URL: <http://www.bacterio.cict.fr/>). *Int J Syst Bacteriol* **47**, 590–592.
- Ferrero, M., Cesena, C., Morelli, L., Scolari, G. & Vescovo, M. (1996). Molecular characterization of *Lactobacillus casei* strains. *FEMS Microbiol Lett* **140**, 215–219.
- Frayne, R. F. (1986). Direct analysis of the major organic components in grape must and wine using high performance liquid chromatography. *Am J Enol Vitic* **37**, 281–287.
- Gevers, D., Huys, G. & Swings, J. (2001). Applicability of rep-PCR fingerprinting for identification *Lactobacillus* species. *FEMS Microbiol Lett* **205**, 31–36.
- Giraffa, G., De Vecchi, P. & Rossetti, L. (1998). Identification of *Lactobacillus delbrueckii* subsp. *lactis* dairy isolates by amplified rDNA restriction analysis. *J Appl Microbiol* **85**, 918–924.
- Hammes, W. P., Weiss, N. & Holzapfel, W. H. (1991). The genera *Lactobacillus* and *Carnobacterium*. In *The Prokaryotes*, 2nd edn, vol. 2, pp. 1535–1594. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Kandler, O. & Weiss, N. (1986). Genus *Lactobacillus* Beijerinck 1901, 212^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1208–1234. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: William & Wilkins.
- Kröckel, L., Schillinger, U., Franz, C. M. A. P., Bantleon, A. & Ludwig, W. (2003). *Lactobacillus versmoldensis* sp. nov. isolated from raw fermented sausage. *Int J Syst Evol Microbiol* **53**, 513–517.
- Leisner, J. J., Vancanneyt, M., Goris, J., Christensen, H. & Rusul, G. (2000). Description of *Paralactobacillus selangorensis* gen. nov., sp. nov., a new lactic acid bacterium isolated from chilli bo, a Malaysian food ingredient. *Int J Syst Evol Microbiol* **50**, 19–24.
- Lonvaud-Funel, A., Joyeux, A. & Ledoux, O. (1991). Specific enumeration of lactic acid bacteria in fermenting grape must and wine by colony hybridization with non-isotopic DNA probes. *J Appl Bacteriol* **71**, 501–508.
- Lortal, S., Valence, F., Bizet, D. & Maubois, J. L. (1997). Electrophoretic pattern of peptidoglycan hydrolases, a new tool for bacterial species identification: application to 10 *Lactobacillus* species. *Res Microbiol* **148**, 461–474.
- Ludwig, W., Strunk, O., Westram, R. & 29 other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

- Matushek, M. G., Bonten, M. J. M. & Hayden, M. K. (1996). Rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. *J Clin Microbiol* **34**, 2598–2600.
- Morotomi, M., Yuki, N., Kado, Y., Kushiro, A., Shimazaki, T., Watanabe, K. & Yuyama, T. (2002). *Lactobacillus equi* sp. nov., a predominant intestinal *Lactobacillus* species of the horse isolated from faeces of healthy horses. *Int J Syst Evol Microbiol* **52**, 211–214.
- Motoyama, Y., Funahashi, W. & Ogata, T. (2000). Characterization of *Lactobacillus* spp. by Ribotyping. *J Am Soc Brew Chem* **58**, 1–3.
- Nigatu, A., Ahrné, S. & Molin, G. (2001). Randomly amplified polymorphic DNA (RAPD) profiles for the distinction of *Lactobacillus* species. *Antonie van Leeuwenhoek* **79**, 1–6.
- Nour, M. (1998). 16S-23S and 23S-5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. *Res Microbiol* **149**, 433–448.
- Patarata, L., Pimentel, M. S., Pot, B., Kersters, K. & Faia, A. M. (1994). Identification of lactic acid bacteria isolated from Portuguese wines and musts by SDS-PAGE. *J Appl Bacteriol* **76**, 288–293.
- Quere, F., Deschamps, A. & Urdaci, M. C. (1997). DNA probe and PCR-specific reaction for *Lactobacillus plantarum*. *J Appl Microbiol* **82**, 783–790.
- Rizzo, A. F., Korkeala, H. & Mononen, I. (1987). Gas chromatography analysis of cellular fatty acids and neutral monosaccharides in the identification of lactobacilli. *Appl Environ Microbiol* **53**, 2883–2888.
- Rodas, A. M., Ferrer, S. & Pardo, I. (2003). 16S-ARDRA, a tool for identification of lactic acid bacteria isolated from grape must and wine. *Syst Appl Microbiol* **26**, 412–422.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schillinger, U., Holzapfel, W. & Kandler, O. (1989). Nucleic acid hybridization studies on *Leuconostoc* and heterofermentative lactobacilli and description of *Leuconostoc amelibiosum* sp. nov. *Syst Appl Microbiol* **12**, 48–55.
- Schleifer, K. H. & Ludwig, W. (1995). Phylogeny of the genus *Lactobacillus* and related genera. *Syst Appl Microbiol* **18**, 461–467.
- Sohier, D. & Lonvaud-Funel, A. (1998). Rapid and sensitive *in situ* hybridization method for detecting and identifying lactic acid bacteria in wine. *Food Microbiol* **15**, 391–397.
- Sohier, D., Coulon, J. & Lonvaud-Funel, A. (1999). Molecular identification of *Lactobacillus hilgardii* and genetic relatedness with *Lactobacillus brevis*. *Int J Syst Bacteriol* **49**, 1075–1081.
- Tanasupawat, S., Shida, O., Okada, S. & Komagata, K. (2000). *Lactobacillus acidipiscis* sp. nov. and *Weissella thailandensis* sp. nov., isolated from fermented fish in Thailand. *Int J Syst Evol Microbiol* **50**, 1479–1485.
- Tompkins, T. A., Stewart, R., Savard, L., Rusell, I. & Dowhanick, T. M. (1996). RAPD-PCR characterization of brewery yeast and beer spoilage bacteria. *J Am Soc Brew Chem* **54**, 91–96.
- Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**, 407–438.
- Ventura, M. & Zink, R. (2002). Specific identification and molecular typing analysis of *Lactobacillus johnsonii* by using PCR-based methods and pulsed-field gel electrophoresis. *FEMS Microbiol Lett* **217**, 141–154.
- Ventura, M., Casas, I. A., Morelli, L. & Callegari, M. L. (2000). Rapid amplified ribosomal DNA restriction analysis (ARDRA) identification of *Lactobacillus* spp. isolated from fecal and vaginal samples. *Syst Appl Microbiol* **23**, 504–509.
- Weiss, N., Schillinger, U. & Kandler, O. (1983). *Lactobacillus trichodes*, and *Lactobacillus heterohiochii*, subjective synonyms of *Lactobacillus fructivorans*. *Syst Appl Microbiol* **4**, 507–511.
- Yoon, J. H., Kang, S. S., Mheen, T. I. & 7 other authors (2000). *Lactobacillus kimchii* sp. nov., a new species from kimchii. *Int J Syst Evol Microbiol* **50**, 1789–1795.
- Zanoni, P., Farrow, J. A. E., Phillips, B. A. & Collins, M. D. (1987). *Lactobacillus pentosus* (Fred, Peterson and Anderson) sp. nov., nom. rev. *Int J Syst Bacteriol* **37**, 339–341.
- Zapparoli, G., Torriani, S. & Dellaglio, F. (1998). Differentiation of *Lactobacillus sanfranciscensis* strains by randomly amplified polymorphic DNA and pulsed-field electrophoresis. *FEMS Microbiol Lett* **166**, 325–332.
- Zhong, W., Millsap, K., Bialkowska-Hobrzanska, H. & Reid, G. (1998). Differentiation of *Lactobacillus* species by molecular typing. *Appl Environ Microbiol* **64**, 2418–2423.
- Zúñiga, M., Pardo, I. & Ferrer, S. (1993). An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. *Int J Food Microbiol* **18**, 37–42.