Differentiation of species of the family *Acetobacteraceae* by AFLP DNA fingerprinting: *Gluconacetobacter kombuchae* is a later heterotypic synonym of *Gluconacetobacter hansenii*

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Amplified fragment length polymorphism (AFLP) DNA fingerprinting was investigated as a tool for fast and accurate identification of acetic acid bacteria (AAB) to the species level. One hundred and thirty five reference strains and 15 additional strains, representing 50 recognized species of the family *Acetobacteraceae*, were subjected to AFLP analysis using the restriction enzyme combination *Apal*/*TaqI* and the primer combination A03/T03. The reference strains had been previously subjected to either DNA–DNA hybridization or 16S–23S rRNA spacer region gene sequence analysis and were regarded as being accurately classified at the species level. The present study revealed that six of these strains should be reclassified, namely *Gluconacetobacter europaeus* LMG 1518 and *Gluconacetobacter xylinus* LMG 1510 as *Gluconacetobacter xylinus* and *Gluconacetobacter europaeus*, respectively; *Gluconacetobacter kombuchae* LMG 23726^T as *Gluconacetobacter hansenii*; and *Acetobacter orleanensis* strains LMG 1545, LMG 1592 and LMG 1608 as *Acetobacter cerevisiae*. Cluster analysis of the AFLP DNA fingerprints of the reference strains revealed one cluster for each species, showing a linkage level below 50 % with other clusters, except for *Acetobacter pasteurianus*, *Acetobacter indonesiensis* and *Acetobacter cerevisiae*. These three species were separated into two, two, and three clusters, respectively. At present, confusion exists regarding the taxonomic status of *Gluconacetobacter oboediens* and *Gluconacetobacter intermedius*; the AFLP data from this study supported their classification as separate taxa. The 15 additional strains could all be identified at the species level. AFLP analysis further revealed that some species harboured genetically diverse strains, whereas other species consisted of strains showing similar banding patterns, indicating a more limited genetic diversity. It can be concluded that AFLP DNA fingerprinting is suitable for accurate identification and classification of a broad range of AAB, as well as for the determination of intraspecific genetic diversity.

Abbreviations: AAB, acetic acid bacteria; AFLP, amplified fragment length polymorphism; ERIC enterobacterial repetitive intergenic consensus sequences; FAFLP, fluorescent amplified fragment length polymorphism; RAPD, randomly amplified polymorphic DNA; REP, Repetitive extragenic palindromic sequences.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of LMG 23726^T is AM999342.

A supplementary figure showing the (GTG)₅-PCR fingerprints of strains belonging to the species of the *Gluconacetobacter xylinus* branch and supplementary tables detailing the DNA–DNA relatedness of the strains of the genera *Acetobacter* and *Gluconacetobacter* as determined in this and previous studies are available with the online version of this paper.

Acetic acid bacteria (AAB) are Gram-negative, coccoid or rod-shaped, obligate aerobic bacteria that have the ability to incompletely oxidize a wide range of carbohydrates, alcohols and sugar alcohols. They are involved in the production of several fermented foods and beverages, either in a beneficial (vinegars, cocoa-based products, Kombucha and nata de coco) or detrimental (spoilage of beer, wine and cider) manner and are also used in the production of commercially important fine chemicals and bacterial cellulose. Recently, some AAB have been

described as human pathogens (Greenberg *et al.*, 2006; Kersters *et al.*, 2006; Tuuminen *et al.*, 2007).

At the time of writing, AAB are classified into ten genera comprising 52 recognized species in the family *Acetobacteraceae* of the class *Alphaproteobacteria*, i.e. the genera *Acetobacter* (19 species), *Acidomonas* (1 species), *Asaia* (4 species), *Gluconacetobacter* (16 species), *Gluconobacter* (7 species), *Granulibacter* (1 species), *Kozakia* (1 species), *Neoasaia* (1 species), *Saccharibacter* (1 species) and *Swaminathania* (1 species) (Cleenwerck *et al.*, 2007, 2008; Dutta & Gachhui, 2007; Ndoye *et al.*, 2007; Malimas *et al.*, 2007, 2008a, b; Cleenwerck & De Vos, 2008; Yamada & Yukphan, 2008).

Multiple studies have shown that accurate identification of AAB isolates is difficult (Moore *et al.*, 2002; Greenberg *et al.*, 2006; Kersters *et al.*, 2006; Tuuminen *et al.*, 2006; Cleenwerck & De Vos, 2008). While identification to the genus level can generally be achieved by 16S rRNA gene sequence analysis and/or some phenotypic tests (Kersters *et al.*, 2006; Cleenwerck & De Vos, 2008; Yamada & Yukphan, 2008), identification to the species level can be problematic (Kersters *et al.*, 2006; Cleenwerck & De Vos, 2008). Species identifications based solely on phenotypic data are not recommended and, for this reason, several authors have advised the use of genotypic data as the basis for species identification of AAB (Cleenwerck & De Vos, 2008).

In the last decade, researchers have therefore increasingly abandoned phenotypic tests or have complemented them by the use of molecular DNA-based methods. DNA–DNA hybridizations and sequence analysis of 16S rRNA genes, 16S–23S rRNA gene spacer regions and genes such as *adhA* [encoding subunit I of pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase] and *recA* (encoding the DNA repair protein RecA) have been applied, as well as DNA fingerprinting techniques such as RFLP analysis of PCR-amplified rRNA (mainly 16S rRNA genes and 16S–23S rRNA gene spacer regions), randomly amplified polymorphic DNA (RAPD) fingerprinting, rep-PCR using the (GTG)₅, REP (repetitive extragenic palindromic sequences) and ERIC (enterobacterial repetitive intergenic consensus sequences) primers, PFGE and plasmid profiling (reviewed by Cleenwerck & De Vos, 2008). However, for many methods, the taxonomic resolution is not completely clear, because its evaluation has often been based on a set of strains with insufficient representatives per taxon and lacking phylogenetic relatives. Nevertheless, it is likely that the resolution power of techniques based on 16S rRNA genes will not reach the species level, especially since high 16S rRNA gene sequence similarities have been found between several species of the family (Cleenwerck & De Vos, 2008). For rapid identification and classification of a broad range of AAB, the most appropriate technique at present appears to be rep-PCR using the (GTG)₅ primer (De Vuyst *et al.*, 2008).

Amplified fragment length polymorphism (AFLP) DNA fingerprinting, a universally applicable technique with a

high discriminatory power and good reproducibility (Janssen *et al.*, 1996; Savelkoul *et al.*, 1999), has proved to be useful for discrimination at the species level and below in various taxa such as the genera *Aeromonas*, *Acinetobacter*, *Campylobacter*, *Xanthomonas* (Savelkoul *et al.*, 1999), *Vibrionaceae* (Thompson *et al.*, 2001), *Bradyrhizobium* (Willems *et al.*, 2001), *Arcobacter* (On *et al.*, 2003) and *Pantoea* (Brady *et al.*, 2007).

In the present study, 150 AAB (Table 1), representing 50 recognized species of the family *Acetobacteraceae*, were analysed by AFLP DNA fingerprinting, with the aim of investigating whether this technique could deliver fast and accurate identification and classification of AAB to the species level. For 135 of these strains, DNA–DNA hybridization data or 16S–23S rRNA gene spacer region sequence analysis data have been reported previously (Urakami *et al.*, 1989; Sievers *et al.*, 1992; Boesch *et al.*, 1998; Sokollek *et al.*, 1998; Franke *et al.*, 1999; Navarro *et al.*, 1999; Lisdiyanti *et al.*, 2000, 2001, 2002, 2006; Schüller *et al.*, 2000; Yamada *et al.*, 2000; Fuentes-Ramírez *et al.*, 2001; Katsura *et al.*, 2001; Cleenwerck *et al.*, 2002, 2007, 2008; Trček & Teuber, 2002; Loganathan & Nair, 2004; Jojima *et al.*, 2004; Yukphan *et al.*, 2004, 2005; Dellaglio *et al.*, 2005; Muthukumarasamy *et al.*, 2005; Sievers & Swings, 2005; Dutta & Gachhui, 2006, 2007; Greenberg *et al.*, 2006; Silva *et al.*, 2006; Takahashi *et al.*, 2006; Camu *et al.*, 2007; Malimas *et al.*, 2007; Ndoye *et al.*, 2007; De Vuyst *et al.*, 2008). Therefore, these strains were assumed to be accurately classified at the species level and were regarded as reference strains for the purpose of this study. The 150 strains used in this study were obtained from the BCCM/LMG Bacteria Collection and the Research Collection of the Laboratory of Microbiology (LM-UGent) or were kindly provided by the German Collection of Microorganisms and Cell Cultures (DSMZ). They were cultivated on GY (5 % glucose, 1 % yeast extract, 1.5 % agar; w/v) or GYAE (5 % glucose, 1 % yeast extract, 1.5 % agar; w/v; 2 % ethanol, 1 % acetic acid; v/v; the latter two components were sterilized separately by filtration and added aseptically to the autoclaved medium) and in some cases on the medium suggested by the provider, by incubation at 28 °C under aerobic conditions.

Fluorescent amplified fragment length polymorphism (FAFLP) DNA fingerprinting of whole genomes was performed on genomic DNA extracted following the method of Wilson (1987) with minor modifications. Briefly, a loopful of cells was washed with RS buffer (0.15 M NaCl, 10 mM EDTA, pH 8.0) and then suspended in a 360 µl mix containing 340 µl 10 mM Tris/HCl, 25 mM EDTA, pH 8.0, 16 µl RNaseA solution (10 mg ml⁻¹; Sigma) and 4.0 µl proteinase K solution (Merck). A 40 µl aliquot of preheated (37 °C) SDS/TE solution (20 % SDS in 10 mM Tris/HCl, 1 mM EDTA, pH 8.0; w/v) was added to the mixture and the whole sample was incubated at 37 °C for 60 min. After visual verification of cell lysis, 120 µl 5 M NaCl was added and mixed by inversion, before the addition of 80 µl CTAB/

Fig. 1. AFLP DNA fingerprints of strains representing 50 recognized species of the family *Acetobacteraceae* (figure has been split over two pages). The dendrogram was constructed with the UPGMA method after calculation of the band pattern similarity (%) using the DICE coefficient. *, strain for which no DNA–DNA hybridization data or 16S–23S rRNA gene spacer region sequence analysis data have (previously) been reported; †, strain reclassified on the basis of data presented in this study; ‡, strain identified on the basis of AFLP DNA fingerprint data obtained in this study.

NaCl solution (10 % hexadecyltrimethylammonium bromide in 0.7 M NaCl; w/v). After 20 min incubation at 65 °C, the lysate was subjected to a phenol/chloroform/isoamylalcohol (49:49:1) extraction using Phase Lock Gel tubes (Eppendorf). A 10 µl aliquot of RNase A solution (10 mg ml⁻¹; Sigma) was added to the carefully transferred aqueous phase and after 15–30 min incubation at 37 °C, the solution was again subjected to a phenol/chloroform/isoamylalcohol (49:49:1) extraction using Phase Lock Gel tubes. The aqueous phase was carefully transferred and mixed with 500 µl ice-cold 100 % 2-propanol and kept on ice for 15 min. Pellets were collected by centrifugation, washed in ice-cold 70 % ethanol, dried and resuspended in 50–200 µl 0.1 × TE solution, depending on the size of the pellet. DNA concentration and purity were estimated by measuring the absorptions at 260 nm, 280 nm and 234 nm. DNA concentration and size were additionally verified by agarose gel electrophoresis using a 1 % (w/v) agarose gel. For *Gluconacetobacter entanii* LTH 4560^T, DNA according to the descriptions of *Gluconacetobacter swingsii* and *Gluconacetobacter rhaeticus* (Dellaglio *et al.*, 2005) was used. Template DNA was prepared using the restriction enzyme combination *ApaI*/*TaqI* essentially as described previously (Janssen *et al.*, 1996). Briefly, 1 µg high-molecular-mass DNA was digested with *TaqI/ApaI*, followed by ligation of the corresponding double-stranded restriction half-site adaptors (Janssen *et al.*, 1996) using T4 ligase (Amersham Pharmacia Biotech). Template DNA was precipitated in a solution containing 100 µl T0.1E (10 mM Tris/HCl, 0.1 mM EDTA, pH 8.0) and used for selective PCR amplification with the primer combination A03/T03. Both these primers consist of a core region (A-, T-primer core sequences, see Janssen *et al.*, 1996) and a guanine extension at the 3'-end. The A03 primer was labelled with the fluorescent label 6-FAM at the 5'-end. The amplification mixture contained 1.5 µl template DNA, 0.5 µl 4 µM hexaprimer, 0.5 µl 1 µM tetraprimer and 7.5 µl Amplification Core Mix (Applied Biosystems). The amplification reactions were performed in a GeneAmp PCR system 9700 thermocycler using the 9600 mode (Applied Biosystems) and the temperature program described previously (Thompson *et al.*, 2001). Selective PCR products were separated using PAGE as described previously (Thompson *et al.*, 2001). Using GENESCAN 3.1 software (Applied Biosystems), the resulting AFLP DNA fingerprints were tracked and normalized, and bands were assigned and sized. Tables with information on the fragments of 50–536 bp were transferred into the

BioNumerics 4.61 software (Applied Maths) for numerical analysis. Fingerprint similarity values were calculated using the DICE coefficient with a position tolerance of 0.2 % to compensate for technical imperfections (optimization tolerance: 0 %), and a dendrogram was constructed using the unweighted pair group method using arithmetic means (UPGMA). The zone between 60 and 500 bp was used for comparison.

AFLP DNA fingerprinting has previously been reported to be a highly reproducible technique, making it very suitable for the generation of databases for identification purposes (Janssen *et al.*, 1996; Savelkoul *et al.*, 1999). In this study, the AFLP DNA fingerprints of 35 strains were generated twice, starting from a new DNA isolation, and genomic DNA of *Gluconacetobacter azotocaptans* LMG 21311^T was included in each set of DNA that was subjected to AFLP analyses. The similarity between fingerprints obtained for the same strain, after assignment of bands, ranged between 80 and 95 %, except for strains producing a lot of cellulose, such as *Gluconacetobacter swingsii* LMG 22125^T and *Gluconacetobacter intermedius* LMG 18909^T (=DSM 11804^T). In these cases, the similarity value was sometimes lower (70 %). Nevertheless, AFLP profiles of the same strain still clustered together when all of the AFLP DNA fingerprints generated were considered, except if other strains generated very similar profiles (with band pattern similarity above the reproducibility level), suggesting that the strains could be clonally related.

The AFLP DNA fingerprints of the 150 strains, as well as an UPGMA dendrogram obtained after cluster analysis of these patterns, are shown in Fig. 1. Most strains (>80 %) generated patterns containing between 40 and 100 bands, evenly distributed in the range of 50–536 bp. Strains generating fewer bands generally belong to the genus *Acetobacter*. For *Acetobacter pasteurianus*, the current restriction enzyme combination and primer combination appeared not to be very suitable, as several strains generated profiles containing mainly larger bands (>200 bp; e.g. A13), while others generated less than 15 bands (e.g. A33).

Nevertheless, the reference strains of a particular species generated profiles that grouped in clusters according to their respective taxonomic designations, with only a few exceptions. Firstly, the species *Acetobacter pasteurianus* and *Acetobacter indonesiensis* were each divided over two AFLP groups (Table 1). Both species have been previously shown to contain genetically diverse strains (Trček & Teuber, 2002; De Vuyst *et al.*, 2008). For *Acetobacter pasteurianus*,

this has also been found to be reflected in the DNA relatedness values which range from 62–90 % (Cleenwerck *et al.*, 2002). Secondly, strains of the species *Acetobacter cerevisiae* (LMG 1599, LMG 1625^T, LMG 1682, LMG 1699) and *Acetobacter orleanensis* (LMG 1545, LMG 1583^T, LMG 1592, LMG 1608), two phenotypically similar and phylogenetically closely related species, as well as *Gluconacetobacter europaeus* LMG 1518, *Gluconacetobacter xylinus* LMG 1510 and *Gluconacetobacter kombuchae* LMG 23726^T (Table 1), did not cluster as expected from their species designation and were, therefore, investigated in more detail (see below). It appeared that six of these strains should be reclassified, namely strains LMG 1545, LMG 1592 and LMG 1608 as *Acetobacter cerevisiae*; LMG 1518 and LMG 1510 as *Gluconacetobacter xylinus* and *Gluconacetobacter europaeus*, respectively; and LMG 23726^T as *Gluconacetobacter hansenii*. After reassignment of these reference strains, the species *Acetobacter cerevisiae* splits up over three AFLP groups (Table 1). Previous studies have suggested that species can be divided over different AFLP clusters (Thompson *et al.*, 2001; Willems *et al.*, 2001).

In total, 54 AFLP groups (A1 to A33, containing more than one strain; S1 to S21, each containing one strain) were delineated; each containing strains of the same species and showing a linkage level below 50 % with other groups. Each species was represented by one AFLP group (except for *Acetobacter pasteurianus*, *Acetobacter indonesienis* and *Acetobacter cerevisiae*; see above). Strain LMG 1531 (S6), previously assigned to *Acetobacter aceti* but removed from this species based on (GTG)₅-PCR fingerprint data and its low DNA–DNA relatedness (<60 %) with true *Acetobacter aceti* strains (De Vuyst *et al.*, 2008), showed a separate position, hereby confirming its status as a novel genotype of *Acetobacter* (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002). Overall, the results with the reference strains revealed the potential of AFLP for the identification and classification of AAB at the species level.

Analysis of the AFLP data of the 15 additional strains proved the taxonomic value of this AFLP approach, as all 15 strains could be identified at the species level. Ten strains clustered according to their original taxonomic assignment; while four strains (*Acetobacter pasteurianus* LMG 1547, *Gluconacetobacter intermedius* LMG 18907 and LMG 18908, and *Gluconacetobacter xylinus* LMG 1521) did not (Table 1); these appeared all to be misclassified (see below). Finally, LMG 23182, deposited in the BCCM/LMG Bacteria Collection as *Acetobacter* sp. (Table 1), could be identified as *Acetobacter pasteurianus*, a result that was expected from other research (E. J. Bartowsky, personal communication).

The AFLP data revealed, as mentioned above, several strains that did not cluster as expected from their species designation. These strains were subjected to additional tests including DNA–DNA hybridizations if appropriate (see Supplementary Tables S1 and S2 in IJSEM Online). DNA–DNA hybridizations were performed following a modi-

fication (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002) of the microplate method described by Ezaki *et al.* (1989) with high-molecular-mass genomic DNA, with A_{260}/A_{280} and A_{234}/A_{260} absorption ratios of 1.8–2.0 and 0.40–0.60 DNA, prepared according to the method of Wilson (1987) with minor modifications (Cleenwerck *et al.*, 2002). The hybridization temperature was 48 °C and hybridizations were performed in the presence of 50 % formamide. Reciprocal reactions (e.g. A × B and B × A) were performed for every pair of DNA samples and their variation was found to be within the limits of this method (Goris *et al.*, 1998). The additional tests revealed ten strains that have to be reclassified (see below).

Strains LMG 1545 (A29), LMG 1592 (A29) and LMG 1608 (A25) were classified as *Acetobacter orleanensis*, mainly on the basis of DNA–DNA hybridization data (Lisdiyanti *et al.*, 2000), however their AFLP DNA fingerprints clustered with those of *Acetobacter cerevisiae* strains, instead of with that of *Acetobacter orleanensis* LMG 1583^T (Fig. 1). DNA–DNA hybridizations performed in this study with strains LMG 1625^T, LMG 1583^T, LMG 1682, LMG 1545 and LMG 1608 confirmed the classification of strain LMG 1682 as *Acetobacter cerevisiae* (DNA relatedness of 88 % with *Acetobacter cerevisiae* LMG 1625^T versus 34 % with *Acetobacter orleanensis* LMG 1583^T), and revealed that strains LMG 1545 and LMG 1608 should be reclassified in *Acetobacter cerevisiae* (see Supplementary Table S1). Strain LMG 1592 should also be reclassified as *Acetobacter cerevisiae* as its AFLP DNA fingerprint showed >80 % band pattern similarity with that of LMG 1545.

Strains LMG 1518 (A14) and LMG 1510 (A16) were classified as *Gluconacetobacter europaeus* and *Gluconacetobacter xylinus*, respectively, on the basis of DNA–DNA hybridization data and (GTG)₅ PCR DNA fingerprint data (De Vuyst *et al.*, 2008). In the present study, AFLP analyses were performed for both strains starting from two newly opened LMG ampoules and on the DNA samples used in the study of De Vuyst *et al.* (2008). The DNA fingerprint data revealed that strains LMG 1518 and LMG 1510 should be reassigned to *Gluconacetobacter xylinus* and *Gluconacetobacter europaeus*, respectively. The previous taxonomic conclusions appear to have been based on erroneous results (suggesting a switch during DNA isolation or strain cultivation).

Strain LMG 23726^T (A20) is the type strain and only strain of *Gluconacetobacter kombuchae* (Dutta & Gachhui, 2007). Its AFLP DNA fingerprint clustered with the AFLP patterns of strains belonging to *Gluconacetobacter hansenii*, its phylogenetically nearest neighbour (99.4 % 16S rRNA gene sequence similarity), indicating that these strains could actually represent the same species and that the names are synonymous. Additional tests confirmed this synonymy. First, a nearly complete 16S rRNA gene sequence of *Gluconacetobacter kombuchae* LMG 23726^T was determined (1441 nucleotides, GenBank accession no. AM999342) following the protocol described by Franz *et al.* (2006)

Table 1. List of AAB used in this study

LMG, BCCM/LMG Bacteria Collection, Ghent University, Belgium; R, Research Collection of the Laboratory of Microbiology LM-UGent, Ghent University, Belgium; DSM, German Collection of Microorganisms and Cell Cultures, Germany; NCIMB, National Collection of Industrial, Marine and Food Bacteria, UK; LMD, Laboratorium voor Microbiologie, Technische Universiteit Delft, The Netherlands; NBRC, NITE Biological Resource Centre, Japan; NRIC, NODAI Culture Collection Center, Tokyo University of Agriculture, Japan. *A.*, *Acetobacter*; *Ac.*, *Acidomonas*; *As.*, *Asaia*; *G.*, *Gluconacetobacter*; *Gl.*, *Gluconobacter*; *Gr.*, *Granulibacter*; *K.*, *Kozakia*; *N.*, *Neosasaia*; *S.*, *Saccharibacter*; *Sw.*, *Swaminathania*.

Strain numbers	Species designation at the time of selection	Year, place and source of isolation	Hybridization group	AFLP group	Proposed species designation
LMG 1496 (=LMG 24WR)	<i>A. aceti</i>		<i>A. aceti</i>	A12	<i>A. aceti</i>
LMG 1504 ^T (=NCIMB 8621 ^T =NBRC 14818 ^T)	<i>A. aceti</i>	1923; beech-wood shavings of vinegar plant	<i>A. aceti</i>	A12	<i>A. aceti</i>
LMG 1535 (=LMG Ch31)	<i>A. aceti</i>	Belgium; vinegar plant	<i>A. aceti</i>	A12	<i>A. aceti</i>
LMG 1531 (=NCIMB 8941)	<i>Acetobacter</i> sp.	Cider	Related to <i>A. aceti</i>	S6	<i>Acetobacter</i> sp.
LMG 1545 (=NBRC 3296=NBRC 3296)	<i>A. orleanensis</i>	1941; Aichi, Japan; film in fermenter of rice vinegar	<i>A. cerevisiae</i>	A29	<i>A. cerevisiae</i>
LMG 1592 (=NCIMB 2224=NBRC 3170)	<i>A. orleanensis</i>	Manufacture of vinegar		A29	<i>A. cerevisiae</i>
LMG 1599 (=NCIMB 6425)	<i>A. cerevisiae</i>	UK; brewers' yeast and beer	<i>A. cerevisiae</i>	A29	<i>A. cerevisiae</i>
LMG 1608 (=NCIMB 8088=NBRC 3223)	<i>A. orleanensis</i>	1932; Netherlands; beer	<i>A. cerevisiae</i>	A25	<i>A. cerevisiae</i>
LMG 1625 ^T (=NCIMB 8894 ^T)	<i>A. cerevisiae</i>	Toronto, Canada; beer (ale) in storage	<i>A. cerevisiae</i>	A25	<i>A. cerevisiae</i>
LMG 1682 (=C101)	<i>A. cerevisiae</i>	Ireland; beer	<i>A. cerevisiae</i>	A29	<i>A. cerevisiae</i>
LMG 1699 (=MARTIN 2)	<i>A. cerevisiae</i>	1977; UK; brewery	<i>A. cerevisiae</i>	S20	<i>A. cerevisiae</i>
LMG 21418 ^T (=4H-1 ^T)	<i>A. cibinongensis</i>	Indonesia; mountain soursop (<i>Annona montanae</i>)	<i>A. cibinongensis</i>	S21	<i>A. cibinongensis</i>
LMG 1572 (=LMG E)	<i>A. estunensis</i>	Bristol, UK; cider	<i>A. estunensis</i>	A15	<i>A. estunensis</i>
LMG 1580 (=LMD 50.6)	<i>A. estunensis</i>	1950; Leiden, Netherlands; beer	<i>A. estunensis</i>	A15	<i>A. estunensis</i>
LMG 1626 ^T (=NCIMB 8935 ^T =NBRC 13751 ^T)	<i>A. estunensis</i>	Bristol, UK; cider	<i>A. estunensis</i>	A15	<i>A. estunensis</i>
R-36330 ^T (=LMG 24244 ^T =985 ^T)	<i>A. fabarum</i>	2006; Ghana; fermented cocoa beans	<i>A. fabarum</i>	A28	<i>A. fabarum</i>
R-36331 (=1145)	<i>A. fabarum</i>	2006; Ghana; fermented cocoa beans	<i>A. fabarum</i>	A28	<i>A. fabarum</i>
R-36458 (=950)	<i>A. fabarum</i>	2006; Ghana; fermented cocoa beans	<i>A. fabarum</i>	A28	<i>A. fabarum</i>
R-36459 (=1039)	<i>A. fabarum</i>	2006; Ghana; fermented cocoa beans	<i>A. fabarum</i>	A28	<i>A. fabarum</i>
R-29336 (=444B)	<i>A. ghanensis</i>	2004; Ghana; fermented cocoa beans	<i>A. ghanensis</i>	A31	<i>A. ghanensis</i>
R-29337 ^T (=LMG 23848 ^T =430A ^T)	<i>A. ghanensis</i>	2004; Ghana; fermented cocoa beans	<i>A. ghanensis</i>	A31	<i>A. ghanensis</i>
R-29338 (=415)	<i>A. ghanensis</i>	2004; Ghana; fermented cocoa beans	<i>A. ghanensis</i>	A31	<i>A. ghanensis</i>
LMG 1571 (=LMD 39.2)	<i>A. indonesiensis</i>		<i>A. indonesiensis</i>	A32	<i>A. indonesiensis</i>
LMG 1588 (=LMD 39.6)	<i>A. indonesiensis</i>		<i>A. indonesiensis</i>	S12	<i>A. indonesiensis</i>
LMG 19824 ^T (=NRIC 0313 ^T =5H-1 ^T)	<i>A. indonesiensis</i>	Indonesia; fruit of zirkak (<i>Annona muricata</i>)	<i>A. indonesiensis</i>	A32	<i>A. indonesiensis</i>
LMG 1617 ^T (=NCIMB 8620 ^T =NBRC 13753 ^T)	<i>A. lovaniensis</i>	1929; Becquevoort, Belgium; sewage on soil	<i>A. lovaniensis</i>	S19	<i>A. lovaniensis</i>
LMG 1746 ^T (=LMG 76.10 ^T)	<i>A. malorum</i>	1976; Ghent, Belgium; rotting apple	<i>A. malorum</i>	S15	<i>A. malorum</i>
LMG 23498 ^T (=RG1 ^T)	<i>A. nitrogenifigens</i>	2004; India; Kombucha tea	<i>A. nitrogenifigens</i>	S5	<i>A. nitrogenifigens</i>
LMG 21952 ^T (=B13 ^T)	<i>A. oeni</i>	2002; Viseu Dão region, Portugal; spoiled red wine	<i>A. oeni</i>	S8	<i>A. oeni</i>
LMG 21417 ^T (=21F-2 ^T)	<i>A. orientalis</i>	Indonesia; canna flower (<i>Canna hybrida</i>)	<i>A. orientalis</i>	A30	<i>A. orientalis</i>
LMG 1547* (=LMD 39.1)	<i>A. pasteurianus</i>		<i>A. orientalis</i>	A30	<i>A. orientalis</i>
LMG 1583 ^T (=NCIMB 8622 ^T =NBRC 13752 ^T)	<i>A. orleanensis</i>	1927; Belgium; beer	<i>A. orleanensis</i>	S13	<i>A. orleanensis</i>
LMG 1262 ^T (=LMD 22.1 t1 ^T)	<i>A. pasteurianus</i>	Netherlands; beer	<i>A. pasteurianus</i>	A13	<i>A. pasteurianus</i>
LMG 1555 (=NCIMB 8163)	<i>A. pasteurianus</i>		<i>A. pasteurianus</i>	A13	<i>A. pasteurianus</i>
LMG 1590 (=LMD 51.1)	<i>A. pasteurianus</i>	Type strain of <i>A. pasteurianus</i> subsp. <i>ascendens</i>		A13	<i>A. pasteurianus</i>

Table 1. cont.

Strain numbers	Species designation at the time of selection	Year, place and source of isolation	Hybridization group	AFLP group	Proposed species designation
LMG 1591* (=LMD 53.6)	<i>A. pasteurianus</i>	Quick vinegar; type strain of <i>A. pasteurianus</i> subsp. <i>paradoxus</i>		A13	<i>A. pasteurianus</i>
LMG 1618* (=NCIMB 8757)	<i>A. pasteurianus</i>	UK; malt vinegar acetifier		A13	<i>A. pasteurianus</i>
LMG 1621* (=NCIMB 8760)	<i>A. pasteurianus</i>	UK; malt vinegar acetifier		A13	<i>A. pasteurianus</i>
LMG 1628 (=NCIMB 9015)	<i>A. pasteurianus</i>			A13	<i>A. pasteurianus</i>
LMG 1629 (=A)	<i>A. pasteurianus</i>	Recife, Brazil; fermented <i>Agave sisalina</i> juice	<i>A. pasteurianus</i>	A33	<i>A. pasteurianus</i>
LMG 1630 (=EQ)	<i>A. pasteurianus</i>	Recife, Brazil; sugar cane bagasse	<i>A. pasteurianus</i>	A33	<i>A. pasteurianus</i>
LMG 1658 (=MM 80)	<i>A. pasteurianus</i>	Myanmar; toddy palm	Border of <i>A. pasteurianus</i>	A33	<i>A. pasteurianus</i>
LMG 1659 (=MM 73)	<i>A. pasteurianus</i>	Myanmar; toddy palm	Border of <i>A. pasteurianus</i>	A13	<i>A. pasteurianus</i>
LMG 1686 (=LMD 31.4)	<i>A. pasteurianus</i>	1928; Netherlands; vinegar from dry raisins	<i>A. pasteurianus</i>	A13	<i>A. pasteurianus</i>
LMG 23182* (=AWRI B253)	<i>Acetobacter</i> sp.	1997; Australia; oxidized Shiraz wine showing spoilage		A13	<i>A. pasteurianus</i>
R-29340 (=165D)	<i>A. pasteurianus</i>	2004; Ghana; fermented cocoa beans	<i>A. pasteurianus</i>	A13	<i>A. pasteurianus</i>
R-29341 (=150)	<i>A. pasteurianus</i>	2004; Ghana; fermented cocoa beans	<i>A. pasteurianus</i>	A33	<i>A. pasteurianus</i>
R-29342 (=406)	<i>A. pasteurianus</i>	2004; Ghana; fermented cocoa beans	<i>A. pasteurianus</i>	A13	<i>A. pasteurianus</i>
LMG 1633	<i>A. peroxydans</i>	1950; Netherlands; ditch water	Border of <i>A. peroxydans</i>	A24	<i>A. peroxydans</i>
LMG 1635 ^T (=NCIMB 8618 ^T =NBRC 13755 ^T)	<i>A. peroxydans</i>	Delft, Netherlands; ditch water	<i>A. peroxydans</i>	A24	<i>A. peroxydans</i>
LMG 21769 (=TNCSEF 36)	<i>A. peroxydans</i>	1999–2000; Thirumangalam Madurai, Tamilnadu, India; <i>Oryza sativa</i> , root tissue	<i>A. peroxydans</i>	A24	<i>A. peroxydans</i>
LMG 21770 (=TNCSEF 49)	<i>A. peroxydans</i>	1999–2000; Villupuram Villupuram, Tamilnadu, India; <i>Oryza sativa</i> , root tissue	<i>A. peroxydans</i>	A24	<i>A. peroxydans</i>
LMG 18848 ^T (=LTH 2458 ^T)	<i>A. pomorum</i>	Esslingen, Germany; submerged cider vinegar fermentation	<i>A. pomorum</i>	S18	<i>A. pomorum</i>
LMG 23690 ^T (=CWBI-B418 ^T)	<i>A. senegalensis</i>	1995; Casamance, Senegal; mango fruit (<i>Mangifera indica</i>)	<i>A. senegalensis</i>	A27	<i>A. senegalensis</i>
R-29339 (=108B)	<i>A. senegalensis</i>	2004; Ghana; fermented cocoa beans	<i>A. senegalensis</i>	A27	<i>A. senegalensis</i>
R-29343 (=420A)	<i>A. senegalensis</i>	2004; Ghana; fermented cocoa beans	<i>A. senegalensis</i>	A27	<i>A. senegalensis</i>
LMG 21419 ^T (=9H-2 ^T)	<i>A. syzygii</i>	Indonesia; fruit of Malay rose apple (<i>Syzygium malaccense</i>)	<i>A. syzygii</i>	S17	<i>A. syzygii</i>
LMG 1663 (=592)	<i>A. tropicalis</i>	UK; fermenting putrefied meat	<i>A. tropicalis</i>	A26	<i>A. tropicalis</i>
LMG 1754 (=LMG 79.18)	<i>A. tropicalis</i>	1979; Ivory Coast; fruit of <i>Ficus capensis</i>	<i>A. tropicalis</i>	A26	<i>A. tropicalis</i>
LMG 19825 ^T (=NRIC 0312 ^T =Ni-6b ^T)	<i>A. tropicalis</i>	Indonesia; coconut juice	<i>A. tropicalis</i>	A26	<i>A. tropicalis</i>
LMG 19826 (=NRIC 0321)	<i>A. tropicalis</i>	Indonesia; lime	<i>A. tropicalis</i>	A26	<i>A. tropicalis</i>
LMG 1667* (=MB 57)	<i>Ac. methanolica</i>	Sludge		A1	<i>Ac. methanolica</i>
LMG 1668 ^T (=MB 58 ^T =IMET 10945 ^T)	<i>Ac. methanolica</i>	Leipzig, Germany; fermentation of methanol with <i>Candida</i> sp. (not sterile)		A1	<i>Ac. methanolica</i>
LMG 1669* (=MB 60)	<i>Ac. methanolica</i>	Sludge		A1	<i>Ac. methanolica</i>
LMG 1735* (=SRI 1214)	<i>Ac. methanolica</i>	Australia; <i>Saccharum officinarum</i> , surface microflora		A1	<i>Ac. methanolica</i>
LMG 21650 ^T (=71 ^T)	<i>As. bogorensis</i>	Bogor, Indonesia; flower of the orchid tree (<i>Bauhinia purpurea</i>)		A4	<i>As. bogorensis</i>

Table 1. cont.

Strain numbers	Species designation at the time of selection	Year, place and source of isolation	Hybridization group	AFLP group	Proposed species designation
LMG 23142 (=87)	<i>As. bogorensis</i>	Bogor, Indonesia; flower of plumbago (<i>Plumbago auriculata</i>)		A4	<i>As. bogorensis</i>
LMG 23143 (=168)	<i>As. bogorensis</i>	Yogyakarta, Indonesia; tape ketan (fermented glutinous rice)		A4	<i>As. bogorensis</i>
LMG 23083 ^T (=AA08 ^T)	<i>As. krungthepensis</i>	Bangkok, Thailand; <i>Heliconia</i> sp., flower		A7	<i>As. krungthepensis</i>
LMG 23139 (=AA06)	<i>As. krungthepensis</i>	Bangkok, Thailand; <i>Heliconia</i> sp., flower		A7	<i>As. krungthepensis</i>
LMG 23140 (=AA09)	<i>As. krungthepensis</i>	Bangkok, Thailand; <i>Heliconia</i> sp., flower		A7	<i>As. krungthepensis</i>
LMG 21651 ^T (=S60-1 ^T)	<i>As. siamensis</i>	Bangkok, Thailand; crown flower (<i>Calotropis gigantea</i>)		A6	<i>As. siamensis</i>
LMG 23136 (=Y85)	<i>As. siamensis</i>	Bogor, Indonesia; flower of spider lily (<i>Crinum asiaticum</i>)		A6	<i>As. siamensis</i>
LMG 23144 (=D4-1)	<i>As. siamensis</i>	Bangkok, Thailand; flower of spider lily (<i>Crinum asiaticum</i>)		A6	<i>As. siamensis</i>
LMG 23145 (=I36)	<i>As. siamensis</i>	Bogor, Indonesia; flower of spider lily (<i>Crinum asiaticum</i>)		A6	<i>As. siamensis</i>
LMG 23146 (=B28S-3)	<i>As. siamensis</i>	Bogor, Indonesia; flower of ixora (<i>Ixora chinensis</i>)		A6	<i>As. siamensis</i>
LMG 21311 ^T (=CFN-Ca54 ^T)	<i>G. azotocaptans</i>	1996; Tapachula Chiapas, Mexico; <i>Coffea arabica</i> L., rhizosphere		A9	<i>G. azotocaptans</i>
LMG 23156 (=UAP-Ca97)	<i>G. azotocaptans</i>	1997; Motozintla Chiapas, Mexico; <i>Coffea arabica</i> L., rhizosphere		A9	<i>G. azotocaptans</i>
LMG 23157 (=UAP-Ca99)	<i>G. azotocaptans</i>	1997; Motozintla Chiapas, Mexico; <i>Coffea arabica</i> L., rhizosphere		A9	<i>G. azotocaptans</i>
LMG 1732 (=SRI 1205)	<i>G. diazotrophicus</i>	Australia, <i>Saccharum officinarum</i> , surface microflora		A3	<i>G. diazotrophicus</i>
LMG 7603 ^T (=Pal 5 ^T)	<i>G. diazotrophicus</i>	Alagoas, Brazil; <i>Saccharum officinarum</i> , root		A3	<i>G. diazotrophicus</i>
LMG 7971 (=PP 4)	<i>G. diazotrophicus</i>	Pernambuco, Brazil; <i>Saccharum officinarum</i> , stem		A3	<i>G. diazotrophicus</i>
LMG 8067 (=PR 20)	<i>G. diazotrophicus</i>	Rio de Janeiro Brazil, <i>Saccharum officinarum</i> , root		A3	<i>G. diazotrophicus</i>
LMG 22174* (=TNCSF 42)	<i>G. diazotrophicus</i>	Thirumangalam Madurai, Tamilnadu, India; <i>Oryza sativa</i> , root tissue		A3	<i>G. diazotrophicus</i>
LMG 22175* (=TNCSF 47)	<i>G. diazotrophicus</i>	Valajabad Kanchipuram, Tamilnadu, India, <i>Oryza sativa</i> , stem tissue		A3	<i>G. diazotrophicus</i>
LTH 4560 ^T	<i>G. entanii</i>	Germany; submerged high-acid spirit vinegar fermentation		S10	<i>G. entanii</i>
LMG 1380 (=NBRC 3261)	<i>G. europaeus</i>	1941; Nishinomiya, Japan; <i>Myrica rubra</i> , fruit		A16	<i>G. europaeus</i>
LMG 1510 (=NCIMB 613)	<i>G. xylinus</i>	Copenhagen, Denmark; vinegar		A16	<i>G. europaeus</i>
LMG 1521* (=NCIMB 7029)	<i>G. xylinus</i>	Nairobi, Kenya; from mixed culture NCTC 6716 isolated from vinegar brew		A16	<i>G. europaeus</i>
LMG 18494 (=Trcek V3)	<i>G. europaeus</i>	1995; Ljubljana, Slovenia; red wine vinegar produced in submerged bioreactor		A16	<i>G. europaeus</i>
LMG 18890 ^T (DES 11 ^T =DSM 6160 ^T)	<i>G. europaeus</i>	Esslingen, Germany; submerged culture vinegar generator		A16	<i>G. europaeus</i>
LMG 20956 (=JK2)	<i>G. europaeus</i>	1995; Ljubljana, Slovenia; cider vinegar produced in industrial submerged bioreactor		A16	<i>G. europaeus</i>

Table 1. cont.

Strain numbers	Species designation at the time of selection	Year, place and source of isolation	Hybridization group	AFLP group	Proposed species designation
LMG 1524 (=NCIMB 8246=NBRC 14916)	<i>G. hansanii</i>	Jerusalem, Israel; vinegar		A20	<i>G. hansanii</i>
LMG 1527 ^T (NCIMB 8746 ^T =NBRC 14820 ^T)	<i>G. hansanii</i>	Cellulose-less mutant 1 derived from NCIB 8745 isolated by M. Aschner, Jerusalem, vinegar		A20	<i>G. hansanii</i>
LMG 1528 (NCIMB 8747=NBRC 14817)	<i>G. hansanii</i>	Cellulose-less mutant 2 derived from NCIB 8745 isolated by M. Aschner, Jerusalem, vinegar		A20	<i>G. hansanii</i>
LMG 23726 (=RG3)	<i>G. kombuchae</i>	2004; India; Kombucha tea fermentation		A20	<i>G. hansanii</i>
LMG 18909 ^T (=TF2 ^T)	<i>G. intermedius</i> †	Switzerland; commercially available tea fungus beverage (Kombucha)		A22	<i>G. intermedius</i>
DSM 11804 ^T (=TF2 ^T)	<i>G. intermedius</i> †	Switzerland; commercially available tea fungus beverage (Kombucha)		A22	<i>G. intermedius</i>
LMG 21312 ^T (=CFN-Cf55 ^T)	<i>G. johannae</i>	1996; Tapachula Chiapas, Mexico; <i>Coffea arabica</i> L., rhizosphere		A11	<i>G. johannae</i>
LMG 23153 (=CFN-Cf75)	<i>G. johannae</i>	1997; Motozintla Chiapas, Mexico; <i>Coffea arabica</i> L., rhizosphere		A11	<i>G. johannae</i>
LMG 23154 (=UAP-Cf76)	<i>G. johannae</i>	1997; Motozintla Chiapas, Mexico, <i>Coffea arabica</i> L., rhizoplane		A11	<i>G. johannae</i>
LMG 1348 (=NCIMB 9417=IAM 1836)	<i>G. liquefaciens</i>	1935; Japan; fruit		A2	<i>G. liquefaciens</i>
LMG 1381 ^T (=NCIMB 9136 ^T =NBRC 12388 ^T)	<i>G. liquefaciens</i>	1935; Japan; <i>Diospyros</i> sp., dried fruit		A2	<i>G. liquefaciens</i>
LMG 1509 (=LMD 53.1)	<i>G. liquefaciens</i>	1950; canal water		A2	<i>G. liquefaciens</i>
LMG 1536 ^T (=NATA-strain)	<i>G. nataicola</i>	Manila, Philippines; nata de coco; nata producing organism		S7	<i>G. nataicola</i>
LMG 1517 (=NCIMB 4940=NBRC 14822)	<i>G. oboediens</i> †			A21	<i>G. oboediens</i>
LMG 1688 (=LMD 22.3)	<i>G. oboediens</i> †			A21	<i>G. oboediens</i>
LMG 1689 (=LMD 29.8)	<i>G. oboediens</i> †			A21	<i>G. oboediens</i>
LMG 18849 ^T (=LTH 2460 ^T)	<i>G. oboediens</i>	Esslingen, Germany; red wine vinegar fermentation		A21	<i>G. oboediens</i>
DSM 11826 ^T (=LTH 2460 ^T)	<i>G. oboediens</i> †	Esslingen, Germany; red wine vinegar fermentation		A21	<i>G. oboediens</i>
LMG 18907* (=JK3)	<i>G. intermedius</i> †	1995; Ljubljana, Slovenia; cider vinegar produced in submerged bioreactor		A21	<i>G. oboediens</i>
LMG 18908* (=JKD)	<i>G. intermedius</i> †	1995; Ljubljana, Slovenia; cider vinegar produced in submerged bioreactor		A21	<i>G. oboediens</i>
LMG 22126 ^T (=DST GL02 ^T)	<i>G. rhaeticus</i>	2003; Val Venosta, South Tyrol, Italy; organic apple juice		S16	<i>G. rhaeticus</i>
LMG 19747 ^T (=SRI 1794 ^T)	<i>G. sacchari</i>	Australia; <i>Saccharum officinarum</i> , surface microflora		A5	<i>G. sacchari</i>
LMG 19748 (=SRI 1853)	<i>G. sacchari</i>	Queensland Australia; mealy bug		A5	<i>G. sacchari</i>
LMG 1582 ^T (=LMD 29.3)	<i>G. saccharivorans</i>	1927; Germany; beet juice		A23	<i>G. saccharivorans</i>
LMG 1584 (=LMD 39.5)	<i>G. saccharivorans</i>			A23	<i>G. saccharivorans</i>
LMG 22125 ^T (=DST GL01 ^T)	<i>G. swingsii</i>	2003; Val Venosta, South Tyrol, Italy; organic apple juice		S4	<i>G. swingsii</i>
LMG 1515 ^T (=NCIMB 11664 ^T =JCM 7644 ^T)	<i>G. xylinus</i>	Mountain ash berries		A14	<i>G. xylinus</i>
LMG 1518 (=NCIMB 5346)	<i>G. europaeus</i>	O. Verona		A14	<i>G. xylinus</i>
LMG 1356 ^T (=NBRC 3250 ^T)	<i>Gl. albidus</i>	1941; Japan; <i>Dahlia coccinea</i> ; <i>A. albidus</i>		A8	<i>Gl. albidus</i>
LMG 1375 t1 (=NBRC 3273)	<i>Gl. albidus</i>	1941; Nishinomiya, Japan; <i>Myrica rubra</i> , fruit		A8	<i>Gl. albidus</i>
LMG 1368 ^T (=NBRC 3267 ^T)	<i>Gl. cerinus</i>	1941; Osaka, Japan; <i>Prunus</i> sp. (cherry)		A19	<i>Gl. cerinus</i>

Table 1. cont.

Strain numbers	Species designation at the time of selection	Year, place and source of isolation	Hybridization group	AFLP group	Proposed species designation
LMG 1376 t1 (=NBRC 3274)	<i>Gl. cerinus</i>	1941; Nishinomiya, Japan; <i>Myrica rubra</i> , fruit.		A19	<i>Gl. cerinus</i>
LMG 1389 (=NBRC 3275)	<i>Gl. cerinus</i>	1941; herbal garden, Kyoto, Japan; <i>Rheum rhabarbarum</i> , flower		A19	<i>Gl. cerinus</i>
LMG 1390 (=NBRC 3276)	<i>Gl. cerinus</i>	1941; herbal garden, Kyoto, Japan; <i>Rheum rhabarbarum</i> , flower; type strain of <i>Gl. asaii</i>		A19	<i>Gl. cerinus</i>
LMG 1357 t1 (=NBRC 3251)	<i>Gl. frateuri</i>	1941; botanical garden, Kyoto, Japan; <i>Dahlia</i> sp., flower		A18	<i>Gl. frateuri</i>
LMG 1358 (=NBRC 3253)	<i>Gl. frateuri</i>	1941; botanical garden, Kyoto, Japan; <i>Dahlia</i> sp., flower		A18	<i>Gl. frateuri</i>
LMG 1365 ^T (=NBRC 2364 ^T)	<i>Gl. frateuri</i>	1941; Osaka, Japan; <i>Fragaria ananassa</i>		A18	<i>Gl. frateuri</i>
LMG 1369 t1 (=NBRC 3268)	<i>Gl. frateuri</i>	1941; Osaka, Japan; <i>Prunus</i> sp. (cherry)		A18	<i>Gl. frateuri</i>
LMG 1371 (=NBRC 3270)	<i>Gl. frateuri</i>	1941; Osaka, Japan; <i>Prunus</i> sp. (cherry)		A18	<i>Gl. frateuri</i>
LMG 1379 (=NBRC 3286)	<i>Gl. frateuri</i>	1941; Osaka, Japan; <i>Eriobotrya japonica</i> , fruit		A18	<i>Gl. frateuri</i>
LMG 1367 ^T t1 (=NBRC 2366)	<i>Gl. kondonii</i>	1941; Osaka, Japan; <i>Fragaria ananassa</i>		S3	<i>Gl. kondonii</i>
LMG 1359 (=NBRC 3462)	<i>Gl. oxydans</i>			A10	<i>Gl. oxydans</i>
LMG 1385 (=NBRC 3293)	<i>Gl. oxydans</i>	1941; Osaka, Japan; <i>Diospyros</i> sp., dried fruit		A10	<i>Gl. oxydans</i>
LMG 1393 (=NBRC 12528)	<i>Gl. oxydans</i>	Delft, Netherlands; Amstel beer; assay of nicotinic acid (=niacin), p-aminobenzoic acid, pantothenic acid; type strain of <i>Gl. oxydans</i> subsp. <i>suboxydans</i>		A10	<i>Gl. oxydans</i>
LMG 1395 (=NBRC 3287)	<i>Gl. oxydans</i>	1941; botanical garden, Kyoto, Japan; <i>Liatriis scariosa</i> ; flower		A10	<i>Gl. oxydans</i>
LMG 1396*	<i>Gl. oxydans</i>	Beer		A10	<i>Gl. oxydans</i>
LMG 1408 ^T (=NBRC 14819 ^T)	<i>Gl. oxydans</i>	Beer		A10	<i>Gl. oxydans</i>
LMG 1424 (=NBRC 3244)	<i>Gl. oxydans</i>			A10	<i>Gl. oxydans</i>
LMG 1674*	<i>Gl. oxydans</i>	1924; Delft, Netherlands; beer		A10	<i>Gl. oxydans</i>
LMG 1486 (=NBRC 3254)	<i>Gl. thailandicus</i>	1941; Osaka, Japan; <i>Fragaria ananassa</i>		A17	<i>Gl. thailandicus</i>
LMG 1487 (=NBRC 3255)	<i>Gl. thailandicus</i>	1941; Osaka, Japan; <i>Fragaria ananassa</i>		A17	<i>Gl. thailandicus</i>
LMG 23137 ^T (=F149-1 ^T)	<i>Gl. thailandicus</i>	Bangkok, Thailand; Indian cork tree (<i>Millingtonia hortensis</i>), flower		A17	<i>Gl. thailandicus</i>
LMG 23138 (=F142-1)	<i>Gl. thailandicus</i>	Bangkok, Thailand; glossy ixora (<i>Ixora lobbii</i>), flower		A17	<i>Gl. thailandicus</i>
DSM 17861 ^T (=CDGNIH1 ^T)	<i>Gr. bethesdensis</i>	2003; Bethesda, United States; patient with chronic granulomatous disease, lymph node		S9	<i>Gr. bethesdensis</i>
LMG 21812 ^T (=Yo-3 ^T)	<i>K. baliensis</i>	1996; Bali, Indonesia; palm brown sugar		S11	<i>K. baliensis</i>
LMG 24037 ^T (=AC28 ^T)	<i>N. chiangmaiensis</i>	2002; Chiang-Mai, Thailand; red ginger (<i>Alpinia purpurata</i>), flower		S2	<i>N. chiangmaiensis</i>
LMG 23170 ^T (=S-877 ^T)	<i>S. floricola</i>	Kanagawa Prefecture, Japan; pollen		S14	<i>S. floricola</i>
LMG 21291 ^T (=PA51 ^T)	<i>Sw. salitolerans</i>	1998; Pichavaram mangrove forest Chidambaram, Tamil Nadu, India; wild rice <i>Porteresia coarctata</i>		S1	<i>Sw. salitolerans</i>

*Strain for which no DNA–DNA hybridization data or 16S–23S rRNA gene ITS sequence analysis data have (previously) been reported.

†The type strains of *G. oboediens* and *G. intermedius* show 63 % DNA relatedness according to Dellaglio *et al.* (2005) and >70 % according to Sievers & Swings (2005) and Lisdiyanti *et al.* (2006).

using the primers *Gamma, *PD, *O, *3, *R, pA, Gamma, 3 and BKL1 (Coenye *et al.*, 1999; Cleenwerck *et al.*, 2007). This revealed that *Gluconacetobacter kombuchae* RG3^T (AY688433) was its nearest neighbour [AY688433, 99.6 % 16S rRNA gene sequence similarity as calculated with the BioNumerics 4.61 software package (Applied Maths) using an open gap penalty of 100 % and a unit gap penalty of 0 %], indicating that strain LMG 23726^T is a subculture of RG3^T. Secondly, DNA–DNA hybridizations between *Gluconacetobacter kombuchae* LMG 23726^T and *Gluconacetobacter hansenii* LMG 1527^T revealed a DNA relatedness value of 81 % in the present study, which is clearly above the 70 % threshold (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002) and much higher than the 25 % DNA relatedness reported by Dutta & Gachhui (2007). Phylogenetically, *Gluconacetobacter kombuchae* and *Gluconacetobacter hansenii* belong to the *Gluconacetobacter xylinus* branch (Dutta & Gachhui, 2007; Yamada & Yukphan, 2008), which contains several species showing ≥ 99.4 % 16S rRNA gene sequence similarity. DNA relatedness values between closely related species of this cluster are generally intermediate (see Supplementary Table S2). Thirdly, the G+C content of DNA prepared for DNA–DNA hybridization was determined for both strains by HPLC according to the method of Mesbah *et al.* (1989) and was 59.4 mol% for LMG 1527^T and 59.5 mol% for LMG 23726^T, the latter being definitely higher than the 55.8 mol% value reported by Dutta & Gachhui (2007). Fourthly, the species description of *Gluconacetobacter hansenii* was emended recently by Lisdiyanti *et al.* (2006); among ten strains previously assigned to this species, only three strains were shown to actually be members of the species (Lisdiyanti *et al.*, 2006). These three strains (LMG 1524, LMG 1527^T and LMG 1528) as well as *Gluconacetobacter kombuchae* LMG 23726^T, were investigated for the phenotypic characteristics that have been reported to enable differentiation of both these species from the other species of the *Gluconacetobacter xylinus* branch. The production of 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from D-glucose was determined by the method described by Gosselé *et al.* (1980) using high pressure anion-exchange chromatography with conductivity detection under ion suppression (Van der Meulen *et al.*, 2006) instead of TLC. Growth on the carbon sources ethanol, sucrose, sorbitol and D-mannitol; growth in the presence of 30 % D-glucose; production of acid from galactitol, and production of cellulose were examined by previously reported methods (Cleenwerck *et al.*, 2002; Lisdiyanti *et al.*, 2006). All four strains produced 2-keto- and 5-keto-D-gluconic acid from D-glucose, produced acid from galactitol and were able to grow on D-sorbitol and D-mannitol as energy sources. Growth on the energy sources ethanol and sucrose was variable (LMG 1527^T grew weakly on ethanol, but not on sucrose), as well as growth in the presence of 30 % D-glucose (LMG 1527^T grew weakly in 30 % D-glucose). Cellulose production was clearly noticed for LMG 23726^T, but could not be confirmed for LMG 1524. In general, most previous phenotypic findings were

confirmed. Fifthly, according to Dutta & Gachhui (2007), the major difference between the type strain of *Gluconacetobacter kombuchae* and related gluconacetobacters was its ability to fix nitrogen. These authors confirmed the presence of the *nifH* gene in *Gluconacetobacter kombuchae* by PCR with the degenerate primers 19F and 407R (Franke *et al.*, 1998) and sequencing of the amplified PCR product (Dutta & Gachhui, 2007). In this study, the four strains all generated a PCR product of the same size (~600 bp) using the degenerate primers used by Dutta & Gachhui (2007) (data not shown), indicating the presence of the *nifH* gene in all of these strains. The data presented clearly show that *Gluconacetobacter kombuchae* is a later heterotypic synonym of *Gluconacetobacter hansenii*, with the name *Gluconacetobacter hansenii* having priority over the name *Gluconacetobacter kombuchae* according to Rules 38 and 42 of the Bacteriological Code (Lapage *et al.*, 1992).

Strain LMG 1547 (A30) was classified as *Acetobacter pasteurianus* by Gosselé *et al.* (1983) based on its position in subphenon E, established on the basis of numerical analysis of 177 phenotypic features. Its AFLP DNA fingerprint showed considerable resemblance (~55 % band pattern similarity and clear visual similarity) to that of *Acetobacter orientalis* LMG 21417^T. DNA hybridizations between both strains revealed 100 % DNA relatedness, proving that strain LMG 1547 is in fact a member of *Acetobacter orientalis*.

Strains LMG 18907 and LMG 18908 (A21) were classified as *Gluconacetobacter intermedius* on the basis of SDS-PAGE data of their soluble proteins (Boesch *et al.*, 1998). However, their AFLP DNA fingerprints showed more similarity with the AFLP patterns of strains of *Gluconacetobacter oboediens*. At present, confusion exists concerning the taxonomic status of *Gluconacetobacter oboediens* and *Gluconacetobacter intermedius*, due to discrepancies in the DNA relatedness values reported for their type strains (Cleenwerck & De Vos, 2008). *Gluconacetobacter oboediens* and *Gluconacetobacter intermedius* were described around the same time in the same year (Boesch *et al.*, 1998; Sokollek *et al.*, 1998) and were hence not compared with each other upon description. Later studies revealed that they were phylogenetically closely related species of the *Gluconacetobacter xylinus* branch (Dellaglio *et al.*, 2005; Lisdiyanti *et al.*, 2006; Yamada & Yukphan, 2008). According to Sievers & Swings (2005) and Lisdiyanti *et al.* (2006), the names should be regarded as synonymous, mainly because a high DNA relatedness value was found between their type strains (76 % and >77 %, respectively). However, according to Dellaglio *et al.* (2005), the type strains shared 63 % DNA relatedness, which was somewhat lower than the 68 % DNA relatedness observed between *Gluconacetobacter oboediens* LMG 18849^T and *Gluconacetobacter europaeus* LMG 18890^T (see Supplementary Table S2), suggesting that both species should remain separate. The AFLP DNA fingerprint data supported the conclusion based on the DNA relatedness of 63 % between the type strains. In this

study, additional tests were therefore performed to clarify the taxonomic status of both species. Firstly, *Gluconacetobacter intermedius* DSM 11804^T and *Gluconacetobacter oboediens* DSM 11826^T, used in the hybridization study of Lisdiyanti *et al.* (2006), were analysed by AFLP and their patterns were found to cluster with those of *Gluconacetobacter intermedius* LMG 18909^T and *Gluconacetobacter oboediens* LMG 18849^T, respectively, with a similarity higher than the reproducibility level. Secondly, the strains of the species of the *Gluconacetobacter xylinus* branch were subjected to (GTG)₅-PCR DNA fingerprinting following a previously described protocol (De Vuyst *et al.*, 2008). Clustering of the (GTG)₅ DNA fingerprints (Supplementary Fig. S1) revealed the same groupings as AFLP DNA fingerprinting. Thirdly, additional DNA–DNA hybridizations with strains LMG 18849^T, LMG 1517, LMG 1688 and LMG 1619 (A21) and LMG 18909^T (A22) revealed DNA relatedness values ranging from 53 to 63 % between *Gluconacetobacter intermedius* LMG 18909^T and strains of AFLP group A21, containing the type strain of *Gluconacetobacter oboediens*, whereas values ranging from 68 to 100 % were obtained between strains of AFLP group A21 (Supplementary Table S2). Together, the data support the conclusion that *Gluconacetobacter intermedius* and *Gluconacetobacter oboediens* should not be regarded as synonymous. Consequently, strains LMG 18907 and LMG 18908 should be reclassified as members of the species *Gluconacetobacter oboediens*. Interestingly, the type strain of *Gluconacetobacter intermedius* was isolated from Kombucha, whereas the strains of AFLP cluster A21 with a known origin, were isolated from vinegar.

Strain LMG 1521 (A16) was classified as *Acetobacter xylinus* (= *Gluconacetobacter xylinus*) on the basis of phenotypic data (Gosselé *et al.*, 1983). However Boesch *et al.* (1998) noticed that this strain reacted with the oligonucleotide probe 23seu, developed for *Gluconacetobacter europaeus*. The AFLP data confirm its classification as *Gluconacetobacter europaeus*.

It is clear from the results presented above that AFLP DNA fingerprinting enables the accurate identification and classification of a broad range of AAB at the species level. Besides this, AFLP analyses appear to be useful for the determination of genetic diversity amongst strains of the same species. In Fig. 1 it can be noticed that the AFLP patterns of strains belonging to a particular species and containing more than 40 bands generally group at a linkage level of 50 % or more. Those with fewer bands generally cluster at a lower linkage level, as can be seen for several species of the genus *Acetobacter*, with three of them being split up in several AFLP groups. Also, Janssen *et al.* (1996) noticed that the linkage levels of some clusters dropped significantly if the number of bands in the AFLP patterns decreased. These data indicate that AFLP DNA fingerprints with less than 40 bands are more discriminatory at the

intraspecies level and are less suitable for species-level identification.

Nevertheless, some conclusions regarding the intraspecific genetic diversity can be made from the data reported. For instance, several species, such as *Gluconacetobacter oxydans* (A10), *Acetobacter pasteurianus* (A13, A33), *Gluconacetobacter frateurii* (A18), *Gluconacetobacter cerinus* (A19), *Acetobacter indonesienis* (S12, A32) and *Acetobacter cerevisiae* (A25, A29, S20) harbour genetically diverse strains, whereas other species, such as *Gluconacetobacter diazotrophicus* (A3), *Acetobacter krungthepensis* (A7), *Gluconacetobacter albidus* (A8), *Gluconacetobacter azotocaptans* (A9), *Gluconacetobacter johannae* (A11), *Acetobacter aceti* (A12), *Acetobacter estunensis* (A15), *Gluconacetobacter thailandicus* (A17) and *Gluconacetobacter hansenii* (A20), harbour strains that generate quite homogeneous AFLP patterns. For the *Gluconobacter* strains, the AFLP data correlate very well with the results of Takahashi *et al.* (2006) based on 16S–23S rRNA gene spacer region sequence analysis data. For the species appearing to have a limited genetic diversity, AFLP analyses of additional strains (if available) and with other primer combinations (such as A01/T03) are advised to gain a deeper understanding of their genetic diversity. A previous study showed that a better insight into the genetic diversity of *Gluconacetobacter diazotrophicus* was obtained when more isolates from sugar cane, as well as from other host plants such as sweet potato and from the mealybug, *Saccharococcus sacchari*, were investigated (Caballero-Mellado *et al.*, 1995). Other studies have stated that highly related strains may be distinguished by AFLP when the appropriate set of selective primers is used (e.g. Janssen *et al.*, 1996).

This study further revealed strains with very similar AFLP DNA fingerprints, grouping together at a linkage level of 80 % or more, e.g. *Gluconacetobacter liquefaciens* LMG 1381^T and LMG 1348 (A2). These strains may be clonally related, especially if they were isolated from the same source at the same site in the same year. In this perspective, it is interesting to note that the four *Gluconacetobacter thailandicus* strains (A17) that showed very similar AFLP patterns had different geographical origins. Two strains were recently isolated from flowers collected in Thailand (LMG 23137^T and LMG 23138), whereas the other two strains were isolated in 1941 from *Fragaria ananassa* in Japan (LMG 1486 and LMG 1487). For all these strains, additional analyses, such as AFLP using other primer combinations or PFGE, are needed to determine whether these strains are indeed clonally related.

In conclusion, the data in this study demonstrate that AFLP DNA fingerprinting permits the accurate identification and classification of a broad range of AAB at the species level. In addition, AFLP DNA fingerprinting appears to be useful for determining intraspecific genetic diversity. The technique can be regarded as valuable for the taxonomy of AAB.

Acknowledgements

This research was funded by the Federal Research Policy (Action for the promotion of and co-operation with the Belgian Coordinated Collections of Microorganisms, BCCM) and the Research Council of the Vrije Universiteit Brussel. The authors wish to acknowledge Katrien Vandemeulebroecke, Leentje Christiaens, Katrien Engelbeen and Cindy Snauwaert for their technical support.

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