Leuconostoc holzapfelii sp. nov., isolated from Ethiopian coffee fermentation and assessment of sequence analysis of housekeeping genes for delineation of *Leuconostoc* species

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A Gram-positive, ovoid lactic acid bacterium, strain LMG 23990^T, was isolated from Ethiopian coffee fermentation. 16S rRNA gene sequence analysis indicated that the novel strain belongs to the genus *Leuconostoc*, with *Leuconostoc citreum* and *Leuconostoc lactis* as the closest neighbours (99.6 and 99.0 % 16S rRNA gene sequence similarity, respectively). Genotypic fingerprinting by fluorescent amplified fragment length polymorphism, whole-cell protein electrophoresis, DNA–DNA hybridizations, comparative sequence analysis of *pheS*, *rpoA*, *atpA*, and physiological and biochemical tests allowed us to differentiate strain LMG 23990^T from all established *Leuconostoc* species. Strain LMG 23990^T (=CCUG 54536^T) therefore represents a novel species, for which the name *Leuconostoc holzapfelii* sp. nov. is proposed.

The genus *Leuconostoc* belongs to the order *Lactobacillales*, an order of Gram-positive bacteria within the phylum *Firmicutes*. Like other lactic acid bacteria, *Leuconostoc* strains produce lactic acid as the major metabolite of sugar fermentation. They are used in the production of fermented products such as cheese, butter, buttermilk, kefir, sourdough and kimchi. At the time of writing the genus *Leuconostoc* comprised 14 species. Recently, several novel *Leuconostoc* species have been described, originating from different types of food. These species include *Leuconostoc inhae* and *Leuconostoc kimchii* from kimchi, a Korean vegetable product (Kim *et al.*, 2000, 2003), *Leuconostoc gasicomitatum* from marinated broiler meat strips (Susiluoto *et al.*, 2003), *Leuconostoc durionis* from tempoyak, fermented durian (Leisner *et al.*, 2005), and *Leuconostoc ficulneum* and *Leuconostoc pseudoficulneum* from ripe figs (Antunes *et al.*, 2002; Chambel *et al.*, 2006).

Strain LMG 23990^T was isolated during an investigation of the microbial populations associated with the fermentation of coffee in Ethiopia. A combined action of lactic acid bacteria and/or endogenous coffee enzymes has been reported to play a role in the mucilage degradation (Vaughn *et al.*, 1958; Arunga, 1973). In this respect *Leuconostoc mesenteroides* was found to solubilize pectic substances (Juven *et al.*, 1985). Whereas the majority of *Leuconostoc* isolates from coffee were identified to the species level by rep-PCR (Caroline, 2005; Böhringer, 2006), this strain did not cluster with any known *Leuconostoc* reference or type species. Subsequent 16S rRNA gene sequence analysis indicated that it might represent a novel *Leuconostoc* species. In the present study additional analyses were performed on reference strains of other *Leuconostoc* species obtained from the BCCM/LMG Bacteria Collection, Ghent, Belgium and DSMZ, Braunschweig, Germany (Table 1). All strains except *Leuconostoc gelidum* strains were cultivated on MRS agar

Abbreviation: FAFLP, fluorescent amplified fragment length polymorphism.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of LMG 23990^T is AM600682. The GenBank/EMBL/DDBJ accession numbers for the *pheS*, *rpoA* and *atpA* gene sequences reported in this paper are AM711136–AM711355, as indicated in Supplementary Figs S1–S6.

Supplementary figures are available with the online version of this paper.

Table 1. List of *Leuconostoc* strains

Species name	Strain no.	Depositor*	Source†
L. carnosum	LMG 18865	J. Björkroth	Cured raw-meat mass (1996, Finland)
L. carnosum	LMG 18866	J. Björkroth	Air (1996, Finland)
L. carnosum	LMG 18867	J. Björkroth	Cured raw-meat mass (1996, Finland)
L. carnosum	LMG 18868	J. Björkroth	Air (1996, Finland)
L. carnosum	LMG 23898^T	CECT	Vacuum-packed beef stored at low temperature (UK)
L. citreum	LMG 9824	DSM	NK
L. citreum	LMG 9849^T	NCFB	Honeydew of rye ear
L. citreum	LMG 11417	NCIMB	Canned tomatoes
L. citreum	LMG 18978	NCIMB	Blood
L. citreum	LMG 22638	M. Zamfir	NK
L. durionis	LMG 22556^T	J. Leisner	Tempoyak made from durian fruit (Malaysia)
L. durionis	LMG 22557	J. Leisner	Tempoyak made from durian fruit (Malaysia)
L. durionis	LMG 22558	J. Leisner	Tempoyak made from durian fruit (Malaysia)
L. fallax	LAB 1671	J. Leisner	Tempoyak made from durian fruit (Malaysia)
L. fallax	LAB 1673	J. Leisner	Tempoyak made from durian fruit (Malaysia)
L. fallax	LAB 1681	J. Leisner	Tempoyak made from durian fruit (Malaysia)
L. fallax	R-35138	(Own isolate)	Fermenting cassava for gari (Benin)
L. fallax	R-35140	(Own isolate)	Fermenting cassava for gari (Benin)
L. fallax	R-35141	(Own isolate)	Fermenting cassava for gari (Benin)
L. fallax	LMG 13177^T	DSM	Sauerkraut (Germany)
L. ficulneum	LMG 21928^T	DSM	Ripe fig (2000, Portugal)
L. fructosum	LMG 9498^T	A. Ledeboer	Flower
L. gasicomitatum	LMG 18811^T	J. Björkroth	Modified-packaged meat strip (Finland)
L. gasicomitatum	LMG 18813	J. Björkroth	Modified-packaged meat strip (Finland)
L. gasicomitatum	LMG 19597	J. Björkroth	Modified-packaged meat strip (Finland)
L. gasicomitatum	LMG 19598	J. Björkroth	Modified-packaged meat strip (Finland)
L. gasicomitatum	LMG 19601	J. Björkroth	Modified-packaged meat strip (Finland)
L. gelidum	R-35295	J. Björkroth	Modified-packaged spoiled pork (Finland)
L. gelidum	R-35296	J. Björkroth	Modified-packaged meat strip (Finland)
L. gelidum	R-35297	J. Björkroth	Organically produced carrots (Finland)
L. gelidum	R-35298	J. Björkroth	Spoiled herring (Finland)
L. gelidum	R-35299	J. Björkroth	EMAP salad (Finland)
L. gelidum	LMG 18297^T	ATCC	Vacuum packaged beef
L. holzapfelii	LMG 23990^T	(Own isolate)	Coffee fermentation (Ethiopia)
L. inhae	LMG 22919^T	DSM	Kimchi (South Korea)
L. kimchii	LMG 23786	MCCM	Raw milk (2004, Morocco)
L. kimchii	LMG 23787	MCCM	Raw milk (2004, Morocco)
L. lactis	LMG 22614	M. Zamfir	Sour cream (2002, Romania)
L. lactis	LMG 23115	P. Svec	Haemoculture (2004, Czech Republic)
L. lactis	LMG 22591	M. Zamfir	Cheese (2002, Romania)
L. lactis (type strain of *L. argentinum*)	LMG 18543	CCUG	Raw milk (Argentina)
L. lactis	LMG 7940	A. Ledeboer	Cheese
L. lactis	LMG 8894^T	A. Ledeboer	Milk (1948)
L. lactis	LMG 22650	M. Zamfir	Cheese (2003, Romania)
L. lactis	LMG 22635	M. Zamfir	Raw cow's milk (2002, Romania)
L. mesenteroides subsp. *cremoris*	LMG 6909^T	NCIB	Hansen's dried cheese starter powder
L. mesenteroides subsp. *cremoris*	LMG 18971	NCIMB	NK
L. mesenteroides subsp. *cremoris*	LMG 18972	NCIMB	Dried cheese starter powder
L. mesenteroides subsp. *dextranicum*	LMG 18973	NCIMB	Fermenting sauerkraut
L. mesenteroides subsp. *dextranicum*	LMG 6908^T	NCIB	(1912)
L. mesenteroides subsp. *dextranicum*	LMG 7954	A. Ledeboer	Fermenting sauerkraut (1928)
L. mesenteroides subsp. *dextranicum*	LMG 11318	NCFB	NK
L. mesenteroides subsp. *dextranicum*	LMG 11320	NCFB	NK
L. mesenteroides subsp. *dextranicum*	LMG 11321	NCFB	Natural starter (1926)
L. mesenteroides subsp. *mesenteroides*	LMG 6893^T	NCIMB	Fermenting olives (1941, USA)
L. mesenteroides subsp. *mesenteroides*	LMG 7939	A. Ledeboer	Slime on root beer
L. mesenteroides subsp. *mesenteroides*	LMG 18967	NCIMB	Silage

Table 1. cont.

Species name	Strain no.	Depositor*	Source†
L. mesenteroides subsp. *mesenteroides*	LMG 19463	V. Perreten	NK
L. mesenteroides subsp. *mesenteroides*	LMG 23111	P. Svec	Haemoculture (2004, Czech Republic)
L. mesenteroides subsp. *mesenteroides*	LMG 8159	CCUG	(1972, Sweden)
L. pseudomesenteroides	LMG 11499		NK
L. pseudomesenteroides	LMG 10779	CCUG	Lactic culture (1990, Sweden)
L. pseudomesenteroides	LMG 11482^T	NCFB	Cane juice
L. pseudomesenteroides	LMG 18969	NCIMB	Fermenting string beans
L. pseudomesenteroides	LMG 22615	M. Zamfir	NK
L. pseudomesenteroides	LMG 23108	P. Svec	Haemoculture (2004, Czech Republic)
L. pseudomesenteroides	LMG 22661	M. Zamfir	NK
L. pseudoficulneum	R-35155	R. Tenreiro	Ripe fig (Portugal)
L. pseudoficulneum	R-35156	R. Tenreiro	Ripe fig (Portugal)
L. pseudoficulneum	R-35157	R. Tenreiro	Ripe fig (Portugal)
L. pseudoficulneum	R-35158	R. Tenreiro	Ripe fig (Portugal)
L. pseudoficulneum	R-35159	R. Tenreiro	Ripe fig (Portugal)
L. pseudoficulneum	R-35160	R. Tenreiro	Ripe fig (Portugal)
L. pseudoficulneum	LMG 23899^T	CECT	Ripe fig (Portugal)

*Abbreviations: ATCC, American type Culture Collection, Manassas, USA; Björkroth J., Helsinki University, Helsinki, Finland; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; CECT, Colleción Espagñola de Cultivos Tipo, Valencia, Spain; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; Ledeboer A., Unilever, Vlaardingen, The Netherlands; Leisner J., Royal Veterinary and Agricultural University, Frederiksberg Copenhagen, Denmark; LMG, Belgian Co-ordinated Collections of Microorganisms/ Laboratory of Microbiology Ghent, Ghent, Belgium; MCCM, Medical Culture Collection Marburg, Marburg, Germany; NCFB, National Collection of Food Bacteria (now NCIMB); NCIB, National Collection of Industrial Bacteria (now NCIMB); NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK; Perreten V., Swiss Federal Institute of Technology Zürich, Zürich, Switzerland; Svec P., Faculty Hospital Brno, Brno, Czech Republic; Tenreiro R., University of Lisbon, Lisbon, Portugal; Zamfir M., Romanian Academy, Bucharest, Romania. LAB and R numbers refer to own isolates from our LMG research collection.
†NK, Not known.

(de Man *et al.*, 1960) at 30 °C for 24 h. *L. gelidum* strains were cultivated at 22 °C for 48 h.

The nearly complete 16S rRNA gene sequence of strain LMG 23990^T was determined as described below. DNA was extracted according to the method of Pitcher *et al.* (1989), as modified for Gram-positive bacteria, as described by Björkroth & Korkeala (1996). PCR products were purified and commercially sequenced at GATC Biotech as described previously (Kostinek *et al.*, 2005). Using FASTA analysis at the EMBL database, the closest related bacteria were identified as members of the genus *Leuconostoc*. Evolutionary distances were calculated using the Jukes & Cantor (1969) evolutionary model and a phylogenetic tree (Fig. 1) was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the BioNumerics software package, version 4.61 (Applied Maths). To evaluate the reliability of the topology of the neighbour-joining tree 500 bootstrap resamplings of

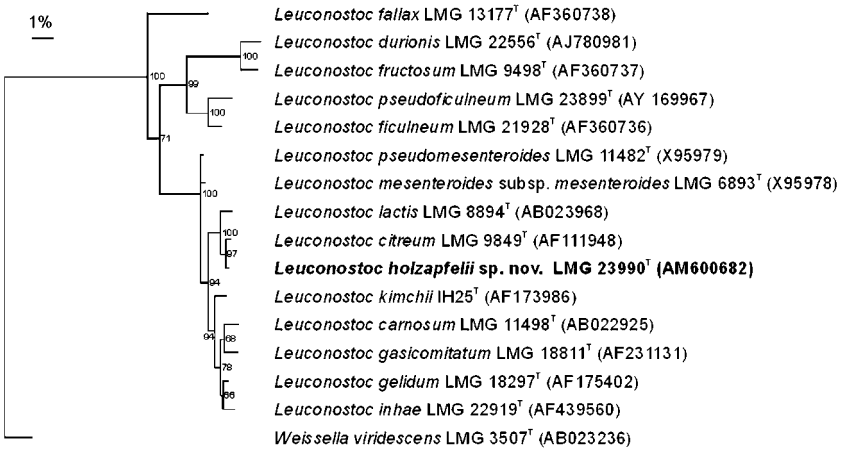

Fig. 1. Phylogenetic neighbour-joining tree based on the 16S rRNA gene sequences showing the relationships of *L. holzapfelii* LMG 23990^T and related species. *Weissella viridescens* LMG 3507^T was used as the outgroup. Numbers at the branching points indicate bootstrap percentage values (>50) based on 500 tree replications.

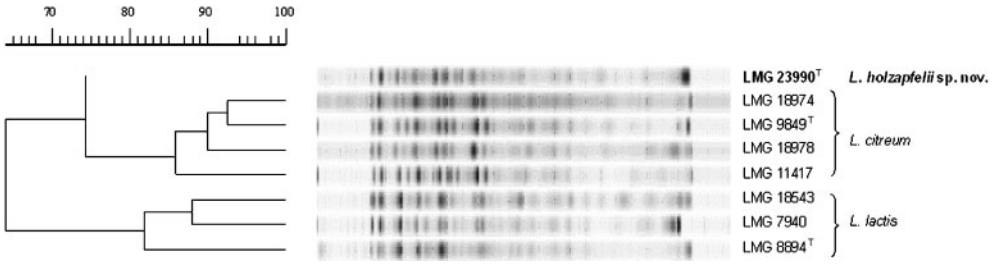

Fig. 2. Whole-cell protein profiles and dendrogram derived from UPGMA linkage of correlation coefficients of *L. holzapfelii* LMG 23990^T, and of *L. lactis* (LMG 8894^T, LMG 7940 and LMG 18543) and *L. citreum* (LMG 9849^T, LMG 11417, LMG 18974 and LMG 18978) reference strains.

the data were performed (Fig. 1). The novel strain belonged to a cluster of species together with *Leuconostoc lactis* and *Leuconostoc citreum*. The 16S rRNA gene sequence similarity values between strain LMG 23990^T and its closest relatives *L. citreum* ATCC 49370^T and *L. lactis* JCM 6123^T were 99.6 and 99.0 %, respectively.

To study the relatedness between strain LMG 23990^T and its nearest phylogenetic neighbours in more detail SDS-PAGE of whole-cell proteins and fluorescent amplified fragment length polymorphism (FAFLP) of genomic DNA were performed. Whole-cell protein extracts and SDS-PAGE analysis were analysed as described by Pot *et al.* (1994). Densitometric analysis, normalization, interpolation of protein profiles and numerical analysis were performed by using the BioNumerics software package, version 4.61 (Applied Maths). The whole-cell protein profile of strain LMG 23990^T was different from those of strains belonging to its two closest phylogenetic neighbours, *L. lactis* and *L. citreum* (Fig. 2).

FAFLP fingerprinting of whole genomes was performed as described by Thompson *et al.* (2001) with the following modifications: *Eco*RI/*Taq*I was used as the restriction enzyme combination, and primer combination E01/T01 (both having an adenosine extension at the 3′-end) was applied for selective PCR. The resulting electrophoretic patterns were tracked and normalized with the GENESCAN 3.1 software package (Applera). Normalized tables of peaks were transferred into BioNumerics software package, version 4.61 (Applied Maths). The FAFLP fingerprint of strain LMG 23990^T was different from those of representatives of its closest phylogenetic neighbours and of other type strains of all established *Leuconostoc* species (Fig. 3).

DNA–DNA hybridizations were performed between strain LMG 23990^T and *L. citreum* DSM 5577^T and *Leuconostoc argentinum* DSM 8581^T, a later synonym of *L. lactis* (Vancanneyt *et al.*, 2006). The total genomic DNA used for these hybridization experiments and for the determination of the DNA base composition was extracted and purified according to the method of Marmur (1961), as modified by Stackebrandt & Kandler (1979). The DNA–DNA relatedness level was determined using the spectrophotometric method of De Ley *et al.* (1970). Hybridization levels of 24

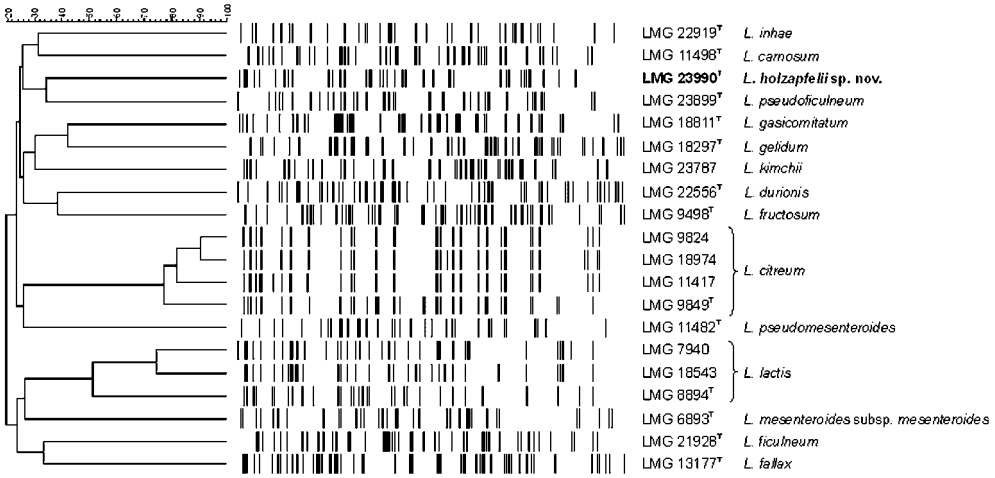

Fig. 3. FAFLP patterns and dendrogram derived from the UPGMA linkage of Dice coefficients of *L. holzapfelii* LMG 23990^T and of type strains of established *Leuconostoc* species.

and 53 % were found between strain LMG 23990^T and *L. citreum* DSM 5577^T, and *L. lactis* DSM 20202^T DSM 8581^T, respectively. These DNA reassociation values were well below the 70 % threshold value recommended for species delineation (Wayne *et al.*, 1987), indicating that strain LMG 23990^T represents a distinct species.

The DNA base composition of strain LMG 23990^T was determined as described by Mesbah *et al.* (1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column. The solvent used was 0.02 M $NH_4H_2PO_4$ (pH 4.0) and 1.5 % (v/v) acetonitrile. Non-methylated lambda phage (Sigma) and *E. coli* LMG 2093 DNAs were used as calibration reference and control, respectively. The DNA G+C content of strain LMG 23990^T was 43.5 mol%, which is within the expected mol% G+C range of the genus *Leuconostoc* (38–44 %).

Recently, novel techniques have been developed to provide a rapid identification of *Leuconostoc* species. Lee *et al.* (2000) and Macian *et al.* (2004) reported identification using multiplex PCR targeting the 16S rRNA genes. Chenoll *et al.* (2003) evaluated the use of rDNA-based techniques such as intergenic spacer region restriction analysis and amplified rDNA restriction analysis. Accurate species identification, however, often requires a polyphasic approach, including 16S rRNA gene sequencing, DNA–DNA hybridizations, SDS-PAGE of whole-cell proteins and FAFLP of genomic DNA. Unfortunately the use of 16S rRNA is not discriminatory enough to differentiate closely related species within the genus *Leuconostoc* and the use of fingerprint patterns is restricted due to difficult inter-laboratory reproducibility. A solution to this problem is offered by the sequencing of housekeeping genes, which is expected to bring a new dimension into the study of genomic relationships at the inter- and intraspecies level (Stackebrandt *et al.*, 2002). Recently, the phylogenetic structure of the *Leuconostoc–Oenococcus–Weissella* clade was evaluated by comparison of 16S rRNA, *dnaA*, *gyrB*, *rpoC* and *dnaK* gene sequence analysis of a restricted set of species within these genera. While some clades were well defined in every gene tree, many other clades were shown to be gene specific (Chelo *et al.*, 2007). In general, the phylogenies obtained with the different genes showed a consistency with the 16S rRNA phylogeny.

Sequence analysis of genes encoding the phenylalanyl-tRNA synthase alpha subunit (*pheS*), RNA polymerase alpha subunit (*rpoA*) and the alpha subunit of ATP synthase (*atpA*) has been successfully applied for the accurate identification of *Enterococcus* (Naser *et al.*, 2005a, b) and *Lactobacillus* species (S. Naser and others, unpublished data). In the present study, we used the primers listed in Table 2 for the amplification and sequencing of the same set of genes. The primer combinations pheS-21-F/pheS-23-R, rpoA-21-F/rpoA-23-R and atpA-20-F/atpA-26-R amplified the target genes of most strains. Where necessary, an alternative primer combination for *rpoA* (rpoA-20-F/rpoA-22-R) and *atpA* (atpA-20-F/atpA-26-R) was used. Amplification conditions and sequencing reactions were performed as described by Naser *et al.* (2005a, b). To assess inter- and intraspecies variation, we included multiple strains per species where possible. Bacterial strains, depositors and their sources are listed in Table 1.

Table 2. Amplification and sequencing primers used in this study

Primer name	Sequence (5′→3′)	Position
pheS-21-F	CAYCCNGCHCGYGAYATGC	557
pheS-23-R	GGRTGRACCATVCCNGCHCC	968
rpoA-20-F	ATGWTNGARWTWGAAAARCC	1
rpoA-21-F	ATGATYGARTTTGAAAAACC	1
rpoA-22-R	ACYTTVATCATNTCWGVYTC	844
rpoA-23-R	ACHGTRTTRATDCCDGCRCG	802
atpA-20-F	TAYRTYGGKGAYGGDATYGC	97
atpA-22-F	GCWCCYGGTRTYATGCARCG	397
atpA-23-R	CGYTGCATRAYACCRGGWGC	397
atpA-24-F	GATGAYYTWTCAAARCAAGC	781
atpA-25-R	GCTTGYTTTGAWARRTCATC	781
atpA-26-R	TTCATBGCYTTRATYTGNGC	1108

The phylogenetic trees of the different genetic loci proved their discriminatory power for species identification of the genus *Leuconostoc* and were roughly in agreement with 16S rRNA gene-based phylogeny. The use of two alternative tree-making methods, the neighbour-joining method (Supplementary Figs S1–S3 available in IJSEM Online) and maximum-parsimony calculations (Supplementary Figs S4–S6), revealed very similar tree topologies. The presence of species with unique positions (e.g. *Leuconostoc fallax*) or identical lineages present in every gene tree, including the 16S rRNA gene-based tree (e.g. the *Leuconostoc fructosum–L. durionis–L. ficulneum–L. pseudoficulneum* lineage or the *L. inhae–L. gasicomitatum–L. gelidum* lineage), illustrates a general consistency of the data. The most divergent line in 16S rRNA analyses is *L. fallax* (Fig. 1). For the genes *pheS*, *rpoA* and *atpA* this observation is not supported, which is in agreement with other sequence analyses (Chelo *et al.*, 2007). According to Chelo *et al.* (2007), who evaluated the evolutionary rates of the 16S rRNA gene, *dnaA*, *gyrB*, *rpoC* and *dnaK* for the *Leuconostoc–Oenococcus–Weissella* clade, this is due to the greater evolutionary rate of the 16S rRNA gene compared to other genes. Philippe *et al.* (2005) demonstrated that in cases of low rates of evolution, there is a tendency for the slow-evolving taxa to resemble outgroup sequences. In particular the difference in branch evolutionary rates between the genes could explain the position of *L. fallax* in 16S rRNA gene phylogeny, as the result of a relatively low rate of evolution in comparison to other *Leuconostoc* species. In all gene phylogenies the clade of *L. ficulneum*, *L. pseudoficulneum*, *L. durionis* and *L. fructosum* resulted in the most peripheral group. The same group was also observed as the most divergent line in the comparative

phylogenies obtained by Chelo *et al.* (2007). For each of the genes studied, species were clearly delineated above 93, 98 and 98 % *pheS*, *rpoA* and *atpA* gene sequence similarity, respectively. For most pairs of species, the intraspecies diversity was consistently smaller than the interspecies similarity towards their nearest neighbours for each of the three genes studied and a clear separation of the species was obtained. The only exception was the differentiation between *L. inhae* and *L. gelidum* in the *rpoA*-based phylogenetic tree (Supplementary Fig. S2). Nevertheless, the separation between the two species was supported by a bootstrap value of 100. In general, the differences between intraspecies diversity and interspecies similarity were smaller for the *rpoA* and *atpA* genes compared with the *pheS* gene. None of the loci examined, nor the concatenated sequences allowed discrimination between the subspecies within *L. mesenteroides*. On the basis of present data, the strains classified as *L. mesenteroides* subsp. *mesenteroides* LMG 8159 and *Leuconostoc pseudomesenteroides* LMG 11499 form a peculiar clade in the three gene phylogenies. These strains should probably be classified as *L. mesenteroides*; however, to formally clarify their taxonomic position DNA–DNA hybridizations should be performed. According to Konstantinidis *et al.* (2006), the minimum number of genes needed for multilocus differentiation between species is three because of potential events of horizontal gene transfer or recombination. In the present study, the concatenated gene sequences indeed proved valuable for the differentiation of all *Leuconostoc* species (Fig. 4).

Morphological, physiological and biochemical tests were performed as described by Schillinger & Lücke (1987). The API 50CHL identification system (bioMérieux) was used to determine the carbohydrate fermentation profile. Results of this characterization are given in the species description. Table 3 gives an overview of the physiological differences between the novel species and the most closely related species. It is evident from these summarized physiological data that strain LMG 23990^T can be distinguished from related *Leuconostoc* species by a combination of acid production tests from sugars.

In conclusion, the results of this polyphasic study demonstrate that strain LMG 23990^T represents a novel *Leuconostoc* species that can be distinguished from its nearest neighbours, for which the name *Leuconostoc holzapfelii* sp. nov. is proposed.

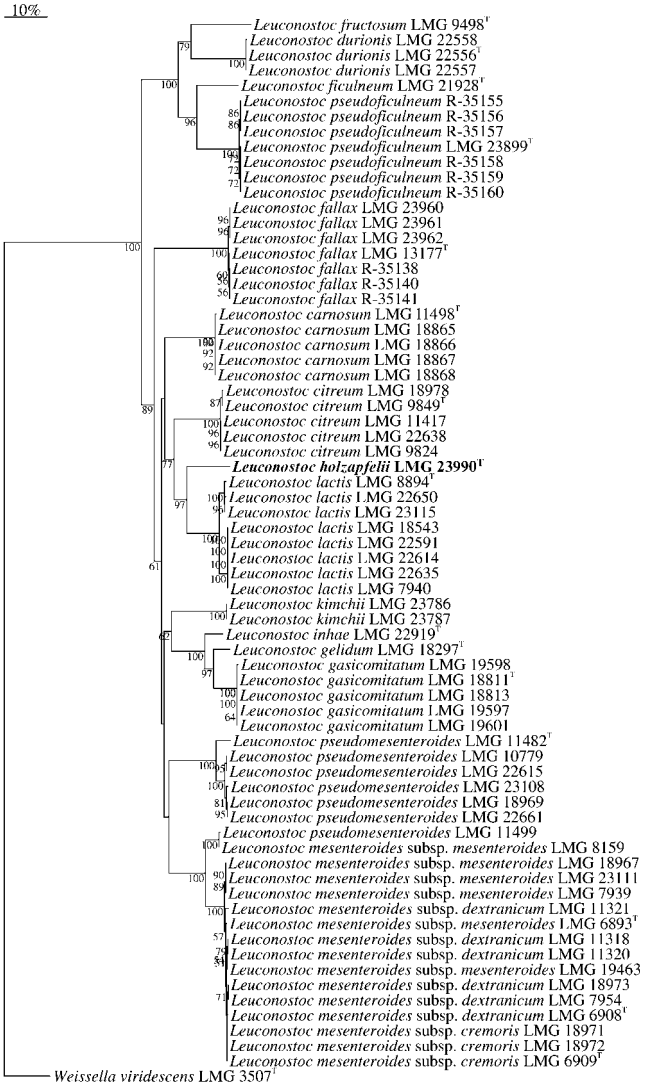

Fig. 4. Concatenated tree based on *pheS*, *rpoA* and *atpA* gene sequences of *Leuconostoc* strains. Distance estimations were obtained by the Jukes & Cantor (1969) model. Bootstrap percentages (>50) after 500 simulations are shown. *Weissella viridescens* LMG 3507^T was used as the outgroup. Bar, 10 % sequence divergence.

Description of *Leuconostoc holzapfelii* sp. nov.

Leuconostoc holzapfelii (hol.za.pfel'i.i. N.L. gen. masc. n. *holzapfelii*, of Holzapfel, in honour of Professor Dr W. H. Holzapfel, in recognition of his outstanding work in the area of lactic acid bacterium taxonomy and physiology).

Cells are Gram-positive, non-motile, ovoid or short rods, approximately 0.8–1.0 × 2.0–3.0 μm in size, and mostly occur in pairs or short chains. Colonies grown on MRS agar at 30 °C for 2 days are approximately 1 mm in diameter, beige, smooth and circular. Facultatively anaerobic, catalase-negative. The D-(-) isomer of lactic acid is produced from glucose with gas formation. Growth occurs at 10 and 37 °C, but not at 4 or 40 °C. Does not grow in the presence of 6.5 % NaCl. Grows at pH 3.9. Ammonia is not produced from arginine. Slime is produced from sucrose. Acid is produced from L-arabinose, galactose, glucose, fructose, mannose, methyl α-D-glucoside, *N*-acetylglucosamine, maltose, melibiose, trehalose, raffinose, D-turanose and gluconate. Acid is not produced from glycerol, erythritol, D-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-D-xyloside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, amygdalin,

Table 3. Phenotypic characterization of strain LMG 23990^T and related *Leuconostoc* species

Species: 1, *L. holzapfelii* LMG 23990^T; 2, *L. citreum* LMG 9849^T; 3, *L. lactis* LMG 8894^T; 4, *L. kimchii* IH25^T; 5, *L. inhae* LMG 22919^T (n=6); 6, *L. gelidum* LMG 18297^T; 7, *L. carnosum* LMG 23898^T; 8, *L. gasicomitatum* LMG 18811^T (n=4); 9, *L. mesenteroides* subsp. *mesenteroides* LMG 6893^T; 10, *L. pseudomesenteroides* LMG 11482^T. Data from Antunes *et al.* (2002), Björkroth *et al.* (2000), Chambel *et al.* (2006), Kim *et al.* (2000, 2003) and this study. Characteristics are scored as: +, more than 90 % of the strains positive; −, more than 90 % of the strains negative; V, variable; ND, not determined; (d), delayed reaction; W, weakly positive.

Acid produced from	1	2	3	4	5	6	7	8	9	10
L-Arabinose	+	+	+	−	+	+	−	+	+	+
Cellobiose	−	+	−	+	+	+	−	+	+	+
Galactose	+	+	+	+	V	−	−	V	+	+
Gluconate	+	+	−	+	V	+	+	V	−	−
Lactose	−	−	+	+	−	−	−	ND	(d)	−
Mannitol	−	+	−	+	+	+	+	−	W	−
Melibiose	+	−	−	−	−	+	V	+	+	+
Raffinose	+	−	−	−	−	+	−	+	+	+
Ribose	−	−	−	+	+	+	V	+	+	+
Salicin	−	+	−	+	V	+	−	−	(d)	V
Trehalose	+	+	−	+	+	+	+	+	+	+
D-Xylose	−	−	−	−	−	+	−	+	(d)	+

arbutin, aesculin, salicin, cellobiose, lactose, sucrose, inulin, melezitose, amygdalin, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluonate or 5-ketogluconate. The DNA G+C content was 43.5 mol%.

The type strain, LMG 23990^T (=CCUG 54536^T), was isolated from coffee fermentation in Ethiopia.

Acknowledgements

This work was funded by the Federal Research Policy [Action for the promotion of and Cooperation with the Belgian Coordinated Collections of Micro-organisms (C3/00/17)]. We thank all depositors of strains used in the study.

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