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3 ***Lactobacillus porcinae* sp. nov. isolated from traditional Vietnamese nem chua**

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23 identification

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25 **Running title:** *Lactobacillus porcinae* sp. nov.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene and *pheS* gene sequences of LMG 26767^T are HE616585 and HE616586, respectively. The accession number for *pheS* gene sequences of *Lactobacillus manihotivorans* LMG 18011, *Lactobacillus camelliae* LMG 24277^T, and *Lactobacillus thailandensis* LMG 24278^T are HE798569, HE798567, HE798568, respectively.

A species diversity study of lactic acid bacteria occurring in traditional Vietnamese nem chua yielded an isolate, LMG 26767^T, that could not be assigned to a validly named species. The isolate was initially investigated by 16S rRNA gene sequence analysis, which revealed that it belonged to the genus *Lactobacillus*, with *Lactobacillus manihotivorans* and *Lactobacillus camelliae* as the closest relatives (98.9% and 96.9% gene sequence similarity towards the type strains, respectively). Comparative (GTG)₅-PCR genomic fingerprinting confirmed the unique taxonomic status of the novel strain. DNA-DNA hybridization experiments, DNA G+C content determination, sequence analysis of the phenylalanyl-tRNA synthase (*pheS*) gene, and physiological and biochemical characterization demonstrated that strain LMG 26767^T (= CCUG 62266^T) represents a novel species, for which the name *Lactobacillus porcinae* sp. nov. is proposed. Biochemically, *Lb. porcinae* can be distinguished from *Lb. manihotivorans* and *Lb. camelliae* by its carbohydrate fermentation profile, absence of growth at 45°C, and production of D- and L- lactate as end products of glucose metabolism.

1 Meat fermentation is an ancient process originally used to extend the shelf life of
2 perishable raw meat. During fermentation complex biochemical and physical
3 reactions take place that result in a significant change of the initial characteristics of
4 the raw materials. Moreover, production of aromatic substances during fermentation
5 defines the sensorial characteristics of the final products which are significantly
6 different from the ones of the raw materials used (Rantsiou & Cocolin, 2008). In
7 Vietnam, fermentation is the main process for preserving meat. Nem chua is a
8 traditional fermented food product that consists of lean ground pork mixed with spices
9 and boiled pig skin cut into thin strings. The meat paste is shaped into cubes which
10 are partly wrapped in a leaf 'Oi' of the plant *Psidium guajava* for decoration and
11 flavour. The cubes are wrapped in banana leaves to provide the anaerobic
12 environment for the fermentation process and to inhibit entry of potentially
13 pathogenic micro-organisms. The fermentation takes place without addition of a
14 starter culture or prior cooking or heating, and proceeds for 2 to 4 days at ambient
15 temperature. Nem chua has a shelf life of five days when preserved at room
16 temperature. However, the shelf life can be prolonged up to one month at refrigerator
17 temperatures (Nguyen *et al.*, 2011).

18 Despite a growing knowledge on the lactic acid bacteria (LAB) ecology of many
19 fermented foods, little is known on the native LAB communities associated with the
20 production of nem chua. Nguyen *et al.* (submitted) recently studied the nem chua
21 LAB communities in various households in Hanoi and Thanhhoa, two cities in
22 Northern Vietnam, through culture-dependent and culture-independent approaches
23 (Nguyen *et al.*, submitted). They reported a dominance of lactobacilli, with
24 *Lactobacillus plantarum*, *Lactobacillus farciminis*, and *Lactobacillus pentosus* as
25 predominant species. The study also yielded a large variety of other LAB, including
26 some less common species, such as *Pediococcus stilesii*, and an isolate, LMG 26767^T,
27 that could not be assigned to a validly named species. In the present study, the
28 taxonomic position of this isolate is determined.

29 Strain LMG 26767^T was isolated from Thanhhoa nem chua in Northern
30 Vietnam from a sample collected after approximately 48 h of fermentation at ambient
31 temperature (about 32 °C); **it was assigned the research collection number R-42633**
32 **originally**. About 25 gram of nem chua (pH 4.4; 42x10⁸ CFU LAB per gram nem chua)
33 was homogenized in 225 ml maximum recovery diluent (MRD) (Oxoid,

1 Erembodegem-Aalst, Belgium) by using a Stomacher Lab Blender 80 (Seward
2 Medical, London, UK), after which 10-fold serial dilutions were prepared in MRD.
3 MRS agar (Oxoid) was used for the isolation of LMG 26767^T. MRS plates were
4 incubated for 48 h at 28 °C in aerobic conditions. The isolate was checked for
5 bacteriological purity by successive plating on MRS agar and stored in MicrobankTM
6 vials (Pro-Lab Diagnostics, Richmond Hill, ON, Canada) at -80 °C until further
7 analysis. Gram stain reaction, cell morphology, catalase activity, and all further
8 experiments were performed by using cultures grown for 48 h in MRS medium at 28
9 °C under aerobic conditions.

10 The phylogenetic position of strain LMG 26767^T was first determined by
11 analysis of its 16S rRNA gene sequence analysis. Genomic DNA was obtained using
12 the phenol-chloroform extraction method described by Gevers and co-workers
13 (Gevers *et al.*, 2001). Quality and purity of the obtained DNA were checked by
14 spectrophotometric measurements at 234, 260, and 280 nm (SpectraMax Plus384,
15 Molecular Devices, California, USA), and visually by electrophoresis of 5 µL DNA
16 mixed with 2 µL loading dye (4 g sucrose and 2.5 mg bromophenol blue dissolved in
17 6 mL TE buffer) on a 1% w/v agarose (Result LE General Purpose Agarose, BIOzym
18 group, Landgraaf, Holland) gel, run for 30 min at 100 V in 1 L TAE buffer (40 mM
19 Tris-acetate, 1 mM EDTA, pH 8.0), flanked by a molecular mass marker
20 (SmartLadder, Eurogentec, Seraing, Belgium). 16S rRNA gene amplification,
21 purification and sequencing were performed as described by De Bruyne *et al.* (De
22 Bruyne *et al.*, 2008), using the following modifications. Sequencing reactions were
23 purified using a BigDye® XTerminatorTM Purification Kit according to the protocol
24 of the supplier (Applied Biosystems). The ARB software package (Ludwig *et al.*,
25 2004) and the corresponding SILVA SSURef 102 database (Pruesse *et al.*, 2007) were
26 used to align the obtained 16S rRNA gene sequence and these of the type strains of all
27 established *Lactobacillus* species, its nearest phylogenetic neighbours (see below).
28 These aligned sequences were imported into the software package MEGA (Molecular
29 Evolutionary Genetics Analysis) version 5.0 (Tamura *et al.*, 2011) and analyzed using
30 the neighbour-joining, maximum-likelihood, and maximum-parsimony methods. The
31 statistical reliability of tree topologies was evaluated by bootstrapping analysis based
32 on 1000 tree replicates. The maximum-parsimony tree and the neighbour-joining tree
33 revealed topologies similar to those obtained in the phylogenetic tree constructed

1 using the maximum-likelihood approach (Fig.1). Sequence similarity calculations
2 performed using the ARB software package indicated that the closest relatives of
3 strain LMG 26767^T were *Lactobacillus manihotivorans* OND 32^T (98.9%) and
4 *Lactobacillus camelliae* MCH3-1^T (96.9%). Lower sequence similarities (< 96.0%)
5 were found towards other validly named species of the genus *Lactobacillus*.

6 The taxonomic position of strain LMG 26767^T was further studied using
7 phenylalanyl-tRNA synthase (*pheS*) gene sequencing analysis (Naser *et al.*, 2005a).
8 Analysis of *pheS* gene sequences proved to be an excellent tool for the identification
9 of *Lactobacillus* isolates and the delineation of novel taxa (Naser *et al.*, 2007; Naser *et al.*,
10 2005b; Švec *et al.*, 2005b; Vancanneyt *et al.*, 2006). The primer combinations
11 *pheS*-21-F (5'-CAYCCNGCHCGYGAYATGC-3') and *pheS*-22-R (5'-
12 CCWARVCCRAARGCAAARCC-3') are used for *pheS* gene amplification for most
13 strains; if they fail to generate an amplicon, the alternative primer set *pheS*-21-F and
14 *pheS*-23-R (5'-GGRTGRACCATVCCNGCHCC-3') can be used. The primers used
15 for amplification of *pheS* of strain LMG 26767^T and *Lb. camelliae* LMG 24277^T were
16 *pheS*-21-F and *pheS*-23-R, whereas primers used for amplification of *pheS* of strains
17 *Lb. manihotivorans* LMG 18011 and *Lb. thailandensis* LMG 24278^T were *pheS*-21-F
18 and *pheS*-22-R. The primers used for sequencing of *pheS* were *pheS*-21-F and *pheS*-
19 23-R. Amplification and sequencing were performed as described by Naser and co-
20 workers (Naser *et al.*, 2005a). *PheS* gene sequences of other *Lactobacillus* reference
21 strains were available from previous studies (Naser *et al.*, 2007) Sequences were
22 subsequently imported into the BioNumerics version 5.1 software package. The
23 software package MEGA version 5.0 (Tamura *et al.*, 2011) was used to align the
24 translated gene sequences and to analyze the nucleotide sequences using the
25 neighbour-joining, maximum-likelihood, and maximum-parsimony methods. The
26 statistical reliability of tree topologies was evaluated by bootstrapping analysis based
27 on 1000 tree replicates. The maximum-parsimony tree and the neighbour-joining tree
28 revealed topologies similar to those obtained in the phylogenetic tree constructed
29 using the maximum-likelihood approach for both analyses (Fig. 2). Strain LMG
30 26767^T again clustered most closely with *Lb. manihotivorans* (88.0% sequence
31 similarity towards both strains LMG 18010^T and LMG 18011) and *Lb. camelliae*
32 (LMG 24277^T at a very low value: 80.0% sequence similarity) (Fig. 2). This low *pheS*
33 gene sequence similarity value strongly suggests that strain LMG 26767^T represents a

1 distinct *Lactobacillus* species. To confirm this, DNA-DNA hybridizations were
2 performed between strain LMG 26767^T and *Lb. manihotivorans* LMG 18010^T, the
3 type strain of its nearest phylogenetic neighbour. DNA was extracted from 0.75-1.25
4 g cell mass using the protocol described by Gevers et al. (Gevers *et al.*, 2001), with
5 the following modifications. Volumes were increased tenfold for large-scale
6 application. After the addition of 20% SDS and glass beads, cells were mixed for 30 s.
7 Subsequently, 16.5 ml TE buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) and 5 ml
8 5 M NaCl were added, followed by 10 min of incubation at 65 °C.
9 Chloroform/isoamyl alcohol extraction and ethanol and RNase treatment were
10 performed as described by Marmur (Marmur, 1961). DNA-DNA hybridizations were
11 performed with biotin-labeled probes in microplate wells (Ezaki *et al.*, 1989), by
12 using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence
13 measurements. The hybridization temperature was 44 °C in the presence of 50%
14 formamide. The average level of DNA-DNA relatedness between strain LMG 26767^T
15 and *Lb. manihotivorans* LMG 18010^T was 39% (the reciprocal values were 38 and
16 40%). This value is well below the threshold of 70% for species delineation,
17 confirming that strain LMG 26767^T represents a novel species in the genus
18 *Lactobacillus*.

19 (GTG)₅-PCR fingerprinting was used as a genotypic tool to distinguish strain
20 LMG 26767^T from its closest relatives, *Lb. manihotivorans* and *Lb. camelliae* as
21 described previously (Gevers *et al.*, 2001; Švec *et al.*, 2005a). In short, PCR
22 amplification was performed as described by Versalovic and co-workers (Versalovic,
23 1994) in a DNA thermal cycler (Perkin Elmer 9600, Massachusetts, USA).
24 Subsequently, PCR amplicons were separated on a 1.5% w/v agarose gel (20 cm x 15
25 cm) in 1 x TAE buffer at 55 V for 16 h. After staining with ethidium bromide,
26 (GTG)₅-PCR profiles were visualized under ultraviolet light and digitally captured
27 using a CCD camera. The resulting fingerprints were analyzed using the BioNumerics
28 software (Applied Maths). Fig. 3 illustrates that the (GTG)₅-PCR profiles of strain
29 LMG 26767^T, and the *Lb. manihotivorans* and *Lb. camelliae* reference strains are
30 clearly different.

31 The mol percentage G + C content of the DNA of strain LMG 26767^T was
32 determined. DNA was enzymically degraded into nucleosides as described by Mesbah
33 and co-workers (Mesbah *et al.*, 1989). The DNA nucleotide mixture was

1 chromatographically analyzed using a Waters Breeze HPLC system. A thermostable
2 XBridge Shield RP18 column was used at 37 °C. The solvent was 0.02 M
3 (NH₄)H₂PO₄ (pH = 4.0) with 1.5% (v/v) acetonitrile. Non-methylated λ phage DNA
4 (Sigma) was used as the calibration reference. The DNA G + C content of strain LMG
5 26767^T was 47.6 mol%, which is similar to the value determined for *Lb.*
6 *manihotivorans* (48.4 mol%) (Morlon-Guyot *et al.*, 1998) and somewhat lower than
7 that of *Lb. camelliae* (51.9 mol%) (Tanasupawat *et al.*, 2007).

8 The preparation of the peptidoglycan and the analysis of the peptidoglycan
9 structure were performed according to Schumann {Schumann, 2011 #3153}. The
10 hydrolysate (6N HCl, 120°C, 15h) contained the peptide L-Lys – D-Asp and the
11 amino acids alanine, glutamic acid, lysine and aspartic acid in the approximate molar
12 ratio: 1.7 Ala : 0.9 Asp : 1.0 Glu : 0.9 Lys. Milder hydrolysis (4N HCl, 100°C, 0.75h)
13 yielded additionally the peptides L-Ala – D-Glu, L-Lys – D-Ala and D-Ala – L-Lys –
14 D-Asp. From these data it was concluded that strain LMG 26767^T shows the
15 peptidoglycan type A4α L-Lys – D-Asp.

16 Finally, the cellular morphology and growth characteristics of strain LMG
17 26767^T were determined in MRS broth (pH = 6.2; Oxoid CM 0359). Growth was
18 tested at 15, 20, 37, 45 and 52 °C, in the presence of 5, 6, 7, and 8% NaCl, and at pH
19 3.9 and 5.4 (at 28 °C) (the pH of nem chua samples normally ranges from 4.3 to 5.0).
20 The production of gas from 2% glucose and 2% gluconate in MRS broth (pH 6.2,
21 without the addition of triammonium citrate) was also determined, along with
22 production of D- and L-lactate from glucose, as determined enzymically by using the
23 R-Biopharm assay. The carbohydrate fermentation pattern of the strains was
24 determined using the API 50 CHL system (bioMérieux) following the manufacturer's
25 instructions (strains were cultivated at 28 °C). A detailed phenotypic description is
26 given below. Characteristics that differentiate the novel species represented by strain
27 LMG 26767^T from *Lb. manihotivorans* include absence of growth at 45°C, acid
28 production from arbutin, no acid production from L-rhamnose, methyl-α-D-
29 glucopyranoside, D-lactose, D-melibiose, and D-raffinose, and production of D- and
30 L- lactate from glucose. Similarly, biochemical differences between the novel species
31 represented by strain LMG 26767^T and *Lb. camelliae* include acid production from D-
32 saccharose and D-trehalose, but not from L-arabinose, D-mannitol, or D-xylose and
33 production of D- and L- lactate from glucose. In conclusion, data from the present

1 study demonstrate that strain LMG 26767^T represents a novel *Lactobacillus* species
2 which can be distinguished from its nearest neighbours, *Lb. manihotivorans* and *Lb.*
3 *camelliae*, by genotypic and phenotypic criteria. We therefore propose to formally
4 classify this strain as *Lactobacillus porcinae* sp. nov., with LMG 26767^T (= CCUG
5 62266^T) as the type strain.

6 **Description of *Lactobacillus porcinae* sp. nov.**

7 *Lactobacillus porcinae* (por'ci.nae L. n. *porcina*, pork; *porcinae*, of pork).

8 Cells are small coccobacilli, 1.5 – 2.0 µm in length and about 1.0 µm wide,
9 and occur singly or in pairs. They are Gram-positive and catalase-negative, do not
10 form spores, and are non motile. After 48 h of incubation on MRS agar at 28 °C,
11 colonies are greyish white, opaque, smooth and circular with a convex elevation,
12 entire margin, and approximately 0.5 – 0.7 mm in diameter. Cells grow well in liquid
13 or on solidified MRS under aerobic and anaerobic conditions and grow at 15, 20 and
14 37 °C, but not at 45 or 52 °C. The maximum NaCl concentration for growth is 6%
15 (w/v). Able to grow at pH values of 3.9 and 5.4. Strain LMG 26767^T produces D and
16 L isomers of lactic acid in a ratio of 1:9 from glucose. Gas is not produced from
17 glucose or gluconate. Acid is produced from D-galactose, D-glucose, D-fructose, D-
18 mannose, N-acetylglucosamine, amygdalin, arbutin, esculin, ferric citrate, salicin, D-
19 cellobiose, D-maltose, D-saccharose, D-trehalose, amidon, and gentiobiose, but not
20 from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-
21 adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-
22 mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, D-
23 lactose, D-melibiose, inulin, D-melezitose, D-raffinose, glycogen, xylitol, D-turanose,
24 D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate,
25 potassium 2-ketogluconate, or potassium 5-ketogluconate.

26 The type strain, LMG 26767^T (=CCUG 62266^T), was isolated from nem chua
27 in Thanhhoa city, Vietnam in 2009. Strain LMG 26767^T was the only *Lb. porcinae*
28 isolate out of a total of 273 LAB that were picked from 10 nem chua samples from
29 Northern Vietnam (Nguyen et al. submitted). The DNA G + C content of the type
30 strain is 47.6 mol% and its peptidoglycan type is A4α L-Lys – D-Asp.

31 **Acknowledgments**

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2 Belgian Technical Cooperation (BTC).

3

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12 **Figures:**

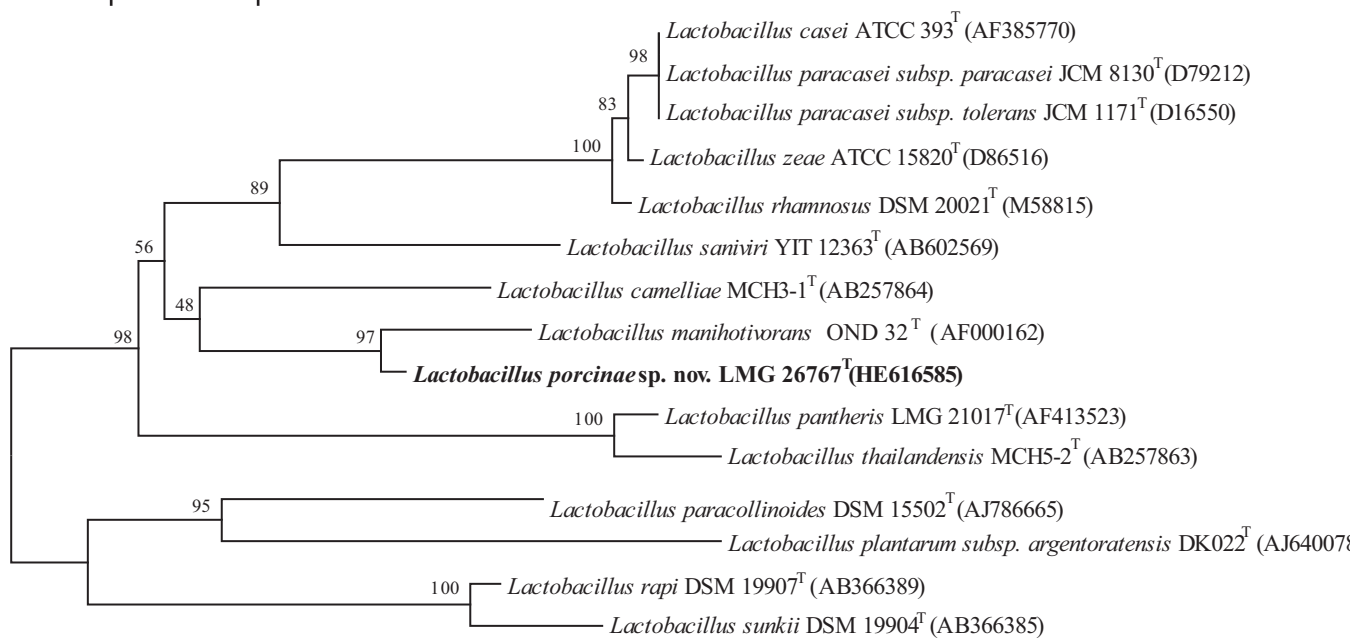
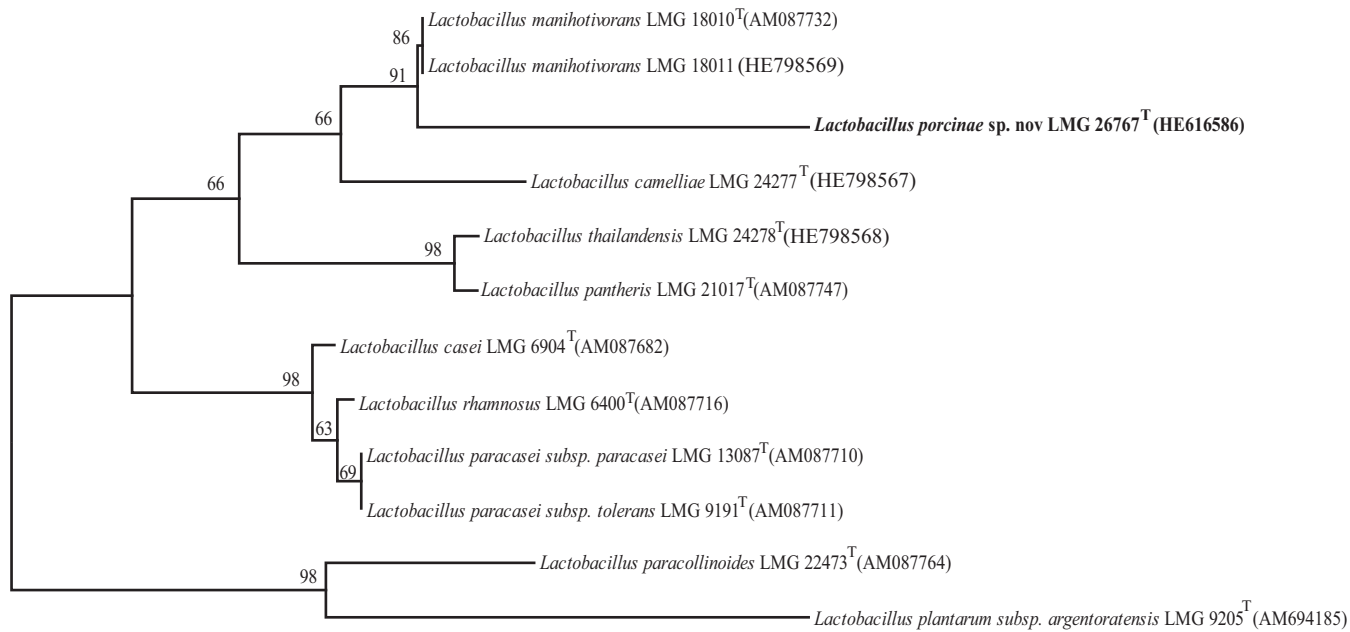
13 **Fig. 1.** Maximum-likelihood tree based on 16S rRNA gene sequences showing the
14 phylogenetic relationships of strain LMG 26767^T among the type strains of its nearest
15 neighbours. Bootstrap values (%) based on 1000 replications are shown at the branch
16 points. The substitution model used is the general time reversible model and the
17 aligned sequence has a length of 1426 bp. The bar indicates 0.005 % sequence
18 divergence.

19 **Fig. 2.** Maximum-likelihood tree based on *pheS* gene sequences showing the
20 phylogenetic relationships of strain LMG 26767^T among the type strains of its nearest
21 neighbours. Bootstrap values (%) based on 1000 replications are shown at the branch
22 points. The substitution model used is the general time reversible model and the
23 aligned sequence has a length of 390 bp. The bar indicates 0.05 % sequence
24 divergence.

25 **Fig. 3.** (GTG)₅-PCR pattern and dendrogram based on the UPGMA linkage of Dice
26 coefficients showing the phylogenetic relationships of strain LMG 26767^T among the
27 type strains of its nearest neighbours.

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