**Bifidobacterium lemurum** sp. nov., from faeces of the ring-tailed lemur (*Lemur catta*)

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Four Gram-positive-staining, microaerophilic, non-spore-forming, fructose-6-phosphate phosphoketolase-positive bacterial strains were isolated from a faecal sample of a 5-year-old ring-tailed lemur (*Lemur catta*). The strains showed a peculiar morphology, resembling a small coiled snake, a ring shape, or forming a little 'Y' shape. The isolated strains appeared identical, and LMC 13T was chosen as a representative strain and characterized further. Strain LMC 13T showed an A3β peptidoglycan type, similar to that found in *Bifidobacterium longum*. The DNA base composition was 57.2 mol% G+C. Almost-complete 16S rRNA, hsp60, rpoB, dnaJ, dnaG, purF, clpC and rpoC gene sequences were obtained, and phylogenetic relationships were determined. Comparative analysis of 16S rRNA gene sequences showed that strain LMC 13T showed the highest similarity to *B. longum* subsp. *suis* ATCC 27533T (96.65 %) and *Bifidobacterium saguini* DSM 23967T (96.64 %). Strain LMC 13T was located in an actinobacterial cluster and was more closely related to the genus *Bifidobacterium* than to other genera in the *Bifidobacteriaceae*. On the basis of these results, strain LMC 13T represents a novel species within the genus *Bifidobacterium*, for which the name *Bifidobacterium lemurum* sp. nov. is proposed; the type strain is LMC 13T (=DSM 28807T =JCM 30168T).

Bifidobacteria are Gram-positive, anaerobic, non-motile and non-spore-forming bacteria and represent one of the large bacterial groups within the class *Actinobacteria*. Members of the genus *Bifidobacterium* are typically found in the gastrointestinal tracts (GIT) of humans and other mammals and the hindguts of honeybees and bumblebees (Biavati & Mattarelli, 2012; Killer et al., 2009, 2011; Turroni et al., 2014; Ventura et al., 2007, 2012). They have also been isolated from waste and dairy products, where the source could have been faecal contamination and intentional probiotic addition, respectively (Mattarelli & Biavati, 2014).

The occurrence and species composition of bifidobacteria in different animals are quite variable; indeed, they are generally host-animal-specific micro-organisms that can be separated into 'human' and 'animal' groups (Ventura et al., 2004). Bifidobacteria are well known for their beneficial effects, and play an important role in maintaining the health of their hosts (Turroni et al., 2011). Thus, the discovery of bifidobacterial diversity in the GIT, together with the isolation and characterization of novel bacterial taxa in different hosts, is important from the viewpoint of their potential to benefit the health of both humans and economically important animals (Killer et al., 2014).

Yildirim et al. (2010) characterized the faecal microbiome from non-human wild primates, and found compelling evidence that, apart from diet, differences in the microbiome species of the different primate families could not be accounted for solely by habitat conditions; in fact, it has been revealed that, in the course of evolution, primate gastrointestinal microbiomes became linked, functionally, to their vertebrate host taxa and are, perhaps, host-specific (Yildirim et al., 2010).

During the study of bifidobacterial distribution in non-human primates, four bifidobacterial strains with the same morphology were isolated from fresh faecal samples of an adult ring-tailed lemur (*Lemur catta*) housed under...
semi-natural conditions at Parco Natura Viva, Verona, northern Italy.

Ring-tailed lemurs are strepsirrhine primates endemic to Madagascar. They are described as generalist feeders, and have a pronounced seasonal foraging strategy that results in periodic dietary changes (Campbell et al., 2000). Such lemurs can best be characterized as opportunistic omnivores with a wide dietary regime, which includes fruit, leaves, leaf stems, flowers, flower stems, spiders, spider webs, caterpillars, cicadas, insect cocoons and sometimes birds (Gould, 2006; Jolly et al., 2006).

Dietary specialization in lemur species is always correlated with significant differences in GIT morphology. Indeed, Lemur catta shows a somewhat enlarged haustrated caecum, a common adaptation to an herbivorous diet. This caecum harbours an intestinal symbiotic microbiota, and it has been assumed that this facilitates plant cell-wall breakdown and leaf fermentation (Campbell et al., 2000; Jolly et al., 2006).

In February 2014, fresh ring-tailed lemur faeces were collected from the ground using a sterile spoon, put into a sterile plastic tube and stored under anaerobic conditions in an anaerobic jar (Merck) at 4 °C. Samples of fresh faeces were collected by the animal-care staff (keepers) during their routine cleaning of the enclosure, and were taken promptly to the laboratory (within 2 h) Samples of the material, of approx. 1–2 g, were serially diluted with peptone water (Merck) supplemented with cysteine hydrochloride (0.5 g l⁻¹), and aliquots of each dilution were inoculated onto TOS agar (Sigma Aldrich). We observed cells of a bacterium with a morphology resembling a small coiled snake or a little ring, very similar to, but smaller than, that of Bifidobacterium aesculapii, a species we recently described in baby common marmoset (Modesto et al., 2014).

A total of four isolates with this morphology were obtained from this one adult ring-tailed lemur subject, and were named LMC 13T, LMC 16, LMC 18 and LMC 19. They were then subcultured on TPY and cells were suspended in a 10 % (w/v) sterile skimmed milk solution supplemented with lactose (3 %) and yeast extract (0.3 %), freeze-dried and kept frozen at −120 °C. For all experiments, the strains were cultivated under anaerobic conditions in anaerobic jars (Merck) and maintained in TPY broth, at pH 6.9 and 37 °C, unless indicated otherwise. The anaerobic atmosphere was obtained using the GasPak EZ Anaerobic Pouch system (BD).

Chromosomal DNA was obtained from the isolates according to the procedure of Rossi et al. (2000), with slight modifications. Briefly, cells of overnight cultures were pelleted and resuspended in 1 ml TE buffer (pH 7.6) containing 50 mg lysozyme ml⁻¹ and then incubated overnight at 37 °C.

For isolate discrimination, molecular typing was performed using enterobacterial repetitive intergenomic consensus sequence (ERIC) PCR with the primer pair ERIC1 (5’-ATGTAAAGCTC-CTGGGGATTCAC-3’) and ERIC2 (5’-AAGTAAGTGAC-TGGGTGAGCG-3’) (Ventura et al., 2003). Each 20 µl reaction mixture contained 3.5 mM MgCl₂, 20 mM Tris/HCl, 50 mM KCl, 200 µM each dNTP (HotStart Taq plus DNA polymerase MasterMix kit; Qiagen), 30 ng DNA template and 2 µM each primer. Amplifications were performed using an Applied Biosystems Veriti thermal cycler with the following temperature profile: 1 cycle at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 4 min; and 1 cycle at 72 °C for 6 min. Aliquots (15 µl each) of each amplification reaction mixture were separated by electrophoresis in 2 % (w/v) agarose gels at a voltage of 7 V cm⁻¹. Gels were stained with ethidium bromide (0.5 µg ml⁻¹) and photographed under 260 nm UV light.

Given that the isolates revealed identical ERIC profiles (see Fig. S1, available in the online Supplementary Material), strain LMC 13T was selected as a representative and characterized further. Morphological, biochemical and molecular characterizations were carried out on this isolate.

The partial 16S rRNA gene of strain LMC 13T was amplified by PCR using the primer pair Bif285 (5’-GAGGGTTCCATTCTGGCTCAG-3’) and Bif261 (5’-AAGGAGGTAGTGATCCAGCCGA-3’) (Kim et al., 2010). Partial hsp60, rpoB, dnaG, dnaJ, clpC and rpoC gene sequences were also obtained using the primer pairs HspF3 (5’-ATCGCCAAGGAGATCGACTG-3’) and HspR4 (5’-AAGGTCCGCGCGATTTGTC-3’), BifF (5’-TCGATCG-GGCACATAGCG-3’) and BifR2 (5’-CGACACTTGGCACAAGC-3’) (Kim et al., 2010), DnaG-uni (5’-CTGTCGCGGTGTTCAACAG-3’) and DnaG-rev (5’-CTCAGATCCGAGTCCG-3’), DnaJ-uni (5’-GAGAAGTTCAAGGACATCTC-3’) and DnaJ-rev (5’-GCTTGGCCCTGTCCCGG-3’), PurF-uni (5’-CATCTGAACTTGGCACAAGC-3’) and PurF-rev (5’-GTGGGTTAGTGGCCTTTG-3’), ClpC-uni (5’-GAGTACCGCAAATCATCGAG-3’) and ClpC-rev (5’-CATCTCATTCCGTGAAAGAC-3’), and RpoC-uni (5’-GTGCACTCGGTCCACAG-3’) and RpoC-rev (5’-CATGCTCAAAACGGAAGA-3’) (Ventura et al., 2006), respectively.

Each PCR mixture (20 µl) contained 1.5 mM MgCl₂, 20 mM Tris/HCl, 50 mM KCl, 200 µM each dNTP (HotStart Taq plus DNA polymerase MasterMix kit; Qiagen), 0.1 µl each primer and 30 or 200 ng DNA template for the 16S rRNA gene and for each housekeeping gene, respectively. Amplifications were performed using a TGradient thermal cycler (Biometra). A touchdown PCR was used to amplify the 16S rRNA gene and all phylogenetic markers (hsp60, rpoB, rpoC, dnaJ, dnaG, clpC and purF), and was performed as follows: initial denaturation (95 °C, 5 min) for HotStartTaq plus activation; 4 cycles of denaturation at 94 °C for 60 s, annealing at 62 °C for 60 s and extension at 72 °C for 90 s; 21 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s and extension at 72 °C for 90 s; and 15 cycles of denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s and extension at 72 °C for 90 s. The PCR was completed with a single elongation step (10 min at 72 °C).
All the resulting amplicons were separated on 2% agarose gels, followed by ethidium bromide staining. PCR fragments were purified using the NucleoSpin gel and PCR clean up kit (Macherey-Nagel) following the manufacturer’s instructions.

To infer a correct phylogeny, the 16S rRNA gene was cloned using an InsTAclone PCR Cloning kit (Fermentas), whereas the partial cloned using an InsTAclone PCR Cloning kit (Fermentas), whereas the partial hsp60, rpoB, dnaG, dnaJ, purF, clpC and rpoC genes were sequenced directly. All sequencing reactions were performed by Eurofins MWG Operon. Assembly of the almost-complete 16S rRNA gene sequence was performed with the BioEdit program (Hall, 1999).

After editing, the closest known relatives of the novel strains were determined by comparison with database entries, and sequences of members of closely related species were retrieved from the EMBL and GenBank nucleotide databases. Pairwise nucleotide sequence similarity values were calculated using the LALIGN program (http://embnet.vital-it.ch/software/LALIGN_form.html), which provides a web-based tool. The sequencing of 15 clones containing 16S rRNA genes did not reveal any heterogeneity of the rRNA operons within the genome of strain LMC 13T. The 16s rRNA gene sequences (about 1400 bp) of strain LMC 13T and its closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned using the CLUSTAL OMEGA program as a web service from the EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/clustalo/) (McWilliam et al., 2013). A phylogenetic tree based on a total of 48 available partial 16S rRNA gene sequences of members of the genus Bifidobacterium was reconstructed with the neighbour-joining method (Saitou & Nei, 1987), and evolutionary distances were computed by Kimura’s two-parameter method (Kimura, 1980) using MEGA version 6.0 (Tamura et al., 2013). The tree was rooted using Mycobacterium tuberculosis H37RvT (Fig. 1). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985), and tree topology was confirmed with the maximum-likelihood method (Cavalli-Sforza & Edwards, 1967) using MEGA version 6.0 (Tamura et al., 2013). Strain LMC 13T showed low sequence similarities to known bifidobacteria (Table 1), and the highest values were found to Bifidobacterium longum subsp. suis ATCC 27533T and Bifidobacterium saguini DSM 23967T (96.65 and 96.64%, respectively); the latter strain belongs to a species described recently from red-handed tamarin (Saguinus midas) by Endo et al. (2012). Based on the neighbour-joining analysis, the novel strain is related phylogenetically to B. longum subsp. suis (Fig. 1). Similar tree topologies were obtained by the maximum-likelihood method (Fig. S2).

Multilocus sequence analysis is a reliable and robust technique for the identification and classification of bacterial isolates to the species level, as an alternative or complement to 16S rRNA gene sequence analysis (Martens et al., 2008). Thus, the phylogenetic location of the novel strain was verified by the analysis of seven additional genetic markers, hsp60, rpoB, dnaG, dnaJ, purF, clpC and rpoC, which have proven to be discriminative for the classification of the genus Bifidobacterium (Jian et al., 2001; Kim et al., 2010; Ventura et al., 2006).

The sequences of the hsp60, rpoB, dnaG, dnaJ, purF, clpC and rpoC genes of strain LMC 13T were amplified and sequenced. Sequences from the type strains of 48 bifidobacterial taxa were retrieved from the public database of the National Center for Biotechnology Information (NCBI). The purF analysis included only 47 type strains as, despite several unsuccessful efforts using different PCR amplification parameters, we could not obtain a specific amplicon from Bifidobacterium actinocoloniforme DSM 22766T, and it was missing from the whole-genome shotgun project (GenBank accession no. JGYK00000000). To complete the phylogenetic study, partial dnaG, purF, rpoC and dnaJ gene sequences were amplified and sequenced directly from Bifidobacterium ascelupii DSM 26737T.

Sequences were aligned using the MAFFT program at CBRC (http://mafft.cbrc.jp/alignment/software/) (Katoh & Standley, 2013). The Gblocks program (version 0.91b), a server tool at the Castresana Lab (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), was then used to eliminate poorly aligned positions and divergent regions of DNA alignments, facilitating the phylogenetic analysis (Talavera & Castresana, 2007).

Approximately 594 bp of the hsp60 gene, 526 bp of the rpoB gene, 933 bp of the dnaG gene, 488 bp of the dnaJ gene, 930 bp of the purF gene, 1171 bp of the rpoC gene and 717 bp of the clpC gene sequences of strain LMC 13T and type strains of related species were used in the analyses. Seven phylogenetic trees were then produced using the individual genes (Figs 2 and S3–S8). The levels of similarity for the eight partial gene sequences obtained from strain LMC 13T and its closest relatives are summarized in Table 1.

The concatenation of gene sequences has been shown to be extremely useful in order to infer bacterial phylogeny (Ventura et al., 2006). For this purpose, an additional tree, including 47 bifidobacterial type strains, was created on the basis of the concatenation of all housekeeping gene sequences that we were able to retrieve or sequence directly (clpC, dnaG, dnaJ, hsp60, rpoC, rpoB and purF). This tree (Fig. 59) was reconstructed with the neighbor-joining method (Saitou & Nei, 1987), and evolutionary distances were computed by Kimura’s two-parameter method (Kimura, 1980) using MEGA version 6.0 (Tamura et al., 2013). The tree was rooted using Mycobacterium tuberculosis H37RvT (Fig. 59). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985). The tree topology was also confirmed by the maximum-likelihood method (Cavalli-Sforza & Edwards, 1967) using MEGA version 6.0 (Tamura et al., 2013) (Fig. S10).

Estimation of the G + C content in bacterial chromosomal DNA of strain LMC 13T was made at the DSMZ (Braunschweig, Germany). DNA was purified on hydroxyapatite according to the procedure of Cashion et al. (1977) and hydrolysed...
enzymically by the method of Mesbah et al. (1989). The resulting deoxyribonucleosides were analysed by HPLC, as described by Tamaoka & Komagata (1984). Strain LMC 13\(^T\) had a DNA G+C content of 57.2 mol%. This value is within the range reported for the genus *Bifidobacterium*, 52–67 mol% (Biavati & Mattarelli, 2012; Killer et al., 2010), and was very similar to that obtained recently from *B. saguini* (57.3 mol%; Endo et al., 2012).

**Fig. 1.** Phylogenetic relationship between strain LMC 13\(^T\) and all species within the genus *Bifidobacterium* based on 16S rRNA gene sequences. The tree was reconstructed by the neighbour-joining method and rooted with *Mycobacterium tuberculosis* H37Rv\(^T\). Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches; values above 70% are given at branching points. Bar, 0.02 substitutions per nucleotide position.
Table 1. Highest similarity between strain LMC 13\textsuperscript{T} and members of related bifidobacterial species

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<th>Related strain</th>
<th>Similarity to strain LMC 13\textsuperscript{T} (%)</th>
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<tr>
<td></td>
<td>16S rRNA</td>
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<tr>
<td>B. longum subsp. suis ATCC 27533\textsuperscript{T}</td>
<td>96.65</td>
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<tr>
<td>B. sagarii DSM 23967\textsuperscript{T}</td>
<td>96.64</td>
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<tr>
<td>B. scardovi DSM 21589\textsuperscript{T}</td>
<td>94.4</td>
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<tr>
<td>B. longum subsp. longum ATCC 15708\textsuperscript{T}</td>
<td>93.0</td>
</tr>
<tr>
<td>B. reuteri DSM 23975\textsuperscript{T}</td>
<td>93.0</td>
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<tr>
<td>B. longum subsp. infantis ATCC 15697\textsuperscript{T}</td>
<td>92.6</td>
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<tr>
<td>B. pullorum DSM 21816\textsuperscript{T}</td>
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<tr>
<td>B. gallinarum LMG 11586\textsuperscript{T}</td>
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<td>B. cuniculi DSM 10738\textsuperscript{T}</td>
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<td>B. saeculare LMG 14934\textsuperscript{T}</td>
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<td>B. subtile LMG 11597\textsuperscript{T}</td>
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<td>B. breve ATCC 15700\textsuperscript{T}</td>
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<td>B. bifidum LMG 11597\textsuperscript{T}</td>
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Morphological, cultural and biochemical characterizations of the isolate according to standard techniques were performed at 37 °C unless otherwise stated. The morphology of cells of strain LMC 13\textsuperscript{T}, as revealed by phase-contrast microscopy, is shown in Fig. 3(a), and morphological characteristics as determined using a scanning electron microscope (SEM) are shown in Fig. 3(b, c). For SEM observations, strains were cultured on TPY agar at 37°C for 48 h under anaerobic conditions. After culturing, a slice of agar was excised and dehydrated with a series of increasing ethanol concentrations (50, 70, 80, 90, 95 and 100 % for 15 min each). The prepared cells were subsequently critical-point-dried in a critical-point dryer apparatus (CPD Emitech K850) using liquid CO\textsubscript{2} as the transitional fluid. Dried samples were mounted on aluminium stubs with silver glue, and coated with gold–palladium film using an ion-sputtering unit (Emitech K500); observations were made in a Philips 515 SEM at 7–10.0 kV.

The temperature range for growth of the strain was tested using anaerobic TPY broth at 20, 25, 30, 35, 37, 40, 42, 45 and 46 °C for 48 h. The sensitivity of the strain to low pH was determined at 37°C in anaerobic TPY broth (at pH 3.5, 4.0, 4.5, 5.0 and 5.5) for 48 h. The ability of the strain to grow under aerobic and microaerophilic conditions (CampyGen; Oxoid) was tested using TPY agar, TPY broth, whole milk at 37°C for 48 h, and TPY broth supplemented with 0.025 % cysteine hydrochloride, inoculated into the API 50CHL test strips and incubated in an anaerobic jar at 37 °C for 5 days. The results are summarized in Table 2.

Bifidobacteria and members of related genera degrade hexoses via the fructose-6-phosphate phosphoketolase (F6PPK) pathway. The key enzyme in this pathway, F6PPK, is considered a taxonomic marker for the identification of members of Bifidobacterium and related genera (Biavati & Mattarelli, 2012). Detection of F6PPK activity was done according to the method described by Scardovi (1986) and modified by Orban & Patterson (2000). All the isolates possessed F6PPK activity (Table 1).

The cell-wall murein composition of strain LMC 13\textsuperscript{T} was examined by the DSMZ, using published protocols (Schumann, 2011). Analysis of partial acid hydrolysates revealed the presence of murein type A3\textsuperscript{b} (L-Orn–L-Ser–L-Ala–L-Thr–L-Ala). This murein type is not unique among members of the genus Bifidobacterium, as it has also been found in B. longum subsp. longum, B. longum subsp. infantis and B. longum subsp. suis, suggesting the relatedness of these species.

According to phylogenetic analyses based on the 16S rRNA gene and on partial hsp60, clpC, rpoC, rpoB, dnaG, dnaJ and purF sequences, and other data, strain LMC 13\textsuperscript{T} is...
genetically and phenotypically distinguishable from the currently recognized species of bifidobacteria, and thus represents a novel species, for which we propose the name Bifidobacterium lemurum sp. nov.

**Description of Bifidobacterium lemurum sp. nov.**

Bifidobacterium lemurum (le.mu’rum. N.L. gen. masc. pl. n. lemurum of/from lemurs, and the genus name of the true
Cells grown in TPY broth are rods, always coiled or ring shaped or having a ‘Y’ shape at the end. They are Gram-positive-staining, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase- and oxidase-negative, indole-negative and microaerophilic. Well-isolated colonies on the surface of TPY agar under anaerobic conditions are white, opaque, smooth and circular with entire edges, while embedded colonies are lens-shaped or elliptical. Colonies reach 1.0–3.0 mm in diameter after 3 days of incubation. The temperature range for growth is 35–46 °C; no growth occurs at 30 or 47 °C. The optimum temperature for growth is 37–42 °C. Grows at pH 5.5–7.0, with optimum growth at pH 6.5–7.0. Grows in milk under both microaerophilic and anaerobic conditions. Acid is produced from D-glucose, L-arabinose, D-ribose, D-xylose, D-galactose, D-mannose, arbutin, cellobiose, maltose, lactose, melibiose, succrose, melezitose, raffinose, glycosgen, D-mannitol, inositol, D-sorbitol, L-rhamnose, amygdalin, salicin, trehalose, potassium 2-ketogluconate and potassium 5-ketogluconate. Acid may or may not be produced from D-fructose, methyl α-D-glucopyranoside, amygdalin and turanose. Acid is not produced from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbitose, L-rhamnose, dulcitol, D-sorbitol, methyl α-D-mannopyranoside, N-acetylgulucosamine, inulin, starch, xylitol, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol or potassium gluconate. Results from the API ZYM test reveal production of leucine arylamidase, acid phosphatase, α- and β-galactosidases and α- and β-glucosidases. Aesculin is hydrolysed. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the B. longum subgroup of the genus Bifidobacterium.

The type strain LMC 13T (=JCM 30168T=DSM 28807T) was isolated from fresh faecal samples of an adult subject of the ring-tailed lemur (Lemur catta), housed in February 2014 under semi-natural conditions in Parco Natura Viva, Verona, northern Italy. The DNA G+C content of the type strain is 57.2 mol%.

**References**


