INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Modesto *et al.*, *Int J Syst Evol Microbiol* 2019;69:2477–2485

DOI 10.1099/ijsem.0.003518

MICROBIOLOGY SOCIETY

Bifidobacterium jacchi sp. nov., isolated from the faeces of a baby common marmoset (*Callithrix jacchus*)

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Abstract

A novel *Bifidobacterium* strain, MRM 9.3^T, was isolated from a faecal sample of a baby common marmoset (*Callithrixjacchus*). Cells were Gram-stain-positive, non-motile, non-sporulating, non-haemolytic, facultatively anaerobic and fructose 6-phosphate phosphoketolase-positive. Phylogenetic analyses based on 16S rRNA genes as well as multilocus sequences (representing *hsp60*, *rpoB*, *clpC*, *dnaJ* and *dnaG* genes) and the core genomes revealed that strain MRM 9.3^T exhibited phylogenetic relatedness to *Bifidobacterium myosotis* DSM 100196^T. Comparative analysis of 16S rRNA gene sequences confirmed the phylogenetic results showing the highest gene sequence identity with strain *B.ifidobacterium myosotis* DSM 100196^T (95.6 %). The average nucleotide identity, amino acid average identity and *in silico* DNA–DNA hybridization values between MRM 9.3^T and DSM 100196^T were 79.9, 72.1 and 28.5 %, respectively. Phenotypic and genotypic features clearly showed that the strain MRM 9.3^T represents a novel species, for which the name *Bifidobacterium jacchi* sp. nov. is proposed. The type strain is MRM 9.3^T (=DSM 103362^T =JCM 31788^T).

Bifidobacteria are one of the most important microbial groups belonging to the phylum *Actinobacteria*, and are common inhabitants of the gastro–intestinal tract of mammals, birds and the hindgut of social insects [1–5]. Particularly, members of this genus are believed to confer a range of health-promoting effects to its host such as maintaining appropriate balance of the gut microbiota, reducing the risk of pathogen infection and modulating the immune system [5–7]. Furthermore, bifidobacteria are known to be abundantly present in those animals that provide parental care to their offspring and several species belonging to this genus are reported to be among the first gut colonizers of newborns [8, 9].

Currently 74 taxa, representing 64 species and 10 subspecies have been formally recognized as members of the genus *Bifidobacterium* (LPSN; www.bacterio.net).

Endo *et al.* [10] have demonstrated the presence and distribution of bifidobacterial species among New World monkeys. They first described two new species of bifidobacteria from the faeces of a common marmoset (*Callithrix jacchus* L.) viz. *Bifidobacterium reuteri* and *Bifidobacterium callitrichos*, and three new species from the faeces of a red-handed tamarin (*Saguinus midas* L.) viz., *Bifidobacterium biavatii*, *Bifidobacterium stellenboschense* and *Bifidobacterium saguini*. In our previous study [11] based on *hsp60* PCR-restriction fragment length polymorphism and 16S rRNA gene sequencing, it has been stated that the bifidobacterial strains isolated from the individual faecal samples of five baby common marmosets constituted different phylogenetically isolated groups of the genus *Bifidobacterium*, thus highlighting the heterogeneous bifidobacterial population in this species of Callithrichidae, which was found to be distinct from the bifidobacterial populations in other primates. In that

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Keywords: new species; *Bifidobacterium*; *Bifidobacterium jacchi*; common marmoset; *Callithrix jacchus*.
Abbreviations: AAI, Amino acid Average Identity; ANI, average nucleotide identity; GGDC, Genome-To-Genome Distance Calculator; isDDH, in silicoDDH; MLSA, Multi-locus sequence analysis.
The GenBank accession number for the 16S rRNA partial gene sequence of strain MRM 9.3^T is KP718960.1. The accession number for the genome is RQSP00000000.
One supplementary table is available with the online version of this article.

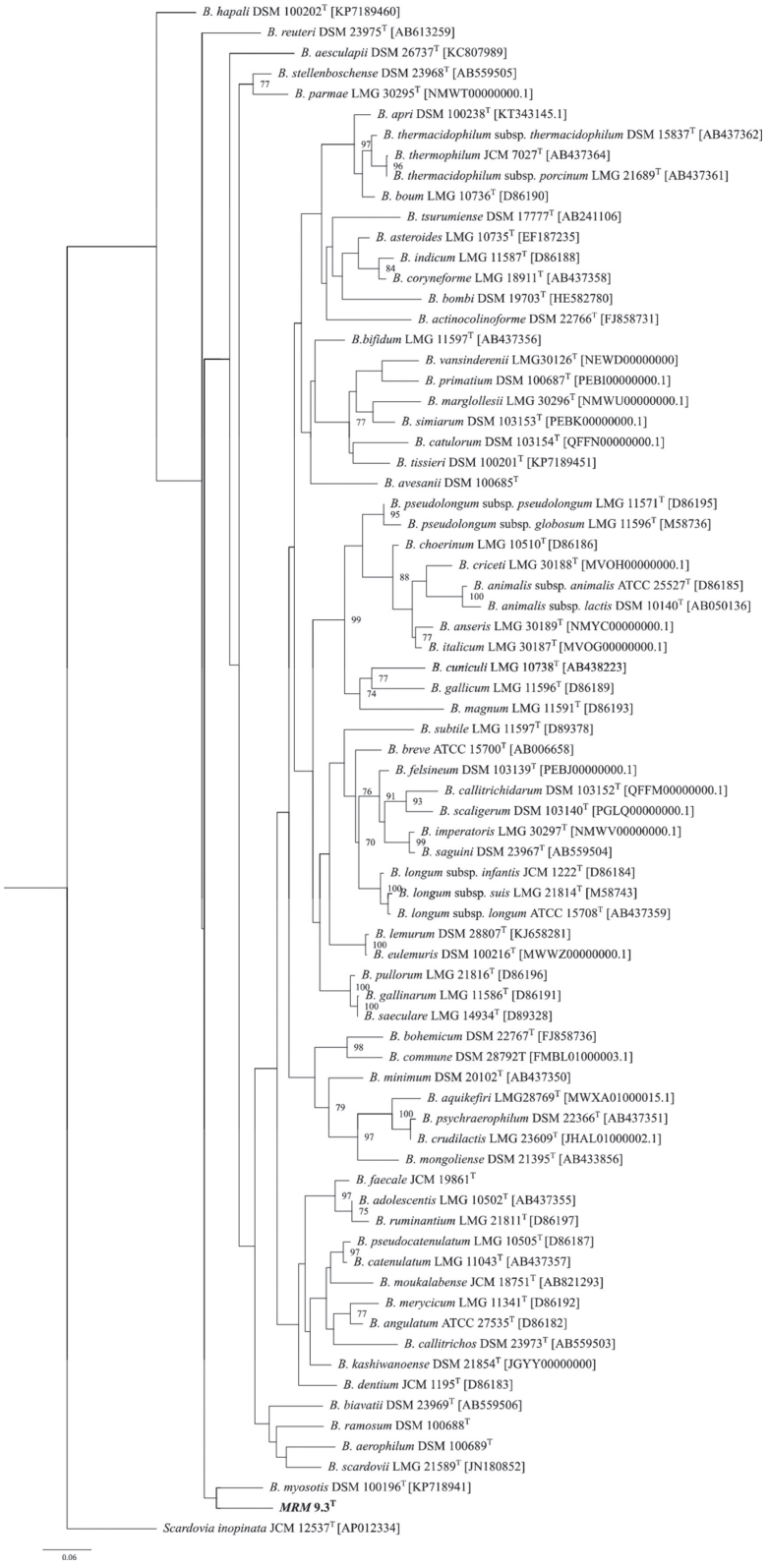

Fig. 1. Phylogenetic tree of the genus *Bifidobacterium* based on 16S rRNA gene sequences, showing the relationship between strain MRM 9.3^T and other members of the genus *Bifidobacterium*. The 16S rRNA gene-based tree was reconstructed by the maximum-likelihood method; the corresponding sequence of *Scardovia inopinata* JCM 12537^T was used as an outgroup. Bootstrap percentages above 70% are shown at node points, based on 1000 replicates of the phylogenetic tree.

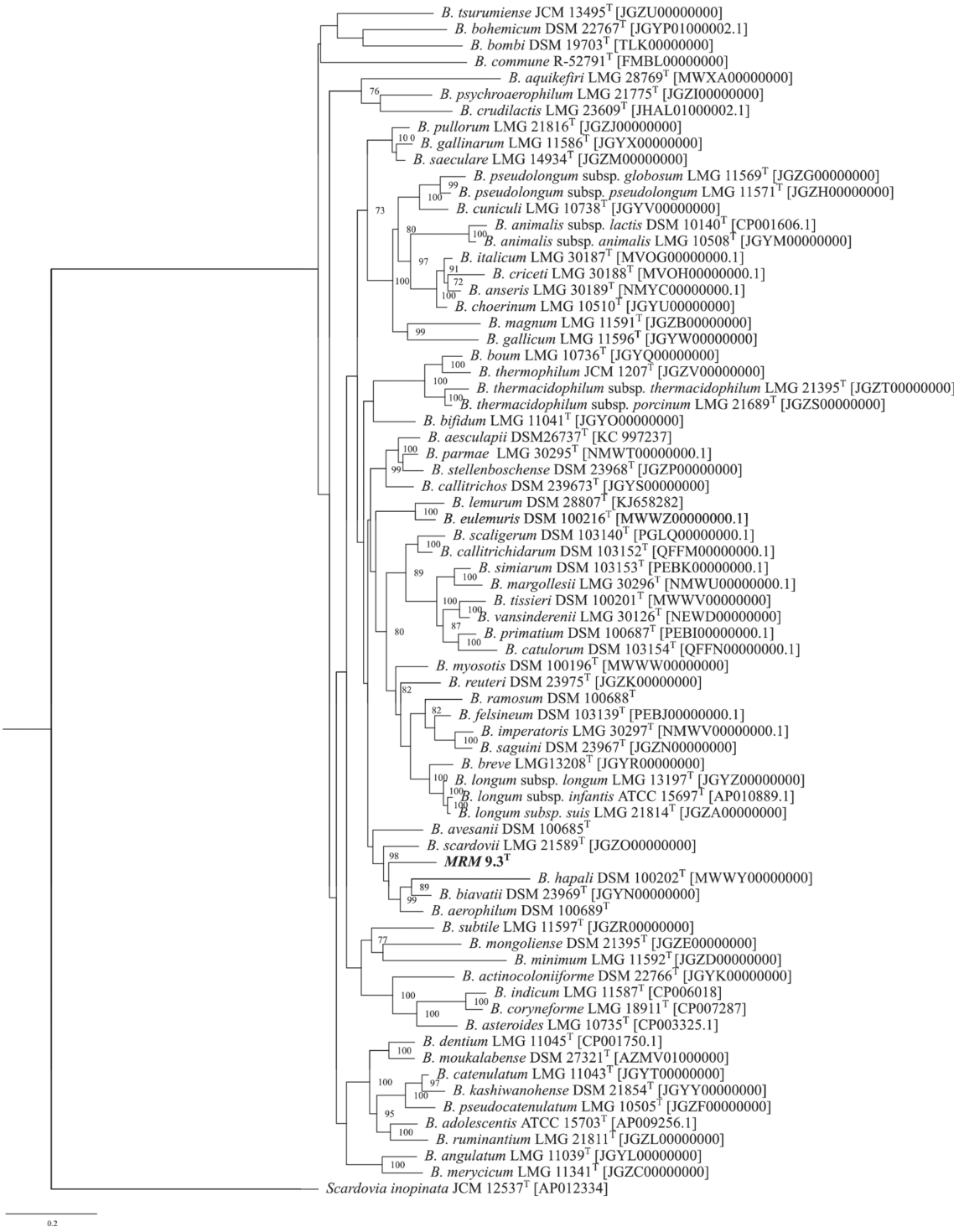

Fig. 2. Phylogenetic three of the genus *Bifidobacterium* based on the concatenation of proteins sequences deduced from the housekeeping genes *clpC*, *dnaG*, *dnaJ*, *hsp60* and *rpoB*, showing the phylogenetic relationships between strain MRM 9.3 and other members of the genus *Bifidobacterium*. The housekeeping gene-based tree was reconstructed by using the maximum-likelihood method, with corresponding sequences of *Scardovia inopinata* JCM 12537^T being used as an outgroup. Bootstrap percentages above 70% are shown at node points, based on 1000 replicates of the phylogenetic tree.

study, we also proposed that these six isolated groups potentially represented novel species of the genus *Bifidobacterium*. Out of them, we have so far described five novel species, viz. *Bifidobacterium aesculapii* [12], *Bifidobacterium myosotis*, *Bifidobacterium tissieri*, *Bifidobacterium hapali* [13] and *Bifidobacterium catulorum* [4].

In the present study, we describe the identification of the novel bifidobacterial isolate, MRM 9.3^T, based on 16S rRNA gene sequences, multi-locus sequences analysis (MLSA; representing *hsp60*, *rpoB*, *clpC*, *dnaJ* and *dnaG* genes) followed by genomic comparison as based on whole-genome sequencing and by phenotypic analysis.

Strain MRM 9.3^T showed rod-shaped cells, 8.0–12.0 µm long and 2.0 µm wide, frequently forming filaments, with irregular contractions along the cells and bifurcations. Cells were cultivated under anaerobic conditions and maintained in TPY broth, pH 6.9, at 37 °C, unless indicated otherwise. Chromosomal DNA from this strain was obtained using a Wizard Genomic DNA Purification kit (Promega) according to the protocol of Michelini *et al.* [13].

The genome of this strain was decoded through a next-generation sequencing (NGS) approach, using a MiSeq platform (Illumina) at Istituto Zooprofilattico Sperimentale (Teramo, Italy). The generated data were depleted of adapter sequences, quality filtered, assembled and annotated through the PATRIC web resources using the RAST server (www.patricbrc.org) [14].

The draft genome size of strain MRM 9.3^T was 2.91 Mb, which contained 2492 predicted protein-coding ORFs.

The G+C content estimation in bacterial chromosomal DNA of strain MRM 9.3^T was performed by HPLC at the DSMZ Identification Service, Braunschweig, Germany, following previously described protocols (Table S1, available in the online version of this article) [15, 16]. The G+C content was 61.5 mol% (Table S1), which is in the range reported for the genus *Bifidobacterium*, i.e. 52–67 mol % [5, 17].

This Whole Genome Shotgun project has been submitted to GenBank under the accession RQSP00000000.

We investigated the phylogenetic relatedness of strain MRM 9.3^T with the other recognized bifidobacterial taxa by inferring the nucleotide sequences of the 16S rRNA gene (Fig. 1), the nucleotide sequences of five housekeeping genes (*hsp60*, *rpoB*, *clpC*, *dnaJ* and *dnaG*) (Fig. 2) as well as the genes constituting the core genome of *Bifidobacterium* species (Fig. 3).

The 16S rRNA gene sequence (1529 bp) of strain MRM 9.3^T and of its closest relatives retrieved from the DDBJ/GenBank/EMBL databases were aligned using CLUSTAL Omega in a CLC Sequence Viewer (1328 nt). A phylogenetic tree based on a total of 74 partial 16S rRNA gene sequences, including those of members of the genus *Bifidobacterium* was reconstructed with the maximum-likelihood method [18] and the evolutionary distances were computed by nucleotide model of GTR CAT. The tree was reconstructed using RaxML version 8.2.7 [19] and rooted with *Scardovia inopinata* JCM 12537^T (Fig. 1). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates and the bootstrap rapid hill climbing algorithm was used. The tree was visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) (Fig. 1).

Strain MRM 9.3^T showed low 16S rRNA gene sequence similarities to known bifidobacteria and the highest values, 95.6 % were found to *B. myosotis* DSM 100196^T and *B. catulorum* DSM 103154^T (94.92 %) Similarity values were obtained using the LALIGN web-based program (http://embnet.vital-it.ch/software/LALIGN_form.html),

The phylogenetic relationships have been confirmed by Maximum-Likelihood analysis, the novel strain was found to be phylogenetically related to *B. myosotis* DSM 100196^T (Fig. 1). MRM 9.3^T and *B. myosotis* DSMZ 100196^T are found in a unique separated cluster in the 16S rRNA gene tree.

MLSA is a reliable and robust technique for the identification and classification of bacterial isolates to the species level and the concatenation of gene sequences has been shown to be extremely useful in order to infer bacterial phylogeny [20].

Thus, the phylogenetic location of the novel strain was verified by the analysis of five housekeeping genes (*hsp60*, *rpoB*, *clpC*, *dnaJ*, *dnaG*), which have proven to be discriminative for the classification of the genus *Bifidobacterium* [20–22].

For this purpose, a phylogenetic tree for 71 bifidobacterial type strains was reconstructed by joining the five coding sequences in the following order: *clpC* (720 bp), *dnaG* (992 bp), *dnaJ* (477 bp), *hsp60* (662 bp) and *rpoB* (500 bp). The resulting in-frame concatenated gene sequences (3154 bp) were aligned with the MAFFT program at CBRC (http://mafft.cbrc.jp/alignment/software/) [23]. The evolutionary distances were computed by nucleotide model GTR CAT, and the phylogenetic tree was reconstructed by using RaxML (version 8.2.7; maximum-likelihood method) [19] with *Scardovia inopinata* JCM 12537^T as the root (Fig. 2). The statistical reliability of the tree was evaluated by bootstrap analysis (rapid hill climbing) of 1000 replicates. The visualization was performed with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

The level of similarity for the partial housekeeping gene sequences of strain MRM 9.3^T in relation to the type strains of its closest phylogenetic relatives was calculated using the LALIGN web-based program (http://embnet.vital-it.ch/software/LALIGN_form.html): the highest values of similarity for the *hsp60*, *rpoB*, *clpC*, *dnaJ* and *dnaG* gene sequences were found to *Bifidobacterium parmae* LMG 30295^T (*hsp60* 92.7 %) and to *Bifidobacterium biavatii* DSM 23969^T (*clpC* 90.6 %, *dnaJ* 81.6 % and *rpoB* 91.1 %, respectively); MLSA also showed the phylogenetic relatedness of this strain to

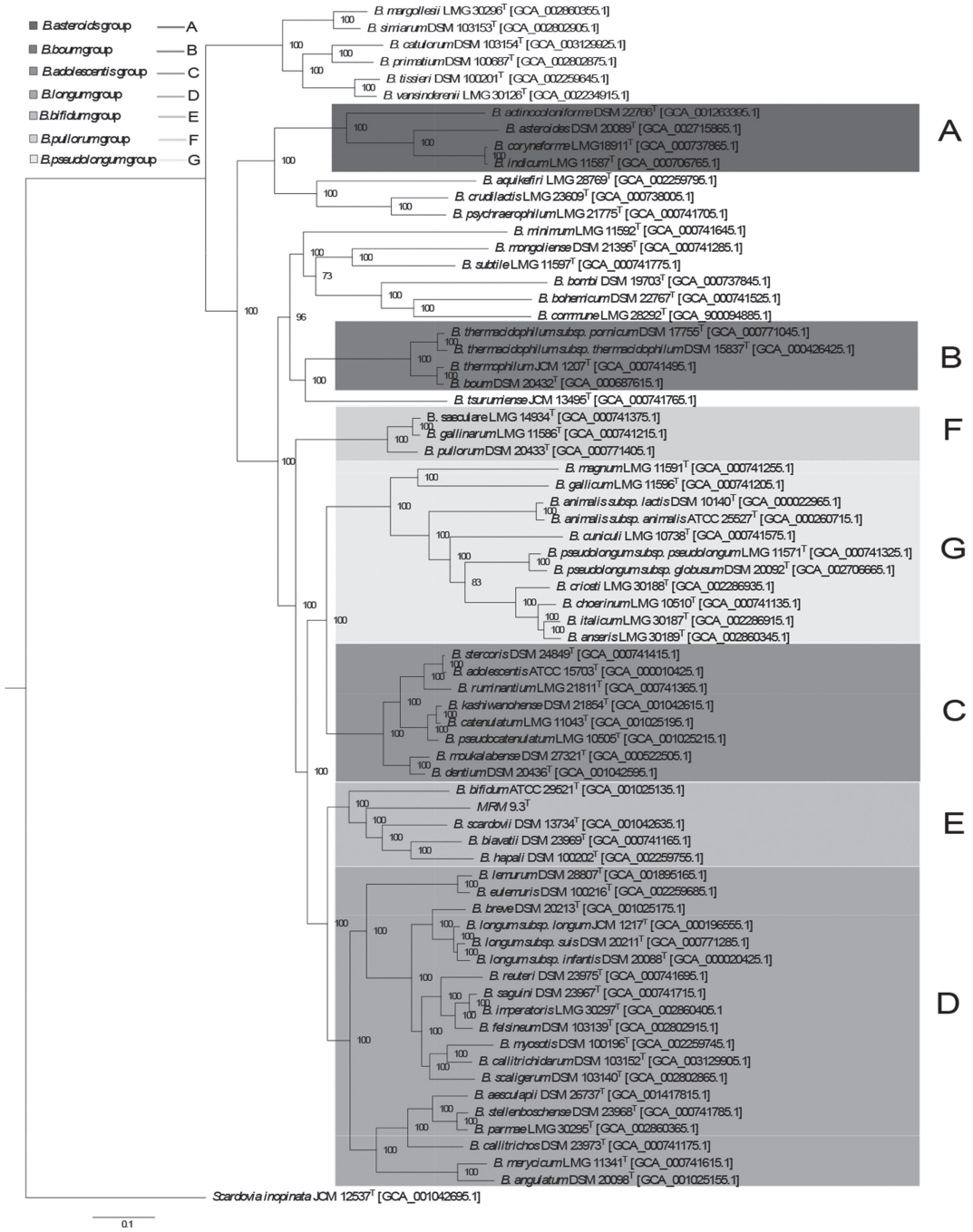

Fig. 3. Phylogenetic tree of the genus *Bifidobacterium* based on the concatenation of 363 shared protein clusters of MRM 9.3^T and other members of the genus *Bifidobacterium*. The core gene-based tree shows the subdivision of the seven phylogenetic groups of the genus *Bifidobacterium* represented by different colours. The phylogenetic tree was built by using the maximum-likelihood method with corresponding sequences of *Scardovia inopinata* JCM 12537^T being used as an outgroup. Bootstrap percentages above 70% are shown at node points, based on 1000 replicates of the phylogenetic tree.

Bifidobacterium scardovii DSM 13734^T(*dnaG* 86.3 %) in the *B. bifidum* LMG 11597 group according to Lugli *et al.* [24].

In order to reconfirm the above phylogenetic analysis, we also reconstructed the phylogenetic tree based on the core genome of *Bifidobacterium* species. A total of 69 type strains of *Bifidobacterium* were annotated with the DFAST program [25], and 363 orthologous genes were identified as the core retained in all genomes. The core protein sequences from each genome were concatenated and aligned using the MAFFT program (version 7.313) [23]. The alignments were

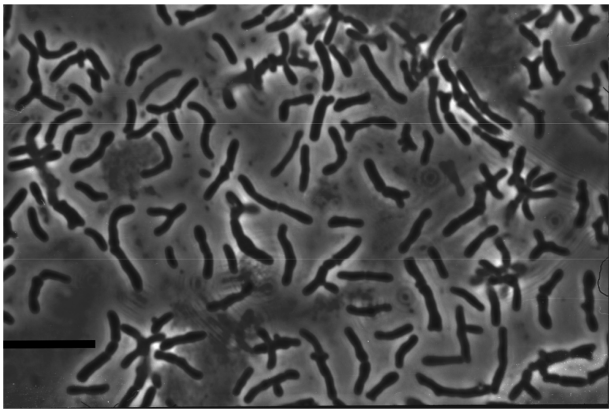

Fig. 4. Cellular morphology of cells grown in TPY broth. Phase-contrast photomicrograph of *Bifidobacterium jacchi* MRM 9.3. Bar, 10 µm.

trimmed using trimAl with the automated1 option [26]. The phylogenetic tree was reconstructed as above. The tree based on the core genome (363 genes) confirmed the position of strain MRM 9.3^{T}as near to *B. scardovii* DSM 13734^T (Fig. 3).

Furthermore average nucleotide identity (ANI) was calculated by using the web server JSpeciesWS (http://jspecies.ribohost.com/jspecieswsl/). This analysis showed that the MRM 9.3^T genome displays the highest level of identity (79.9 %) with chromosomal sequences of *B. myosotis* DSM 100196^T. In this context, it should be noted that two strains displaying an ANI value $\leq$95 % are considered to belong to two distinct species [27].

Amino acid average identity (AAI) was also calculated by using the AAI calculator web tool (http://enve-omics.ce.gatech.edu/aai/index). The value achieved was 72.1 % vs *B. myosotis* DSM 100196^T.

Furthermore, *in silico* DDH (*is*DDH) was also carried out using GGDC [Genome-To-Genome Distance Calculator (GGDC) version 2.1], the most accurate known tool for calculating DDH-analogous values, developed at DSMZ and available at http://ggdc.dsmz.de/ggdc.php#. The threshold value of $\leq$70 % is generally accepted for separated prokaryote species. The value achieved was 28.5 % vs *B. myosotis* DSM 100196^T.

Morphological, cultural and biochemical characterization of the strains were performed at 37 °C unless otherwise stated, according to Modesto *et al.* [2].

The morphology of cells of strain MRM 9.3^T, as revealed by phase-contrast microscopy, is shown in Fig. 4. Optimal growth conditions of the strain was determined in TPY broth after 24 h of incubation at 37 °C in anaerobic conditions. Growth at 22, 25, 30, 35, 37, 40, 42, 45 and 48 °C was tested. Sensitivity to low pH was screened at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5. The ability of the strain to grow under aerobic and microaerophilic conditions (CampyGen, Oxoid) was also verified in TPY broth after 48 h of incubation at 37 °C. For strain MRM 9.3^T best growth conditions were obtained in TPY broth, pH 7 at 37 °C and it was able to survive and grow in microaerophilic and in aerobic conditions. Haemolytic activity was determined in Columbia blood agar (Biolife) at 37 °C under anaerobic conditions for 48 h [28]. Gram-staining, motility assay, catalase and oxidase activities were performed according to Modesto *et al.* [12]. Strain MRM 9.3^T and the related species, *B. myosotis* DSM 100196^T, was also investigated for substrate utilization and enzyme production with API 50 CHL and Rapid ID 32A test kits (bioMérieux). Results are summarized in Table 1.

Bifidobacteria and members of related genera possess fructose-6-phosphate phosphoketolase (F6PPK), the enzyme-degrading hexose via the F6PPK pathway, which is considered a taxonomic marker for identification of *Bifidobacterium* and related genera [5]. Detection of F6PPK activity was carried out according to the method described by Orban and Patterson [29]. Strain MRM 9.3^T possessed F6PPK activity.

Following the protocol of Schumann [30], the cell-wall murein composition of the strain MRM 9.3^T was examined by the DSMZ Identification Service.

The total hydrolysate of the peptidoglycan (4 N HCl, 16 h at 100 °C) revealed the presence in strain MRM 9.3^T of the amino acids ornithine, alanine, glutamic acid and serine. Quantitative analysis according to Protocol 10 [30] revealed the following approximate molar amino acid ratio: 2.6 Ala; 1.0 Ser; 0.8 Orn; 3.7 Glu; 0.2 Thr; 0.2 Asp; 0.1 Lys. Dinitrophenylation could not be carried out as the obtained amount of peptidoglycan was not sufficient for this analysis. Two-dimensional TLC of the partial hydrolysate (4 N HCl, 100 °C,

Table 1. Differential phenotypic characteristics of strain MRM 9.3^T and its phylogenetically related species *Bifidobacterium myosotis* DSM 100196^T, *Bifidobacterium scardovi* DSM 13734^T and *Bifidobacterium catulorum* DSM 103154^T

Data are from this study. +, Positive; −, negative; w, weakly positive. All strains produced acid from L-arabinose, D-glucose, lactose, maltose, sucrose and trehalose. All strains produced arginine arylamidase, α-galactosidase, β-galactosidase, α-glucosidase, histidine arylamidase, leucine arylamidase, phenylalanine arylamidase, proline arylamidase and serine arylamidase. None of the strains produced acid from D-adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, erithritol, D-fucose, L-fucose, glycerol, glycogen, inositol, inulin, D-lyxose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate, L-rhamnose, salicin, D-sorbitol, L-sorbose, starch, D-tagatose, L-xylose and xylitol. None of the strains produced α-arabinosidase, α-fucosidase, β-galactosidase 6 phosphate, β-glucosidase, β-glucuronidase, glutamic acid decarboxylase, glutamyl glutamic acid arylamidase and pyroglutamic acid arylamidase.

	Strain MRM 9.3^T	***Bifidobacterium myosotis* DSM 100196^T**	***Bifidobacterium scardovii* DSM 13734^T**	***Bifidobacterium catulorum* DSM 103154^T**
Enzymatic activity:				
Urease	−	+	w	−
Arginine dihydrolase	−	+	−	−
N-Acetyl-β-glucosaminidase	+	−	w	−
Alkaline phosphatase	w	−	−	+
Leucyl glycine arylamidase	−	+	−	w
Tyrosine arylamidase	+	+	+	+
Alanine arylamidase	−	+	w	−
Glycine arylamidase	w	+	+	+
Fermentation:				
D-Ribose	+	−	−	+
D-Xylose	−	+	−	+
Methyl β-D-xylopyranoside	+	w	−	+
D-Galactose	+	w	−	−
D-Fructose	+	w	w	w
D-Mannose	+	w	+	−
D-Mannitol	+	w	−	+
N-Acetyl glucosamine	w	−	w	−
Amygdalin	−	w	w	−
Arbutin	+	−	−	−
Cellobiose	−	+	+	−
Melibiose	w	+	+	−
Melezitose	−	w	w	+
Raffinose	+	−	+	−
Gentiobiose	−	w	−	−
Turanose	w	w	+	+
Hydrolysis of aesculin	−	+	w	+
Temperature range for growth (°C)	20–48	20–48	22–48	22–48
Optimum temperature for growth (°C)	37	42	37.6	37
pH range for growth	4.0−7.5	4.0−7.5	4–8	4–8
Optimum pH for growth	7	7	7	7
DNA G+C content (mol%)	61.5*	65.1†	60.1‡	61.7§
Peptidoglycan type	L-Orn – L-Ser – L-Ala*	L-Glu– L-Ala– L-Lys†	L-Lys–L-Ser–L-Ala‡	L-Orn (Lys)–L- Ser§

*Data from this study.

†Data from Michelini *et al.* [13].

‡Data from Hoyles *et al.* [32]

§Data from Modesto *et al.* [4].

45 min) of the peptidoglycan revealed the presence of the peptides L-Ala – D-Glu, L-Ala – D-Ala, L- Orn – D-Ala, L-Orn – L-Ser and D-Ala - L-Orn – L-Ser. From the data obtained, it appears that strain MRM 9.3^T displays a A3β peptidoglycan of the following structure: L-Orn – L-Ser – L-Ala.

On the basis of the phenotypic and chemotaxonomic characterization, as well as molecular-based methods, phylogenetic analysis based on the 16S rRNA gene sequences, MLSA based on the concatenated five housekeeping gene sequences, and whole-genome-based comparisons, strain

MRM 9.3^T was genetically and phenotypically discernible from the currently recognized species of bifidobacteria; thus, according to minimal standard guidelines [31], it represents a novel taxon for which the name *Bifidobacterium jacchi* is proposed.

DESCRIPTION OF *BIFIDOBACTERIUM JACCHI* SP. NOV.

Bifidobacterium jacchi [jac'chi. L. gen. n. *jacchi*, based on the specific epithet of the marmoset *Callithrix jacchus* (from L. masc. n. *Iacchus=Bacchus*)].

Cells are Gram-stain-positive, non-motile, asporogenous, non-haemolytic, F6PPK-positive, catalase-negative, oxidase-negative and indole-negative. Cells, when grown in TPY broth, are rods of various shapes, 8.0–12.0 µm long and 2.0 µm wide, forming a branched structure with 'Y' at the both side. Well-isolated colonies grown on the surface of TPY agar under anaerobic conditions are white, opaque, smooth and circular with entire edges, while the embedded colonies are lens-shaped or elliptical. Colonies reach 1.0–2.0 mm in diameter after 3 days of incubation. Cells can grow under aerobic and microaerophilic conditions. Cells can grow in the range 22–48 °C. Cells grow at pH 4.0–7.5. Optimal conditions of growth occur at pH 7 and 37 °C. Using API 50 CHL system, acids are produced from D-glucose, L-arabinose, D-ribose, methyl β-D-xylopyranoside, D-galactose, lactose, D-fructose, D-mannitol, D-mannose, arbutin, maltose, sucrose, trehalose and raffinose; produced weakly from *N*-acetyl-glucosamine, melebiose and turanose; but not from other carbohydrates. Activity was observed for α- and β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, arginine arylamidase, proline arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, histidine arylamidase, and serine arylamidase. Activity was also observed weakly for alkaline phosphatase and glycine arylamidase. Aesculin is not hydrolysed. No reduction of nitrates was recognized. Cells are negative for urease. The peptidoglycan type is L-Orn – L-Ser – L-Ala.

The type strain, MRM 9.3^T (=DSM 103362^T=JCM 31788^T), was isolated from the faeces of a baby common marmoset. The DNA G+C content of the type strain is 61.5 mol%.

Funding information
Computational analysis was performed on the NIG supercomputer at the Research Organization of Information and Systems (ROIS). Short-term Research Abroad & long-term Internship Program 2018 cost was supported by SOKENDAI. M. A. and M. S. are also supported by KAKENHI 17K19248.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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