June 23, 2019
SUNDAY – EEB-527

Using Whole-Genome Sequencing to Revise the Classification of *Bifidobacterium* and *Gardnerella* Genera

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Abstract

Most commonly known for their probiotic properties, Bifidobacteria are Gram-positive, rod-shaped, anaerobic bacteria often found in the digestive tracts of various mammals and insects. Belonging to the same family as *Bifidobacterium*, *Gardnerella* comprises a single species (*G. vaginalis*) implicated in bacterial vaginosis, whose taxonomic status has often been disputed. Various phylogenetic trees of the *Bifidobacteriaceae* family place *G. vaginalis* centrally within the *Bifidobacterium* genus on the basis of 16S rRNA sequences. Additionally, the discrimination between *Gardnerella* and *Bifidobacterium* has proven difficult in the laboratory. In this study, we aim to elucidate the taxonomic position of *G. vaginalis* and revise the classification of the *Bifidobacterium* genus through whole-genome sequencing (WGS) of 62 type strains of *Bifidobacterium* and the type strain of *G. vaginalis*. To infer their taxonomic relationship to each other, we measured their whole-genome distance and placement within the phylogenetic tree. Within the *Bifidobacterium* genus, comparative WGS analyses showed close relatedness between seemingly distinct species, and conversely, dissimilarity among the subspecies. We propose the reclassification of *B. faecale* as a later heterotypic synonym of *B. adolescentis*. Similarly, we propose the unification of *B. gallinarum* and *B. saeculare* as *B. gallinarum* subsp. *gallinarum* and *B. gallinarum* subsp. *saeculare*, and the unification of *B. catenulatum* and *B. kashiwanohense* as *B. catenulatum* subsp. *catenulatum* and *B. catenulatum* subsp. *kashiwanohense*. In the *B. longum* subspecies group, we propose the reclassification of *B. longum* subsp. *infantis* as its own species, *B. infantis*, and the reclassification of *B. longum* subsp. *suillum* as *B. longum* subsp. *suis*. Additionally, insufficient relatedness between *B. pseudolongum* subsp. *pseudolongum* and *B. pseudolongum* subsp. *globosum*, and between *B. animalis* subsp. *animalis and B. animalis* subsp. *lactis*, indicate the subspecies should be reclassified as their own species. We suggest amending the classification of *B. pseudolongum* subspecies group to *B. pseudolongum* and *B. globosum*, and of *B. animalis* subspecies group to *B. animalis* and *B. lactis*. Distance- and gene-based phylogenetic trees of the type strains position *G. vaginalis* in the midst of the *Bifidobacterium* genus, further corroborating the close relatedness between the two genera and providing additional justification for reclassifying *Gardnerella vaginalis* as a species of *Bifidobacterium*.

Materials and Methods

Bacterial Strains, DNA Extraction, and Genome Sequences Included in the Analysis. The type strains for each species and subspecies of *Bifidobacterium* and *Gardnerella* were obtained from the American Type Culture Collection (ATCC), NIAID's BEI Resources (www.beiresources.org), Leibnitz Institute Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ), Riken BRC/Japan Collection of Microorganisms (JCM), and Belgian Co-ordinated Collections of Microorganisms (BCCM). A total of 69 type strains were grown as recommended by the manufacturers, and gDNA was extracted using the QIAGEN® MagAttract® High-Molecular Weight (HMW) system. Additionally, existing genomes from GenBank were also used in the analysis. Together, the 134 genomes from the strains sequenced and those from GenBank compose the main dataset for the genomic analysis.

Whole-Genome Sequencing (WGS). DNA was prepared using the Nextera® XT Library Preparation Kit (Illumina®) and sequenced using Illumina MiSeq® v3 flow cells (2×300). Resultant paired-end reads underwent contamination detection by using the One Codex microbial genomics read-based identification algorithm. Read pairs were then adapter trimmed and quality filtered, then used for *de novo* genome assembly using SPAdes 3.12.0.

Calculation of Genomic Distances. For independent corroboration of the results, two algorithmic approaches were used. Genomic distance based on digital DNA-DNA hybridization (dDDH) was calculated with the Genome-to-Genome Distance Calculator (GGDC) v2.1 using the recommended Formula 2.[1,2] Average nucleotide identity (ANI) was calculated using OrthoANIu.[3] The species delineation thresholds used were ≥70% via dDDH and ≥96% via ANI.[4,5] A dDDH distance of ≥70-79.9% was considered to represent different subspecies of the same species, whereas ≥80% was considered to represent the same subspecies of the same species (or no subspecies in the case of species without multiple subspecies).[4] No subspecies delineation threshold based on ANI values currently exists. The calculated dDDH values were used as the basis for a phylogenetic tree as described previously.[6]

Phylogenetic tree construction. To calculate phylogenetic trees by using constituent genomes, we calculated maximum likelihood species trees via UBCG. UBCG automatically extracts 92 pre-defined bacterial core genes from all provided genome assemblies, creates a multiple sequence alignment (MSA) for each gene, and concatenated all MSAs into a single supermatrix. UBCG was run using default settings. The resultant supermatrix was used as input for RAxML maximum likelihood species tree estimation. RAxML was run using the GTRGAMMA model of nucleotide evolution with the option to calculate 100 bootstrap trees, which were used to calculate branch support values. Each species tree was rooted at the outgroup, and branches with support values less than 70 were collapsed to show only statistically supported branches.

References

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Results

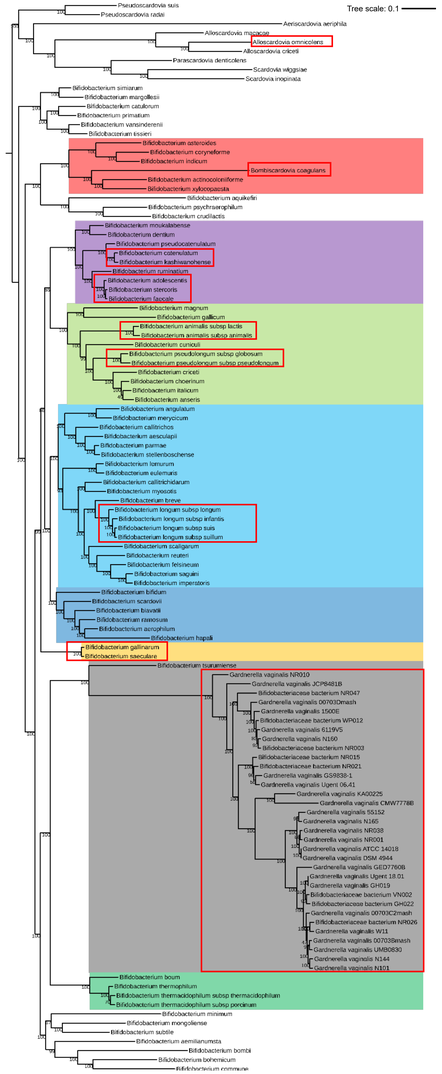

Figure 1. Phylogenomic tree of the *Bifidobacteriales* family. The name on each leaf represents the strain's existing taxonomic identification. The red boxes indicate the reclassifications identified. The colored background boxes correspond to the clades defined by Lugli, *et al.* 2017.[7]

Table 1. Genomic distances (dDDH percentages) between the strains examined that justify a taxonomic reclassification. *Mycobacterium smegmatis* was used as an outgroup.

dDDH Values (Formula 2)	Reclassification
G. vaginalis 350 GVAG	*Alloscardovia omnicolens*
G. vaginalis 476 GVAG	*Alloscardovia omnicolens*
G. vaginalis 842 GVAG	*Alloscardovia omnicolens*
G. vaginalis 1036 GVAG	*Alloscardovia omnicolens*
Alloscardovia omnicolens	*Alloscardovia omnicolens*
Bifidobacterium asteroides	*Bifidobacterium* 1
B. catenulatum	*B. catenulatum* subsp. *catenulatum*
B. kashiwanohense	*B. catenulatum* subsp. *kashiwanohense*
B. stercoris	*B. adolescentis*
B. faecale	*B. adolescentis*
B. animalis subsp. *animalis*	*B. animalis*
B. animalis subsp. *lactis*	*B. lactis*
B. pseudolongum subsp. *globosum*	*B. globosum*
Bifidobacterium subsp. *pseudolongum*	*B. pseudolongum*
B. longum subsp. *longum*	*B. longum* subsp. *longum*
B. longum subsp. *suillum*	*B. longum* subsp. *suis*
B. longum subsp. *infantis*	*B. infantis*
B. gallinarum	*B. saeculare* subsp. *gallinarum*
B. saeculare	*B. saeculare* subsp. *saeculare*
G. vaginalis 1400E	*B. vaginalis* subsp. 1
G. vaginalis NR002	*B. vaginalis* subsp. 2
G. vaginalis NR018	*B. vaginalis* subsp. 3
Bifidobacteriaceae bacterium NR018	*Bifidobacterium* 2 subsp. 1
Bifidobacteriaceae bacterium VN002	*Bifidobacterium* 2 subsp. 2
Bifidobacteriaceae bacterium WP012	*Bifidobacterium* 2 subsp. 3
G. vaginalis 6119V5	*Bifidobacterium* 2 subsp. 4
G. vaginalis N160	*Bifidobacterium* 2 subsp. 5
Bifidobacteriaceae bacterium NR036	*Bifidobacterium* 2 subsp. 6
Bifidobacteriaceae bacterium NR053	*Bifidobacterium* 3 subsp. 1
Bifidobacteriaceae bacterium NR021	*Bifidobacterium* 3 subsp. 2
Bifidobacteriaceae bacterium WP025	*Bifidobacterium* 3 subsp. 3
G. vaginalis 0288A	*Bifidobacterium* 3 subsp. 4
G. vaginalis N172	*Bifidobacterium* 3 subsp. 5
Bifidobacteriaceae bacterium GH006	*Bifidobacterium* 3 subsp. 6
Bifidobacteriaceae bacterium NR013	*Bifidobacterium* 4 subsp. 1
Bifidobacteriaceae bacterium NR038	*Bifidobacterium* 4 subsp. 2
Bifidobacteriaceae bacterium WP022	*Bifidobacterium* 4 subsp. 3
G. vaginalis UGent06.41	*Bifidobacterium* 4 subsp. 4
Bifidobacteriaceae bacterium NR053	*Bifidobacterium* 5
Bifidobacteriaceae bacterium NR021	*Bifidobacterium* 6
Bifidobacteriaceae bacterium NR025	*Bifidobacterium* 7
Bifidobacteriaceae bacterium NR040	*Bifidobacterium* 8
G. vaginalis 00703Dmash	*Bifidobacterium* 9
G. vaginalis 1500E	*Bifidobacterium* 10
G. vaginalis 55152	*Bifidobacterium* 11
G. vaginalis GMV77778B	*Bifidobacterium* 12
G. vaginalis GED7760B	*Bifidobacterium* 13
G. vaginalis JCP8481B	*Bifidobacterium* 14
G. vaginalis AS0025	*Bifidobacterium* 15
G. vaginalis N165	*Bifidobacterium* 16
G. vaginalis NR001	*Bifidobacterium* 17
G. vaginalis UMB0830	*Bifidobacterium* 18
G. vaginalis W11	*Bifidobacterium* 19
G. vaginalis 1500E	*Bifidobacterium* 20
	Bifidobacterium 21

Type strain | Non-Type Strain | Subspecies Circumscription | Species Circumscription

dDDH Interpretation:	Same species and subspecies	Same species, different subspecies	Different species
	80-100%	70-80%	0-70%

Conclusions

Table 1 shows the pairwise dDDH results obtained for those items that will require a taxonomic reclassification as a new species or subspecies. The phylogenomic tree is shown in Figure 1; the proposed taxonomic changes based upon whole-genome comparison are summarized below (following the order found in the phylogenetic tree shown in Figure 1).

- dDDH values indicated close relatedness between *Alloscardovia omnicolens* and *G. vaginalis* 350_GVAG, *G. vaginalis* 476_GVAG, *G. vaginalis* 842_GVAG, and *G. vaginalis* 1036_GVAG (dDDH: 88.4-90.6%), suggesting that these misclassified *G. vaginalis* strains are actually isolates of *A. omnicolens*.
- *Bombiscardovia coagulans* is nestled within the *Bifidobacterium* species. The isolate of this single species should be reclassified as *Bifidobacterium coagulans* comb. nov.
- *B. kashiwanohense* and *B. catenulatum* showed a dDDH >73%, suggesting they are a single species but different subspecies. For this reason, these should be reclassified as *B. catenulatum* subsp. *catenulatum* and *B. catenulatum* subsp. *kashiwanohense* subsp. nov., respectively.
- *B. stercoris* and *B. adolescentis* were defined as separate species by 16S rRNA sequencing and multi-locus sequence analysis.[8] However, we observed dDDH: >80% and ANI: >97.6 along with *B. faecale*, suggesting that they are all a single species. These three species should be reclassified as *B. adolescentis.*
- GGDs between *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* are distinct enough (dDDH 65%) to be separated into two species, resulting in *B. lactis* sp. nov. Similarly, *B. pseudolongum* subsp. *pseudolongum* and *B. pseudolongum* subsp. *globosum* should be separated, resulting in *B. globosum* sp. nov.
- The subspecies of *B. longum* have seen many revisions in taxonomy over the past five decades. Our data shows confirms two existing subspecies: *B. longum* subsp. *longum* and *B. longum* subsp. *suis*. *B. longum* subsp. *suillum* is a later heterotypic synonym of *B. longum* subsp. *suis*. In the case of *B. longum* subsp. *infantis* the reclassification is not obvious. The dDDH values of 62-68% suggest this can be classified as a separate species, but the phylogenetic tree (Figure 1) suggests it should remain as a separate subspecies.
- *B. saeculare* and *B. gallinarum* show dDDH values of 72%, suggesting these are subspecies, so they should be classified as *B. saeculare* subsp. *gallinarum* subsp. nov. and *B. saeculare* subsp. *saeculare* subsp. nov.
- The type strain of *G. vaginalis*, as well as several other proposed isolates that could represent new species of *Gardnerella*,[9] all clade together in a very distinct branch among many other species of *Bifidobacterium*. This suggests that the genus *Gardnerella* should be reclassified as part of *Bifidobacterium*. Among the isolates included in this study, we identified at least 21 new proposed species of *Bifidobacterium*. Interestingly, all the isolates in this branch seem to be pathogenic, suggesting a common ancestral origin that originated the whole clade (Figure 1; grey section).

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