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Comparative genomics reveals robust phylogroups in the genus *Lactobacillus* as the basis for reclassification

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Running head: genome-based taxonomy of genus *Lactobacillus*

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Abstract

The genus *Lactobacillus* includes over 200 species that are widely used in fermented food preservation, biotechnology or that are explored for beneficial effects on health. Naming, classifying and comparing lactobacilli has been challenging due to the high level of phenotypic and genotypic diversity they display, and because of the uncertain degree of relatedness between them and associated genera. The aim of this study was to investigate the feasibility of dividing the genus *Lactobacillus* into more homogeneous genera/clusters, exploiting genome-based data. The relatedness of 269 species belonging primarily to the families Lactobacillaceae and Leuconostocaceae was investigated through phylogenetic analysis (ribosomal proteins and housekeeping genes) and the assessement of the Average Amino acid Identity (AAI) and, the Percentage of Conserved Proteins (POCP). For each sub-generic group that emerged, conserved signature genes were identified. Both distance-based and sequence-based metrics showed that the *Lactobacillus* genus was paraphyletic and revealed the presence of 10 methodologically consistent subclades, which were also characterized by distinct distribution of conserved signature orthologues. We present two ways to reclassify lactobacilli - a conservative division into two subgeneric groups based on presence/absence of a key carbohydrate utilization gene, or a more radical subdivision into 10 groups that satisfy more stringent criteria for genomic relatedness.

Importance

Lactobacilli have significant scientific and economic value but their extraordinary diversity means they are not robustly classified. The 10 homogeneous genera/subgeneric entitites we identify here are characterised by uniform patterns of the presence/absence of specific sets of genes which offer potential as discovery tools for understanding differential biological features. Reclassification/sub-division of the genus *Lactobacillus* into more uniform taxonomic nuclei will also provide accurate molecular markers that will be enabling for regulatory approval applications. Re-classification will facilitate scientific communication related to lactobacilli and prevent mis-identification issues, which are still the major cause of mislabelling of probiotic and food products reported worldwide.

Keywords: *Lactobacillus*, taxonomy, phylogeny, comparative genomics, reclassification.

INTRODUCTION

The genus *Lactobacillus* includes 232 species (as reported in http://www.bacterio.net/lactobacillus.html), a number which is rising continuously as novel species are described every year. Lactobacilli are Gram-positive bacteria, mostly non-motile, catalase-negative, non-spore-forming and rod-shaped (although coccobacilli are observed). They populate nutrient-rich habitats associated with food, feed, soil, plants, animals (both vertebrates and invertebrates) and humans (1) and are mainly characterized by a fermentative metabolism but some evidence of respiration (2), with lactic acid as the main product.

Lactobacilli are key players in industry, food, and human and animal health-related fields: they contribute to fermented food production, to food texture and its preservation, they deliver pure lactic acid from raw carbohydrates for onward conversion to bioplastics, and some strains are marketed as probiotics, meaning they exhibit health benefits beyond the basic nutritional value. In addition, lactobacilli are also being explored as therapeutics and delivery systems for vaccines (1, 3, 4, 5).

From a food regulatory viewpoint, 84 *Lactobacillus* species are certified for safe, technological and beneficial use by the European Food and Feed Cultures Association (6), 36 species have Qualified Presumption of Safety (QPS) status according to the European Food Safety Authority (EFSA) (7) and 12 species are Generally Recognised as Safe (GRAS) according to the U.S. Food and Drug Administration (FDA) (http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices) (8).

The economic value of lactobacilli is substantial: the probiotics and direct-fed microbials markets, in which lactobacilli play an essential role, are projected to reach a value of USD 64 and 1.4 billion by 2022, respectively (www.marketsandmarkets.com, 2017). Continued or indeed enhanced levels of economic exploitation of lactobacilli will benefit from a rigorous comparative genomics framework, such as the documentation of endogenous or transmissible antibiotic resistance elements across the genus (Campedelli et al., this issue [submitted]).

From a taxonomic perspective, the primary distinction between members of the genus *Lactobacillus* has historically been based on physiological characteristics until the first proposal of introducing 16S rRNA gene sequence analysis in 1991 (9). Thus far, analysis of 16S rRNA gene similarity is combined with the analysis of carbohydrate fermentation profile, according to which lactobacilli are divided into homofermentative (use of hexose and production of lactic acid), facultatively heterofermentative (use of pentose/hexose and production of lactic acid and other products) and obligately heterofermentative (use of pentose/hexoses and production of lactic acid, side products and CO_2) (10). However, the expansion of the *Lactobacillus* genus since its first description, the presence of overlapping characteristics, together with the threshhold ambiguity associated with 16S rRNA sequence comparison, has led to frequent taxonomic changes, mis-identification issues for strains and species at short phylogenetic range, and for clade distinction at long phylogenetic range (11-14). Further, the comparative analysis of the genome sequences of almost all *Lactobacillus* type strains and historically related genera (3, 4) revealed an overall level of genomic diversity associated with that between members of a bacterial order, and the currently defined genus *Lactobacillus sensu lato* encompasses members of genera *Pediococcus* (Lactobacillaceae family), *Convivina, Fructobacillus*, *Leuconostoc*, *Weissella*, and *Oenococcus* (family Leuconostocaceae). The extreme diversity of the genus *Lactobacillus* and its polyphyletic structure strongly suggest that this taxonomic arrangement should be formally re-evaluated. Hence, the aim of the present study was to understand the evolutionary relationships within the families Lactobacillaceae and Leuconostocaeae and to provide a robust genome-based framework for a novel taxonomic scheme for the genus *Lactobacillus*. Genomics provides bacterial taxonomists with powerful evolutionary information which has been successfully employed for the identification and classification of prokaryotic species as well as elucidating diagnostic components in different taxonomic groups (15, 16). Here we interrogated the genome sequences of 222 strains of *Lactobacillus* and associated genera through the application of distance-based metrics, *viz*. the Average Nucleotide Identity (ANI), the Average Amino acid Identity (AAI) (17) and the Percentage of Conserved Proteins (POCP) (18), and sequence-based methods, namely phylogenetic and network analyses based on 29 ribosomal proteins and 12 established phylogenetic markers. With respect to previous observations, which were based essentially on maximum likelihood of 73 core genes (3), here we i) integrated information derived from distance-based methods to obtain a consensus on delineated clades; ii) reduced the number of genes for multilocus sequence analysis, and deeply investigated the phylogenetic signal by means of split decomposition; iii) revealed the presence of clade-specific genes. The data obtained illustrate the feasibility and advisability of dividing the current genus *Lactobacillus* into a number of more homogeneous genera, and provide the basis for the development of future taxonomic procedures which should be robust and straitghtforward.

RESULTS

Multilocus sequence analysis (r/MLSA) defines 10 discrete clades within the lactobacilli

We constructed phylogenetic trees for selected strains belonging to the genus *Lactobacillus* and related genera based on multilocus sequence analysis of 29 ribosomal proteins (rMLSA) and 12 phylogenetic markers (MLSA) as shown in Figure 1 (panels A and B, respectively). Both trees are characterized by high bootstrap values, which indicate that the proteins selected are reflective of robust evolutionary relatedness between taxa and clades. The trees showed that lactobacilli branch in several clades (defined by colors in both trees) and are intermixed with genera *Pediococcus*, *Fructobacillus*, *Leuconostoc*, *Oenococcus* and *Weissella*. This supports previous observations on the paraphyly of the genus *Lactobacillus* which is taxonomically non-cohesive.

At long phylogenetic range, the individual *Lactobacillus* species are split into Cluster I (46% of all lactobacilli, bootstrap value: 100% in both trees) and Cluster II (54% of lactobacilli, bootstrap value: 98% in rMLSA and 100% in MLSA trees; Figure 1A and 1B) which are consistent in branching order and composition across the two trees. Cluster I includes six highly supported phylogroups, whose nomenclature we assigned based on their description in previous studies (3, 4, 11, 12) and are the following: i) *Lactobacillus delbrueckii* group (orange), ii) *Lactobacillus alimentarius* group (red), iii) *Lactobacillus perolens* group (green), iv) *Lactobacillus casei* group (grey), v) *Lactobacillus sakei* group (dark pink) and vi) *Lactobacillus coryniformis* group (light pink). Cluster II comprises four phylogroups, namely, i) *Lactobacillus salivarius* group (violet), ii) *Lactobacillus reuteri* and *Lactobacillus* *vaccinostercus* groups, which can be collapsed in a single phylogroup (brown), iii) *Lactobacillus fructivorans*, *Lactobacillus brevis*, *Lactobacillus buchneri* and *Lactobacillus collinoides* groups, which form a unique phylogroup that we designate *L. buchneri* (the first species described within this group) (light grey), and iv) *Lactobacillus plantarum*-group (light blue). Remarkably, Cluster II also includes the Leuconostocaceae family and the genus *Pediococcus*, which is a sister branch of the expanded *L. buchneri* group in both trees.

For those species not clustered in phylogroups, two couples emerged: *Lactobacillus concavus*-*Lactobacillus dextrinicus*, which are peripheral in Cluster I, and *Lactobacillus rossiae*-*Lactobacillus siliginis*, which are associated to Leuconostocaceae in Cluster II, in both trees. *Lactobacillus selangorensis* represents a single line of descent and it is the sole inconsistency between the two trees: it belongs to Cluster I in both trees, but it is associated to the *L. casei* phylogroup in the ribosomal protein tree (Figure 1A), and to the *L. sakei* group in the other phylogenetic tree (Figure 1B).

The paraphyletic nature of the *Lactobacillus* genus was also corroborated by the split decomposition analysis (Supplementary Figure S1A and S1B): the 10 phylogroups were recapitulated in both the phylogenetic structures, in which pediococci and leuconostocs were interspersed. Interconnecting networks were also revealed, indicating the occurrence of events more complicated than speciation in the evolution of the genus *Lactobacillus* and, more generally, of the families Lactobacillaceae and Leuconostocaceae.

Selection of distance-based methods to assess genetic relatedness

ANI, AAI and POCP values were calculated across the 222 genome sequences to assess their genetic relatedness. The majority of ANI values obtained were below the 75-80% range (Figure S2), meaning that the genomes are distantly related, and indicating that ANI calculation was not appropriate for the current dataset (16, 19). Thus only AAI and POCP were considered in the present study since they provide much more robust resolution.

AAI and POCP metrics support the phylogenetic analysis

AAI and POCP clusterings are shown in Figure 2. Their statistical robustness is supported by the high bootstrap values at the nodes. The dendrograms substantiate the conclusions from the phylogenetic analysis: the genus *Pediococcus* and the family Leuconostocaceae are clustered within the genus *Lactobacillus*; further, lactobacilli are branched in almost the same phylogroups observed in the phylogenetic trees. In detail, *Lactobacillus* species are split in two clusters in both the dendrograms: Cluster I comprises just the *L. delbrueckii* phylogroup, while Cluster II contains all the other species, including Leuconostocaceae (which is peripheral in Cluster II in both the graphics) and pediococci. In the dendrogram based on AAI values, *L. perolens*, *L. casei*, *L sakei* and *L. coryniformis* phylogroups form a single subclade in Cluster II, while the *L. salivarius* phylogroup is associated with *L. reuteri-vaccinostercus*, *L. buchneri* and *L. plantarum* phylogroups and the *Pediococcus* genus (Figure 2A). In the POCP dendrogram, *L. perolens*, *L. casei*, and *L. sakei* phylogroups form a single clade together with the *Pediococcus* genus, while *L. coryniformis* is associated with the *L. reuteri-vaccinostercus*, *L. buchneri* and *L. plantarum* phylogroups (Figure 2B).

In contrast to the phylogenetic analysis, the *L. reuteri-vaccinostercus* and *L. buchneri* groups are split into their original group composition and intermixed. *L. concavus-L. dextrinicus* and *L. selangorensis* are associated to *L. sakei* phylogroup, while *L. rossiae-L. siliginis* are clustered with *L. vaccinostercus* group in both dendrograms.

Identification of conserved signature genes within *Lactobacillus* phylogroups

To investigate the functional differences in phylogroups established with distance-based (AAI, POCP) and sequence-based methods (MLSA), a large-scale orthology analysis was performed. This led to the identification of 15 orthologs which were selected as putative clade specific-genes based on their pattern of presence/absence among the phylogroups (Table 1, Table 2, Table S3). One of the key genes was the glycolytic phosphopfructokinase (*pfk*, QTS_863) which is present in all the members of *L. delbrueckii*, *L. alimentarius*, *L. perolens*, *L. casei*, *L. sakei*, *L. salivarius*, *L. plantarum*, *L. coryniformis* phylogroups, in *L.*

concavus-dextrinicus and in the *Pediococcus* genus, while it is lacking in all the members of *L. reuteri*, *L. vaccinostercus*, the expanded *L. buchneri* group, *L. rossiae-L. siliginis* and all the Leuconostocaceae. The presence-absence pattern of Pfk seems to have an impact on the carbohydrate metabolism of these species. In fact, members within the Pfk-lacking group (Table 2) were classified as obligately heterofermentative (3, 12), with the rest being facultatively heterofermentative or homofermentative. Taking the presence-absence pattern of Pfk as a reference, the distribution of nine other signature genes is distinct in species belonging to different phylogroups in the Pfk-positive group (Table 1). Four of them have been associated to a function and they belong to different Clusters of Orthologous Genes (COGs, Table 1) while five of these genes are annotated as hypothetical proteins and lack conserved domains. Interestingly, QTS_569, a Zinc-dependent peptidase, is present in all the Pfk-positive species, except members of *L. delbrueckii* group, which, on the other hand, are the only species within the Pfk-positive group with QTS_2524, a hypothetical protein (profile A, Table 1). Furthermore, QTS_4707, another hypothetical protein, seems to be specific to the *L. alimentarius* group (profile B). Presence-absence profiles of these nine genes (reported in Table 1) are almost unique for each Pfk-positive phylogroup, the *Pediococcus* genus included; the only exception is the couple *L. concavus-L. dextrinicus* which has the same profile as the *L. sakei* phylogroup (profile E), characterized by the presence of QTS_569, the Zinc-dependent peptidase, and QTS_898, a protein annotated as a cell division inhibitor, and the absence of the rest of the genes.

Regarding the Pfk-negative group, the differential distribution of seven genes uniquely describes the members of most of the groups (Table 2). Six genes out of seven have been annotated and were found to belong to six COGs (Table 2), while only one gene is annotated as encoding a hypothetical protein. Species belonging to *L. reuteri* and *L. vaccinostercus* clades have the same pattern, one displayed also by *L. rossiae-L. siliginis* (profile A), which is characterized by the absence of QTS_898, the cell division inhibitor, and QTS_2490, a hypothetical protein. Members of the *L. fructivorans*, *L. buchneri* and *L. collinoides* groups display all the genes except QTS_2490 (profile B), which is, instead, present in *L. brevis* group members (profile C). Interestingly, the species belonging to the Leuconostocaceae family have a completely different profile compared to other Pfk-negative groups as they lack all the genes under consideration (profile D).

DISCUSSION

One of the overall aims of this study was to stop the never-ending expansion of *Lactobacillus* as a heterogeneous clade (1, 3, 4, 11, 12, 20). We used two methods with a phylogenetic component (MLSA of ribosomal proteins and a set of housekeeping genes) and two which were phylogeny-independent (AAI and POCP). MLSA affords higher resolution of the phylogenetic relationships of species within a genus and genera within a family (16, 21), and successfully resolved the complex taxonomic structure of genera *Escherichia* and *Shigella* and the family Enterobacteriaceae (22-24). Housekeeping protein-coding genes used for MLSA are believed to evolve at a slow but constant rate and have a better resolution power compared to the 16S rRNA gene; ribosomal proteins are usually syntenic and co-located in the same genomic area, thus avoiding binning errors which could perturb the geometry of the tree (19, 21, 25). The phylogenetic trees we generated confirmed the paraphyletic nature of the genus *Lactobacillus* (first observed with a 16S rRNA gene-based phylogeny and a smaller dataset of genome sequences, (11, 12, 13)), where Leuconostocaceae and pediococci branched from the lactobacilli as subgroups. The topologies of the trees obtained here confirmed the phylogenomic topology inferred from 73 core proteins (3) and from 172 core genes shared by 174 genomes of lactobacilli and pediococci (1, 4). Each phylogenomic reconstruction revealed the association of obligately heterofermentative lactobacilli with Leuconostocaceae (displaying the same metabolism) and their separation from the homofermentative and facultatively heterofermentative *Lactobacillus* species (4). Ten historically recognized *Lactobacillus* subgroups could also be identified from our analysis (1, 3, 4, 11, 12, 26, 27), which updates the phylogroupings which we described with Sun and colleagues (3).

Only five *Lactobacillus* species remained outside the phylogroups: two couples, namely *L. rossiae*-*L. siliginis* and *L. concavus*-*L. dextrinicus*, and *L. selangorensis*. These species were not clustered within any other *Lactobacillus* phylogroups using other datasets ranging from 16S rRNA gene to core genes (1, 3, 4, 12). Interestingly, *L. dextrinicus* was first described as *Pediococcus dextrinicus* (28) while *L. selangorensis* constituted the sole species of the genus *Paralactobacillus* (29). Both species were later reclassified as *Lactobacillus* species based on MLSA of the 16S rRNA gene and other housekeeping genes (30, 31).

Furthermore, 10 consistent subgroups were defined, namely i) *L. delbrueckii* (named after the type species of *Lactobacillus*) which comprises also the peripheral species *L. amylophilus*, *L. amylotrophicus* and *L. floricola*; ii) *L. alimentarius*; iii) *L. perolens*: iv) *L. casei*; v) *L. sakei* (without *L. selangorensis*); vi) *L. coryniformis*; vii) *L. salivarius*; viii) *L. plantarum*; ix) *L. reuteri*, which includes also *L. vaccinostercus*-related species; and x) *L. buchneri*, which encompasses members of *L. brevis*, *L. fructivorans* and *L. collinoides* groups (the group was given the name *L. buchneri* since it was the first species described within the phylogroup).

The inferred subgroups were largely corroborated by AAI and POCP analysis, which were rigorously applied to lactobacilli in the present project. AAI analysis has shown excellent potential to improve the classification of higher taxa (e.g. the Enterobacteriaceae family, (32)); POCP was proposed by Qin and colleagues (18) as a complementary approach to AAI, and it is calculated using all the proteins of the genomes to be compared. The ANI was also applied to the dataset since it has been officially recommended as a substitute for DNA-DNA hybridization and has been used in more than 30 classifications (19), but most of ANI values fell below the 75-80% range (as also observed by Zheng and colleagues (4)), showing the extremely wide genetic diversity of strains under study and making this method unreliable for the present dataset. This method gives robust resolution to genomes that have 80 – 100% ANI and/or share at least 30% of their gene content, a scenario which typically occurs within species belonging to the same genus (but it is clearly not applicable to lactobacilli); if two strains have a distant genetic relationship, only a small proportion of the whole-genome DNA sequence is considered for ANI calculation and the majority of DNA information is discarded due to the lack of homology (18, 33). In fact, such strains could then be ascribed to different genera as the low values render comparison as essentially impossible.

Despite relatively high intra-group AAI and POCP values, some inconsistencies in the phylogenetic trees among the obligately heterofermentative groups emerged. Specifically, the *L. vaccinostercus*-related species were separated from the *L. reuteri* group and the *L. buchneri* group was split into its original subclades (*L. fructivorans*, *L. brevis*, *L. collinoides* and *L. buchneri* groups). In the light of this incongruence, genome sequences were further explored to identify signature genes which could assist in the definition of supported *Lactobacillus* subgroups. A set of 15 genes was thus identified, whose presence/absence pattern was specific for the 10 phylogroups. The most discriminative gene was the phosphofructokinase (*pfk*) which was present in all the homofermentative and facultatively heterofermentative lactobacilli and absent in the obligately heterofermentative lactobacilli (and Leuconostocaceae). Production of CO_2 differentiates obligately from facultatively heterofermentative metabolism (13). The *pfk* gene distribution represents the first element in *Lactobacillus* taxonomy in which phylogenetic clustering, genome-based analysis and phenotypic (metabolic) analysis come to an agreement. The other retrieved genes could not be attributed to specific functions nor to unambiguous phenotypic traits. Nevertheless they represent a biological signature, which, together with robust phylogenetic groupings, can be used for the definition of cohesive taxonomic entities within the genus *Lactobacillus* and thus used as diagnostic tools. Furthermore, given their crucial position at the branch points that occurred during the evolution of lactobacilli, they provide a resource to be functionally explored from which new important information on these bacteria may be uncovered (32, 34).

A summary of the data from sequence-based and distance-based methods (Table 3) combining the analysis of orthologous gene presence/absence crystallizes two scenarios for the formal reclassification of the *Lactobacillus* genus. The first scenario consists of splitting the genus into two groups, based on the presence/absence of *pfk*, groups that are relatively consistent with pylogenetic trees based on ribosomal proteins, housekeeping genes and core genes and congruent with carbohydrate fermentation profiles. However these two subgeneric groups are still characterized by POCP and AAI values that would not meet the criteria for genus delineation (species should share at least 55-60% AAI and 50% POCP to be considered within the same genus; (18, 33)). A second scenario envisages the proposal of the ten subgroups that emerged from the phylogenetic analysis as nuclei of novel genera within lactobacilli: the subgroups are consistent in the different trees, they were mainly recapitulated by 16S rRNA-based sequence analysis (including also species for which a genome sequence is not available, Supplementary Figure S3), most of them share values of POCP and AAI higher than 50% and 55-60%, respectively, and they are also characterized by distinct gene distributions (Table 3). In this scenario, some questions remain unanswered: the first challenge regards the *L. delbrueckii*, *L. alimentarius* and *L. perolens* groups, whose intragroup diversity changes when peripheral species are considered. For instance, the exclusion of *L. floricola*, *L. amylophilus* and *L. amylotrophicus* from the *L. delbrueckii* group increases intragroup AAI and POCP values from 52.1 and 46.4%, to 59.3 and 52.9%, respectively, thus allowing this group to meet the criteria suggested for genus delineation based on distance-based metrics (the same situation applies for the *L. perolens* and *L. alimentarius* groups). For the clade composed by members of the expanded *L. buchneri* group (*L. fructivorans*, *L. brevis*, *L. buchneri* and *L. collinoides* members), a consistent phylogenetic inference faces unmet criteria in distance-based methods (particularly POCP, which is 45.9%) and a differential distribution of “clade-specific” genes (i.e. members of *L. brevis* have a different gene presence/absence pattern compared to the other species).

Those challenges suggest that, besides the improvements that genome analyses deliver, genomics-derived thresholds should not be used in isolation or be applied agnostically. Indeed, formal reclassifications should be proposed on the basis of the results of polyphasic study (10) to ensure that diversity of *taxa* is coherently described by names at the different taxonomic ranks. *De facto*, thresholds (i.e. AAI and POCP) are useful to uniformally delineate taxonomic ranks among phylogenetic lineages, but they should be applied flexibly and other factors such as other genomic markers (e. g. clade specific proteins, or conserved amino acids within essential protein sequences (Zhang et al. 2018)), the phenotype, (e.g. carbohydrate fermentation pattern, or chemotaxonomic markers (35)), the ecology and the niche-adaptation should be included in the analysis of all taxonomic ranks, including species (1, 36). A valuable case towards this perspective is given by Zhang and colleagues which showed a clear link between the

Lactobacillus phylogenetic clusterings, their vancomycin sensitive/resistant phenotype and the sequence composition of Ddl dipeptide ligase enzyme (Zhang et al., 2018).

Notwithstanding these caveats, data reported here represent a significant further step towards the splitting of the genus *Lactobacillus* into more homogeneous genera: they demonstrate a very robust evolutionary backbone at the basis of a possible renovated classification scheme, and this is of utmost importance to guarantee stability of names of future taxa, once they are delineated, as this is one of essential points in nomenclature (37). Indeed, until a complete revaluation of phenotypic coherency of groups proposed here is performed, no reclassification is advisable; Principle 1 of the Bacteriological Code (37) suggests avoiding the useless creation of names, a condition that could occur if genomic thresholds are strictly applied (for instance, if all the peripheral species of groups in Table 3 were unhelpfully proposed as novel genera) and without considering the broad effect this reclassification could have for the scientific community and *Lactobacillus* users such as legislative bodies, regulatory agencies, microbial safety assessors (Campedelli *et al*., in preparation), probiotic and fermented food manufacturers.

The pragmatic genome-based approach applied here to the genus *Lactobacillus* sheds light on the feasibility of creating a renovated taxonomic scheme in which at least ten homogenous genera/clusters could accommodate the existing species and those still to be discovered. An open discussion among other experts, such as the Lactic Acid Bacteria scientific and industrial community and members of the Subcommittee of Taxonomy of genus *Lactobacillus* (35) is now advocated in order to proceed towards the formal proposal of the reclassification of the genus *Lactobacillus*.

MATERIALS AND METHODS

Dataset

The list of 222 genome sequences belonging to the genus *Lactobacillus* and related genera that were used in the present study are shown in Table S1. A further 47 strains for which the genome sequences were not available were included based on their 16S rRNA gene sequences (Table S1).

Multilocus sequence analysis based on 29 ribosomal proteins and 12 phylogenetic markers and phylogenetic tree construction.

A Maximum Likelihood phylogeny was built from 29 ribosomal proteins and 12 housekeeping markers which were chosen based on their use in published multilocus sequence typing schemes and their presence in the 222 genomes (Table S2) (38).

Amino-acid sequences were aligned, concatenated and the phylogeny was inferred using the PROTCATWAG model in RAxML v8.0.22 and rooted using *Atopobium minutum* DSM 20584^T, *Atopobium rimae* DSM 7090^T, *Kandleria vitulina* DSM 20405^T and *Olsenella uli* DSM 7084^T. Bootstrapping was carried out using 100 replicates.

SplitsTree4 (39) was applied to detect conflicting signals (possible horizontal gene transfer events), which are then displayed as networks instead of bifurcating trees.

16S rRNA gene-based phylogeny

16S rRNA phylogenetic analysis for each subgroup were carried out with the MEGA v7.0.26 (40) software package using Jukes-Cantor as the distance model. The neighbor-joining (41) and minimum-evolution (42) methods were used for tree reconstruction. The statistical reliability of the phylogenetic tree topology was evaluated using bootstrapping with 1000 replicates (43).

Distance-based methods: ANI, AAI, POCP.

The ANI, AAI and POCP values across the genomes were calculated according to methods proposed by Konstantinidis *et al*., (17, 44), and Qin *et al*. (18). In detail, the ANI between two genomes was calculated as the mean identity of all BLASTN (v. 2.2.26+) matches based on 1kb fragments which showed more than 30% overall sequence identity over an alignable region of at least 70% of total length (45). We used a command line version of the AAI software (http://enve-omics.ce.gatech.edu/aai/) that takes two FASTA files of predicted genes as input, identifies reciprocal best BLAST hits and calculates the AAI score based on these orthologs(17). For POCP, an in-house script was written following the formula of Qin et al. 2014, which uses two-way BLAST to calculate a POCP score: (C1 + C2)/(T1 + T2) * 100 where C = number of conserved proteins (identity >= 40% and aligned length of query >= 50%) and T = total number of proteins; 1 and 2 refer to input files 1 and 2, respectively(18). The in-house script has been deposited on figshare with the following digital object identifier: https://doi.org/10.6084/m9.figshare.4577953.v1. Amino acid sequences used in AAI and POCP were predicted using a combination of three software – Glimmer3 (v3.02) (46), GeneMark.HMM (v1.1) (47) and MetaGene (48) – where a gene sequence predicted by at least one software was included in the dataset. Statistics and visualization were carried out in R v3.1.1 (https://www.r-project.org/) using 'pvclust' (49).

Ortholog prediction and identification of clade-specific genes

Orthologs were predicted using QuartetS where two sequences from separate genomes were considered to be orthologs if they were bi-directional best hits (BBH) of each other, had >=30% identity and >=25% alignment length. QuartetS also differentiates paralogs from orthologs by building quartet gene trees that include two sequences from a third genome. The output from QuartetS was a table with 222 genomes as columns and 34,257 clusters of orthologs as rows where the presence of a sequence for a particular ortholog was represented as 1 and its absence as 0. This table therefore provided a sequence presence/absence distribution for each ortholog that was used to predict clade-specific genes. The random forest algorithm (50) was used to predict clade-specific genes from the R package randomForest. The software was run in an iterative manner using default parameters where all orthologs having a Gini index of zero at each iteration were removed. The remaining 90 genes gave an out-of-bag error rate of zero, which is random forest's internal method of cross-validation. This suggested that the subset of orthologs contained potential clade-specific genes. These clade-specific genes were identified in R and further manual assessment was carried out to exclude potential false positives, including the alignment of sequences back to genomes using TBLASTN.

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Figure legends

Figure 1.

Phylogenetic trees based on the amino acid sequences of 29 ribosomal protein (1A) and 12 phylogenetic markers (1B). Clusters I and II are indicated in the tree. Leu: Leuconostocaceae; Ped: *Pediococcus*. The phylogeny was inferred using the PROTCATWAG model in RAxML v8.0.22 and rooted using *Atopobium minutum* DSM 20584^T, *Atopobium rimae* DSM 7090^T, *Kandleria vitulina* DSM 20405^T and *Olsenella uli* DSM 7084^T. Bootstrapping was carried out using 100 replicates and values are indicated on the nodes.

Figure 2.

Dendrograms depicting the genome relatedness based on the Average Amino acid Identity (AAI, 2A) and the Percentage of Conserved Proteins (POCP, 2B) calculations. Colours refer to the same phylogroups indicated in Figure 1. L_delb: *L. delbrueckii* group; L_alim: *L. alimentarius* group; L_per: *L. perolens* group; L_cas: *L. casei* group; L_sak: *L. sakei* group; L_coryn: *L. coryniformis* group; L_saliv: *L. salivarius* group; L_reut: *L. reuteri* group; L_buch: *L. buchneri* group; L_plan: *L. plantarum* group. Leu: Leuconostocaceae; Ped: *Pediococcus*. Statistics and visualization were carried out in R v3.1.1 (https://www.r-project.org/) using ‘pvclust’ (50-Suzuki and Shimodaira, 2006).

Table 1: Details of signature proteins for species with Pfk (6-phosphofructokinase)

Genes	NCBI annotation	Locus tag	COG	*L. delbrueckii*	*L. alimentarius*	*L. perolens*	*L. casei*	*L. sakei*	*L. salivarius*	*L. plantarum*	*L. coryniformis*	*L. concavus – L. dextrinicus*	*L. selangorensis*	*Pediococcus*
QTS_863	6-phosphofructokinase	lp_1898[a]	COG0205G	+	+	+	+	+	+	+	+	+	+	+
QTS_569	Zn-dependent peptidase	lp_2306[a]	COG0612R	-	+	+	+	+	+	+	+	+	+	+
QTS_898	Cell division inhibitor	lp_2316[a]	COG0850D	-	+	+	+	+	+	+	+	+	+	-
QTS_1754	Transcription termination factor Rho	lp_0511[a]	COG1158K	-	-	-	-	-	+	+	+	-	-	+
QTS_2490	Hypothetical protein	LBA0167[b]	n.d.	+*	-[†]	-[§]	-	-	-	-	-	-	+	-
QTS_2524	Hypothetical protein	LBA0844[b]	n.d.	+*	-	-	-	-	-	-	-	-	-	-
QTS_2525	S1 Family RNA-binding protein	LBA0276[b]	COG1098R	+	+	+[§§]	-	-	-	+	-	-	-	-[‡]
QTS_3870	Hypothetical protein	LSEI_1730[c]	n.d.	-	-	+	+	-	-	-	+	-	+	-
QTS_4397	Hypothetical protein	LSEI_0696[c]	n.d.	-	-	-	+	-	-	-	+	-	+	-
QTS_4707	Hypothetical protein	FC67_GL001143[d]	n.d.	-	+	-	-	-	-	-	-	-	-	-
			Profile	A	B	C	D	E	F	G	H	E	I	L

Locus tags: [a]*Lactobacillus plantarum* WCFS1; [b]*Lactobacillus acidophilus* NCFM; [c]*Lactobacillus paracasei* ATCC 334; [d]*Lactobacillus alimentarius* DSM 20249. COGs: D. Cell cycle control, cell division, chromosome partitioning; G. carbohydrate transport and metabolism; K. Transcription; R. General function prediction only. n.d.: not determined. *absent in *L. floricola*; †present in *L. mellifer* and *L. mellis*; §present in *L. composti*; §§absent in *L. composti*; ‡: present in *P. claussenii*.

Table 2: Details of signature proteins for species without Pfk (6-phosphofructokinase)

Genes	NCBI annotation	Locus tag	COG	*L. reuteri*	*L. vaccinostercus*	*L. fructivorans*	*L. brevis*	*L. buchneri*	*L. collinoides*	*L. rossiae – L. siliginis*	*Leuconostocaceae*
QTS_863	6-phosphofructokinase	lp_1898[a]	COG0205G	-	-	-	-	-	-	-	-
QTS_494	Thiamine biosynthesis protein Thil	LVIS_RS17650[b]	COG0301HJ	+	+	+	+	+	+	+	-
QTS_497	tRNA methyltransferase	LVIS_RS18530[b]	COG0482J	+	+	+	+	+	+	+	-
QTS_502	Transcriptional regulator NrdR	LVIS_RS16605[b]	COG1327K	+	+	+	+	+	+	+	-
QTS_509	tRNA uridine 5-carboxymethylaminomethyl modification protein	LVIS_RS22810[b]	COG0445J	+	+	+	+	+	+	+	-
QTS_514	DNA replication intiation control protein YabA	LVIS_RS14505[b]	COG4467L	+	+	+	+	+	+	+	-
QTS_898	Cell division inhibitor	LVIS_RS17610[b]	COG0850D	-	-	+	+	+	+	-	-
QTS_2490	Hypothetical protein	LVIS_RS11970[b]	n.d.	-	-	-	+	-	-	-	-
			Profile	A	A	B	C	B	B	A	D

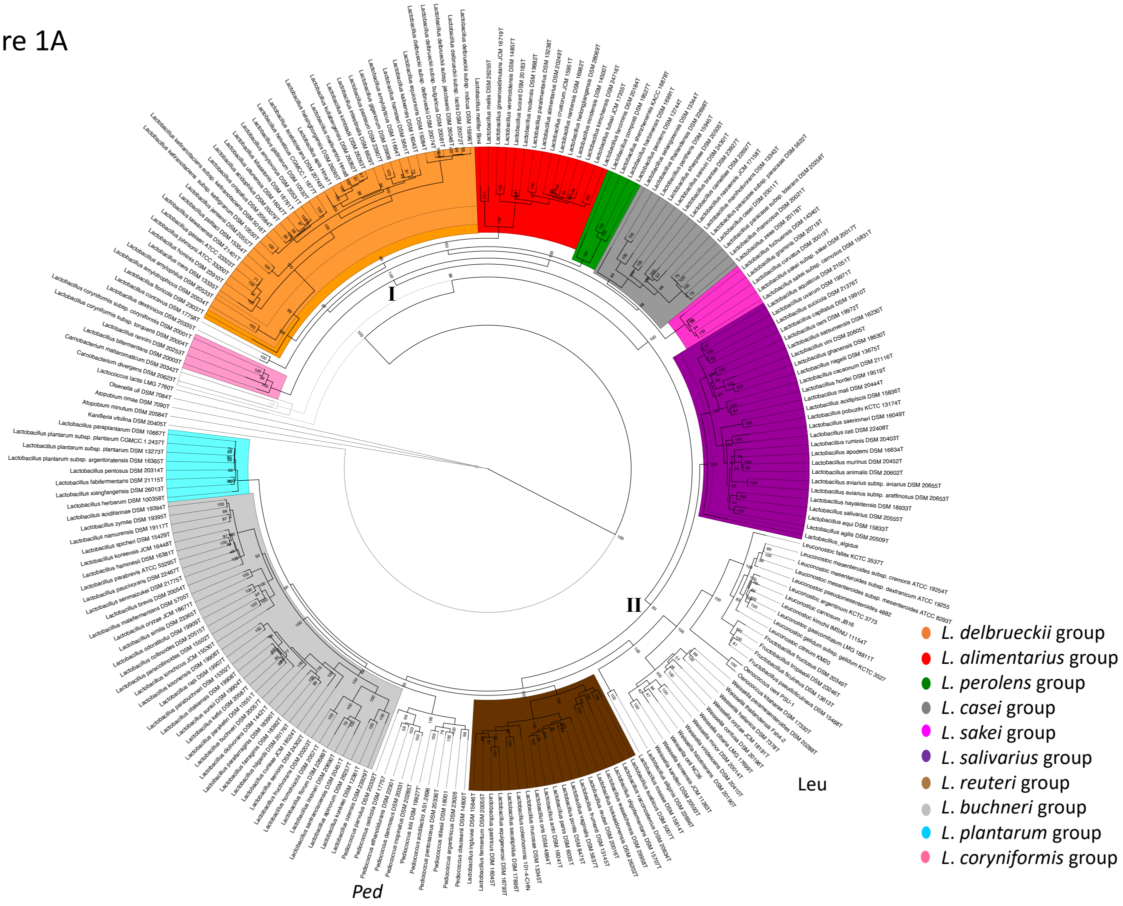
Locus tags: [a]*Lactobacillus plantarum* WCFS1; [b]*Lactobacillus brevis* ATCC 367; COGs: D. Cell cycle control, cell division, chromosome partitioning; G. carbohydrate transport and metabolism; H. Coenzyme transport and metabolism; J. Translation, ribosomal structure and biogenesis; K. Transcription; L: Replication, recombination and repair. R. General function prediction only. n.d.: not determined.

Table 3: Combination of distance-based and sequence-based data with the analysis of signature proteins for each phylogroup

Phylogroups	No. of species	AAI%*		POCP%*		pfk	QTS_569	QTS_898	QTS_1754	QTS_2490	QTS_2425	QTS_2525	QTS_3870	QTS_4397	QTS_4707
L. delbrueckii	35	52.1	**59.3**[a]	46.4	**52.9**[a]	+	-	-	-	+	+	+	-	-	-
L. alimentarius	21	52.8	**68.4**[b]	44.6	**62.4**[b]	+	+	+	-	-	-	+	-	-	+
L. perolens	4	**55.9**	**72.9**[c]	48	**67.8**[c]	+	+	+	-	-	-	+	-	-	+
L. casei	16	**59.3**		**55.2**		+	+	+	-	-	-	-	+	+	-
L. sakei	4	**76.7**		**75.2**		+	+	+	-	-	-	-	-	-	-
L. plantarum	9	**76.5**		**76**		+	+	+	+	-	-	+	-	-	-
L. coryniformis	5	**62.5**		**61.1**		+	+	+	+	-	-	-	+	+	-
L. salivarius	27	**56.1**	**61.1**[d]	**53.5**	**59.3**[d]	+	+	+	+	-	-	-	-	-	-
L. concavus- *L. dextrinicus*	2	**72.7**		**70.9**		+	+	+	-	-	-	-	-	-	-
L. selangorensis	1					+	+	+	-	+	-	-	+	+	-
							QTS_494	QTS_497	QTS_502	QTS_509	QTS_514	QTS_898	QTS_2490		
L. reuteri	23	**63.2**	**57.6**[e]	**62**	**51**[e]	-	+	+	+	+	+	-	-		
L. vaccinostercus		**68.9**		**69**		-	+	+	+	+	+	-	-		
L. fructivorans	48	**58.3**	**56.1**[f]	**58.3**	45.9[f]	-	+	+	+	+	+	+	-		
L. brevis		**74.6**		**70.8**		-	+	+	+	+	+	+	+		
L. buchneri		**63.3**		**55.6**		-	+	+	+	+	+	+	-		
L. collinoides		**62.07**		**62.2**		-	+	+	+	+	+	+	-		
L. rossiae- *L. siliginis*	2	**73.7**		**67.3**		-	+	+	+	+	+	-	-		

Numbers in bold are values > 55-60% ANI and >50% POCP which are the thresholds empirically taken as genus delineation. *lower percentages within a single phylogroup; [a]: AAI and POCP values for *L. delbrueckii* group without considering peripheral species (*L. amylophilus*; *L. amylotrophicus*, *L. floricola*); [b]: AAI and POCP values for *L. alimentarius* group without considering peripheral species (*L. mellifer*, *L. mellis*); [c]: AAI and POCP values for *L. perolens* group without considering peripheral species (*L. composti*); [d]: AAI and POCP values for *L. salivarius* group without considering peripheral species (*L. algidus*): [e]: AAI and POCP values considering members of *L. reuteri* and *L. vaccinostercus* groups; [f]: AAI and POCP values considering members of *L. fructivorans*, *L. brevis*, *L. buchneri*, *L. collinoides* groups.

Figure 1A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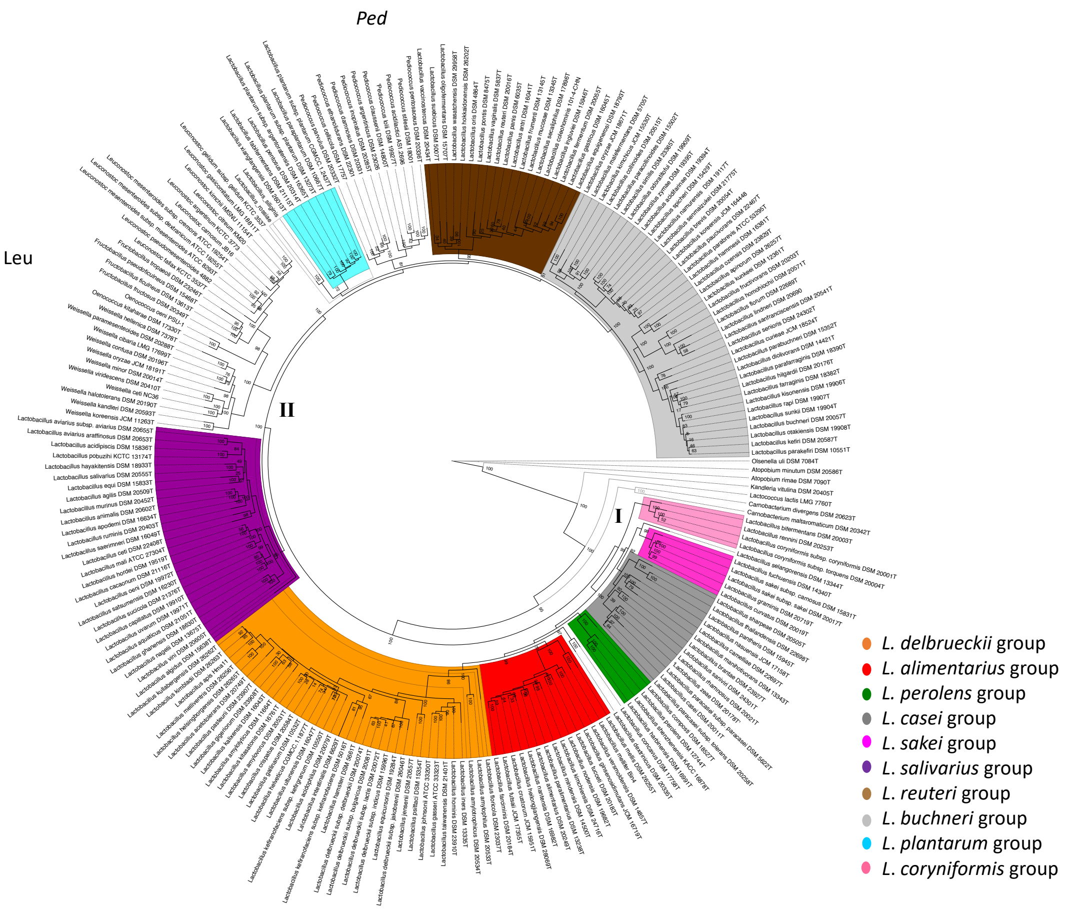
● L. reuteri group
● L. buchneri group
● L. plantarum group
● L. coryniformis group

Figure 1B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● L. casei group
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● L. reuteri group
● L. buchneri group
● L. plantarum group
● L. coryniformis group

Figure 2A

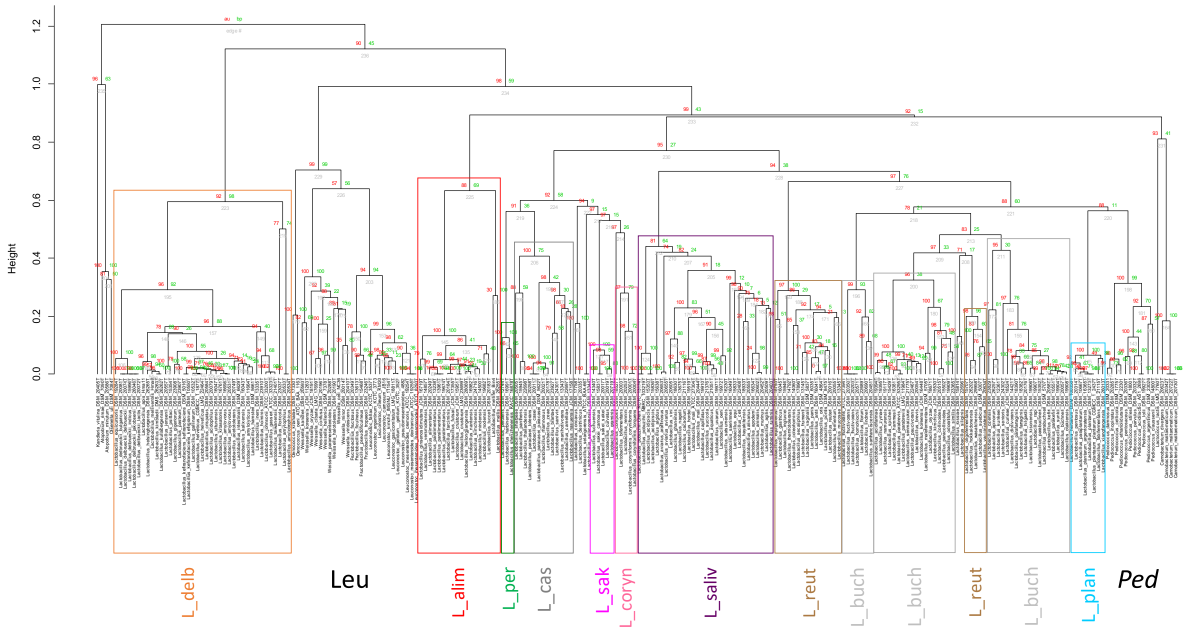
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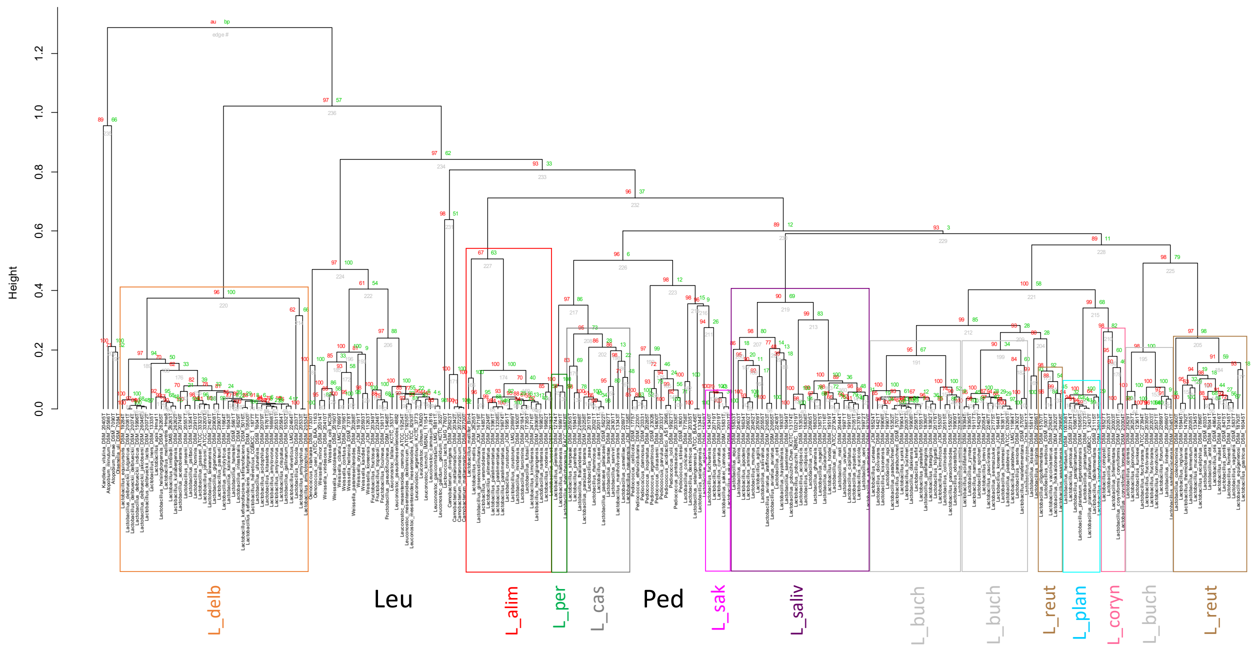
Figure 2B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