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Phylogenetic Analysis of *Burkholderia* Species by Multilocus Sequence Analysis

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Abstract *Burkholderia* comprises more than 60 species of environmental, clinical, and agro-biotechnological relevance. Previous phylogenetic analyses of 16S rRNA, *recA*, *gyrB*, *rpoB*, and *acdS* gene sequences as well as genome sequence comparisons of different *Burkholderia* species have revealed two major species clusters. In this study, we undertook a multilocus sequence analysis of 77 type and reference strains of *Burkholderia* using *atpD*, *gltB*, *lepA*, and *recA* genes in combination with the 16S rRNA gene sequence and employed maximum likelihood and neighbor-joining criteria to test this further. The phylogenetic analysis revealed, with high supporting values, distinct lineages within the genus *Burkholderia*. The two large groups were named A and B, whereas the *B. rhizoxinica*/*B. endofungorum*, and *B. andropogonis* groups consisted of two and one species, respectively. The group A encompasses several plant-associated and saprophytic bacterial species. The group B comprises the *B. cepacia* complex (opportunistic human pathogens), the *B. pseudomallei* subgroup, which includes both human and animal pathogens, and an assemblage of plant pathogenic species. The distinct lineages present in *Burkholderia* suggest that each group might represent a different genus. However, it will be necessary to analyze the full set of *Burkholderia* species and explore whether enough phenotypic features exist among the different clusters to propose that these groups should be considered separate genera.

Abbreviations

MLSA	Multilocus sequence analysis
BCC	*Burkholderia cepacia* complex
ML	Maximum likelihood
NJ	Neighbor-joining

Introduction

The genus *Burkholderia*, a β-proteobacteria group, was created to accommodate seven species from the *Pseudomonas* ribosomal RNA group II [51, 89]. Eventually, many more species were described and included in this new genus. Others were removed or reclassified, such as *Burkholderia pickettii* (now *Ralstonia pickettii*) and *B. solanacearum* (now *Ralstonia solanacearum*) [90], or *B. cocovenenans*, which was a synonym of *B. gladioli* [17, 41] and *B. vandii*, a synonym of *B. plantarii* [17]. Currently, *Burkholderia* comprises more than 60 species, which are distributed in diverse habitats. For example, several species are important components of the rhizosphere [22]. Others have been found in water, plant roots, or legume nodules, or can be opportunistic pathogens zon plants or humans [22, 77]. Among the pathogenic

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Burkholderia, *B. mallei* and *B. pseudomallei* are extremely important because they are the etiologic agents of known diseases such as glanders and melioidosis, respectively [10, 87]. The *Burkholderia cepacia* complex (BCC; 17 species up to now) is another important group that includes many opportunistic pathogens, which may be found in patients with cystic fibrosis [38].

In 2001, two reports presented a totally different view of *Burkholderia*. First, the genus was rich in nitrogen-fixing species [28], and second, several nitrogen-fixing species were found to nodulate legume plants [48]. This last feature was striking because previously legumes were thought to be nodulated only by α-proteobacteria. Today, eight *Burkholderia* species that elicit effective nodule formation on legume roots have been reported and more are in the pipeline awaiting description [37, 46]. Recently, strains from *B. fungorum* were found to nodulate *Phaseolus vulgaris*, although ineffectively [30].

As the number of described *Burkholderia* species has increased, 16S rRNA sequence analyses have revealed two sub-lineages within the genus [8, 37, 53, 54, 58]. These two groups have been recovered with different phylogenetic reconstruction methods using 16S rRNA sequences [52], Multilocus sequence analysis (MLSA) with *gyrB* and *rpoB* genes sequences [2] or seven housekeeping genes [63], or *recA* gene sequences [73]. A phylogenetic analysis of the *acdS* gene in *Burkholderia* species also revealed the same two clusters [49]. One of these sub-lineages contains the BCC opportunistic human pathogens, some environmental species, the *B. pseudomallei* group, as well as plant pathogenic species, which have been also detected in human infections [43, 60, 86]. The other sub-lineage comprises soil, water, and plant-associated species, which thrive in rhizospheres, live as endophytes, or nodulate legumes. Many species are diazotrophs and so far, evidence for pathogenicity is lacking, although a few reports exist of single strains of *B. xenovorans*, *B. tropica*, and *B. fungorum* that were isolated from animal or human clinical samples [18, 24, 35]. Additionally, the phylogenetic analysis of 617 genes, from the *Burkholderia* core genome, demonstrated that the BCC–*B. pseudomallei*–plant pathogen group was clearly separated from the cluster of environmental species [69].

Not only MLSA but also Multilocus sequence typing (MLST) have been used for epidemiologic and population genetics studies, delineation of species, and assignment of strains to defined bacterial species in *Burkholderia* [2, 40, 80, 82]. Different housekeeping genes have been useful for this purpose and an MLST database exists for BCC members (http://pubmlst.org/bcc/).

In view of these findings, we performed MLSA of a set of *Burkholderia* species (77 *Burkholderia* type and reference species) using four housekeeping genes, *atpD*, *gltB*, *lepA*, and *recA,* combined with the 16S rRNA gene sequence, to explore the positioning of the *Burkholderia* species in a phylogenetic analysis. In this report, we present evidence that the genus *Burkholderia* is composed of distinctly different phylogenetic lineages.

Materials and Methods

Bacterial Strains

A set of *Burkholderia* species was analyzed by MLSA. Several species with sequenced genomes were also chosen for the analysis. The set of *Burkholderia* species analyzed included a total of 77 *Burkholderia* species, 51 type species and 26 reference species. Additionally, type and reference strains from *Cupriavidus* (3) and *Ralstonia* (4) species were included. A list of strains used in this study is presented in Additional file 1.

atpD, *gltB*, *lepA*, and *recA* Gene Sequencing

The housekeeping genes were randomly selected based on the BCC MLST Database (http://pubmlst.org/bcc/). The following set of primers was designed to obtain DNA fragments in the range of 850–1,100 bp. These DNA fragments were more than twice as long as the ones used in MLST for the BCC because we wished to retrieve more information for the phylogenetic analysis. For the ATP synthase beta chain (*atpD*), the primers atpD-F2 (5′-CCACCAGCACAAGCCGCT-3′) and atpD-R (5′-ATCCG CTCGTCGTCGGCG-3′) were used. For the glutamate synthase large subunit (*gltB*), the primers gltB-F (5′-CTGCGCTCGAAGATCAAGCAGGG-3′) and gltB-R (5′-TGCGCACCGGCTGGATGAACG-3′) were utilized. For the GTP binding protein (*lepA*), the primers lepA-F2 (5′-TGGTTCGACAACTACGTCGG-3′) and lepA-R (5′-ATCAGCATGTCGACCTTCAC-3′) were employed, and for recombinase A (*recA*), BUR1 and BUR2 primers were used [52]. The PCR was performed as described previously [28]. The DNA fragments on each strand were sequenced with the primers used in the initial PCR amplification by Macrogen (www.macrogen.com). The housekeeping genes from some *Burkholderia* species were retrieved from the NCBI Genome Database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome). The accession numbers are provided in Additional file 1 and displayed on the phylogenetic trees.

Phylogenetic Inference

Data processing and phylogenetic analyses were performed as described elsewhere [77]. In brief, nucleotide sequences

of the housekeeping genes were translated and aligned using Muscle 3.57 [25]. The resulting multiple-sequence alignments of proteins were used as masks to generate the corresponding nucleotide codon alignments using custom Perl scripts. Individual alignments were concatenated using *ad hoc* Perl scripts. Models of nucleotide substitution were selected by the Akaike information criterion, using MODELTEST3.7 [56, 57]. Among-site rate variation was modeled by a gamma distribution, approximated with four rate categories [91], with each category being represented by its mean. ML trees were inferred for each dataset under the models of nucleotide substitution selected by the Akaike information criterion [57], using PhyML v3.0 [36]. Tree searches were initiated from a BioNJ seed tree, retaining the best tree among those found with NNI and TBR branch swapping. The robustness of the ML topologies was evaluated using a recently developed Shimodaira–Hasegawa (SH)-like test [6] for branches implemented in PhyML v3.0. In brief, the test assesses whether the branch being studied provides a significant likelihood gain, in comparison with the null hypothesis that involves collapsing that particular branch, but leaving the rest of the tree topology the same. The SH-like procedure was chosen for assessing bipartition significance because the test is nonparametric and much less liberal than the diverse (parametric) approximate-likelihood ratio tests that are also implemented in that program. The resulting SH-like *P* values, therefore, indicate the probability that the corresponding split is significant. The ML phylogenetic trees were visualized with the program *MEGA* version 5 [66]. The alignment dataset was also used for NJ analysis. The NJ trees were constructed based on Tamura–Nei distances, using 1,000 bootstrap replicates with the program *MEGA* version 5.

Diazotrophy Test

The capacity to fix nitrogen by most *Burkholderia* species was tested by acetylene reduction activity [28], by *nifH* gene amplification [53, 55, 70], or by collecting information from the literature.

Results and Discussion

Phylogenetic Inference of 16S rRNA Gene Sequences

All *Burkholderia* species were included in the analysis of the 16S rRNA gene sequence by ML (Fig. 1). The phylogenetic analyses resulted in two large clusters; one (designated as group A) comprised the plant-associated and saprophytic species. The second cluster (group B) contained the BCC opportunistic human pathogens, the *B. pseudomallei* group, and the plant pathogenic species. *Burkholderia andropogonis*, *B. rhizoxinica*, and *B. endofungorum* were placed at the edge of group A. A distance analysis (using *MEGA* version 5) of the 16S rRNA sequences from group A and B, *B. rhizoxinica*/*B. endofungorum* and *B. andropogonis* was carried out. The analysis showed a larger dissimilarity of 4 % between group A and B. The intra-group 16S rRNA similarity values were 98.7 % among the species of the group B and 96.0 % for the group A. Also, the 16S rRNA similarity value of *B. andropogonis* with the different groups was <96: 95.5 % with group B and 93.8 % with group A. Similarly, the *B. endofungorum*/*B. rhizoxinica* group was 96.1 % identical to the group B and 95.1 % to group A. The *B. endofungorum*/*B. rhizoxinica* group was found to be 95.1 % similar to *B. andropogonis*. This evidence clearly indicates that the genus *Burkholderia* consists of different bacterial lineages. Generally, values below ~95 % 16S rRNA gene sequence similarity indicate different genera [67].

Furthermore, the incongruent position of *B. andropogonis* was previously reported by Viallard et al. [84] based on analysis of 16S rRNA gene. To investigate this species in greater detail, fifteen 16S rRNA gene sequences from the NCBI database belonging to different strains of *B. andropogonis* were included in an NJ tree with the rest of the *Burkholderia* species (data not shown). The analysis showed that the *B. andropogonis* strains clustered outside of groups A, B and *B. endofungorum*/*B. rhizoxinica* groups, and distantly from *Cupriavidus*/*Ralstonia* species. The phylogenetic position of this species and the 16S similarity percentages among *B. andropogonis* and the other groups suggest that this species might represent a new genus. This may also be the case for *B. endofungorum* and *B. rhizoxinica*, but these species were not analyzed further because both species were described with only single strains.

The 16S sequence genes were also analyzed by NJ. The analysis showed that the genus split in different lineages, interestingly group A was further divided in two groups and *B. andropogonis* was placed outside from all clusters (Additional file 2).

Phylogenetic Inference of Individual and Concatenated Housekeeping Genes

The set of *Burkholderia* species analyzed in this study was selected from our in-house *Burkholderia* collection and some of the other species were gathered from the LMG culture collection (BCCM/LMG, Belgium). Whenever possible, more than one strain from each *Burkholderia* species was selected to perform robust species phylogeny estimation by MLSA. A phylogenetic analysis of the individual *atpD*, *gltB*, *lepA*, and *recA* sequences under the ML criterion revealed two distinct lineages within the

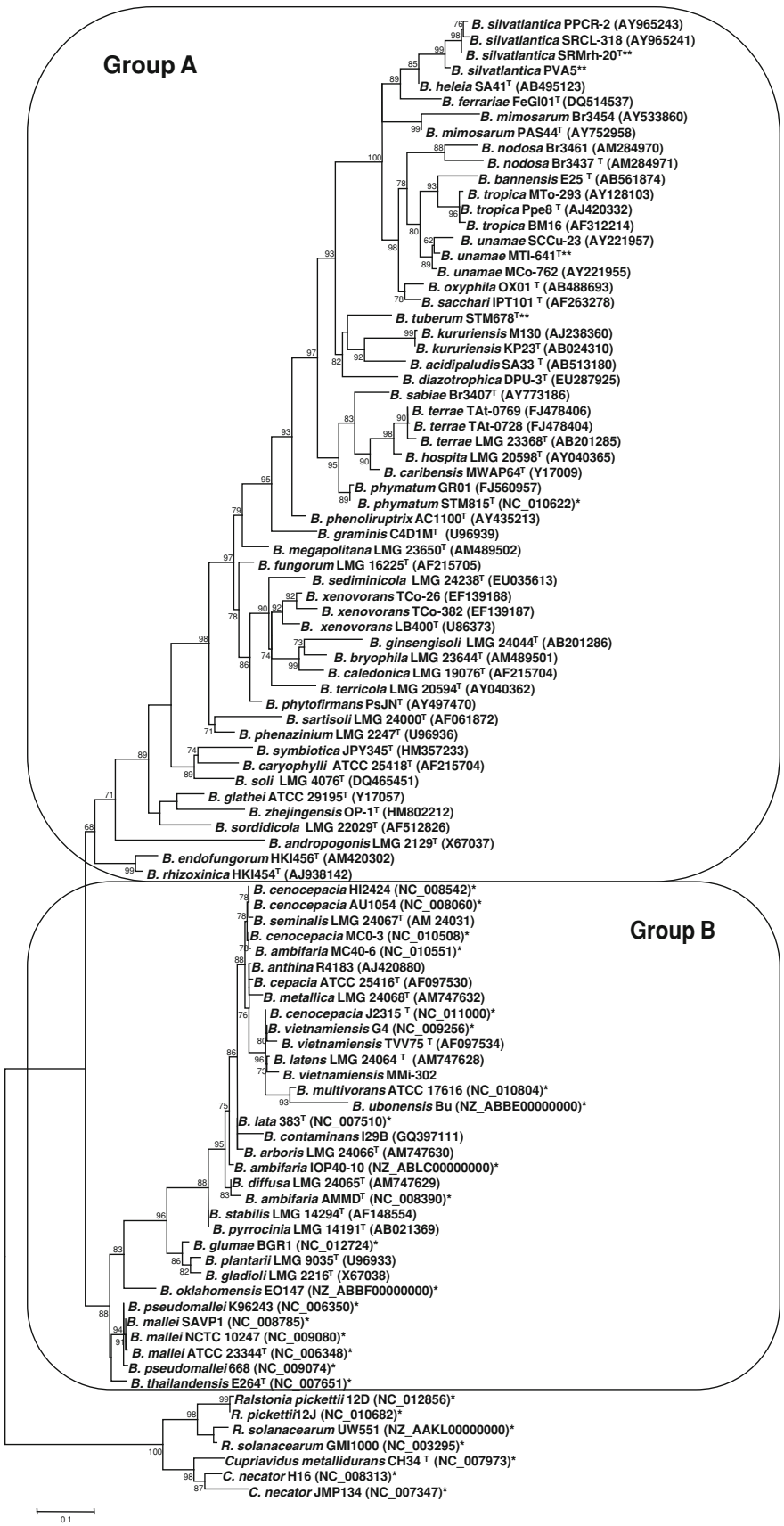

Fig. 1 Maximum likelihood tree inferred from the 16S rRNA gene, showing the phylogenetic relationships among *Burkholderia* species. The *bar* represents the number of expected substitutions per site under the GTR + G model. *One asterisk* indicates sequences obtained from the Genome database at NCBI. *Two asterisks* indicate sequences obtained from an ongoing genomic project. In *parenthesis* are accession numbers at NCBI

genus *Burkholderia*, the same groups A and B found in the analysis of 16S rRNA gene. However, in the *gltB* and *lepA* phylogenetic trees, group A was further split in two distinct lineages (Additional file 3–6). The dataset was also analyzed under the NJ criterion (Additional files 7–10), again showing two main groups in the *atpD* and *lepA* trees and three clusters in the *gltB* and *recA* trees. Moreover, the position of certain *Burkholderia* species was not clear. Generally, *B. andropogonis*, *B. soil*, and *B. rhizoxinica* were found to be not related to the two main groups.

A highly resolved ML species tree was estimated from the five gene concatenated dataset (Fig. 2). Only those strains having the full set of sequenced genes were included in this analysis. The results showed the two clusters, which are designated as group A and B. However, *B. soli*, *B. rhizoxinica*, *B. kururiensis*, and *B. glathei* were positioned outside these two main clusters, which indicates that more strains from these species must be analyzed to define their actual position in the phylogenetic trees. *Burkholderia andropogonis* was not included in the concatenated tree because the *recA* gene sequence was not successfully amplified and hence not examined. The concatened dataset analyzed by NJ showed three groups with group A further split in two clusters (Additional file 11).

GC Content Analysis

Previously, Gyaneshwar et al. [37] reported that some of the species included in the group A have a lower GC content than the species in the pathogenic group B. We expanded upon this analysis, and surveyed the literature and various databases to learn the GC content of all the currently described *Burkholderia* species (Additional file 12, 13). The analysis of the GC content of the different groups showed a substantial divergence between the group B (67.1 $\pm$ 1.0 %) compared to the group A (62.9 $\pm$ 1.3 %), *B. andropogonis* (59.0 %), and the *B. rhizoxinica*/*B. endofungorum* (60.7 %) cluster. Evidently, the group B GC content is higher than any of the other *Burkholderia* groups. Tindall et al. [67] observed that, with some exceptions, GC content may be fairly constant in a bacterial group. Taken together, this observation strongly suggests that the groups observed in *Burkholderia* consist of distinct bacterial lineages.

Diazotrophy Test

The ability to fix nitrogen is well known in *B. vietnamiensis* [31], the only member of the BCC within the group B that has unequivocally been shown to perform this activity. Starting in 2001 and continuing, the genus *Burkholderia*, especially members of the group A, is rich in nitrogen-fixing species [8, 28, 48, 53, 54, 58, 74]. Previously, *B. ferrariae*, a member of the group A, was found to have a *nifH* gene (GenBank:EF158799) [47] and the capacity of this species to fix nitrogen was confirmed by acetylene reduction activity (ARA) in the present study (Additional file 1). However, Aizawa et al. [5] did not detect ARA activity either in *B. ferrariae* or in *B. acidipaludis* or *B. bannensis*, although a *nifH* sequence was detected by PCR analysis. Thus it is still unknown whether the *nif* operon is complete or whether these strains actually fix nitrogen. One possibility to explain the difference in the reports about *B. ferrariae* diazotrophy is that the growth medium used to test ARA by Aizawa et al. [5] was not optimal. We found that using different medium [28] we could detect that *B. ferrariae* is a diazotroph. A similar situation could exist for both *B. acidipaludis* and *B. bannensis*. Other *Burkholderia* species not tested in this study by MLSA are also diazotrophs; such as *B. heleia*, *B. symbiotica*, and *B. diazotrophica*, the latter two species are able to nodulate *Mimosa* spp. plants [3, 61, 62]. However, *B. oxyphila*, *B. rhizoxinica*, and *B. endofungorum* have not been reported to be diazotrophs. *Burkholderia andropogonis* was found to be ARA-minus in this study. In conclusion, approximately half of the species from the group A are able to fix nitrogen as either free-living bacteria or in symbiotic associations.

Conclusions

The results presented in this report show that the genus *Burkholderia* is composed of different lineages. A glimpse of this observation was showed by Ussery et al. [69] where 56 *Burkholderia* genomes where analyzed in a phylogenetic study. Vandamme and Dawyndt [79] came to similar conclusions using ML to examine 100,000 base positions randomly extracted from single-copy core genes of the available *Burkholderia* genomes released up to date.

As new *Burkholderia* species are continually being described, the presence of different lineages will become more obvious. However, to propose a split of the *Burkholderia* genus at this time may be premature. The presence of two and sometimes three main groups plus *B. andropogonis* and *B. endofungorum*/*B. rhizoxinica* shows that the genus is evolving over time such that different bacterial lineages are developing. To understand this process better, the entire assemblage of species must be thoroughly analyzed, which means that both phylogenetic and physiological/biochemical traits need to be examined as well.

One example is the pathogenicity shown by the species of the group B compared to the species of the group A. Preliminary experiments found no pathogenic activity toward *Caenorhabditis elegans* or HeLa cells after testing *B. unamae*, *B. phytofirmans*, *B. tuberum*, and two strains of

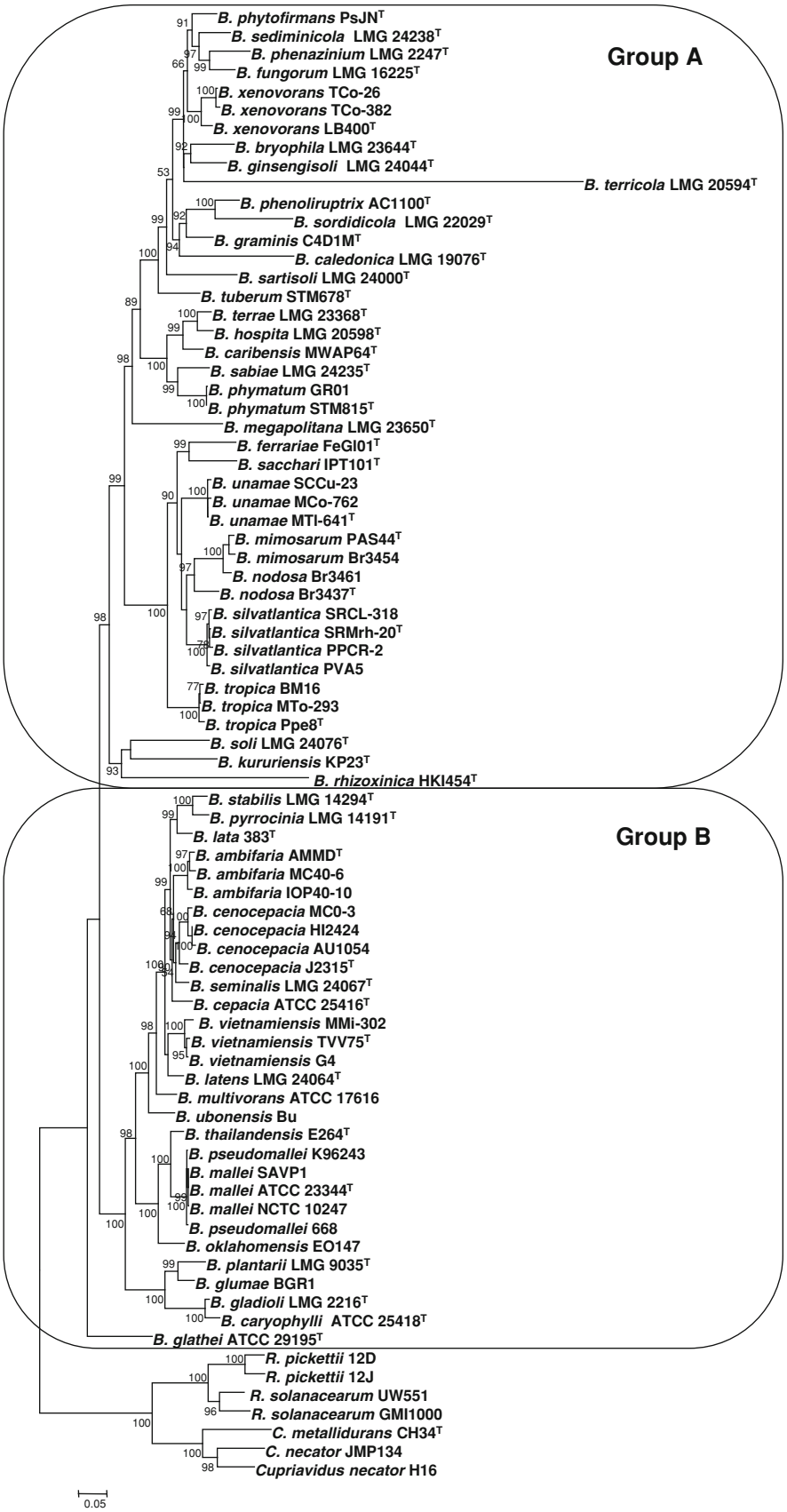

Fig. 2 Maximum likelihood species tree inferred from the concatenated alignment *atpD–gltB–lepA–recA*–16S rRNA genes, showing the phylogenetic relationships among *Burkholderia* species. The *bar* represents the number of expected substitutions per site under the GTR + G model

B. silvatlantica (A. Angus and A.M. Hirsch, ms. in prep.). In any case, differential phenotypic characteristics may not be as relevant at the genus level as they are for the description of new species because phenotypic traits may be inconsistent when large populations are studied [85]. Nevertheless, it is critical to analyze the full set of *Burkholderia* species, including more strains from each species as well as including extra housekeeping genes in the analysis.

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