Housekeeping gene *gyrA*, a potential molecular marker for *Bacillus* ecology study

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Abstract

Bacillus is a ubiquitous microorganism and is one of the most commercially important species widely used in industry, agriculture and healthcare. *Bacillus* is relatively well understood at the single-cell level; however, molecular tools that determine diversity and ecology of *Bacillus* community are limited, which limits our understanding of how the *Bacillus* community works. In the present study, we investigated the potential of the housekeeping gene *gyrA* as a molecular marker for determining the diversity of *Bacillus* species. The amplification efficiency for *Bacillus* species diversity could be greatly improved by primer design. Therefore, we designed a novel primer pair *gyrA*3 that can detect at least 92 *Bacillus* species and related species. For *Bacillus amyloliquefaciens*, *Bacillus pumilus,* and *Bacillus megaterium*, we observed that the high variability of the *gyrA* gene allows for more detailed clustering at the subspecies level that cannot be achieved by the 16S rRNA gene. Since *gyrA* provides better phylogenetic resolution than 16S rRNA and informs on the diversity of the *Bacillus* community, we propose that *gyrA* gene may have broad application prospects in the study of *Bacillus* ecology.

KEYWORDS: Molecular marker**,** 16S rRNA, Housekeeping *gyrA* gene, SNP, *Bacillus* diversity

Introduction

Microbial communities in soil are known to be one of the largest reservoirs of biological diversity and have been extensively studied (Timmis & Ramos, 2021). Current advances in high-throughput DNA sequencing of a portion of the small subunit of ribosomal RNA (16S and 18S rRNA) form the backbone of most studies of soil microbial ecology (Klindworth et al., 2013). For bacteria, the 16S rRNA gene is usually preferred, because it contains both highly conserved and hypervariable regions (Peer, Chapelle, & Wachter, 1996), and especially because comprehensive reference databases have been compiled for comparison (Cole et al., 2014; McDonald et al., 2012; Quast et al., 2013; Yoon et al., 2017). However, 16S rRNA amplicon sequencing also has many shortcomings: first, 16S rRNA evolves slowly and is highly conserved, making it a poor marker for distinguishing between closely related strains. Second, chimera formation during PCR is high because 16S rRNA variability is very low (Pinto & Raskin, 2012; Sun, Jiang, Wu, & Zhou, 2013). Third, the number of 16S rRNA copies in different species is highly variable, and single nucleotide polymorphisms (SNPs) at the single-cell level may result in an overestimation of diversity (Johnson et al., 2019). Fourth, the similarity between species can be very high, making it difficult to delineate species in cluster analysis, and different clustering levels lead to different results (Eren et al., 2013). Therefore, new complementary taxonomic markers for genetic and bioinformatic analysis need to be

66 developed to study microbial diversity in more detail, especially at the subspecies
67 level.

68 *Bacillus* is one of the most intensely studied bacterial genus comprising at least 200
69 species (Mandic-Mulec, Stefanic, & van Elsas, 2016). It is a heterogeneous bacterial
70 taxon that is ubiquitous in various ecological niches and widely used in medicine,
71 industry and agriculture. Although there have been many in-depth studies on *Bacillus*
72 model species, community-level studies on *Bacillus* in soil and other habitats lag
73 behind. Because sequence of 16S rRNA within *Bacillus* species is often similar, the
74 definition and delineation of bacterial species based on the 16S rRNA gene
75 comparison among related species in the genus *Bacillus* is unclear. Therefore,
76 identification and typing of *Bacillus* isolates based on the 16S rRNA gene alone
77 cannot provide accurate results and it is important to explore and use other genes as
78 molecular markers to assess the diversity of the *Bacillus* community (Mandic-Mulec
79 et al., 2016).

80 Housekeeping genes are potential candidates for assessing microbial diversity because
81 they have been shown to elicit higher phylogenetic resolution than the 16S rRNA
82 gene, such as the *rpoB* gene, *gyrA* gene, and *gyrB* gene, etc.(Chun & Bae, 2000;
83 Hurtle et al., 2004; Kasai, Tamura, & Harayama, 2000; Levican, Collado, & Figueras,
84 2013; Ménard, Buissonnière, Prouzet-Mauléon, Sifré, & Mégraud, 2016; Stefanic et
85 al., 2012; Stefanic, Kraigher, Lyons, Kolter, & Mandic-Mulec, 2015; Stefanic &
86 Mandic-Mulec, 2009; Yamamoto et al., 2000). Typically, there are only one or two
87 copies of housekeeping genes per genome, and the use of low-copy number genes

compared to high-copy number genes could lead to more accurate diversity analysis by avoiding overestimation of diversity due to SNPs in different gene copies. Indeed, several studies have tested the *rpoB* gene and the *gyrB* gene as molecular markers to analyze the diversity of bacterial communities by amplicon sequencing. The results showed that housekeeping gene sequencing provided a more accurate description of bacterial community composition than 16S rRNA sequencing under certain conditions (Ogier, Pagès, Galan, Barret, & Gaudriault, 2019; Poirier et al., 2018; Vos, Quince, Pijl, de Hollander, & Kowalchuk, 2012).

The housekeeping gene *gyrA*, encoding DNA gyrase subunit A, is essential for DNA replication and present in all bacteria (Cozzarelli, 1980). Analyses of *gyrA* identity provided higher phylogenetic resolution than the 16S rRNA gene for tested *Bacillus* isolates (Chun & Bae, 2000; Ménard et al., 2016). Specifically, partial *gyrA* gene sequences was used for phylogenetic analysis and species identification of seven *Bacillus* strains, including *B. amyloliquefaciens*, *B. atrophaeus*, *B. licheniformis*, *B. mojavensis*, *B. subtilis*, *B. subtilis subsp. Spizizenii*, and *B. vallismortis* (Chun & Bae, 2000). Moreover, the *gyrA* gene sequences provided a good marker for *B. subtilis* and *B. amyloliquefaciens* and showed better discriminatory potential between these two closely related species than the *rpoB* gene (Chun & Bae, 2000). The *gyrA* gene has been also successfully used to detect intraspecific diversity of *B. subtilis* isolates from soil microscale (Stefanic and Mandic Mulec, 2009) and tomato rhizosphere isolates (Oslizlo et al., 2015). Overall, these works suggest that *gyrA* has a good potential to be used as a molecular marker for microbial ecology studies of *Bacillus* genus and

related species, however, to the best of our knowledge, the available primer pairs used in studies indicated above, have not been used for amplicon based community analyses of *Bacillus* species.

In this study, we first compared the rate of variation of the16S rRNA and *gyrA* genes between 20 *Bacillus* species and then designed a new primer pair that specifically target *gyrA* (*gyrA*3). We then evaluate their performance by using *in silico* PCR, testing their efficiency on *Bacillus* isolates and performed SNP analysis of 16S rRNA and *gyrA* genes for selected species that were available in the NCBI database. Finally, verified *gyrA*3 primers to differentiate species and strains of *Bacillus* mock community, and compared the obtained results with those targeting 16S rRNA. Our results suggest that the *gyrA* gene is a useful molecular marker for identification of *Bacillus* isolates and describing the diversity of the *Bacillus* community.

Results

The *gyrA* gene of the *Bacillus* genus shows higher variation rates than 16S rRNA

The housekeeping gene *gyrA* is considered to be more variable than 16S rRNA and has been used as a molecular tool for classification and identification of *Bacillus subtilis* species (Borshchevskaya, Kalinina, & Sineokii, 2013; Chun & Bae, 2000). To determine the specific variation divergence of 16S rRNA and *gyrA* gene sequences in the genus *Bacillus*, two alignments with sequences of the entire 16S rRNA and *gyrA* genes from 20 *Bacillus* species (361 genomes) (Table S1) were used to analyze nucleotide diversity. The nucleotide diversity (Pi) of 16S rRNA and *gyrA* gene calculated by the DnaSP software was 0,039 and 0.491, respectively (Fig. 1A,B blue

line). The results showed that the degree of interspecies variation was significantly higher for the *gyrA* gene than for the 16S rRNA gene.

Intraspecific variation analysis of the two genes was performed using the same approach but focusing on three *Bacillus* species: *Bacillus amyloliquefaciens* (88 genomes), *Bacillus licheniformis* (71 genomes) and *Bacillus pumilus* (97 genomes) (Table S1). The intraspecific nucleotide diversity (Pi) of 16S rRNA was again lower than the intraspecific nucleotide diversity of *gyrA* gene sequences in all three species: *B. amyloliquefaciens* (Pi_{16S}=0.0014; Pi_{gyrA}=0.0244), *B. licheniformis* (Pi_{16S}=0.00024; Pi_{gyrA}=0.0021) and *B. pumilus* (Pi_{16S}=0.00136; Pi_{gyrA}=0.0344) (Fig. 1A,B nonblue lines). In *B. amyloliquefaciens*, *B. licheniformis* and *B. pumilus*, the degree of variation of the *gyrA* gene was significantly higher than that of 16S rRNA. In conclusion, the *Bacillus gyrA* gene shows higher variation rates than 16S rRNA, hence we propose that *gyrA* represents a promising molecular marker for analyses of *Bacillus* community diversity analyses and the diversity of *Bacillus* isolates.

First comparative tests of three primer pairs for the detection of *Bacillus* species

As indicated above *Bacillus* isolates have been already analyzed by primers targeting *gyrA*, however the specificity of these primers has not been investigated broadly (Ansaldi, Marolt, Stebe, Mandic-Mulec, & Dubnau, 2002; Roberts, Nakamura, & Cohan, 1994). In order to satisfy the amplicon sequencing requirements, we designed a new primer pair (*gyrA*3) (Fig. 2A), and compared its amplification potential in colony PCR and virtual PCR with the previously designed primers, referred to here as *gyrA*1 (Stefanic & Mandic-Mulec, 2009) and *gyrA*2 (De Clerck et al., 2004).

First, we selected seven strains of different *Bacillus* species: *Lysinibacillus fusiformis*, *Paenibacillus polymyxa*, *Bacillus pumilus*, *Bacillus velezensis*, *Bacillus megaterium*, *Bacillus cereus* and *Bacillus subtilis* (Fig. 2B) to perform PCR amplification with primer pairs *gyrA*1, *gyrA*2 and *gyrA*3. The PCR amplification results showed that *gyrA*1 detected only *B. subtilis*; *gyrA*2 detected *B. subtilis*, *B. velezensis* and *L. fusiformis*; whereas *gyrA*3 performed much better and detected all *Bacillus* species included in the analysis (Fig. 2B and Fig. S1).

For *in-silico* PCR analysis, we constructed a *gyrA* gene library containing 5062 full-length *gyrA* gene sequences from 226 *Bacillus* species obtained from the National Center for Biotechnology Information (NCBI) (Table S2). Our criteria for positive *in-silico gyrA* PCR amplification were annealing of the primer pair with at least 18 bases match (forward primer and reverse primer). The results showed that only 8 *Bacillus* species were amplified in-silico by *gyrA*1, 9 *Bacillus* species were amplified by *gyrA*2 (Fig. S2), while 55 *Bacillus* species were amplified by *gyrA*3 (Fig. 2C). The majority of sequences amplified by *gyrA*1 and *gyrA*2 belonged to *B. subtilis*, whereas *gyrA*3 demonstrated broader diversity as evidenced by the amplification of seven species and *in-silico* PCR (Fig. 2B,C and Table S3).

Specificity range of the *gyrA*3 primer pair by using PCR and *in silico* PCR

Because the *gyrA*3 primer pair performed better than the previously reported primers, we next combined analysis of the *in-silico* amplified *gyrA* genes with PCR analysis of *Bacillus* isolates from our laboratory culture collection. Virtual *gyrA*3 PCR amplicons from 55 different *Bacillus* species from the NCBI database were evenly distributed

among the branches of the phylogenetic tree (Fig. 3 orange and green).

Next, we used 127 *Bacillus* strains and related genera (*Paenibacillus*, *Lysinibacillus*, *Aneurinibacillus*, *Virgibacillus*, *Brevibacillus*, *Halobacillus*, *Fictibacillus*) from our laboratory culture collection to amplify their *gyrA* genes with a *gyrA*3 primer pair (Table S4). The results showed that 28 *Bacillus* species and 16 *Bacillus*-related species could be amplified by the *gyrA*3 primers (Fig. 3 blue and green). These *Bacillus* species, marked in green in the phylogenetic tree, 7 species were detectable by both methods (Fig. 3 green). In summary, the primer pair *gyrA*3 can potentially detect 76 *Bacillus* species and as many as 16 species from related genera (Fig. 3).

The *gyrA* gene provides better intraspecific phylogenetic resolution than the 16S rRNA gene among certain species

Compared to 16S rRNA, the molecular evolution rate of *gyrA* gene sequences is faster (Timmis & Ramos, 2021), so we hypothesized that *gyrA* might provide better phylogenetic resolution at the subspecies level. To analyze the intraspecific variability of the 16S rRNA and the *gyrA* gene, we downloaded the genomes of four *Bacillus* species, each of which had more than 100 genomes available in the NCBI database, including *B. amyloliquefaciens* (116 genomes), *B. pumilus* (140 genomes), *B. megaterium* (117 genomes) and *B. anthracis* (226 genomes) (Table S5). We did not include analysis the of *B. subtilis* genomes because templates for *gyrA* primers have already been developed and applied for analyses of this species (Ansaldi et al., 2002; De Clerck et al., 2004; Roberts et al., 1994; Stefanic & Mandic-Mulec, 2009).

In *B. amyloliquefaciens*, the 480 bp long 16S rRNA region (V3-V4 region) contained

12 variable base sites (Fig. 4A and Fig. S3A), which were detected in only 4 of 116 genomes of this species (Fig. 4A), and the SNP frequency at variable sites within the 4 genomes ranged from 0.21%-1.46% (Fig. S3C red column). In contrast, the 500-nucleotide *gyrA* region (positions 350-850) contained 59 variable sites (Fig. 4B and Fig. S3B). The variable sites were detected in 109 of 116 genomes (Fig. 4B), and the frequency of SNPs at the variable sites ranged from 0.4% to 6.4% (Fig. S3C blue column).

In *B. pumilus*, alignment of the 16S rRNA V3-V4 region revealed 21 variable base sites (Fig. 4C and Fig. S4A), but again only in 11 out of 140 genomes (Fig. 4C). The frequency of SNPs at variable sites ranged from 0.21% to 3.54% (Fig. S4C red column). In contrast, in the *gyrA* gene 130 of the base sites were variable (Fig. 4D and Fig. S4B) and these were found in 87 of 140 genomes, with SNP frequencies at variable sites ranging from 0.2% to 15.8% (Fig. S4C blue column). However, in *B. pumilus* nearly 50% of the genomes examined had 100% identity in the *gyrA* gene, indicating high degree of relatedness between genomes that may require sequencing of additional marker genes for clonality verification and strain typing.

We also observed that the variation of the *gyrA* gene is quite different in different *Bacillus* species, which could be a bias of the NCBI database or a property of certain species. For example, *B. megaterium*, showed lower diversity with 3 bases of variation in the 16S rRNA alignment region (V3-V4 region) (Fig. S5A and Fig. S6A). Although 48 of 117 genomes showed polymorphism, the maximum SNP frequency at variable sites of *B. megaterium* genomes was only 0.42% (Fig. S6C red column). The

gyrA gene was again more polymorphic with 58 bases of variation (Fig. S5B and Fig. S6B) occurring in 99 of 117 genomes, with SNP's frequencies at variable sites ranging from 0.2% to 2.8% (Fig. S6C blue column).

The variation divergence between the two genes of *Bacillus anthracis* was much lower than in the three *Bacillus* species described above. Although we identified 13 variable base sites in the 16S rRNA V3-V4 region and 65 variable base sites in the *gyrA* gene region (Fig. S5C,D and Fig. S7A,B), these single nucleotide polymorphisms (SNPs) occurred in only 7 and 18 of 226 genomes, respectively. Moreover, the SNP frequencies at variable sites in 7 and 18 of *B. anthracis* genomes were also low: 0.21%-1.04% and 0.2%-7.4%, respectively (Fig. S7C).

Overall, our results showed that within *Bacillus* species the frequency of SNPs in the *gyrA* gene was consistently much higher than in the 16S rRNA (Fig. 4 and Fig. S5). We therefore suggest that the *gyrA* gene provides better resolution than 16S rRNA for identification and typing of *Bacillus* isolates at the subspecies level. This is particularly true for *B. amyloliquefaciens*, *B. pumilus* and *B. megaterium* but less so for *B.* anthracis (Table 1).

The resolution power of *Bacillus* mock community *gyrA* amplicon sequencing

Our results above suggest that the primer pair *gyrA*3 could be used as a molecular marker for diversity analysis of *Bacillus*. Next, we aimed to design a mock community to test the efficacy of the *gyrA*3 primer and used the general 16S rRNA primers (V3-V4) as a positive control. For better comparison, we designed two additional primer pairs, *gyrA*4 and *gyrA*5, which are very close to the position of the

242 *gyrA*3 in the *gyrA* gene (Fig. S8A). We selected eight strains belonging to four species
243 (*B. altitudinis*, *B. licheniformis*, *B. velezensis* and *Lysinbacillus pakistanensis*) and
244 successfully amplified their *gyrA* gene by *gyrA*3, *gyrA*4 and *gyrA*5 primer pairs in a
245 routine PCR for selected strains (Fig. 5A). Next, we artificially mixed eight indicated
246 strains in equal proportions to obtain a mock community and then retested the
247 resolution power of the three *gyrA* primer pairs and the 16S rRNA-specific primers.
248 Our goal was to test whether the primer pair is suitable for determining the diversity
249 of mock community (Fig. 5). Sequencing of the 16S rRNA amplicons showed that
250 16S rRNA primers could distinguish only five units, as strains LY1 and LY18, LY37
251 and LY43, and LY39 and LY48 had identical V3-V4 nucleotide sequence (Fig. 5B).
252 The *gyrA* primer pairs were capable of resolving six units, but *gyrA*4 and *gyrA*5
253 produced amplicons of variable abundance and preferentially amplified LY35 and
254 LY2, respectively(Fig. 5C-E). In comparison, primers *gyrA*3 also amplified six
255 fragments but the relative abundance of these amplicons was more uniform (Table S6)
256 with the exception of LY2 strain (Fig. 5C). Our data suggest that *gyrA*3 has potential
257 for Illumina amplicon sequencing of more complex *Bacillus* communities.

258 **Discussion**

259 It is believed that the diversity of microorganisms in nature is immense, and therefore
260 its detection still remains a challenge (Widder et al., 2016). Molecular tools (e.g., for
261 specific amplification of marker genes) combined with high-throughput sequencing
262 are expected to open the door to the vast diversity of microorganisms (Klindworth et
263 al., 2013). Here, we systematically investigated the potential of the *gyrA* gene as a

marker gene for taxonomic typing of *Bacillus* isolates and assessment of *Bacillus* community composition by amplicon sequencing. The novel *gyrA*3 primer pair is capable of detecting 76 *Bacillus* species by virtual and colony PCR; hence, our results suggest that the *gyrA* gene is a good phylogenetic marker for detecting intra- and interspecific diversity of the genus *Bacillus*.

Sequencing of 16S rRNA amplicons on the Illumina platform has been the mainstream method of studying microbial communities, but this gene also shows shortcomings, especially when we aim to determine diversity within a genus or species (Amir et al., 2017; Callahan, McMurdie, & Holmes, 2017; Callahan et al., 2016; R. Edgar, 2016a, 2016b; R. C. Edgar, 2013). This is particularly true for *Bacillus* species, which exhibit very low interspecific variability (Vos et al., 2012). Although 16S rRNA is widely used as a molecular marker for bacterial community analyses (Clarridge, 2004), its amplicon sequencing can only describe community diversity at the genus level (Gupta et al., 2019). In contrast, faster evolution and consequently higher diversity of the *gyrA* gene (Timmis & Ramos, 2021) suggests that this gene might provide higher phylogenetic resolution than the 16S rRNA gene within the genus *Bacillus*.

In our study, the differential effect of the *gyrA* gene on *Bacillus* interspecies and several *Bacillus* species was better than that 16S rRNA (Fig. 1). Moreover, the results of comparative sequence analysis (including the 16S rRNA and the *gyrA* gene regions) of the three species: *B. amyloliquefaciens*, *B. pumilus*, and *B. megaterium*, support this prediction, as we find wider range of SNPs in the *gyrA* genes than in the 16S

rRNA (Fig. 4, Fig. S5 and Table 1). Our results are consistent with findings that housekeeping genes, including *gyrA*, evolve much faster than 16S rRNA genes and are suitable for the identification and typing of closely related species (Poirier et al., 2018) and for the intraspecific resolution of isolates, as previously shown for *B. subtilis* (Ansaldi et al., 2002; Roberts et al., 1994).

To date, there have been only a few reports in which conserved genes (e.g. *rpoB* and *gyrB*) have been used as templates for amplicon sequencing of microbial communities (Ogier et al., 2019; Poirier et al., 2018; Vos et al., 2012). These reports show that sequencing of conserved protein coding genes provides a more accurate description of bacterial community composition than 16S rRNA sequencing. Specifically, the *rpoB* gene has been used in addition to the 16S rRNA molecular marker for high-throughput sequencing studies of species diversity in the *Proteobacteria* phylum (Vos et al., 2012).

Although it remains to be investigated whether *gyrA* is suitable as a molecular marker for the majority of *Bacillus* species, we identified here its resolution power for 76 *Bacillus* species (Fig. 3) and propose that the diversity of the *Bacillus* community can be more accurately assessed by combining 16S rRNA and *gyrA* amplicon sequencing.

As a molecular marker, the housekeeping gene *gyrA* also has some limitations. Due to the high variability of *gyrA* gene sequences, it was difficult to design universal primers that can amplify the entire *Bacillus* genus, as has been previously described for the *Escherichia* genus (Johnning, Kristiansson, Fick, Weijdegård, & Larsson, 2015). Although *gyrA*3 significantly improved the amplification efficiency of *Bacillus*

species compared to previous primers, not all *Bacillus* species could be detected (Fig. S2 and Fig. 3). On the other hand, the *gyrA* amplicon region we selected was well suited for resolving subspecies of *B. amyloliquefaciens*, *B. pumilus*, and *B. megaterium*. For *B. anthracis*, which is known for its low diversity (Lista et al., 2006) intraspecific resolution was limited (Fig. 4 and Fig. S5). Using amplicon sequencing, *gyrA*3 also had its own blind area in the detection, for example, it was not good at distinguishing between LY1 and LY18, LY39 and LY48 (Fig. 5). This could be due to the extremely high similarity of the *gyrA* genes in selected genomes, some of which have even identical *gyrA* sequences. However, the *gyrA*3 distinguished very well two strains with highly similar *gyrA* genes such as LY37 and LY43, which 16S rRNA did not (Fig. 5). Although primers for amplicon sequencing of the *gyrA* gene had certain limitations, such as not amplifying all selected targets or not reflecting the abundance of added DNA, this study has put forward the advantages that *gyrA*3 covers the broadest diversity of *Bacillus* species reported to date.

In summary, this study investigated the application of the *gyrA* gene as a molecular marker in *Bacillus* subspecies typing and high-throughput sequencing of the *Bacillus* mock community. The greater ability of *gyrA*-based analyses to distinguish *Bacillus* strains at the subspecies level should increase resolution and provide more reliable results for the ecological studies of the genus *Bacillus*. We believe that the primer pair will have broad applications in the *Bacillus* research.

Materials and Methods

Strains and culture condition

The 127 strains used in the study were strains of *Bacillus* and related genera of *Bacillus*, most of which were isolated from soil. Some strains labelled with ACCC are deposited in the Agricultural Culture Collection of China (http://www.accc.org.cn) (Table S4).

All strains were grown at 30 □ in low-salt Luria-Bertani medium (LB), containing 10 g tryptone, 5 g yeast extract, and 3 g NaCl per litre.

Nucleotide diversity (Pi) analysis

For interspecies analysis, the whole sequences of the 16S rRNA and the *gyrA* genes of 20 *Bacillus* species (361 genomes) were downloaded from the NCBI genome database (Table S1). For intraspecies analysis, three species were selected and whole sequences of 16S rRNA and *gyrA* gene were downloaded from the database: *Bacillus amyloliquefaciens* (88 genomes), *Bacillus licheniformis* (71 genomes) and *Bacillus pumilus* (97 genomes) (Table S1). Alignment of these sequences was conducted using online alignment tool Kalign on EMBL (https://www.ebi.ac.uk/Tools/msa/). Subsequently, nucleotide diversity (Pi) was estimated with DnaSP 6 (v.6) using a window size of 100 bp and a step size of 10 bp (Rozas et al., 2017).

DNA extraction of strains, PCR and gel electrophoresis

Genomic DNA was extracted using the Omega Bacterial DNA Kit D3350 (Omega, Bio-tek, Norcross, GA, USA), and the concentration and quality of DNA were determined using a NanoDrop 2000 spectrophotometer (Wilmington, DE, USA).

The reaction mixture for PCR amplification was prepared in 25 μL containing 1 μL of DNA, 2 μL of each primer (the primer pairs *gyrA*1 were *gyrA*1-F:

5′-CAGTCAGGAAATGCGTACGTCCTT-3’ (Roberts et al., 1994) and *gyrA*1-R: 5′-GTATCCGTTGTGCGTCAGAGTAAC-3’ (Ansaldi et al., 2002), the primer pairs *gyrA*2 were *gyrA*2-F: 5′-CAGTCAGGAAATGCGTACGTCCTT-3’ (Roberts et al., 1994) and *gyrA*2-R: 5′-CAAGGTAATGCTCCAGGCATTGCT-3’ (Roberts et al., 1994), the primer pairs *gyrA*3 were *gyrA*3-F: 5′-GCDGCHGCNATGCGTTAYAC-3’ and *gyrA*3-R: 5′-ACAAGMTCWGCKATTTTTTC-3’, the primers for the 16S rRNA gene were 27F: 5′-AGAGTTTGATCCTGGCTCAG-3’ and 1492R: 5′-GGTTACCTTGTTACGACTT-3’), 12.5 μL Green Taq Mix (http://www.vazyme.com), and 7.5 μL deionized water.

PCR was performed under the following conditions: Predenaturation at 94 □ for 5 min; denaturation at 94 □ for 30 s; annealing at 50 □ for 30 s; elongation at 72 □ for 40 s (35 cycles); and elongation at 72 □ for 7 min.

In Silico PCR

The *gyrA* sequence database was constructed as a virtual community, containing 5062 full-length sequences from the National Center for Biotechnology Information (NCBI) belonging to 226 *Bacillus* species (Table S2) (the method for obtaining the database sequences is described in detail in the SNP analysis section below). First, the degenerate primer pair *gyrA*3 was converted to primers that do not contain degenerate bases, and the converted *gyrA*3 is listed in Table S7. Subsequently, the primer pairs *gyrA*1, *gyrA*2 and the converted *gyrA*3 were aligned with the *gyrA* database using NCBI-blast+ software (v.2.9.0). The match at 18 bases of both, the forward and reverse primer, was considered amplifiable by the primer pair.

Phylogenetic analysis

In this study, phylogenetic analysis of genes was performed using MEGA (v.5.05) (Tamura et al., 2011) for Neighbor-Joining and the reliability of clades was tested using 1000 bootstrap replicates. Furthermore, annotation and beautification of trees was performed using programs available at the iTol online site (https://itol.embl.de) (Tamura et al., 2011).

SNP analysis

A total of 734 available genomes of 4 different *Bacillus* species were downloaded from the NCBI database using the NCBI-genome-download script (https://github.com/kblin/ncbigenome-download/) (Table S5) including 124 genomes of *B. amyloliquefaciens*, 167 genomes of *B. pumilus*, 150 genomes of *B. megaterium* and 293 genomes of *B. anthracis*. Then, the location information of 16S rRNA and *gyrA* gene sequences on the genomes were determined by aligning 16S rRNA and *gyrA* sequences of the same species using NCBI-blast+ (v.2.9.0) and the 16S rRNA / *gyrA* gene sequences were extracted using the Fasta Extract tool in TBtools (v1.0986853) (Chen et al., 2020). After filtration, genomes that did not carry complete 16S rRNA and *gyrA* genes, were removed and 600 genomes were retained, including 116 genomes of *B. amyloliquefaciens*, 140 genomes of *B. pumilus*, 117 genomes of *B. megaterium* and 226 genomes of *B. anthracis* (Table S5).

The alignment of the 16S rRNA (base sites:330-810) and *gyrA* genes (base sites:350-850) within each species was performed using the L-INS-I method of MAFFT (v7.487) (https://mafft.cbrc.jp/alignment/software/). The two gene sequences

(16S rRNA, *gyrA*) on the same genome were selected as representative sequences (the top sequence of each figure was the representative sequence), and the base mismatch sites on other sequences were marked with color after comparison with the representative sequence. The genomes of the representative sequences in different species were GCF_017815555.1 (*B. amyloliquefaciens*), GCF_014269965.1 (*B. pumilus*), GCF_002577645.1 (*B. megaterium*) and GCF_000007845.1 (*B. anthracis*) (Fig. 4 and Fig. S5).

Construction of the DNA-based *Bacillus* mock community, amplicon sequencing and data analysis

For the *Bacillus* mock community, we selected 8 strains with known genome sequences. Genomic DNA from 8 strains was extracted and its quality and quantity determined. Eight genomic DNAs were pooled in equal amounts after being diluted to approximately the same concentrations.

The hypervariable region V3-V4 of the 16S rRNA gene was amplified with the universal primers 338F: 5′-CCTACGGRRBGCASCAGKVRVGAAT-3’ and 806R: 5′-GGACTACNVGGGTWTCTAATCC-3′. The *gyrA* gene from 8 strains of the mock community was amplified with primer pairs *gyrA*3 (see above), *gyrA*4 (F:5′-TAYGCRATGAGYRTHATYGT-3’ and R: 5′-TTBGTNGCCATHCCDACMGC-3’), and *gyrA*5 (F:5′-GCDGCNGCVATGCGTTAYAC-3’ and R: 5′-CGNAGRTYBGTAATDCCDTC-3’). Sequencing was performed on an Illumina Miseq PE300 instrument.

Raw data were processed using the UPARSE pipeline (http://drive5.com/usearch/manual/uparse_pipeline.html) (R. C. Edgar, 2013) to obtain the ZOTUs table. This included truncating and assembling, removing double end primers, filtering low-quality sequences, finding unique sequences, denoising, and creating the ZOTUs with 100% similarity, which were then used to create the ZOTUs table. Denoising (error-correction) of the amplicon reads was performed by using the Unoise3 algorithm (R. Edgar, 2016b) to identify and correct reads with sequencing errors and remove chimeras.

The eight strains of the mock community with known complete 16S rRNA and *gyrA* gene sequences were used to annotate the ZOTUs (Dataset S2 and S3). The annotation of ZOTUs sequences was performed with NCBI-blast+ (v.2.9.0). The ZOTUs of the same species were pooled into a single unit after annotation. Finally, we used box plots to show the community structure and the characteristics of the different primer pairs during sequencing. The box plots were drawn using R (v.4.0.3).

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Competing Interests

The authors declare that they have no conflicts of interest.

Author contributions

YL and ZX designed the study; YL performed the experiments. YL, YM and PS analysed the data and created the figures. YL, PS and ZX wrote the first draft of the paper; PS, ZX, RZ, QS and IM revised the paper.

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Figure legends:

Figure 1 Sliding window analysis of nucleotide variability [Pi (π)] along the sequence of the *gyrA* gene (**A)** and 16S rRNA (**B)** in *Bacillus*. 361 and 256 of *gyrA* and 16S sequences from 20 species and three species (*B. amyloliquefaciens* (n=88), *B. licheniformis* (n=71) and *B. pumilus* (n=97)) were aligned with Kalign method and nucleotide diversity was determined in DnaSP (v.6) (Rozas et al., 2017) using a window size of 100-bp, a step size of 10-bp, and points based on the mid-point of each window (i.e., the first point is at position 200). The blue lines display the *Bacillus* inter-species nucleotide diversity of the two genes and the orange, grey and yellow lines display the nucleotide diversity of two genes in *B. amyloliquefaciens*, *B. licheniformis and B. pumilus*, respectively. The sequence length of *gyrA* gene for analysis was 2450 bp, and that of 16S rRNA was 1540bp.

Figure 2 Display of the ability of the three primer pairs to amplify *gyrA* gene for seven *Bacillus* strains. (**A)** The position of *gyrA* amplicons obtained by primer pairs *gyrA*1, *gyrA*2 and *gyrA*3. (**B)** PCR amplification of seven *Bacillus* strains using three *gyrA* gene primer pairs. The orange square indicates that the target gene of *gyrA* was amplified by the indicated primer pair, and the white square indicates that it was not. The phylogenetic tree was constructed based on the complete 16S rRNA (1358bp) (Dataset S1) by using Neighbor-Joining method in MEGA 5.05 software. The reliability of clades was tested by the 1000 bootstrap replications. (**B)** Display of the results of the computer simulation amplification ability of the three *gyrA* gene primer pairs using the *Bacillus* database. The white columns represent number of sequences in the database that were matched by the primers, while the grey columns represent number of *Bacillus* species identified by the primer pair (Table S3). The database sequences were downloaded from NCBI genome database (Table S2).

Figure 3 The amplification ability of the primer pair *gyrA*3 of *Bacillus* species and related species performed by computer simulation and *in situ* amplification. The *Bacillus* species indicated by the orange color (48 species) represent the species in the database that were matched by *gyrA*3 only in virtual PCR. The *Bacillus* species in

blue color represented strains that can be amplified by *gyrA*3 only by colony PCR. These strains included 21 *Bacillus* species and 16 *Bacillus*'s related genera species. The *Bacillus* species in green color (7 species) represent strains that can be amplified by two methods: virtual PCR and colony PCR. The *Bacillus* species in black color (2 species) represent strains that cannot be amplified in virtual PCR nor by colony PCR. The phylogenetic tree was constructed based on 16S rRNA gene (1302bp).

Figure 4 Alignment of the 16S rRNA and *gyrA* sequences of *B. amyloliquefaciens* (116 genomes) and *B. pumilus* (140 genomes). Sequences were aligned using L-INS-I method of MAFFT (v7.487). Analysis involved positions along 16S rRNA from 330-810 and along *gyrA* from 350-850. On the left side of the graph GCF reference numbers of genomes were displayed, with reference sequence GCF_017815555.1 displayed at the top for B. *amyloliquefaciens* and GCF_014269965.1 for *B. pumilus*. The display of base site variation was drawn using MEGA (v.5.05). The colored line (red or blue) indicates a variation of specific base sites as compared to the reference sequence.

Figure 5 The results of amplicon sequencing for the mock community including eight *Bacillus* strains. (**A)** PCR amplification of eight *Bacillus* strains by *gyrA* gene primer pairs. The orange square indicates positive and white square unsuccessful amplification. The amplicon sequencing results of 16S rRNA gene (**B)** and *gyrA* gene using primer pairs *gyrA*3 (**C)**, *gyrA*4 (**D)**, and *gyrA*5 (**E)** for the mock community. The relative abundance represented by reads numbers for each unit as indicated on X axis. The phylogenetic tree was contracted based on the complete *gyrA* genes (2469bp) (Dataset S3) by using Neighbor-Joining method in MEGA 5.05 software. The reliability of clades was tested by the 1000 bootstrap replications. The box plots were drawn by ggplot2 R package (v.3.2.1).

Figure S1 The agarose gel electrophoresis of DNA amplification products of three *gyrA* gene primer pairs: *gyrA*1 **(A)**, *gyrA*2 **(B)** and *gyrA*3 **(C)**. 1-7 indicates strains LY18, LY25, LY37, FZB42, SXL408, SXL277 and ACCC01043.

Figure S2 Alignment of the 16S rRNA and *gyrA* sequences of *B. megaterium* (117 genomes) and *B. anthracis* (226 genomes). Sequences were aligned using L-INS-I method of MAFFT (v7.487). Analysis involved positions along 16S rRNA from 330-810 and along *gyrA* from 350-850. On the left side of the graph GCF reference numbers of genomes were displayed, with reference sequence GCF_002577645.1 displayed at the top for *B. megaterium* and GCF_000007845.1 for *B. anthracis*. The display of base site variation was drawn using MEGA (v.5.05). The colored line (red or blue) indicates a variation of specific base sites as compared to the reference sequence.

Figure S3 Polymorphisms in the 16S rRNA and *gyrA* gene's regions of *B. amyloliquefaciens* (116 genomes). **(A)** The proportion of variation at different base

sites along 16S rRNA gene V3-V4 region. **(B)** The proportion of variation at the different base positions of the *gyrA* gene region. **(C)** The proportion of variants along 16S rRNA (red column) and *gyrA* gene (blue column) region in different genomes.

Figure S4 Polymorphisms in the 16S rRNA and *gyrA* gene's region of *B. pumilus* (140 genomes). **(A)** The proportion of variation at different base sites along the 16S rRNA gene **(B)** and the *gyrA* gene. **(C)** The proportion of variable base sites in 16S rRNA (red column) and *gyrA* (blue column) sequences in different genomes.

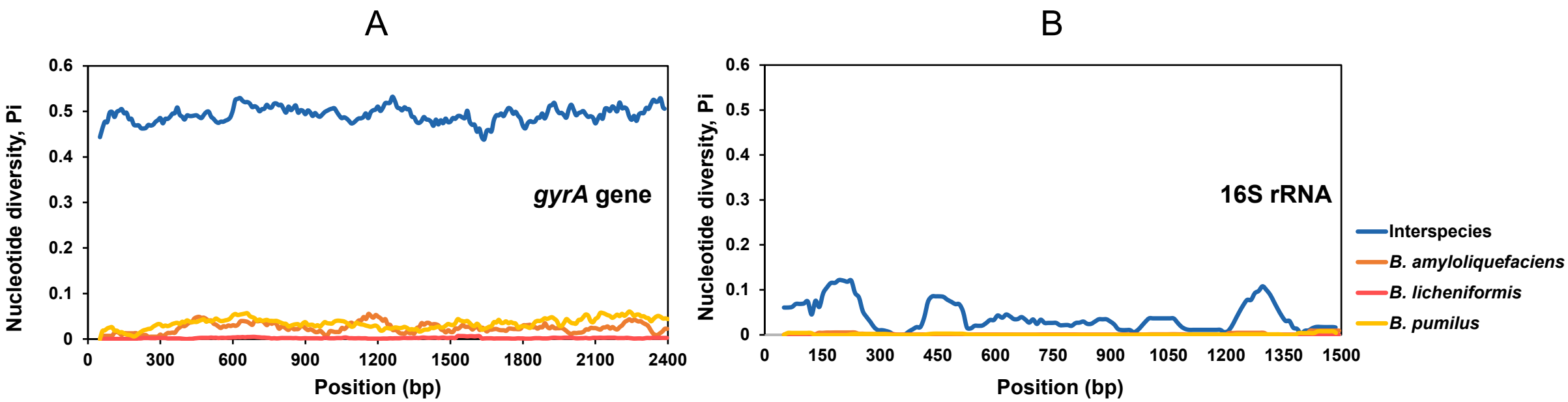
Figure S5 Polymorphisms in the 16S rRNA and *gyrA* gene's regions of *B. megaterium* (117 genomes). **(A)** The proportion of variation at different base sites along 16S rRNA gene V3-V4 region. **(B)** The proportion of variation at the different base positions of the *gyrA* gene region. **(C)** The proportion of variants along 16S rRNA (red column) and *gyrA* gene (blue column) region in different genomes.

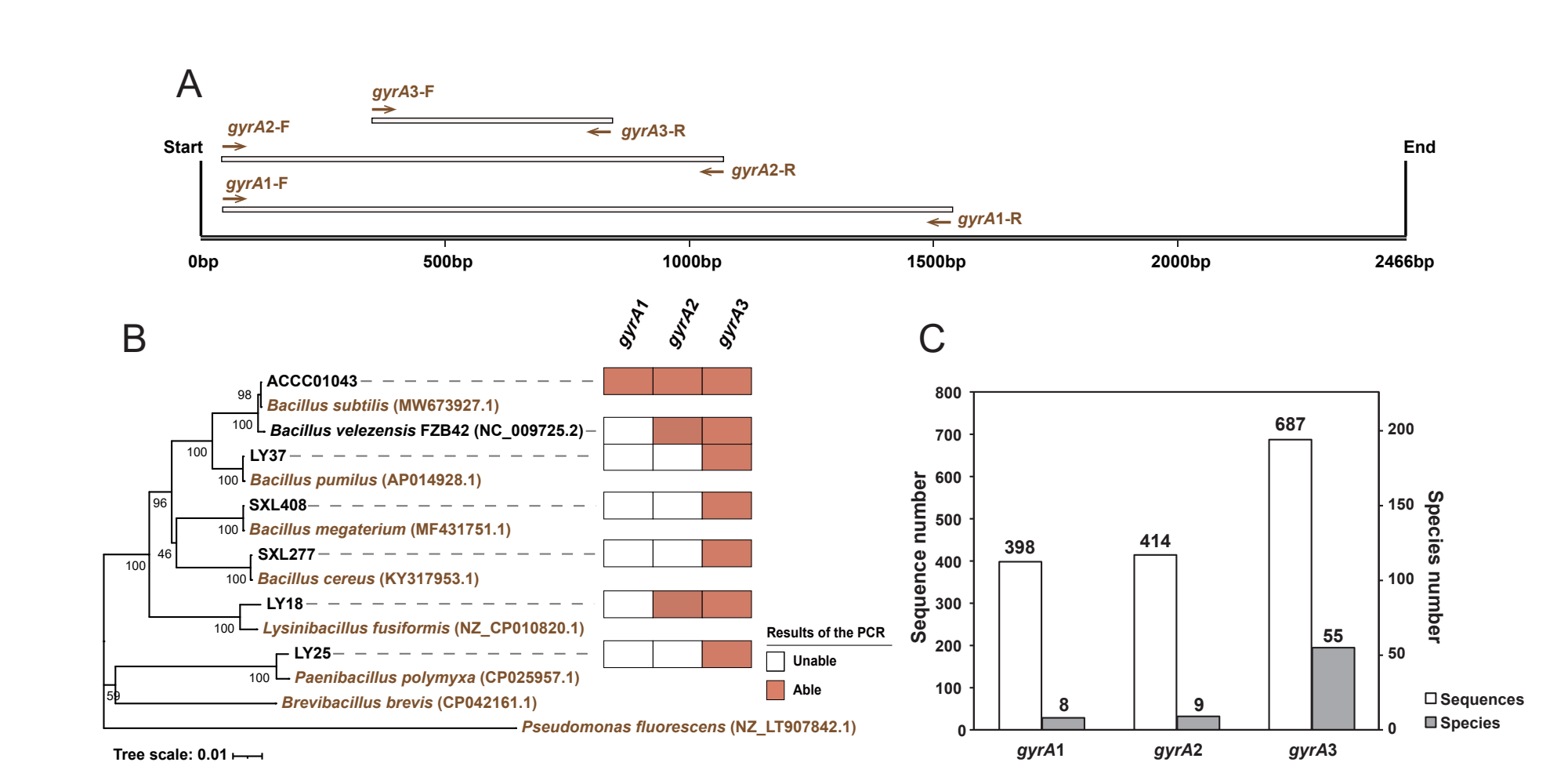
Figure S6 Polymorphisms in the 16S rRNA and *gyrA* gene's region of *B. anthracis* (226 genomes). **(A)** The proportion of variation at different base sites along the 16S rRNA gene **(B)** and the *gyrA* gene. **(C)** The proportion of variable base sites in 16S rRNA (red column) and *gyrA* (blue column) sequences in different genomes.

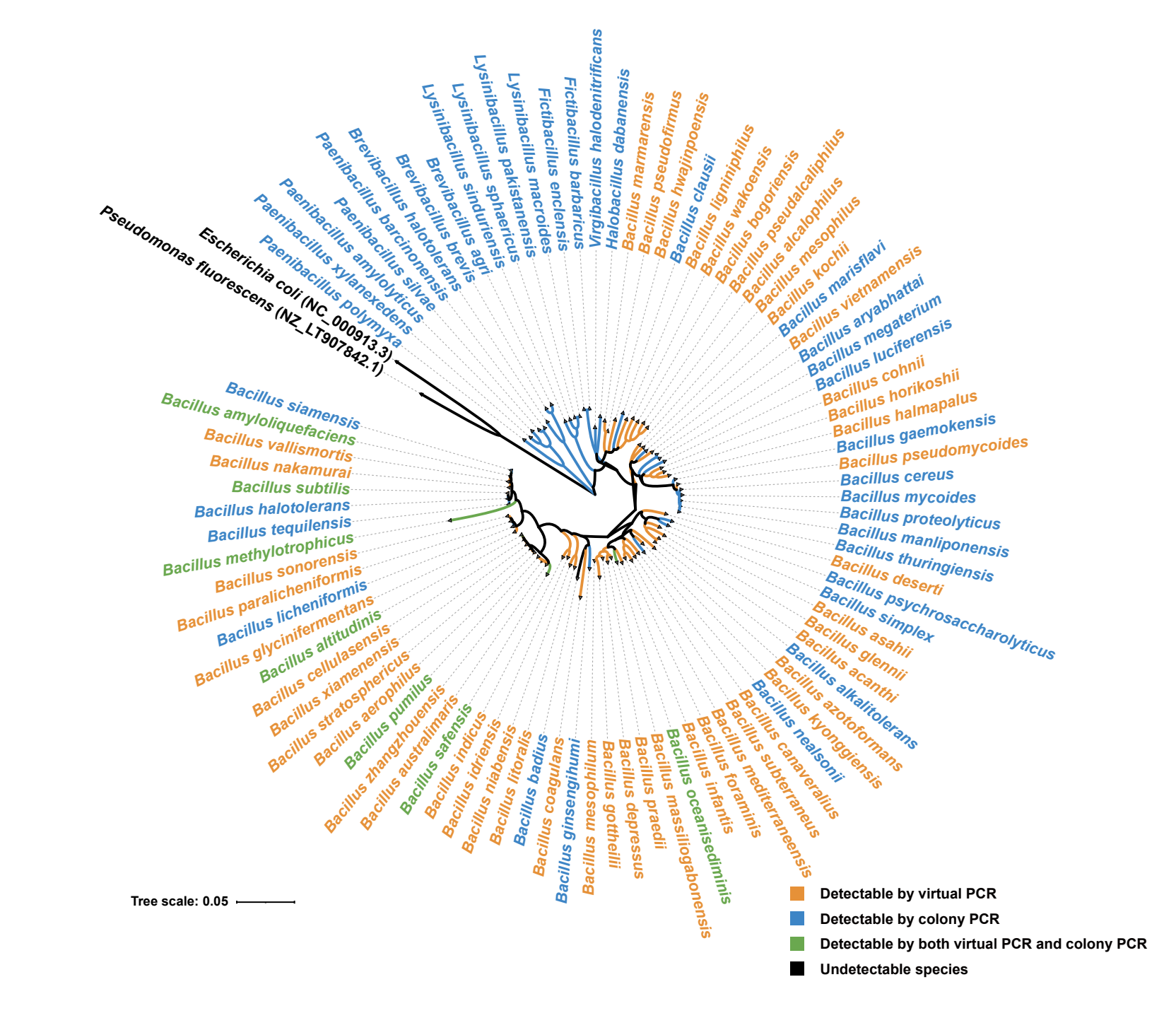
Figure S7 Description of *gyrA* gene primer pairs for amplicon sequencing. (**A)** The position of *gyrA* amplicons obtained by primer pairs *gyrA*3, *gyrA*4 and *gyrA*5. (**B)** PCR amplification of eight *Bacillus* strains by *gyrA* gene primer pairs. The orange square indicates positive and white square unsuccessful amplification. The phylogenetic tree was contracted based on the complete *gyrA* genes (2469bp) (Dataset S3) by using Neighbor-Joining method in MEGA 5.05 software. The reliability of clades was tested by the 1000 bootstrap replications.

Table 1 Polymorphisms of the 16S rRNA and *gyrA* gene

Species	Gene	Gene length (bp)	Number of variable sites	Total number of genomes	Genomes number with variable bases	Range of genomes variable sites	Range of genomes variable sites (%)
Bacillus amyloliquefaciens	16S	480	12	116	4	1-7	0.21-1.46
	gyrA	500	59		109	2-32	0.4-6.4
Bacillus pumilus	16S	480	21	140	11	1-17	0.21-3.54
	gyrA	500	130		87	1-79	0.2-15.8
Bacillus megaterium	16S	480	3	117	48	1-2	0.21-0.42
	gyrA	500	58		99	1-14	0.2-2.8
Bacillus anthracis	16S	480	13	226	7	1-5	0.21-1.04
	gyrA	500	65		18	1-37	0.2-7.4


A
B
Nucleotide diversity, Pi
0.6
0.5
0.4
0.3
0.2
0.1
0
0
300
600
900
1200
1500
1800
2100
2400
Position (bp)
gyrA gene
16S rRNA
0
150
300
450
600
750
900
1050
1200
1350
1500
Interspecies
B. amyloliquefaciens
B. licheniformis
B. pumilus

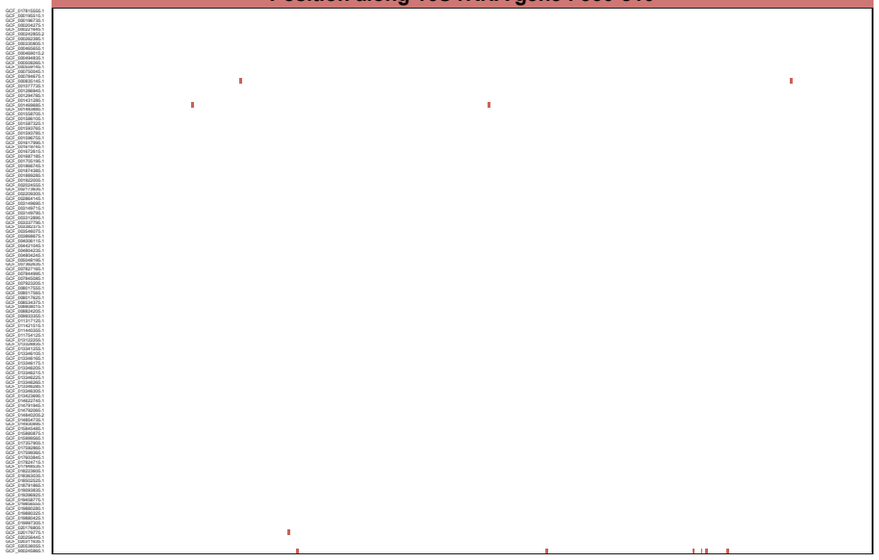

A
gyrA3-F
gyrA2-F
gyrA3-R
Start
End
gyrA2-R
gyrA1-F
gyrA1-R
0bp
500bp
1000bp
1500bp
2000bp
2466bp
B
gyrA1
gyrA2
gyrA3
ACCC01043
Bacillus subtilis (MW673927.1)
Bacillus velezensis FZB42 (NC_009725.2)
LY37
Bacillus pumilus (AP014928.1)
SXL408
Bacillus megaterium (MF431751.1)
SXL277
Bacillus cereus (KY317953.1)
LY18
Lysinibacillus fusiformis (NZ_CP010820.1)
LY25
Paenibacillus polymyxa (CP025957.1)
Brevibacillus brevis (CP042161.1)
Pseudomonas fluorescens (NZ_LT907842.1)
98
100
100
100
96
100
46
100
100
100
59
Results of the PCR
Unable
Able
Tree scale: 0.01
C
Sequence number
Species number
800
700
600
500
400
300
200
100
0
200
150
100
50
0
398
8
414
9
687
55
gyrA1
gyrA2
gyrA3
Sequences
Species


Pseudomonas fluorescens (NZ_LT907842.1)
Escherichia coli (NC_000913.3)
Paenibacillus xylanexedens
Paenibacillus polymyxa
Paenibacillus amylolyticus
Paenibacillus barcinonensis
Paenibacillus silvae
Brevibacillus halotolerans
Brevibacillus brevis
Lysinibacillus agri
Lysinibacillus sinduriensis
Lysinibacillus sphaericus
Lysinibacillus pakistanensis
Lysinibacillus macroides
Fictibacillus enclensis
Fictibacillus barbaricus
Virgibacillus halodenitrificans
Halobacillus dabanensis
Bacillus marmarensis
Bacillus pseudofirmus
Bacillus hwajinpoensis
Bacillus clausii
Bacillus ligniniphilus
Bacillus wakoensis
Bacillus bogoriensis
Bacillus pseudalcaliphilus
Bacillus alcalophilus
Bacillus mesophilus
Bacillus kochii
Bacillus marisflavi
Bacillus vietnamensis
Bacillus aryabhattai
Bacillus megaterium
Bacillus luciferensis
Bacillus cohnii
Bacillus horikoshii
Bacillus halmapalus
Bacillus gaemokensis
Bacillus pseudomycoides
Bacillus cereus
Bacillus mycoides
Bacillus proteolyticus
Bacillus manliponensis
Bacillus thuringiensis
Bacillus deserti
Bacillus psychrosaccharolyticus
Bacillus simplex
Bacillus asahii
Bacillus glennii
Bacillus acanthi
Bacillus alkalitolerans
Bacillus azotoformans
Bacillus kyonggiensis
Bacillus nealsonii
Bacillus canaveralius
Bacillus subterraneus
Bacillus mediterraneensis
Bacillus foraminis
Bacillus infantis
Bacillus oceanisediminis
Bacillus massiliogabonensis
Bacillus praedii
Bacillus depressus
Bacillus gottheilii
Bacillus mesophilum
Bacillus ginsengihumi
Bacillus coagulans
Bacillus badius
Bacillus litoralis
Bacillus niabensis
Bacillus idriensis
Bacillus indicus
Bacillus safensis
Bacillus australimaris
Bacillus zhangzhouensis
Bacillus pumilus
Bacillus aerophilus
Bacillus stratosphericus
Bacillus xiamenensis
Bacillus cellulasensis
Bacillus altitudinis
Bacillus glycinifermentans
Bacillus licheniformis
Bacillus paralicheniformis
Bacillus sonorensis
Bacillus methylotrophicus
Bacillus tequilensis
Bacillus halotolerans
Bacillus subtilis
Bacillus nakamurai
Bacillus vallismortis
Bacillus amyloliquefaciens
Bacillus siamensis
Tree scale: 0.05
Detectable by virtual PCR
Detectable by colony PCR
Detectable by both virtual PCR and colony PCR
Undetectable species

Bacillus amyloliquefaciens

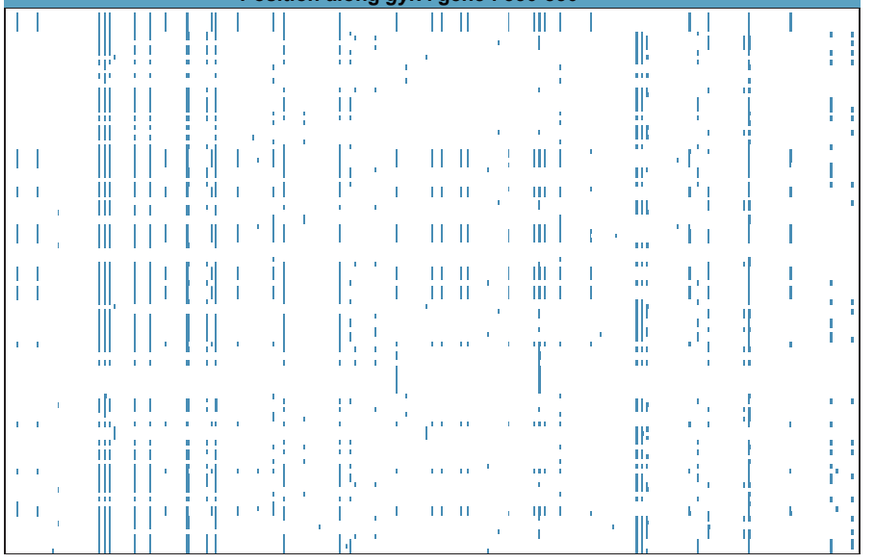
A

Position along 16S rRNA gene : 330-810



B

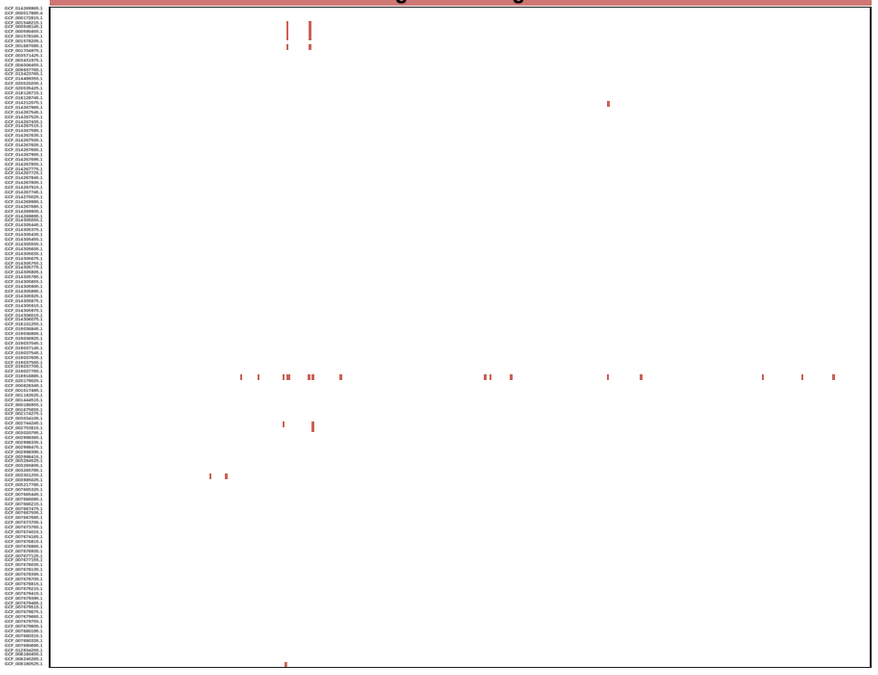
Position along *gyrA* gene : 350-850



Bacillus pumilus

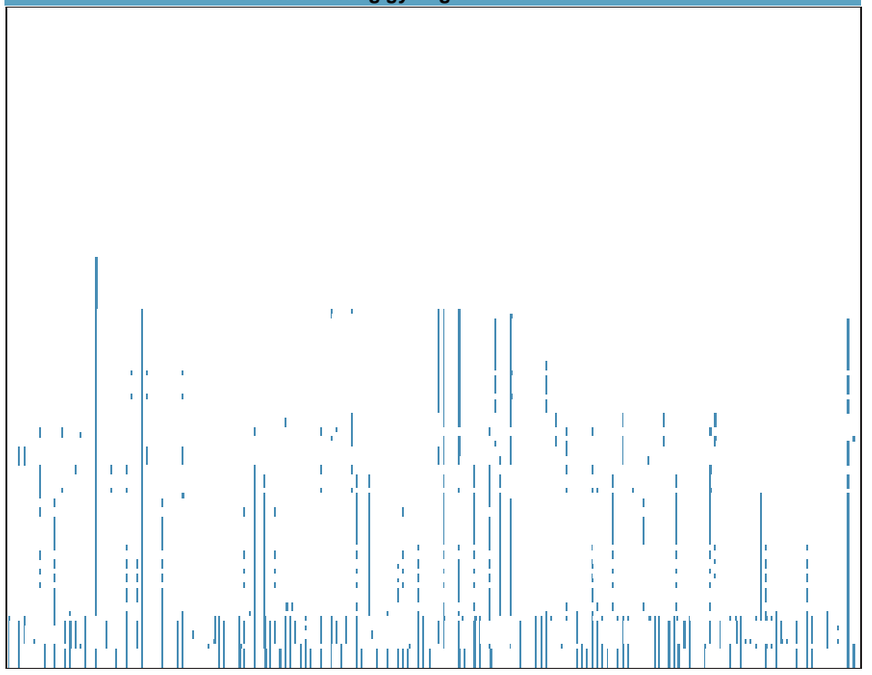
C

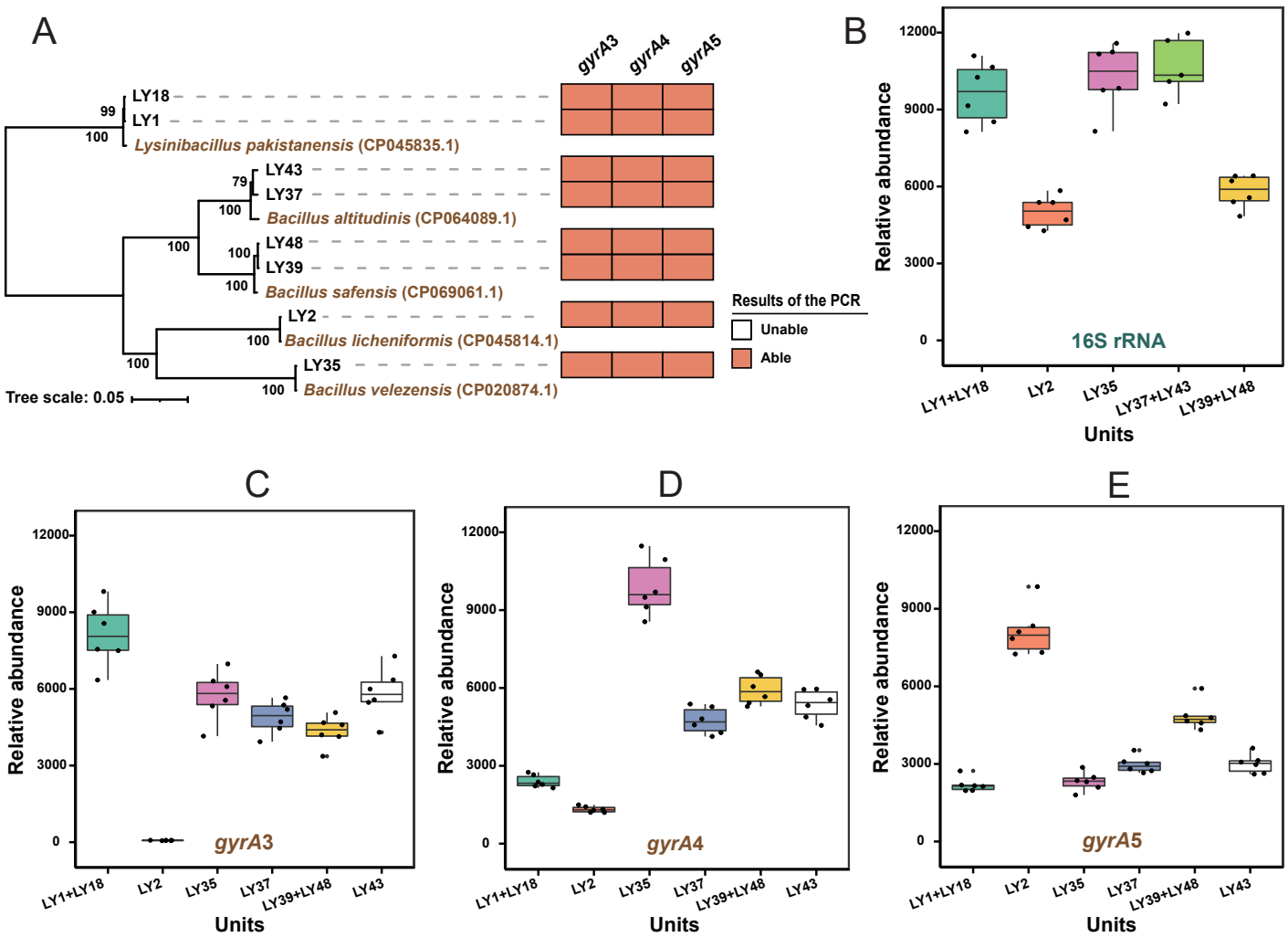
Position along 16S rRNA gene : 330-810



D

Position along *gyrA* gene : 350-850




A
gyrA3
gyrA4
gyrA5
LY18
LY1
Lysinibacillus pakistanensis (CP045835.1)
LY43
LY37
Bacillus altitudinis (CP064089.1)
LY48
LY39
Bacillus safensis (CP069061.1)
LY2
Bacillus licheniformis (CP045814.1)
LY35
Bacillus velezensis (CP020874.1)
99
100
79
100
100
100
100
100
100
Tree scale: 0.05
Results of the PCR
Unable
Able
B
Relative abundance
16S rRNA
LY1+LY18
LY2
LY35
LY37+LY43
LY39+LY48
Units
C
gyrA3
D
gyrA4
E
gyrA5
LY43
LY37
LY39+LY48
0
3000
6000
9000
12000