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# *gyrA* AND *rpoB* AS ALTERNATIVE MARKERS FOR BACTERIAL IDENTIFICATION

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**Abstract:** We evaluated the effectiveness of gyrA and rpoB as alternative markers for species-level bacterial identification when the 16S rRNA gene is insufficient, using *in silico* analysis and molecular genetic methods.

**Keywords:** 16S rRNA, gyrA, rpoB, nucleotide diversity

**Annotatsiya:** Bakteriyalarni tur darajasida identifikatsiya qilishda 16S rRNA geni yetarli bo‘lmagan holatlar uchun muqobil *gyrA* va *rpoB* markerlarining samaradorligi in silico tahlil va molekulyar-genetik usullar yordamida o‘rganildi.

**Kalit so‘zlar:** 16S rRNA, gyrA, rpoB, nukleotid diversligi

**Аннотация:** Эффективность альтернативных маркеров *gyrA* и *rpoB* для идентификации бактерий на видовом уровне в случаях, когда ген 16S рРНК недостаточен, оценивалась с помощью *in silico* анализа и молекулярно-генетических методов.

**Ключевые слова:** 16S рРНК, gyrA, rpoB, нуклеотидное разнообразие

The 16S rRNA gene is the most widely used marker for bacterial identification and phylogenetic analysis. However, because it is highly conserved, present in multiple copies per genome, and divergent among those copies, the 16S rRNA gene may be insufficient to reliably discriminate closely related species. Consequently, single-copy, protein-coding genes that evolve faster than 16S rRNA are being considered as alternative markers, notably *gyrA*, which encodes the DNA *gyra*se A subunit, and *rpoB*, which encodes the RNA polymerase β subunit.

For the *in silico* analysis, complete genomes of *Bacillus* representatives were retrieved from the NCBI GenBank database, and the 16S rRNA, *gyrA*, and *rpoB* sequences were extracted from them. Intraspecific variability was assessed in a total of 230 genomes of *B. subtilis* (93), *B. cereus* (79), and *B. safensis* (58). Interspecific variability was examined in a dataset of 283 genomes representing 17 species. Sequences were aligned with MUSCLE, nucleotide diversity (π) was calculated in DnaSP using a 10-bp sliding window, and the distribution of SNPs along the genes was analyzed. In the interspecific comparison, *gyrA* exhibited the highest nucleotide diversity ($\pi \approx 0.15$–$0.28$), *rpoB* showed intermediate values ($\pi \approx 0.06$–$0.27$), and the 16S rRNA gene was characterized by very low diversity ($\pi \approx 0.01$–$0.03$, reaching $\approx 0.11$ only in isolated variable regions). Within species, the 16S rRNA gene was nearly invariant ($\pi \approx 0$), π in *rpoB* did not exceed 0.03, and π in *gyrA* rose to ≈ 0.09, with the highest value observed in *B. cereus*.

Five bacterial isolates (N1, N2, N3, N4, and N5) were used in this study. Genomic DNA was extracted from the isolates by the Marmur method. The 16S rRNA gene was amplified by PCR with the universal primers 27F and 1492R under the following conditions: initial denaturation at 95 °C for

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3 min; 30 cycles of denaturation at 95 °C for 40 s, annealing at 56 °C for 40 s, and extension at 72 °C for 1.5 min; and a final extension at 72 °C for 7 min. Amplification products were verified by electrophoresis on a 0.8% agarose gel and sequenced on a Sanger sequencer. The resulting sequences were compared against the NCBI BLAST database, and the isolates were identified to the genus level.

A fragment of the *gyrA* gene was amplified by PCR with the degenerate primers:

*gyrA*3-F (5′-GCDGCHGCNATGCGTTAYAC-3′)

*gyrA*3-R (5′-ACAAGMTCWGCKATTTTTTC-3′)

PCR was initially performed under the following conditions: initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 40 s, annealing at 56 °C for 40 s, and extension at 72 °C for 40 s; and a final extension at 72 °C for 7 min. However, owing to the high degeneracy of the primers and the low Tm of the reverse primer, these conditions yielded nonspecific products. A stepwise annealing program was therefore applied, consisting of 2 cycles each at 50, 51, 52, 53, and 54 °C (10 cycles in total), followed by 10 cycles at 55 °C and 10 cycles at 56 °C (30 cycles in total). The denaturation and extension steps were left unchanged. Amplification products were verified by electrophoresis on a 0.8% agarose gel.

A fragment of the *rpoB* gene was amplified by PCR with the degenerate primers:

rpoB_F (5′-GGYTWYGAAGTNCGHGACGTDCA-3′)

rpoB_R (5′-TGACGYTGCATGTTBGMRCCCATMA-3′)

PCR was performed under the following conditions: initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 40 s, annealing at 56 °C for 40 s, and extension at 72 °C for 40 s; and a final extension at 72 °C for 7 min. Amplification products were verified by electrophoresis on a 0.8% agarose gel.

The *in silico* analysis showed that, in the genus *Bacillus*, *gyrA* and *rpoB* have higher nucleotide diversity than the 16S rRNA gene (π: *gyrA* ≈ 0.15–0.28, *rpoB* ≈ 0.06–0.27, 16S rRNA ≈ 0.01–0.03). Whereas the 16S rRNA gene was nearly invariant within species, variability was detected in both *gyrA* and *rpoB*. These genes are therefore promising supplementary markers to 16S rRNA for discriminating closely related species. The conditions for amplifying *gyrA* and *rpoB* fragments were optimized. *gyrA* and *rpoB* can serve as effective supplementary markers for identifying closely related species that 16S rRNA cannot resolve.

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