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Molecular Characterization of Lipolytic Isolate BL23: Evaluating the Efficacy of 16S rRNA Gene as a Single Marker for Bacterial Taxonomy

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ABSTRACT

Isolate BL23 is a lipolytic bacterium isolated from cashew nut shell waste in Southeast Sulawesi; however, its specific identity remains unknown. The goal of this study was to identify isolate BL23 involving 16S rRNA gene as a single marker. Genomic DNA extraction of sample was performed using the Presto Mini gDNA Bacteria Kit, while 16S rRNA sequences was amplified involving a pair primer of 63F and 1387R with amplicon target was approximately 1300 bp. Bidirectional DNA sequencing was performed using the Sanger method. The results showed that the partial sequence of the 16S rRNA gene from BL23 was found to have a G+C content of 50.87% and an A+T content of 49.13%. These values are consistent with those commonly observed within the genus Staphylococcus. Sequence alignment revealed that the partial 16S rRNA gene (1327 bp) of strain BL23 exhibited high similarity to Staphylococcus haemolyticus (99.77%), S. taiwanensis (99.70%), and S. borealis (99.70%). The Phylogenetic analysis and genetic distance clustered isolate BL23 most closely with S. haemolyticus, but these molecular data necessitated supplementary phenotypic characterization to confirm the taxonomic identity. These data demonstrate that the 16S rRNA gene is difficult to use as a single molecular marker for bacterial taxonomy, although it is useful for initial identification.

Keywords : Identification, Lipolytic Bacteria, 16S rRNA, Staphylococcus, Taxonomy

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INTRODUCTION

Isolate BL23 is a lipolytic bacterium isolated from cashew nut shell waste in Southeast Sulawesi; however, its specific identity remains unknown. Lipolytic bacteria are organisms that produce lipase enzymes, which break down triacylglycerols into glycerol and free fatty acids (Syah et al. 2023). These enzymes are valuable for various biotransformation in the detergent, biodiesel synthesis, and pharmaceutical fields (Mota et al. 2022; Parandi et al. 2022). Bacterial lipases possess a broad substrate spectrum, making them highly effective catalysts for diverse biotransformation.

Each bacterial species possesses specific catalytic capabilities; therefore, determining the identity of a bacterium is essential prior to its future application. Fundamentally, bacterial identification can be conducted through conventional methods involving morphological data, biochemical assays, and physiological tests. However, this approach has limitations, primarily concerning inconsistencies in character expression that depend on environmental growth factors, as well as the extensive data required for species determination. Consequently, molecular techniques have emerged as a more robust and efficient alternative for high-precision species determination.

The 16S rRNA gene is widely recognized as the primary molecular marker for the initial taxonomic classification of lipolytic bacteria; however, incorporating supplementary housekeeping

genes can improve the sensitivity of identification (Syah 2022; Syah et al. 2024). Sized at approximately 1,500 bp, the gene features highly conserved regions within a single species, alongside variable domains that facilitate differentiation across genera. Despite its utility, using the 16S rRNA gene as the sole molecular marker presents challenges. High sequence similarity among closely related species often complicates definitive taxonomic resolution, as exemplified by Syah (2022), who reported a greater than 99% 16S rRNA gene sequence similarity between Ralstonia pickettii and Ralstonia mannitolytica.

Molecular identification of lipolytic bacteria primarily relies on 16S rRNA gene metrics, specifically sequence similarity, genetic distance, and phylogenetic topology. According to established taxonomic standards, a similarity threshold of at least 99%, coupled with minimal genetic distance and the formation of a monophyletic clade, is required for species-level assignment. Therefore, characterizing the 16S rRNA gene of isolate BL23 is imperative to resolve its taxonomic identity with precision.

RESEARCH METHODS

Materials and Sample Sources

The main reagents consisted of Go Taq Green Master Mix (Promega, USA), Primer 63F, Primer 1387R, Nuclease Free Water (NFW), Luria Bertani (LB), Isopropanol, Presto Mini gDNA Bacteria Kit (Geneaid, Taiwan), Ethidium Bromide, Tris Acetic EDTA (TAE), Agarose, Distilled Water, Loading Dye (Promega), Ladder 1 Kb (Promega, USA), and Alcohol 70%. Isolate BL23, a lipolytic bacterium used in this study, was isolated from cashew nut shell waste in Southeast Sulawesi.

DNA Genomic Extraction and Electrophoresis

To prepare the sample, a single colony of isolate BL23 was grown in Luria Bertani (LB) broth for 16 hours at ambient temperature. This culture served as the substrate for genomic DNA extraction, which was performed using the Presto Mini gDNA Bacteria Kit (Geneaid, Taiwan) following the provided instructions. The integrity of the isolated DNA was assessed via 1% (w/v) agarose gel electrophoresis. A mixture containing 4 µl of genomic DNA and 1 µl of 6x loading dye was loaded into the wells of a 1% (w/v) agarose gel. Electrophoresis was performed in 1x TAE buffer at 100 V and 400 mA for 60 min. Following electrophoresis, the gel was stained with ethidium bromide for 10 min and destained in distilled water for 5 min. Genomic DNA bands were visualized using a Bio-Rad Gel Doc system under UV illumination, where successful extraction was confirmed by clear, high-molecular-weight bands near the wells.

Amplification of 16S rRNA Gene

The 16S rRNA gene of isolate BL23 was amplified using a Bio-Rad thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The final 10 µl PCR mixture contained genomic DNA template (0.5 µl), forward primer 63F (1 µl), reverse primer 1387R (1 µl), GoTaq® Green Master Mix (2x concentration, 5 µl), and nuclease-free distilled water (2.5 µl) to achieve the final volume. The components were briefly mixed by gentle pipetting and centrifuged briefly in a microcentrifuge to collect the reaction mixture at the bottom of the tube.

The PCR thermal cycling profile was carried out with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. A final extension step was performed at 72°C for 7 min. The amplification targeted a fragment of approximately 1300 bp (Syah, 2022). The resulting PCR products were validated via electrophoresis on a 1% (w/v) agarose gel; notably, no additional loading dye was required as the GoTaq® Green Master Mix already contains a density agent and tracking dyes.

DNA Sequencing

Bidirectional DNA sequencing was performed using the Sanger method. Prior to sequencing, the 16S rRNA gene amplicons were purified using the ExoSAP-IT™ enzymatic method. Specifically, 5 µl of PCR product was treated with a mixture of 1 µl Exonuclease I (Exo I) and 1 µl Shrimp Alkaline Phosphatase (SAP). The reaction mixture was briefly centrifuged and incubated at 37°C for 15 min for enzymatic digestion, followed by inactivation at 80°C for 15 min.

The purified products were subsequently used as templates for cycle sequencing using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Each sequencing reaction (final volume ~25 µl) consisted of 17.3 µl nuclease-free water, 4.42 µl BigDye Buffer, 1.26 µl BigDye Terminator Ready Reaction Mix, 1.01 µl of either primer 63F or 1387R, and 0.25 µl of the purified 16S rRNA template.

Cycle sequencing was performed using the following thermal profile: initial denaturation at 96°C for 3 min; followed by 35 cycles of 96°C for 30 s, 55°C for 30 s, and 60°C for 1 min; and a final extension at 60°C for 5 min. The resulting cycle sequencing products were subsequently purified via a standard precipitation method. Specifically, 18 µl of a S-adenocylmethionine (SAM) and 4 µl of X-terminator beads were added to each reaction. The mixture was vortexed for 30 s, pulse-centrifuged at 1000 rpm for 2 min, and the supernatant transferred to a fresh sequencing microtube. Capillary electrophoresis was then performed using an Applied Biosystems Hitachi 3130 Genetic Analyzer.

Bioinformatics Analysis

Sequence chromatograms were analyzed and edited using the BioEdit software package (v7.0.9.0). Low-quality regions at the 5′ and 3′ ends of the raw sequences, characterized by poor signal intensity or overlapping peaks, were manually trimmed. High-quality sequence data were identified by the presence of distinct, well-resolved fluorescent peaks with minimal background noise and no signal overlap.

Sequence alignment and homology analysis were conducted using the Basic Local Alignment Search Tool (BLAST) via the National Center for Biotechnology Information (NCBI) platform. Taxonomic identification was determined based on two primary parameters: Query Coverage and the Expectation value (E-value). Alignments with 100% query coverage and an E-value of 0.0 (or approaching zero) were prioritized as indicators of high-confidence homology, although these values were interpreted in the context of sequence length and alignment depth (Syah et al. 2024).

Genetic distances were calculated using the Kimura 2-Parameter (K2P) model within the MEGA 6.0 software package. A phylogenetic tree was subsequently inferred using the Maximum Likelihood (ML) method. The robustness of the internal branches was assessed with 1,000 bootstrap replicates. The phylogeny was reconstructed using 39 reference species from the genus Staphylococcus; to minimize phylogenetic bias, all sequences were trimmed to a uniform length prior to analysis.

RESULTS AND DISCUSSION

Genomic DNA Extraction

Electrophoretic analysis on a 1% (w/v) agarose gel revealed distinct fluorescent bands under UV illumination, confirming the successful extraction of genomic DNA from isolate BL23 (Fig. 1). The observed fluorescence resulted from the intercalation of ethidium bromide into the DNA double helix, with the signal intensity serving as a qualitative indicator of DNA concentration. The integrity and yield of the extracted genomic DNA provided a high-quality template, which was essential for the subsequent amplification of the 16S rRNA gene.

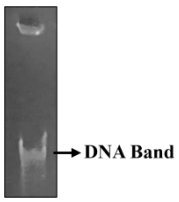

Figure 1. DNA Genomic of BL23 on 1% Agarose Gel Under UV Irradiation

According to the protocol provided by New England Biolabs, DNA template concentrations between 1 ng and 1 µg are required for effective Taq-mediated amplification. Increasing the DNA concentration may decrease specificity by promoting non-specific product formation, especially during extended thermocycling. To prioritize fidelity, it is recommended to use higher initial DNA concentrations while reducing the total number of cycles.

Amplification of 16S rRNA Gene

Visualization of the PCR products on a 1% agarose gel confirmed the presence of a target fragment at approximately 1300 bp relative to the 1 kb molecular weight marker (Fig. 2). The successful amplification of this partial 16S rRNA gene fragment aligns with established literature using the 63F and 1387R primers, which typically generate amplicons of this size (Azizah et al. 2025). Furthermore, the absence of non-specific bands suggests a high degree of primer specificity and reaction optimization.

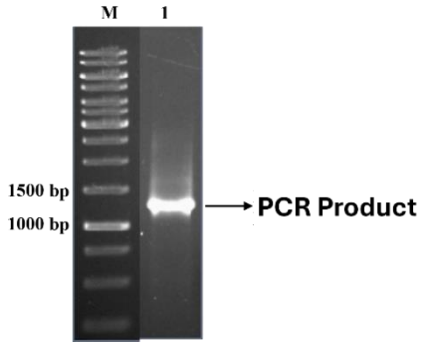

Figure 2. Amplification Product of 16S rRNA Gene on 1% Electrophoresis Gel, M : DNA Ladder 1 Kb; 1 : BL23 Isolate

Electrophoretic analysis was performed to verify PCR product integrity; a single, clear band confirmed that the samples were free from degradation and non-specific amplification (Fig. 2). To satisfy Sanger sequencing requirements, template concentrations were normalized based on fragment length, following the general guideline of 1.5 ng/µL per 100 bp (microsynth.com). For amplicons between 1000 and 2000 bp, a target concentration of 30 ng/µL was maintained to ensure high-quality sequencing traces.

Sequence Analysis of 16S rRNA Gene

Analysis of the resulting chromatogram revealed that the sequenced 16S rRNA gene fragment from strain BL23 was 1327 bp in length, representing a partial sequence (Fig. 3). While a complete bacterial 16S rRNA gene typically measures approximately 1500 bp (Saul et al. 1994; de Los Santos Villalobos et al. 2019), the observed length is dependent on the specific primer set utilized. The partial sequence of the 16S rRNA gene from BL23 was found to have a G+C content of 50.87% and an A+T content of 49.13%. These values are consistent with those commonly observed within the genus *Staphylococcus*.

Sequence alignment revealed that the partial 16S rRNA gene of strain BL23 exhibited high similarity to *Staphylococcus haemolyticus* (99.77%), *S. taiwanensis* (99.70%), and *S. borealis* (99.70%) (Table 1). Specifically, the sequence differed by only three nucleotides from *S. haemolyticus* and four nucleotides from both *S. taiwanensis* and *S. borealis*. Although these values exceed the threshold for species delineation-defined by Janda and Abbott (2007) as a minimum similarity of 99% and ideally above 99.5% for sequences exceeding 1300 bp. Definitive species identification remains challenging due to the identical similarity scores across multiple taxa.

Staphylococcus borealis and *S. taiwanensis* were originally delineated from *S. haemolyticus* despite nearly identical 16S rRNA gene sequences (99.86%, 99.87%, respectively). They were identified as distinct species through polyphasic analysis, which revealed an average nucleotide identity (ANI) of <95% and inferred DNA–DNA hybridization (DDH) of <70%. These genomic thresholds, combined with specific phenotypic traits, justify their status as separate taxa despite their high 16S rRNA sequence conservation (Pain et al. 2020; Lin et al. 2022; Zong et al. 2020). Moreover, phenotypic characterization remains a vital tool for delineating these closely related taxa; for instance, *S. borealis* differs from *S. haemolyticus* in its pigmentation, urease expression, and cellular fatty acid composition (Pain et al., 2020). Similarly, *S. taiwanensis* NTUH-S172T is distinguished from *S. haemolyticus* by its urease-positive status and from *S. borealis* by its nitrate reduction capacity (Lin et al. 2022).

Current bacterial taxonomy has evolved significantly, transitioning from a reliance on single molecular markers, such as the 16S rRNA gene, to a polyphasic approach that incorporates

phenotypic traits. For instance, *Staphylococcus argenteus* sp. nov. and *Staphylococcus schweitzeri* sp. nov. were proposed as novel species despite possessing 16S rRNA sequences and phenotypic characteristics nearly identical to *Staphylococcus aureus*. This reclassification was necessitated by genomic and biochemical metrics, specifically Average Nucleotide Identity (ANI) values below 95% and in silico DNA-DNA Hybridization (dDDH) values below 70%, alongside differences in peptidoglycan composition and distinct clinical and ecological niches (Tong et al. 2015).

Table 1. Multiple Sequences Alignment Score Using BLASTn

No	Genus Staphylococcus	Accesion Number	Perc. Identity (%)
1	*Staphylococcus haemolyticus*	L37600.1	99.77
2	*Staphylococcus taiwanensis*	NR_181843.1	99.70
3	*Staphylococcus borealis*	NR_181247.1	99.70
4	*Staphylococcus devriesei*	NR_116627.1	98.8
5	*Staphylococcus hominis*	L37601.1	98.72
6	*Staphylococcus lugdunensis*	MK015782.1	98.57
7	*Staphylococcus epidermidis*	NR_156818.1	98.42
8	*Staphylococcus cohnii* strain PL436	MK015771.1	98.34
9	*Staphylococcus pseudoxylosus*	NR_180150.1	98.34
10	*Staphylococcus saprophyticus subsp. saprophyticus*	NR_074999.2	98.34
11	*Staphylococcus xylosus*	LT797529.1	98.27
12	*Staphylococcus caeli*	NR_180106.1	98.27
13	*Staphylococcus nepalensis strain PL440*	MK015777.1	98.12
14	*Staphylococcus epidermidis*	L37605.1	98.04
15	*Staphylococcus aureus*	LN681573.1	98.04
16	*Staphylococcus warneri*	L37603.1	98.04
17	*Staphylococcus succinus*	MK015781.1	98.04
18	*Staphylococcus schweitzeri* strain DSM 28300	MF678907.1	98.04
19	*Staphylococcus gallinarum* strain PCM 2439	MK015772.1	97.97
20	*Staphylococcus roterodami*	NR_175559.1	97.97
21	*Staphylococcus argenteus*	NR_181984.1	97.89
22	*Staphylococcus saccharolyticus*	L37602.1	97.67
23	*Staphylococcus durrellii*	NR_181502.1	97.59
24	*Staphylococcus ratti* strain CCM 9025	-	97.52
25	*Staphylococcus equorum*	LT797519.1	97.44
26	*Staphylococcus simulans*	MK015778.1	97.36
27	*Staphylococcus muscae*	NR_104762.1	97.29

28	*Staphylococcus lloydii*	NR_181501.1	97.29
29	*Staphylococcus chromogenes*	MK015779.1	97.21
30	*Staphylococcus piscifermentans*	NR_116436.1	97.14
31	*Staphylococcus agnetis* strain PL439	MK015776.1	97.14
32	*Staphylococcus debuckii*	NR_174273.1	97.07
33	*Staphylococcus petteenkofer i*	NR_114596.1	96.99
34	*Staphylococcus condimenti*	NR_116435.1	96.99
35	*Staphylococcus rostri* strain DSM 21968	MF678902.1	96.84
36	*Staphylococcus pseudolugdunensis*	NR_115938.2	96.84
37	*Staphylococcus intermedius* strain PCM 2405	MK015768.1	96.69
38	*Staphylococcus pseudintermedius*	NR_042284.1	96.76
39	*Staphylococcus schleiferi*	NR_037009.1	96.61

Several species previously assigned to the genus *Staphylococcus* have undergone reclassification into the genus *Mammaliicoccus*, including *M. fleurettii*, *M. lentus*, *M. sciuri*, *M. stepanovicii*, and *M. vitulinus*. This genus was differentiated from *Staphylococcus* based on comprehensive analyses of 16S rRNA gene sequences, core-genome phylogenies, and overall genome relatedness indices (OGRI) (Madhaiyan et al. 2020).

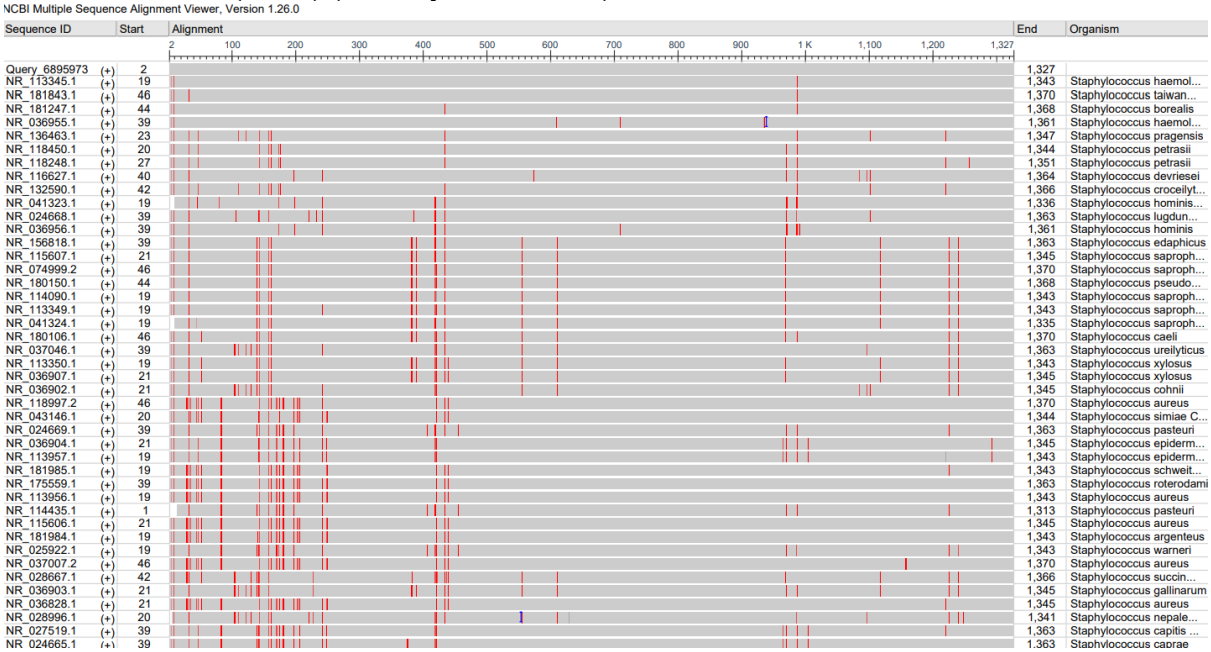

Fig. 3 The BLASTn Multiple Sequence Alignment (MSA) Viewer with Variation Site (Red)

Utilizing the BLASTn Multiple Sequence Alignment (MSA) Viewer, we identified sequence variations within nucleotides 90–319 (regions V1–V2) and 499–525 (region V3) (Fig. 3). These coordinates include a 63-nucleotide downstream offset necessitated by the use of the 63F universal primer. The high variability within these regions provides distinctive molecular markers for species-

level differentiation, consistent with the framework established by Shahi et al. (2017). Furthermore, López-Aladid (2023) recently validated the utility of the V1–V2 hypervariable regions for high-resolution taxonomic identification, particularly within complex clinical such as sputum samples.

Genetic Distance Analysis

Genetic distance analysis using the Kimura 2-parameter (K2P) method revealed minimal divergence between isolate BL23 and *Staphylococcus haemolyticus* (0.001), *Staphylococcus borealis* (0.002), and *Staphylococcus taiwanensis* (0.002) (Fig. 4). These results correlate with high sequence similarity among the three species. The close relationship is consistent with recent taxonomic revisions where *S. borealis* and *S. taiwanensis* were differentiated from the *S. haemolyticus* group [Pain et al., 2020; Lin et al., 2022]. This indicates that isolate BL23 shares a very close evolutionary lineage with these three species. Furthermore, analysis of the partial 16S rRNA gene (1,327 bp) showed that genetic distances up to 0.029 fall within the established range for the *Staphylococcus* genus. While the genetic distance value alone is insufficient for definitive species identification, the data strongly suggest that isolate BL23 belongs to one of these three closely related species. The Kimura 2-parameter (K2P) method estimates genetic distance by accounting for the differing rates of transition and transversion mutations.

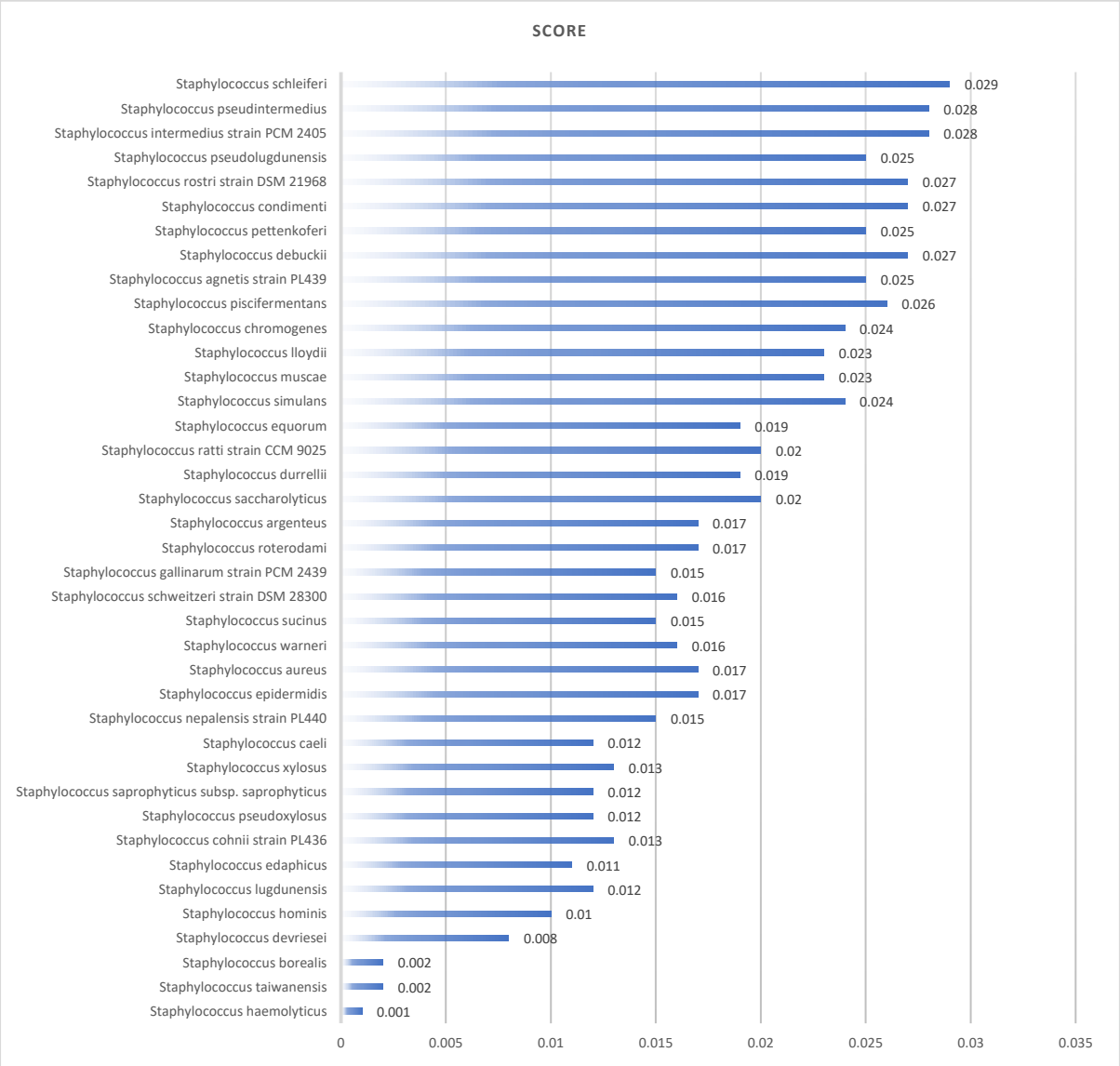

Figure 4. Genetic Distance of BL23 compared to other species of genus *Staphylococcus*

Phylogenetic Relationship

Phylogenetic reconstruction demonstrated that isolate BL23 forms a monophyletic clade with *Staphylococcus haemolyticus*, clustering in a subclade that includes *S. taiwanensis* and *S. borealis* (Fig. 5). This strong genetic association suggests BL23 shares a recent common ancestor with these species. The observed high relatedness is consistent with the fact that *S. taiwanensis* and *S. borealis* were recently reclassified from the original *S. haemolyticus* group due to their high 16S rRNA gene sequence similarity (>99%).

These findings are further supported by corresponding of small genetic distances and high sequence similarity. The clustering pattern indicates that Isolate BL23 is genetically very similar to these three closely related species. Determining its exact species requires further analysis, such as whole-genome sequencing or specific phenotypic tests, as 16S rRNA analysis alone has limitations in distinguishing highly similar species. The clustering patterns remained consistent across different phylogenetic reconstruction methods, including Neighbor-Joining, UPGMA, and Minimum Evolution (ME).

Important considerations must be addressed prior to phylogenetic tree reconstruction to ensure accuracy. These include the type of sequence data (i.e., partial or complete sequences) and the algorithmic model selected for analysis. When utilizing partial sequences, it is critical to ensure that the sequence length is uniform across all samples and reference sequences after alignment. Variations in sequence length can significantly influence the accuracy of the resulting phylogenetic tree. Conversely, using complete sequences generally negates the requirement for manual length adjustments or trimming, simplifying the data preparation stage.

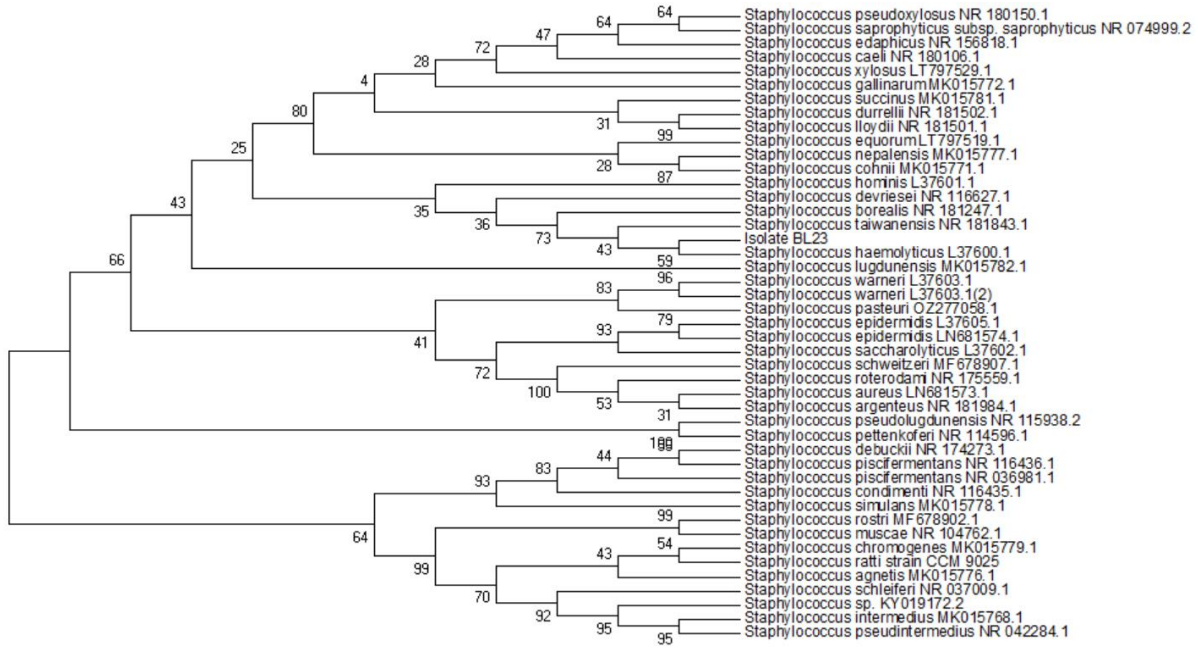

Figure 5. Phylogenetic tree was reconstructed using Maximum Likelihood and Kimura 2-Parameter with 1000 bootstrap replicates

Phylogenetic reconstruction methods are broadly categorized into two groups: distance-based methods, such as Neighbor-Joining (NJ), UPGMA, and Minimum Evolution (ME) (Balaban et al., 2022), and character-based methods, such as Maximum Likelihood (ML) (Felsenstein, 1973). Distance-based methods represent the simplest approach, utilizing the results of a distance matrix analysis between species to determine evolutionary distance and clustering (Mount, 2008). The probability of finding the best tree using the NJ approach decreases drastically as the number of sequences increases (Zou et al., 2024). Despite limitations, NJ methods are applicable to large datasets (Desper et al., 2006; Tamura et al., 2004), while UPGMA sequentially groups or compares the most similar taxa and is generally not recommended for analyzing definitive evolutionary history.

Character-based methods analyze specific substitution patterns within DNA sequences to evaluate multiple topological hypotheses, ultimately selecting the optimal tree based on defined

statistical or parsimonious criteria. Maximum Likelihood approaches utilize explicit models of nucleotide substitution to evaluate the likelihood of various topologies, identifying the optimal tree through a rigorous search of the phylogenetic space (De Maio et al. 2023). Moreover, Maximum Parsimony method examines individual site variations to reconstruct ancestral states, evaluating competing hypotheses to identify the tree requiring the fewest evolutionary transitions (Tamura et al. 2011).

CONCLUSION

While the 16S rRNA gene sequence of isolate BL23 exhibited high similarity to Staphylococcus haemolyticus (99.77%), definitive species-level identification remained inconclusive due to comparable similarity scores with Staphylococcus borealis (99.7%) and Staphylococcus taiwanensis (99.7%). The Phylogenetic analysis and genetic distance calculations clustered isolate BL23 most closely with S. haemolyticus, but these molecular data necessitated supplementary phenotypic characterization to confirm the taxonomic identity. These data demonstrate that the 16S rRNA gene is difficult to use as a single molecular marker for bacterial taxonomy, although it is useful for initial identification.

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