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Original Article

Molecular Identification and Phylogenetic Characterization of Actinomycetes from Libyan Soils via 16S rRNA Gene Sequencing

Khalia Esmail*[1], Adham Alshuwayhidi[2]

[1]*Department of Genetic Engineering, College of Medical Technology, Derna, Libya*
[2]*Department of Laboratory Medicine, College of Medical Technology, Derna, Libya*
Email. *khaliarmd6666@gmail.com*

Abstract
Molecular and genetic analysis of actinomycetes is crucial because these soil bacteria produce the majority of natural antibiotics. This investigation sought to identify actinomycetes using the 16S rRNA gene sequencing technique. Actinomycetes were isolated from soil samples collected near Derna, northeastern Libya. Starch casein medium supplemented with tetracycline and nystatin was employed to isolate actinomycete colonies. Eight isolates were obtained from twenty soil samples collected. DNA was extracted, the 16S rRNA gene was amplified via universal primers 27F/1492R, and the products were sequenced. Sequences were processed using bioinformatics software, compared against the NCBI database, and used to construct a phylogenetic tree in MEGA X via the maximum parsimony method. Five of the eight obtained samples represent sequence identity percentages ranging from 99.28% to 99.63%, which is similar to three registered strains: *Streptomyces monomycini* ATHUBA 220, *Streptomyces olivaceiscleroticus* CSSP709, and *Streptomyces olivaceiscleroticus* JCM 4805. These isolates were successfully deposited in GenBank under accession numbers. One isolate was morphologically similar to *Streptomyces* but was later characterized as *Alterluteimonas muca* MHLX1A. The other two isolates were identified as *Streptomyces ramulosus* NBRC 12812 and *Streptomyces monomycini* ATHUBA 220. However, their sequence similarity was below 98.5% due to insufficient sequencing data, signifying they may represent novel species. Libyan soils exhibit a high diversity of actinomycetes; consequently, the use of advanced genetic markers – such as DNA-DNA hybridization, multi-locus sequence analysis, and whole-genome sequencing – is recommended for identifying novel strains and searching for their bioactivity.
Keywords. Actinomycetes, Streptomyces, 16S rRNA, Gene Sequencing, Phylogenetic Tree, Libyan Soil.

Introduction

Actinomycetes are recognized as prokaryotes that have a Gram-positive cell wall and resemble fungi in their filamentous morphology, slow growth, and production of exospores [1,2]. They are mainly found in soil environments, thriving within a pH range of 5.0 to 9.0. The majority of soil actinomycetes are obligate aerobes, depending on oxygen to support their growth and metabolic activities [3]. Their DNA compositions have high G+C content related to their unique, complicated genetic constitution [4-6].

Actinomycetes are responsible for producing about 70–80% of antimicrobial agents from microbial origin, with the bulk from Streptomyces species [7,8]. They additionally yield an assortment of secondary bioactive compounds including antitumor and antifungal agents, as well as key industrial enzymes [9,10]. Ecologically, actinomycetes have a vital role in the degradation of organic matter, assisting in nutrient recycling, and sustaining biogeochemical cycles essential for environmental health [11-13]. These bacteria also release a diverse set of volatile organic substances [11], among which geosmin contributes to the classic earthy smell of wet soil [14,15].

Traditional microbiological identification often lacks the sensitivity to detect genetic differences between closely related strains [16]. Advances in molecular biology have improved the techniques to identify and classify closely related actinomycetes according to their genetic structure [17]. The 16S rRNA gene is found in all prokaryotic cells and has a highly conserved nature; the gene's size of approximately 1500 base pairs makes it a suitable target for bioinformatics analyses. It is the most valued gene in bacterial taxonomy and phylogenetic studies [18-20]. In view of limited molecular studies in Libya, this study aims to molecularly identify and characterize actinomycetes strains isolated from Libyan soils utilizing 16S rRNA gene sequencing.

Methods

Study area

Samples were collected from the city of Derna and its surrounding forests. As represented in (Figure 1), Derna is located in northeastern Libya at 32.7676° N latitude and 22.6397° E longitude. Samples were randomly collected from the Al-Fataih, Bab Tobruq, Karsa, and Ras Al-Hilal regions, coded as A, B, C, and D, respectively. Sampling encompassed both rhizosphere and bulk soils.

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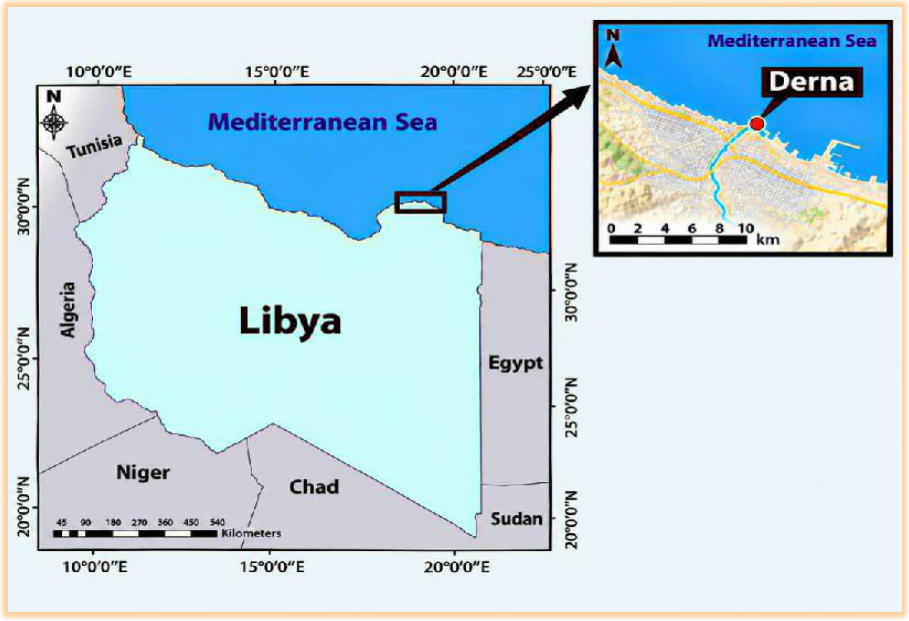


Figure 1. A map of the study area

Soil sampling and processing

Soil samples were compiled and treated as reported in the literature [5,21]. Twenty soil samples were collected using a sterile stainless-steel spoon from a depth of 5–15 cm, transferred into clean polyethylene bags, and stored in a refrigerator at 4 °C for 24 hours. Large particles were crushed using asterile pestle, and foreign particles and plant debris were removed employing a sieve. The soil samples were then spread on aluminum foil at room temperature for 3-7 days and turned over daily to dry and kill fast-growing Gram-negative bacteria. The pH of all samples was measured using pH strips. Ten grams of soil were suspended in 90 mL of 0.9% NaCl and shaken at 200 rpm for 20 minutes. A 1 mL aliquot of this mixture was then serially diluted into 9 mL volumes of 0.9% NaCl up to a 10^{-5} dilution.

Media and culture

Starch casein agar (SCA, Accumix, Microxpress, Goa, India) was prepared according to the manufacturer's instructions and autoclaved at 121 °C for 15 min, then allowed to cool to 50–55 °C. The antimicrobial compounds tetracycline (10 mg/L) and nystatin (50 mg/L) were then added. This was followed by gentle mixing and pouring into Petri dishes under sterile conditions, within a laminar flow hood. An aliquot of 0.1 mL of all sample dilutions was spread by sterile loops and incubated at 28 ◦C for 10-18 days; the resulting colonies of actinomycetes were Gram-stained and examined by a light microscope. Colonies were subcultured three times through streaking on SCA medium and incubated for 7-14 days at 30°C as a final purity check.

Isolation of genomic DNA

Actinomycetes colonies of the strains S1, S2, S3, S4, S5, S6, S7, and S8 were retrieved employing a sterile inoculating loop and suspended in 1.5 mL microcentrifuge tubes containing 0.5 mL sterile distilled water. The tubes were centrifuged at 10,000 rpm for 10 min, and the supernatant was discarded. The resulting pellet was resuspended in 0.5 mL of InstaGene Matrix (Bio-Rad, USA), incubated at 56 °C for 30 min, and subsequently heated at 100 °C for 10 min. The obtained supernatant after heat treatment was used as the DNA template for amplification by PCR.

Amplification of 16S rRNA sequences

PCR reactions were initiated by adding 1 µL of template DNA to a 20 µL reaction mixture containing the universal bacterial primer pair 27F/1492R. Amplification was performed exploiting ABI 7500 Real-Time PCR System, Assiut University Mycological Centre, Faculty of Science, Assiut University, Assiut, Egypt. The PCR amplification consisted of 35 cycles under the conditions: denaturation at 94 °C for 45 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s. The expected amplicon size was approximately 1,400 bp for actinomycetes. Each PCR run included a positive control (E. coli genomic DNA) and a negative control to ensure reliability. PCR products were purified from residual primers and unincorporated dNTPs using the Montage PCR Clean-Up Kit (Millipore), yielding purified amplicons suitable for sequencing.

Sequencing of amplified 16S rRNA gene

Purified PCR products (~1,400 bp) were sequenced at SolGent company, Daejeon, South Korea using primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (TAC GGY TAC CTT GTT ACG ACT T) [22]. Sequencing reactions were carried out

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with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA), and products were resolved on an Applied Biosystems 3730XL automated DNA sequencing system to generate high-quality sequence data for downstream analysis.

Phylogenetic tree construction

The assembly of the phylogenetic tree was conducted as reported by other studies [23-27]. Contiguous sequences of the actinomycete strains S1, S2, S3, S4, S5, S6, S7, and S8 were aligned by MAFFT (version 6.861b) with the most similar sequences downloaded from GenBank. Alignment gaps and parsimony-uninformative characters were treated by BMGE**.** Maximum parsimony (MP) phylogenetic analysis was performed employing MEGA X (version 10.2.6). The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications. Utilizing Model test 3.7's Akaike Information Criterion (AIC), the optimum nucleotide substitution model for MP analysis was selected. The phylogenetic tree was visualized using MEGA X (version 10.2.6), and the resulting tree was edited and saved as a TIF.

Results

Isolation of actinomycetes colonies from soil samples

Out of the twenty soil samples collected from four regions, eight isolates of actinomycetes were obtained from soils of the regions labelled A, C, and D with pH ranging from 8 to 9. However, no colonies were obtained from region B, with a pH spanning between 7 and 8. The morphological characteristics of the colonies on SCA media are displayed in (Table 1), and (Figure 2).

Table 1. Cultural characteristics of Actinomycete isolates on SCA (S: sample).

Sample source	Isolate Code	Colony shape	Size (mm)	Aerial mycelium color	Substrate mycelium color	Soluble pigment	Texture	Adhesion
A	S1	Wrinkled, flat	4-5	Gray	Dark brown	Yellow	Powdery	Moderate
	S2	Circular, raised	2-3	Gray	Brown	-	Powdery	Strong
	S3	Wrinkled, raised	2-3	Gray	Dark brown	Pale yellow	Powdery	Strong
C	S4	Wrinkled, raised	3-4	White	White	-	Chalky	Strong
	S5	Circular, raised	1-2	White	White	-	Powdery	Strong
	S6	Wrinkled, flat	1-2	White	Cream	-	Powdery	Moderate
D	S7	Wrinkled, raised	4-5	White	White	-	Powdery	Strong
	S8	Circular, raised	3-4	White	White	-	Powdery	Strong

Phylogenetic tree

As shown in (Figure 4), the phylogenetic tree illustrates the evolutionary relationships among the analyzed strains, featuring clear rooting, clade separation, and nodal support values. It is rooted with *Alterluteimonas muca* (strains MHLX1A and S2) as the basal taxon and *Nocardiopsis dassonvillei* DSM 43111 as the outgroup. The Major clades in the tree uncovered two lineages. These are lineage A, comprising *S. hygroscopicus*, *S. amphotericinicus*, and *S. endus*, altogether clustering with strong support (0.994). Reference strains of *S. ramulosus* originate from a sister branch (0.961). Lineage B is a larger, more diverse clade including *S. rimosus*, *S. chrestomyceticus*, *S. sclerotialus*, *S. monomycini*, *S. ochraceiscleroticus*, *S. olivaceiscleroticus*, and *S. niger*. Although strain S4 was referred to S. ramulosus, it clustered in Lineage A. The phylogenetic tree of the current investigation revealed its clustering within Lineage B on a long branch (0.3258). *S. monomycini* S7 and *S. olivaceiscleroticus* S8 formed a strongly supported branch (node = 1); however, S8 demonstrated a longer branch length. Furthermore, analysis of the unresolved nodes in the subclade encompassing *S. niger* and *S. olivaceiscleroticus* reference strains revealed near-identical sequences and a lack of phylogenetic resolution. The tree uncovered robust statistical support for major clades, with confidence values for deep nodes ranging between 0.968 and 0.994. Most of the terminal branches were extremely short (0.00000001–0.003), except for the elongated branches of S4 and S8.

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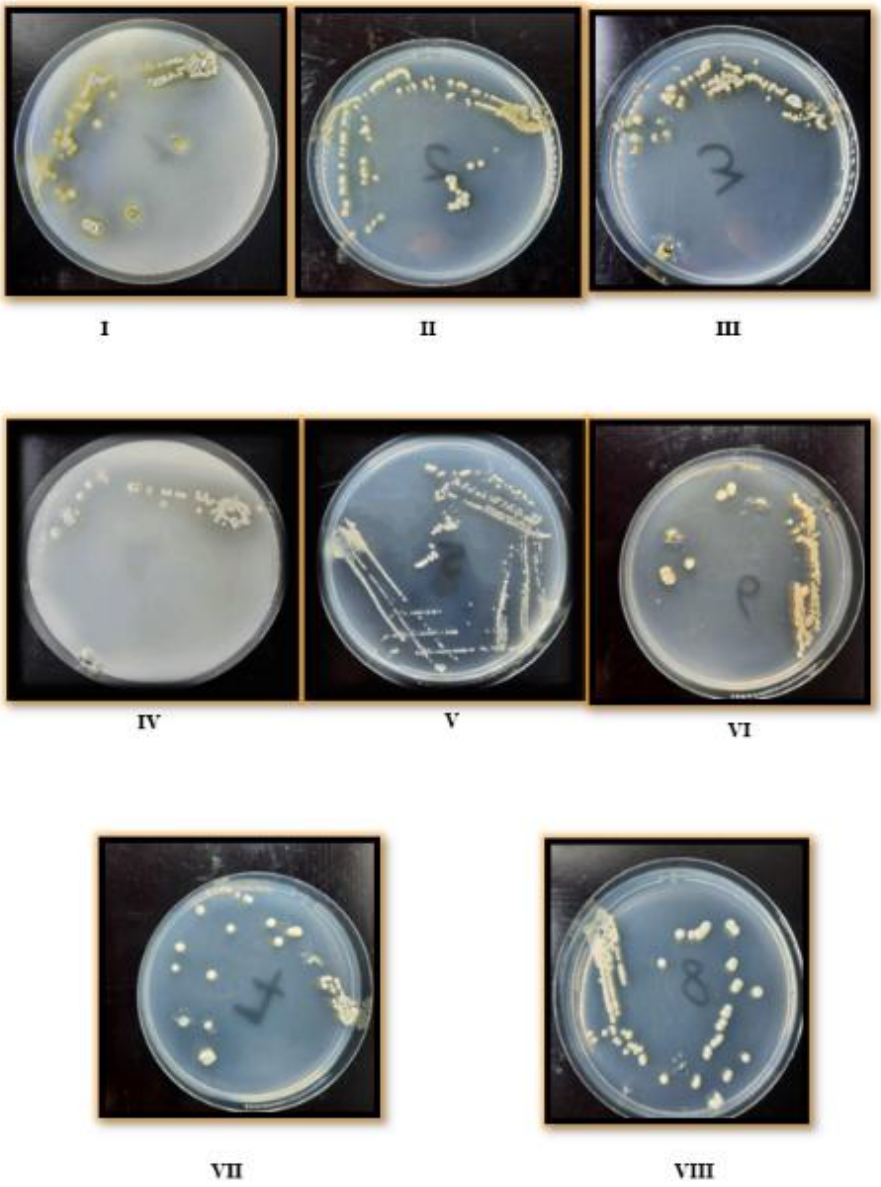


Figure 2. Photographic images of actinomycetes isolates (eight samples) on SCA from A, C, and, D regoins enlighteneing the morphological differences among the eight isolates. Image I: Isolate S1; image II: Isolate S2; Image III: Isolate S3; Image IV: Isolate S4; Image V: Isolate S5; Image VI: Isolate S6; ImageVII: Isolate S7, and Image VIII: Isolate S8

Microscopic examination of the isolates revealed Gram-positive filamentous branched hyphae, as presented in (Figure 3).

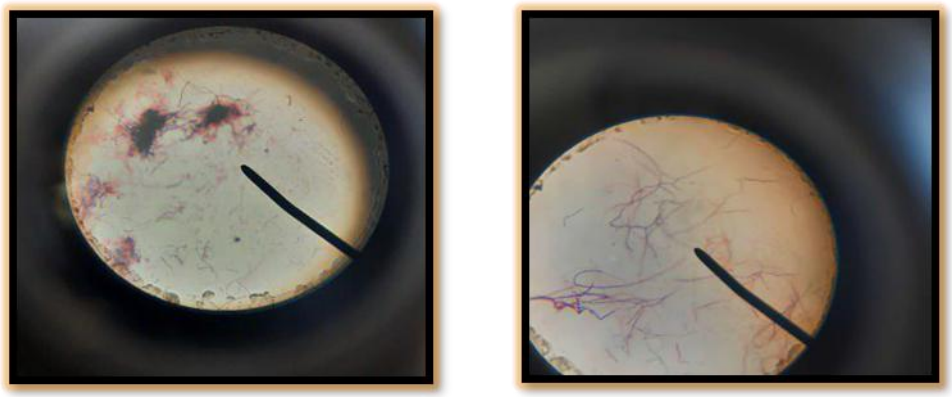


Figure 3. Branched hyphae of actinomycetes under a light microscope. left image: magnification 40x; right image: 100 x immersion oil.

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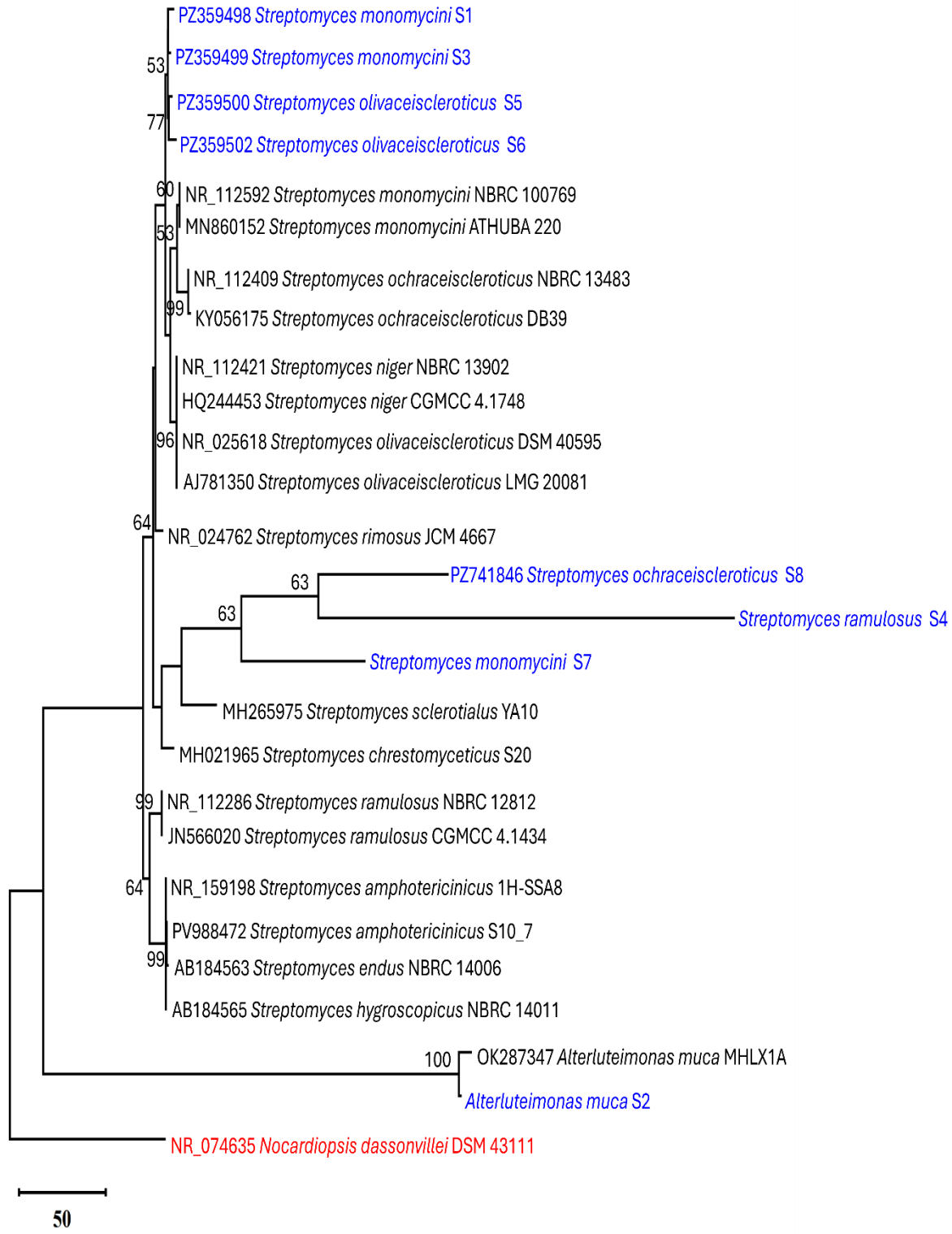


Figure 4. phylogenetic tree of isolates. Maximum parsimonious phylogenetic tree generated based on 16S RNA sequences of actinomycetes strains S1, S3, S4, S5, S6, S7, and S8 and Alterluteimonas muca strain S2 (in blue color) compared to the most similar species in GenBank. Bootstrap support values (1000 replications) ≥50% are indicated near the respective nodes. The tree is rooted to Nocardiopsis dassonvillei DSM 43111 as outgroup (in red color).

Molecular identification of isolates

A megablast search of the NCBI GenBank nucleotide database was performed using the 16S rRNA sequences of strains S1–S8. The similarity percentage between the isolates and their closest references strains ranged between 96.86% and 99.63%, and the sequences were deposited in GenBank under Accession Number listed in (Table).

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Table 2. BLAST- based similarity analysis of the isolates.

Code of isolate	Source of soil sample	Region	Closest strain	Similarity (%)	Accession number of studied isolate
S1	Ononis natrix	Al-Fataih, Derna	*Streptomyces monomycini* ATHUBA 220	99.63%	PZ359498
S2	Lyceum barbarum	Al-Fataih, Derna	*Alterluteimonas muca* MHLX1A	96.86%	Not deposited (insufficient sequence quality)
S3	Hyssopus officinalis	Al-Fataih, Derna	*Streptomyces monomycini* ATHUBA 220	99.43%	PZ359499
S4	Ceratonia siliqua	Karsa forest, Derna	*Streptomyces ramulosus* NBRC 12812	98.22%	Not deposited (insufficient sequence quality)
S5	Ceratonia siliqua	Karsa forest, Derna	*Streptomyces olivaceiscleroticus* CSSP709	99.49%	PZ359500
S6	Pistachia atlantica	Karsa forest, Derna city	*Streptomyces olivaceiscleroticus* CSSP709	99.28%	PZ359502
S7	Pistachia atlantica	Ras Al-Hilal Forest, Derna	*Streptomyces monomycini* ATHUBA 220	97.47%	Not deposited (insufficient sequence quality)
S8	Olea europaea	Ras Al-Hilal Forest, Derna	*Streptomyces olivaceiscleroticus* JCM 4805	99.54%	PZ741846

Strains S1 and S3 showed the highest similarity to *Streptomyces monomycini* ATHUBA 220 (GenBank accession MN860152), with identities of 99.63% (1364/1369) and 99.43% (1385/1393), and gap values of 4/1369 and 6/1393, respectively. Their sequences were deposited in GenBank under accession numbers PZ359498 and PZ359499. Strains S5 and S6 were closely related to *Streptomyces olivaceiscleroticus* CSSP709 (GenBank accession NR_115405), with identities of 99.49% (1371/1378) and 99.28% (1371/1381), and gap values of 3/1378 and 8/1381, respectively. These sequences were submitted to GenBank as PZ359500 and PZ359502. Strain S8 showed 99.24% identity (891/907; gaps = 10/907) to *Streptomyces olivaceiscleroticus* JCM 4805 (GenBank accession MT760617), and its sequence was banked under accession number PZ741846 as *Streptomyces olivaceiscleroticus*. Strain S2 exhibited 96.86% identity (1381/1383; gaps = 0/1383) to rod-shaped bacteria *Alterluteimonas muca* MHLX1A (GenBank accession OK287347); however, its sequence was not accepted for GenBank submission due to insufficient quality. Strain S7 was most similar to *Streptomyces monomycini* ATHUBA 220 (GenBank accession MN860152), with 97.47% identity (808/829; gaps = 7/829), while strain S4 matched *Streptomyces ramulosus* NBRC 12812 (GenBank accession NR_112286), with 98.22% identity (716/729; gaps = 10/729). Due to low sequence quality, the sequences of strains S4 and S7 were not deposited in GenBank.

Discussion

SCA is considered a suitable medium for the selective isolation of soil actinomycetes. The pH value of the positive sample sites A, C, and D fluctuated from 8 to 9. These observations were in coherence with previous reports [5]. However, no colonies were obtained from region B, where pH ranged between 7 and 8. The application of selective growth media and soil pretreatments with $CaCO_3$, heat, and phenol has been indicated by various studies to enhance the isolation of diverse *Streptomyces* species [28-31]. According to previous studies, preliminary identification of *Streptomyces* isolates relied on characteristic macroscopic and microscopic features, including tough or leathery substrate mycelium, chalky or powdery aerial mycelium with diverse pigmentation (such as white, gray, brown, or pink), and Gram-positive filamentous morphology [32-34]. In addition, all actinomycete isolates were obtained from rhizosphere soils as documented in earlier reports [35].

The assembled phylogenetic tree is rooted with *Alterluteimonas mucae (strains MHLX1A and S2)* designated as the basal taxon. *Nocardiopsis dassonvillei DSM 43111* formed a distinct branch between the root and the Streptomyces ingroup, confirming its role as a closely related outgroup. This choice is reinforced by previous investigations that used *Nocardia* as an outgroup when deducing evolutionary relationships among species closely related to *Streptomyces* [36]. The phylogenetic tree revealed that all studied isolates except for S2 were clustered into major clades that divided by deep

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nodes with robust support (0.968–0.994), indicating the high reliability of the inferred relationships. Moreover, the length of most terminal branches was extremely short (0.00000001–0.003), which is consistent with the strong sequence conservation nature of the16S rRNA gene among isolates, as reported previously [18,19], except for the elongated branches of S4 and S8. Strain S4, which typically references *S. ramulosus* strains in Lineage A, was characterized deep within Lineage B on a long branch (0.3258), suggesting divergence or possible misidentification. Meanwhile, S7 and S8 closely clustered with *S. monomycini* and *S. olivaceiscleroticus,* respectively, forming a strongly supported branch (node = 1). However, S8 displayed a longer branch length, indicating greater genetic divergence, which implies it may be a novel strain [37].

16S rRNA gene sequences of the isolates were compared with the NCBI GenBank nucleotide database. S1 and S3 isolates from region A showed the highest sequence similarity to *Streptomyces monomycini* ATHUBA 220 strain, with identity values of 99.63% and 99.43%, respectively. In the meantime, isolates S5 and S6 from region C showed sequence identities ranging between 99.49% and 99.28%, which closely associate them with Streptomyces olivaceiscleroticus strain CSSP709. Besides, region D isolate S8 uncovered a sequence identity of 99.54% to *Streptomyces olivaceiscleroticus* JCM 4805. As substantiated by several studies, 16S rRNA gene sequencing proved to be a vital tool for the identification of actinomycetes in the current research [38-40]. The isolates were identified, and nucleotide sequences were successfully submitted to GenBank.

Isolate S2, obtained from region A, was initially misinterpreted as a *Streptomyces* species based on its filamentous phenotype. However, genetic analysis revealed a 96.86% identity to *Alterluteimonas muca MHLX1A*. A similar taxonomic discrepancy has also been noted by other investigators [41]. Isolates S4 from region C and S7 from region D exhibited 98.22% and 97.47% 16S rRNA gene sequence similarity to Streptomyces ramulosus NBRC 12812 and Streptomyces monomycini ATHUBA 220, respectively. Since the sequence details for strains S2, S4, and S7 were imperfect, their precise taxonomic placement remains uncertain; thus, their sequences were excluded from GenBank submission, a constraint also documented by other analyses [37]. As previously noted, attaining accurate identification requires the deployment of complementary genomic methods, including DNA–DNA hybridization, multilocus sequence analysis (MLSA), and whole-genome sequencing (WGS) [37, 42, 43].

Conclusion and Recommendation

This study represents the first investigation in Eastern Libya, and potentially the entire country, to focus on the molecular identification and phylogenetic characterization of soil actinomycetes using 16S rRNA gene sequencing. Our findings demonstrate that the soil in Derna and its surrounding regions is a rich source of Streptomyces species. Furthermore, insufficient 16S rRNA gene sequence matches for certain isolates strongly suggest the presence of novel species, which may hold significant biotechnological potential. To ensure definitive taxonomic identification, future research should employ advanced polyphasic and genomic approaches, such as DNA–DNA hybridization, multilocus sequence analysis (MLSA), and whole-genome sequencing (WGS). Additionally, screening these actinomycete isolates for their capacity to produce novel bioactive compounds remains highly warranted.

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Conflicts of Interest

The authors have no competing interests to disclose.

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