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# Isolation, Identification and Phylogeny of *Actinobacteria* from Island Soils Using Different Isolation Methods

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**Highlights**

- This article focuses on the actinobacterial biodiversity of island soils.
- The effectiveness of different isolation methods in isolating actinobacteria was investigated.
- The potential of the isolated actinobacteria strains to be new species was determined.

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*Molecular typing*
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**Abstract**

*Actinobacteria* are one of the most frequently studied prokaryotic groups within the *Bacteria* domain. In this study, soil samples collected from the islands of Burgazada, Büyükada, Gökçeada, Heybeliada, and Kınalıada were used to isolate, identify, and analyze the phylogeny of *Actinobacteria*. For the isolation studies, three different isolation methods and 11 different selective media were employed. As a result, a total of 103 bacterial strains were isolated. The molecular identification of the isolated strains was conducted using 16S rRNA gene region sequence analyses. These analyses revealed that the isolates belonged to 12 different genera within the *Actinobacteria* phylum. Comparison of the 16S rRNA gene sequences of the isolates with their closest relatives in the EzBioCloud database indicated a sequence similarity ranging between 95.76% and 100%. The isolation studies demonstrated that the standard dilution plate method was more effective for isolating both diverse genera and potential novel species. The data obtained through this method showed that 22 of the isolated strains, belonging to 8 different genera, have the potential to represent novel species. In conclusion, this study highlights that island soils are an important source for the discovery of new *Actinobacteria* species. Furthermore, it emphasizes the significance of such isolation studies in uncovering the rich biotechnological potential of *Actinobacteria*.



## 1. INTRODUCTION

*Actinobacteria*, gram-positive prokaryotes with a high proportion of guanine and cytosine (G+C) in their genome, live in a variety of terrestrial and aquatic ecosystems, including extreme environments such as acidic-alkaline, low-high temperature, high salt concentration, high radiation, low humidity and low nutrient content. These aerobic bacteria, which form spores and filaments, often have a mycelial life cycle and form the dominant group of the soil microbiome. Their ability to be found in almost all habitats is due to the spore's ability to produce extracellular hydrolytic enzymes and secondary metabolites [1,2].

The islands are of great importance for the Earth's biodiversity as they make significant contributions to global biodiversity, although their surface measurements are small [3]. Most of the biodiversity studies of the islands have focused on macroorganisms. However, the information about the interactions of microbial communities on the islands with each other and with other living things is quite limited [4]. The identification of microbial communities on the islands and the eclarification of symbiotic relationships help us to understand the effects of environmental changes such as habitat fragmentation on microbial communities and therefore the functions undertaken by these communities [5]. Determination of the biodiversity of *Actinobacteria* in these habitats plays an important place in current systematic studies. In the study from South Shetland Island, 36 actinobacteria species identified by 16S rRNA gene region

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sequence analyses were isolated from 15 different soil samples using 5 different selective media. According to the results of 16S rRNA gene region sequence analysis, it was determined that these actinobacterial isolates belonged to 10 different genera (*Brachybacterium, Brevibacterium, Dermacoccus, Kocuria, Micromonospora, Micrococcus, Microbacterium, Rhodococcus, Rothia* and *Streptomyces*) [6]. In another isolation study conducted from Sichang Island, Chonburi Province of Thailand, 6 soil samples were taken and the culture-based method was used to obtain *Actinobacteria*. As a result of 16S rRNA gene region sequence analysis, 55 actinobacteria strains isolated using only humic acid vitamin (HV) agar medium were determined to be members of 3 different genera (*Nocardia, Saccharothrix* and *Streptomyces*) [7]. In recent years, *Streptomyces boninensis* K11-0400$^T$ [8] and *Agromyces seonyunensis* MMS17-SY077$^T$ [9] have been introduced to the literature as new *Actinobacteria* species from different island soils.

In this study, the biodiversity and molecular typing of actinobacteria strains isolated from the soil of the Prince Islands (Burgazada, Büyükada, Heybeliada and Kınalıada) in the Marmara Sea and Gökçeada in the Aegean Sea were investigated by 16S rRNA gene region sequence analysis.

## 2. MATERIAL AND METHODS

### 2.1. Collection of Soil Samples and Isolation of *Actinobacteria*

Soil samples were collected from selected locations of Burgazada, Büyükada, Gökçeada, Heybeliada, and Kınalıada at a depth of 10-20 cm under sterile conditions (Table 1) and were stored at +4 °C until the isolation date.

***Table 1.*** *Locality and Geographical coordinates of soil samples*

| **Büyükada** **June 2012*** | **Heybeliada** **June 2012*** | **Gökçeada** **November 2014*** | |
|---|---|---|---|
| 40°51'53.16"K 29° 7'33.34"D | 40°52'29.57"K 29° 5'28.00"D | 40°10'39.89"K 25°51'24.35"D | 40°11'35.66"K 25°52'31.56"D |
| 40°51'40.64"K 29° 6'43.73"D | 40°52'26.36"K 29° 5'44.06"D | 40° 8'59.98"K 25°51'40.55"D | 40° 8'35.77"K 25°56'45.74"D |
| **Burgazada** **June 2012*** | **Kınalıada** **June 2012*** | | |
| 40°52'52.24"K 29° 3'49.74"D | 40°54'26.09"K 29° 2'54.95"D | 40°14'8.80"K 25°54'22.79"D | 40° 9'2.58"K 25°55'27.56"D |
| 40°53'11.72"K 29° 3'23.02"D | 40°54'46.94"K 29° 2'31.42"D | | |

* Date of collection of soil samples

Standard dilution plate [10], sucrose centrifugation [11] and 1.5% phenol [12] methods were used in the isolation of *Actinobacteria*. Before insulation, 25 g was taken from each soil sample and dried at room temperature for 15 days. After drying, 1 g of soil samples were weighed and placed in previously prepared sterile bottles containing 9 ml of ringer solution and glass beads. After half an hour of shaking, 1 ml of homogeneous solution taken from these $10^{-1}$ solutions under sterile conditions was transferred to glass tubes containing 9 ml of sterile ringer solution and a $10^{-2}$ dilution was prepared. This procedure was repeated for the $10^{-3}$ solution under the same conditions and 0.2 ml of homogeneous solution from each dilution was inoculated into antibiotic-added selective media (Table 2). For the $10^{-2}$ solution to be used in the sucrose centrifugation method, 1 ml was taken from the $10^{-1}$ solutions prepared in the dilution plate method with the help of an automatic pipette and added to the tubes containing sterile 9 ml of 20% sucrose solution. Then these tubes were centrifuged (30 min, 240xg at room temperature) and 0.2 ml was taken from the upper part of the gradient formed in the tubes and passaged into the selective isolation media (Table 2). For the $10^{-2}$ solution to be used in the 1.5% phenol method, 1 ml was taken from the $10^{-1}$ solutions prepared in the dilution plate method with the help of an automatic pipette and added to the tubes with sterile 9 ml of 1.5% phenol solution. After this mixture was incubated for 30 minutes at 28 °C, 0.2 ml of homogeneous mixture taken by automatic pipette from $10^{-2}$ dilution was spread to selective isolation mediums (Table 2)

by swab. Two plates were prepared from each dilution prepared by different isolation methods and incubated at 28 °C for 14-21 days.

***Table 2.*** *Selective media and antibiotics used in isolation*

| No | Media | Antibiotics |
|---|---|---|
| **1** | Humic Acid-Vitamin Agar [13] | Cycloheximide (50 µg /ml)<br>Rifampicin (5 µg/ml)<br>Nalidixic acid (10 µg/ml) |
| **2** | Bennettss agar [14] | |
| **3** | Czapeks dox agar [15] | Nystatin (50µg/ml)<br>Cycloheximide (50µg/ml) |
| **4** | International Streptomyces Project-2 Medium (ISP2 agar) [16] | Nystatin (50µg/ml)<br>Rifampicin (5µg/ml) |
| **5** | International Streptomyces Project-5 Medium (ISP5 agar) [17] | |
| **6** | Starch Casein agar [18] | Nystatin (50µg/ml)<br>Nalidixic acid (10 µg/ml) |
| **7** | SM1 Stevenson's agar [18] | Nystatin (50µg/ml)<br>Cycloheximide (50µg/ml)<br>Neomycin sulfate (50µg /ml) |
| **8** | SM2 Stevenson's agar [18] | |
| **9** | SM3 agar - Gauze's agar [18] | Nystatin (50µg/ml)<br>Nalidixic acid (10µg/ml) |
| **10** | Tryptone yeast extract agar [15] | Cycloheximide (50 µg /ml)<br>Rifampicin (5 µg/ml)<br>Nalidixic acid (10 µg/ml) |
| **11** | Tryptone yeast extract vitamin agar [15] | |

### 2.2. Selection, Purification and Stocking of *Actinobacteria* Isolates

The samples transferred to different selective media by consecutive dilutions were left for incubation at 28 °C for 14-21 days, and actinobacterial-like colonies were selected according to their morphological character in these isolation plates. The identified actinobacteria and similar colonies are cycloheximide (50 µg/ml) supplemented glucose yeast extract agar (medium no. 54; DSMZ) [19], tryptone yeast extract agar (medium no. 680; DSMZ) [15] and NZ-Amin agar (medium no. 554; DSMZ) was transferred to the surface by line planting method. After 14 days of incubation at 28 °C, pure isolates were obtained from the transferred plates. Properties of isolates in fattening media, such as development, spore color, air micellar color and soluble pigment color, were determined using the ISCC-NBS color catalog proposed by Kelly (1964) [20]. Isolates were enumerated, pure cultures were made and transferred to autoclaved screw-capped tubes containing 25% glycerol with the help of a core and stored at -80 °C.

### 2.3. Genomic DNA Isolation, 16S rRNA Gene Region Sequence Analyses and Phylogenetic Analyses

Genomic DNA isolation of the isolates was performed using PureLink® Genomic DNA Isolation Kit (Invitrogen, USA). Two universal primers 27F- (5′-AGAGTTTGATC(AC)TGGCTCAG-3′) and 1492R-(5′-ACGG(CT)TACCTTGTTACGACTT-3′) were used for PCR amplification (Thermo Fisher, USA) of the 16S rRNA gene region [21,22]. The sequencing process of 16S rRNA gene regions, which was amplified, was performed by Macrogen Europe using 5 primers (Table 3). The chromatogram files in ABI format obtained after the sequencing process were combined using Chromas version 2.6.6 and sequences of 16S rRNA gene regions in FASTA format of each strain were obtained. These sequences that were used to identify the isolates at the genus level and the closest types were determined using the EzBioCloud database (https://www.ezbiocloud.net/) [23]. MEGA 11 program was used in phylogenetic analyses of actinobacterial strains [24]. The phylogenetic dendrograms were constructed using the Neighbour-Joining method [25] and the Jukes-Cantor phylogenetic distance matrix [25].

***Table 3.*** *Oligonucleotide primers used for 16S rRNA sequencing and* PCR amplification

| Primer code | Sequences (5'-3') | Base length | References |
|---|---|---|---|
| 518F | CCAGCAGCCGCGGTAAT | 17 | [26] |
| MG5F | AAACTCAAAGGAATTGACGG | 20 | [27] |
| 800R | TACCAGGGTATCTAATCC | 18 | [27] |
| 27F | AGAGTTTGATCMTGGCTCAG | 20 | [28] |
| 1525R | AAGGAGGTGWTCCARCC | 17 | [28] |

## 3. THE RESEARCH FINDINGS AND DISCUSSION

### 3.1. Isolation of *Actinobacteria*

For the isolation study, the lands of Büyükada (2), Burgazada (2), Heybeliada (2) and Kınalıada (2) were collected from 8 different localities, namely from coastal and forest areas. Gökçeada lands, on the other hand, were taken from 6 different locations, taking into account the diversity of habitats (coast, forest, agricultural area, pasture, settlement). The effect of these factors on the determination of actinobacterial biodiversity was also investigated by preferring different methods, media and localities in the isolation of soil samples. A total of 11 different mediums (Table 2) containing different antibiotics were used in the isolation of *Actinobacteria*. Three different isolation methods (standard dilution plate [10], sucrose centrifugation [11] and 1.5% phenol [12]) and 4 different media were used in the isolation of *Actinobacteria* from the lands of Büyükada, Burgazada, Heybeliada and Kınalıada. In the isolation of *Actinobacteria* from Gökçeada lands, only the standard dilution plate method and 7 different media were used.

A total of 103 *Actinobacteria* strains were isolated in the isolation study. Of these, 85 of them were isolated by standard dilution plate method, 11 were isolated by 1.5% phenol method and 7 by sucrose centrifugation method. The distribution of the isolates according to the media is given in Figure 1. 41 isolates were obtained from Gökçeada, 25 isolates from Heybeliada, 22 isolates from Kınalıada, 11 isolates from Burgazada and 4 isolates from Büyükada.

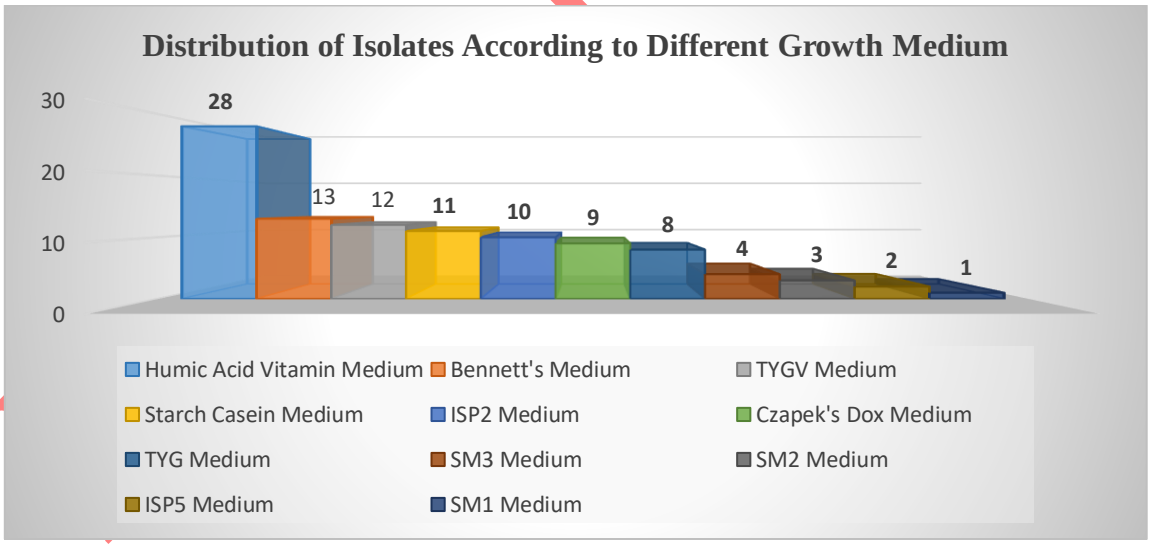


***Figure 1.*** *Distribution of isolates according to different growth medium*

### 3.2. Molecular Typing and Phylogenetic Analysis of Isolates

The molecular identification of 103 *Actinobacteria* strains was based on sequence analysis of the 16S rRNA gene. To determine the types to which the strains belong and the degree of kinship with the closest types, the sequences of the 16S rRNA gene region were compared with the data in the EzBioCloud server database. In the strains to be performed with phylogenetic analyses, those with sufficient length (≥1300) of the 16S rRNA gene region sequences (76 actinobacterial isolates) were preferred [29]. Accordingly, it was determined that the strains belonged to 12 different genera of *Actinobacteria* (Figure 2). It was determined

that the isolated strains belonged to 7 different species in Gökçeada, 6 in Heybeliada, 5 in Kınalıada, 4 in Burgazada and 3 in Büyükada. Strains belonging to 9 genera were isolated from soil samples taken from two localities in Büyükada, Burgazada, Heybeliada and Kınalıada using three different methods and 4 different media. In the isolation study conducted in Gökçeada using only the dilution plate method and 7 different mediums, strains belonging to 7 different types of actinobacteria were isolated. Of these strains, 49 were members of *Streptomyces*, 17 of *Micromonospora*, 10 of *Nonomuraea*, 6 of *Nocardia*, 5 of *Actinomadura*, 4 of *Saccharopolyspora*, 3 of *Rhodococus*, 3 of *Kribbella*, 2 of *Agromyces*, 2 of *Microbispora*, 1 of *Amycolatopsis* and 1 of *Geodermatophilus* (Figure 2). According to 16S rRNA gene region sequence analyses, the types of strains are closest related are given in Tables 4-7. The previously recommended minimum 16S rRNA gene similarity value for the identification of a new bacterial species was 97% [30]. However, according to the study conducted by Chun and colleagues in 2018, this value was re-updated and proposed to 98.7% [31]. However, there are also species in the literature that are over 98.7% similar to the closest type species and are described as new species [22, 31-33].

***Table 4.*** *16S r RNA sequence analysis results of strains isolated from Büyükada and Burgazada soil*

| Isolate | Closest type strain | Similarity (%) | Nucleotid difference |
|---|---|---|---|
| B2F13 | *Actinomadura bangladeshensis* 3-46-b3$^T$ | 98.89 | 16/1442 |
| Z2R53 | *Geodermatophilus daqingensis* WT-2-1$^T$ | 99.93 | 1/1443 |
| Z1R8 | *Micromonospora noduli* GUI43$^T$ | 99.86 | 2/1429 |
| Z1R61 | *Micromonospora salmantinae* PSH03$^T$ | 99.65 | 5/1430 |
| Z1F35 | *Micromonospora tulbaghiae* DSM 45142$^T$ | 99.86 | 2/1437 |
| Z2R54 | *Nonomuraea lycopersici* NEAU-DE8(1)$^T$ | 99.17 | 12/1440 |
| Z1R24 | *Nonomuraea turkmeniaca* DSM 43926$^T$ | 98.87 | 16/1416 |
| Z1R34 | *Streptomyces ureilyticus* YC419$^T$ | 98.21 | 26/1449 |
| Z1R7 | *Streptomyces burgazadensis* Z1R7$^T$ | 95.76 | 65/1441 |

***Table 5.*** *16S r RNA sequence analysis results of strains isolated from Kınalıada soil*

| Isolate | Closest type strain | Similarity (%) | Nucleotid difference |
|---|---|---|---|
| K1R23 | *Agromyces neolithicus* 23-23$^T$ | 98.75 | 18/1439 |
| K2R23 | *Agromyces neolithicus* 23-23$^T$ | 98.89 | 16/1439 |
| K2F73 | *Micromonospora chaiyaphumensis* DSM 45246$^T$ | 99.44 | 8/1437 |
| K2F72 | *Micromonospora luteifusca* GUI2$^T$ | 99.09 | 13/1427 |
| K2R35 | *Micromonospora musae* MS1-9$^T$ | 99.64 | 5/1391 |
| K2R33 | *Micromonospora palomenae* NEAU-CX1$^T$ | 99.86 | 2/1428 |
| K2R55 | *Micromonospora saelicesensis* Lupac 09$^T$ | 99.93 | 1/1437 |
| K2S19 | *Micromonospora saelicesensis* Lupac 09$^T$ | 99.93 | 1/1437 |
| K2S20 | *Micromonospora saelicesensis* Lupac 09$^T$ | 99.79 | 3/1437 |
| K2R49 | *Micromonospora trifolii* NIE79$^T$ | 99.93 | 1/1429 |
| K2R47 | *Micromonospora zamorensis* DSM 45600$^T$ | 100.00 | 0/1439 |
| K2R37 | *Nocardia jinanensis* NBRC 108249$^T$ | 99.37 | 9/1439 |

***Table 6.*** *16S r RNA sequence analysis results of strains isolated from Gökçeada soil*

| Isolate | Closest type strain | Similarity (%) | Nucleotid difference |
|---|---|---|---|
| AI238 | *Actinomadura montaniterrae* CYP1-1B$^T$ | 98.96 | 15/1440 |
| AYDS4 | *Actinomadura montaniterrae* CYP1-1B$^T$ | 99.86 | 2/1440 |
| AS21 | *Actinomadura napierensis* B60$^T$ | 99.11 | 12/1344 |
| AI239 | *Actinomadura napierensis* B60$^T$ | 99.18 | 11/1344 |
| ZIRC94 | *Amycolatopsis kentuckyensis* NRRLB-24129$^T$ | 99.86 | 2/1443 |
| AZ5 | *Microbispora rosea* subsp. *rosea* ATCC 12950$^T$ | 99.10 | 13/1443 |
| EZ3 | *Microbispora rosea* subsp. *rosea* ATCC 12950$^T$ | 100.00 | 0/1431 |
| EA55 | *Nocardia gipuzkoensis* 234509$^T$ | 100.00 | 0/1441 |
| AS26 | *Nocardia nova* NBRC 15556$^T$ | 99.24 | 11/1439 |
| EC52 | *Nocardia rhamnosiphila* NRRL B-24637$^T$ | 99.65 | 5/1438 |
| ZİZ37 | *Nonomuraea candida* HMC10$^T$ | 98.78 | 17/1396 |
| ADS25 | *Nonomuraea indica* DRQ-2$^T$ | 99.28 | 10/1396 |
| YILB32 | *Saccharopolyspora antimicrobica* DSM 45119$^T$ | 99.51 | 7/1434 |
| YILB29 | *Saccharopolyspora antimicrobica* DSM 45119$^T$ | 99.52 | 7/1444 |
| ZEYS56 | *Saccharopolyspora elongata* 7K502$^T$ | 100.00 | 0/1445 |
| ZES61 | *Saccharopolyspora shandongensis* 88$^T$ | 99.22 | 11/1417 |
| YILC25 | *Saccharopolyspora shandongensis* 88$^T$ | 99.72 | 4/1417 |
| EC51 | *Streptomyces ambofaciens* ATCC 23877$^T$ | 99.72 | 4/1449 |
| EI2125 | *Streptomyces ambofaciens* ATCC 23877$^T$ | 99.72 | 4/1449 |
| BI255 | *Streptomyces antimycoticus* NBRC 12839$^T$ | 99.93 | 1/1445 |
| ZEZ7 | *Streptomyces antimycoticus* NBRC 12839$^T$ | 99.72 | 4/1445 |
| ZIRS29 | *Streptomyces aureocirculatus* NRRL ISP-5386$^T$ | 99.52 | 7/1448 |
| ZIRI57 | *Streptomyces aureocirculatus* NRRL ISP-5386$^T$ | 99.52 | 7/1450 |
| ZIRI269 | *Streptomyces coeruleorubidus* ISP 5145$^T$ | 99.01 | 14/1412 |
| BI519 | *Streptomyces filipinensis* NBRC 12860$^T$ | 99.09 | 13/1436 |
| BI245 | *Streptomyces filipinensis* NBRC 12860$^T$ | 99.31 | 10/1445 |
| BARZ15 | *Streptomyces flaveolus* NBRC 3715$^T$ | 100.00 | 0/1444 |
| ZEI231 | *Streptomyces melanosporofaciens* DSM 40318$^T$ | 100.00 | 0/1449 |
| EZ11 | *Streptomyces rubrogriseus* LMG 20318$^T$ | 100.00 | 0/1448 |
| ES24 | *Streptomyces rubrogriseus* LMG 20318$^T$ | 100.00 | 0/1448 |
| ES109 | *Streptomyces rubrogriseus* LMG 20318$^T$ | 100.00 | 0/1448 |
| YI12 | *Streptomyces samsunensis* M1463$^T$ | 100.00 | 0/1425 |
| ZEYZ1 | *Streptomyces seymenliensis* B1041$^T$ | 98.76 | 18/1449 |
| ZEYZ14 | *Streptomyces umbrinus* NBRC 13091$^T$ | 99.59 | 6/1446 |
| AS59 | *Streptomyces virginiae* NRRL ISP-5094$^T$ | 99.58 | 6/1444 |
| AB77 | *Streptomyces virginiae* NRRL ISP-5094$^T$ | 99.59 | 6/1446 |

***Table 7.*** *16S r RNA sequence analysis results of strains isolated from Heybeliada soil*

| Isolate | Closest type strain | Similarity (%) | Nucleotid difference |
|---|---|---|---|
| H3R5 | *Kribbella karoonensis* Q41$^T$ | 99.59 | 6/1447 |
| H2R4 | *Kribbella shirazensis* UTMC 693$^T$ | 99.29 | 10/1404 |
| H2R22 | *Kribbella turkmenica* 16K104$^T$ | 98.96 | 15/1442 |
| H2F23 | *Micromonospora coriariae* DSM 44875$^T$ | 100.00 | 0/1439 |
| H2R10 | *Micromonospora coriariae* DSM 44875$^T$ | 100.00 | 0/1439 |
| H2F8 | *Micromonospora inositola* DSM 43819$^T$ | 98.71 | 18/1395 |
| H3F6 | *Micromonospora yasonensis* DS3186$^T$ | 98.96 | 15/1438 |
| H3F25 | *Micromonospora yasonensis* DS3186$^T$ | 98.89 | 16/1437 |
| H3S3 | *Nocardia takedensis* NBRC 100417$^T$ | 98.85 | 16/1395 |
| H3S24 | *Nocardia takedensis* NBRC 100417$^T$ | 99.38 | 9/1441 |
| H2R21 | *Nonomuraea purpurea* 1SM4-01$^T$ | 99.10 | 15/1444 |
| H1R4 | *Nonomuraea candida* HMC10$^T$ | 98.14 | 26/1396 |
| H2R11 | *Nonomuraea candida* HMC10$^T$ | 99.36 | 9/1398 |
| H2R3 | *Nonomuraea insulae* H2R21$^T$ | 99.31 | 10/1442 |
| H2R16 | *Nonomuraea insulae* H2R21$^T$ | 99.86 | 2/1444 |
| H2R15 | *Nonomuraea lycopersici* NEAU-DE8(1)$^T$ | 99.03 | 14/1441 |
| H3R2 | *Rhodococcus wratislaviensis* NBRC 100605$^T$ | 99.51 | 7/1441 |
| H1R6 | *Streptomyces lannensis* TA4-8$^T$ | 99.65 | 5/1446 |
| H1R9 | *Streptomyces lannensis* TA4-8$^T$ | 99.72 | 4/1446 |

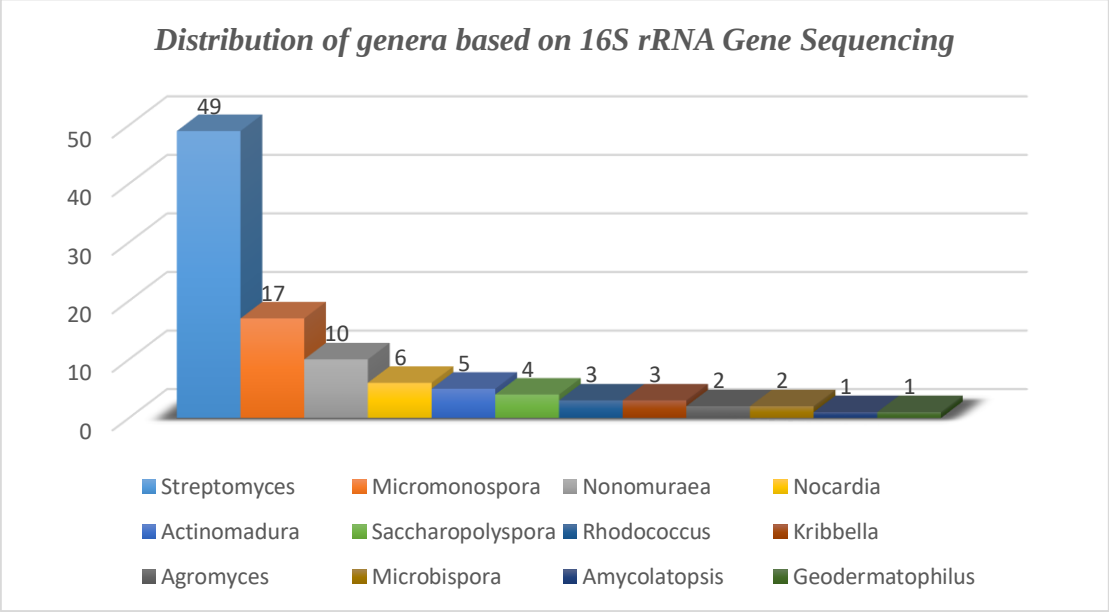


***Figure 2.*** *Distribution of genera based on 16S rRNA Gene Sequencing*

In this study, strains with similarity values between the lowest 95.76 % and the highest 100% in terms of sequence similarity of the 16S rRNA gene region were isolated (Tables 4-7). Of these strains, the Z1R7 strain [21], which has a similarity of the 16S rRNA gene region of 95.76% with the *Streptomyces specialis* GW41-1564T type, and the H2R21 strain, which is 99.1% similar to the *Nonomuraea purpurea* 1SM4-01T type type [22] were introduced to the literature by polyphasic taxonomy. Apart from these, the strains with the lowest 16S rRNA gene region sequence similarity were strain H1R4 (PP331372) with 98.14% similarity with *Nonomuraea candida* HMC10$^T$ type strain and strain Z1R34 (PP331421) with 98.21% similarity with

*Streptomyces ureilyticus* YC419$^{T}$ type strain. Isolates that are similar between 98.71% and 99.18% with the closest strains were *Micromonospora* sp. H2F8 (PP331440) (98.71%), *Agromyces* sp. K1R23 (PQ808885) (98.75%), *Streptomyces* sp. ZEYZ1 (KU497646) (98.76), *Nonomuraea* sp. ZİZ37 (KU497655) (98.78), *Nocardia* sp. H3S3 (PP331419) (98.85%), *Nonomuraea* sp. Z1R24 (PQ808136) (98.87%), *Actinomadura* sp. B2F13 (KP027413) (98.89%), *Agromyces* sp. K2R23 (PQ808886) (98.89%), *Micromonospora* sp. H3F25 (PQ807782) (98.89%), *Actinomadura* sp. AI238 (KU497647) (98.96%), *Kribbella* sp. H2R22 (PQ808888) (98.96%), *Micromonospora* sp. H3F6 (PP331449) (98.96%), *Streptomyces* sp. ZIRI269 (KU497661) (99.01%), *Nonomuraea* sp. H2R15 (PQ808135) (99.03%), *Micromonospora* sp. K2F72 (PP331492) (99.09%), *Streptomyces* sp. BI519 (KU497662) (99.09%), *Microbispora* sp. AZ5 (KU497650) (99.10), *Actinomadura* sp. AS21 (KU497648) (99.11%), *Nonomuraea* sp. Z2R54 (PQ808874) (99.17%) and *Actinomadura* sp. AI239 (KU497649) (99.18%). The closest types of these strains and nucleotide differences are given in Table 8.

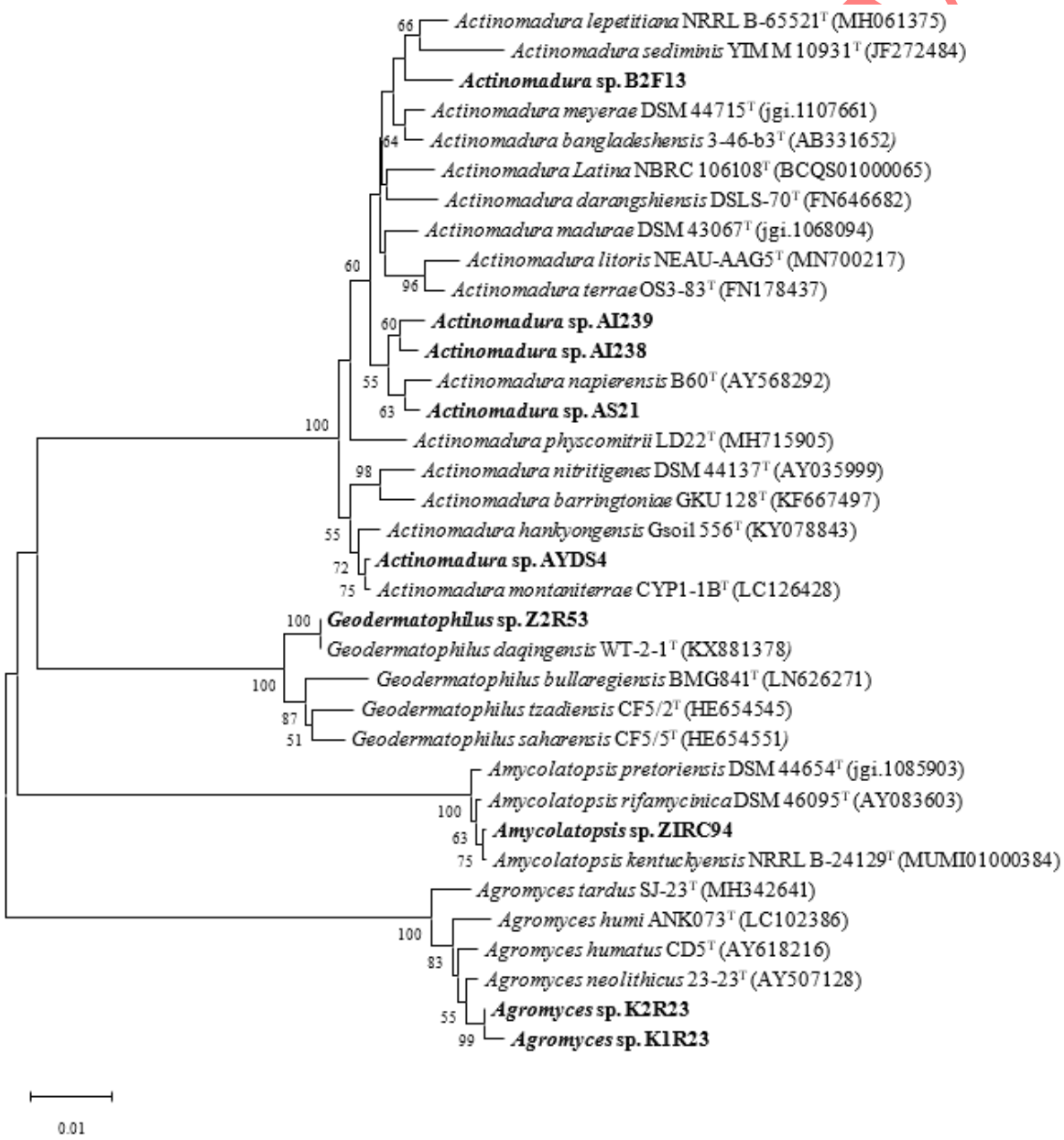


***Figure 3.*** *Neighbor-joining [25] phylogenetic tree of Actinomadura, Agromyces and Geodermatophilus strains based on 16S rRNA gene sequence analysis using MEGA 11 software. Bootstrap values above 50% are shown in the dendrogram*

In the dendrogram shown in Figure 3, strain B2F13 clustered with *Actinomadura bangladeshensis* 3-46-b3$^{T}$, the closest type species with 98.89% 16S rRNA gene region sequence similarity. Strains AI238, AS21,

AI239, which show less than 99.2% sequence similarity with the closest type species, also cluster with *Actinomadura napierensis* B60$^{T}$. Strains K1R23 and K2R23 clustered with the closest type species, *Agromyces neolithicus* 23-23$^{T}$. Accordingly, strains B2F13, AI238, AS21, AI239, K1R23 and K2R23 have the potential to be new species considering the previous studies [32-34].

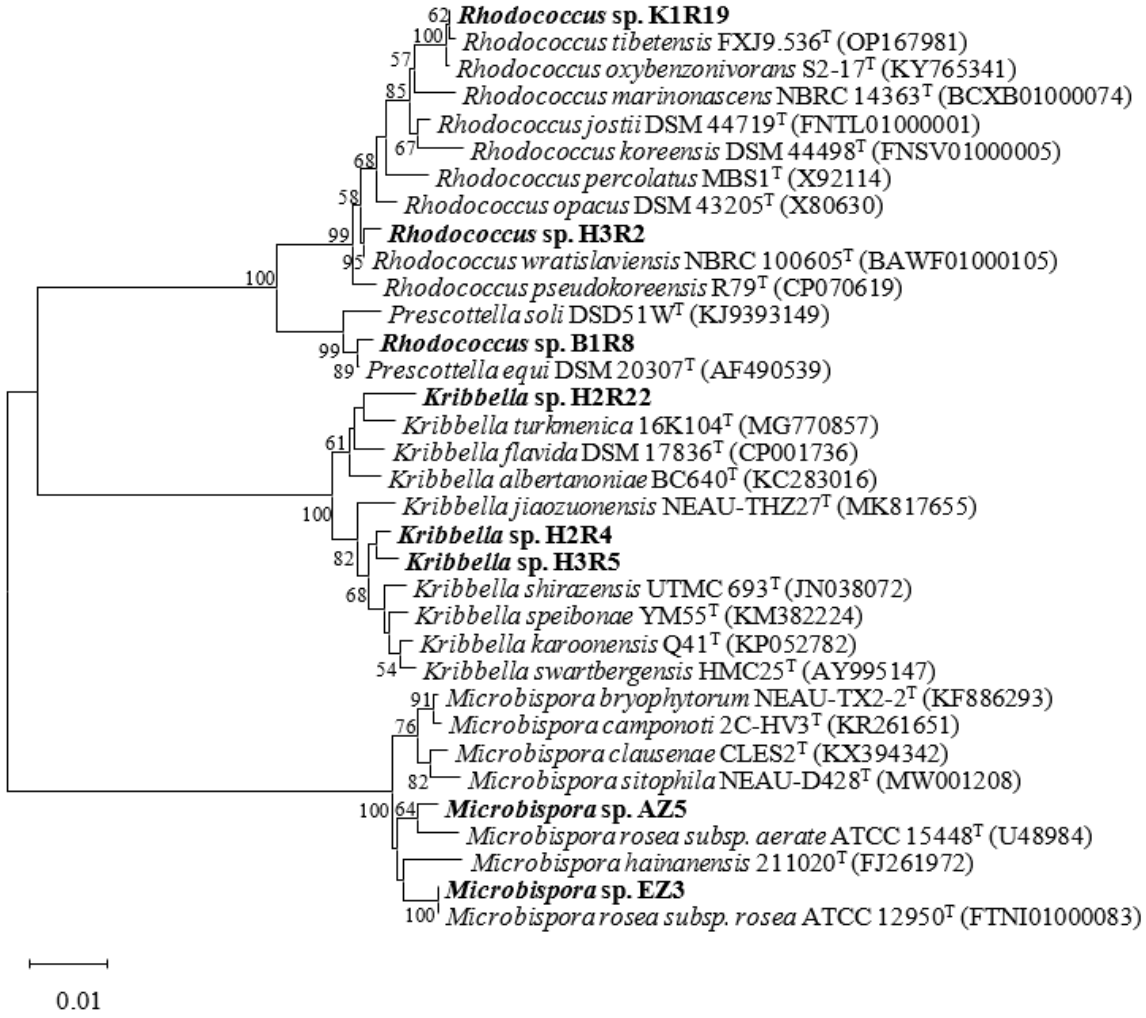


***Figure 4.*** *Neighbor-joining [25] phylogenetic tree of Kribbella, Microbispora and Rhodococcus strains based on 16S rRNA gene sequence analysis using MEGA 11 software. Bootstrap values above 50% are shown in the dendrogram*

In the dendrogram constructed using the neighbor-joining method (Figure 4), the H2R22 strain formed a nearest cluster with the closest type species *Kribbella turkmenica* 16K104$^{T}$ with 98.98% sequence similarity. Similarly, strain AZ5 formed a tree topology with the type species *Microbispora rosea* subsp. *rosea* ATCC 12950$^{T}$ with 99.1% sequence similarity, indicating that both strains have the potential to be new species.

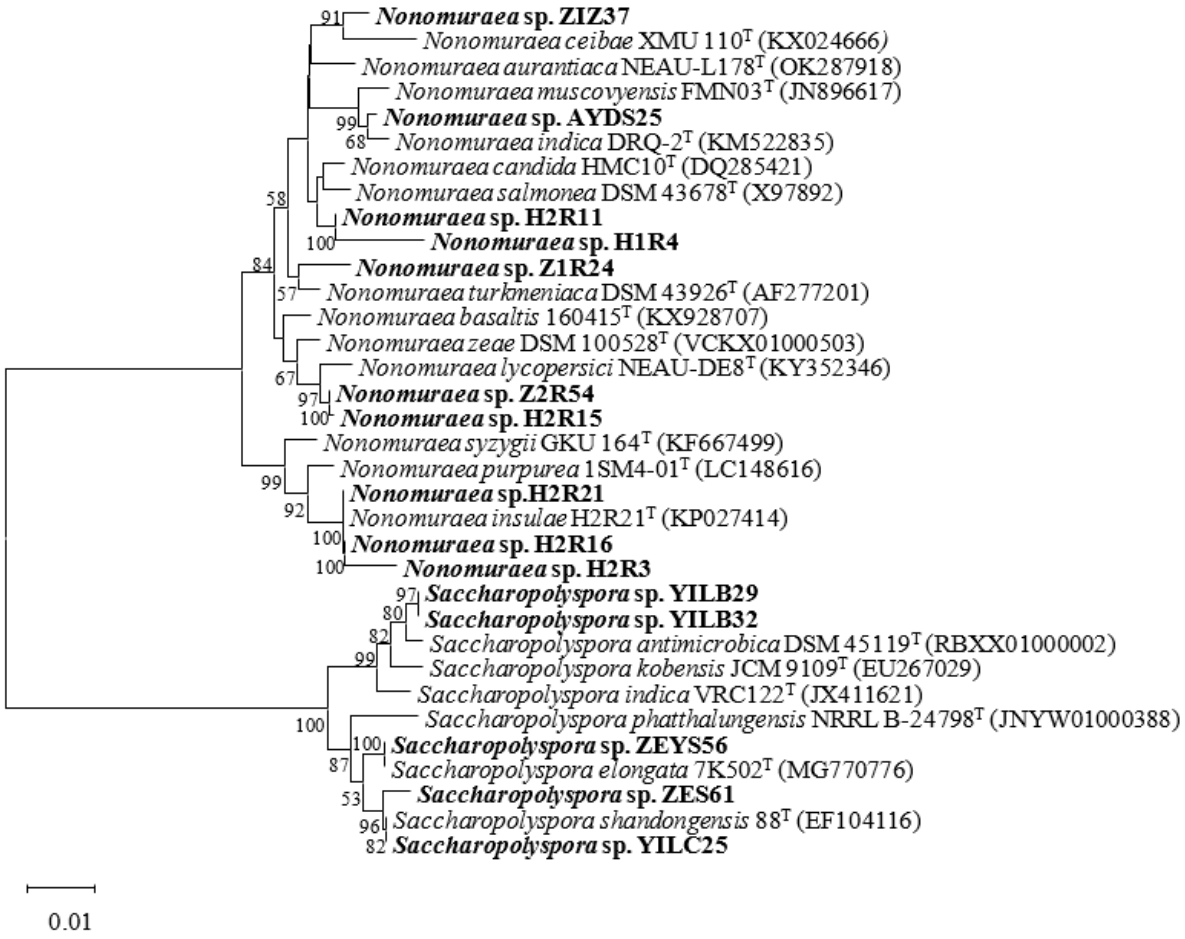


***Figure 5.*** *Neighbor-joining [25] phylogenetic tree of Nonomuraea and Saccharopolyspora strains based on 16S rRNA gene sequence analysis using MEGA 11 software. Bootstrap values above 50% are shown in the dendrogram*

In the phylogenetic dendrogram created with *Nonomuraea* and *Saccharopolyspora* strains (Figure 5), isolates H1R4, ZIZ37, Z1R24, H2R15 and Z2R24 have the potential to be new species according to both the 16S rRNA gene region sequence similarity rates and the topology they show with the closest type species in the phylogenetic dendrogram. The 14-17 nucleotide difference between the closest type strains in terms of 16S rRNA gene region sequences of these strains also supports this potential.

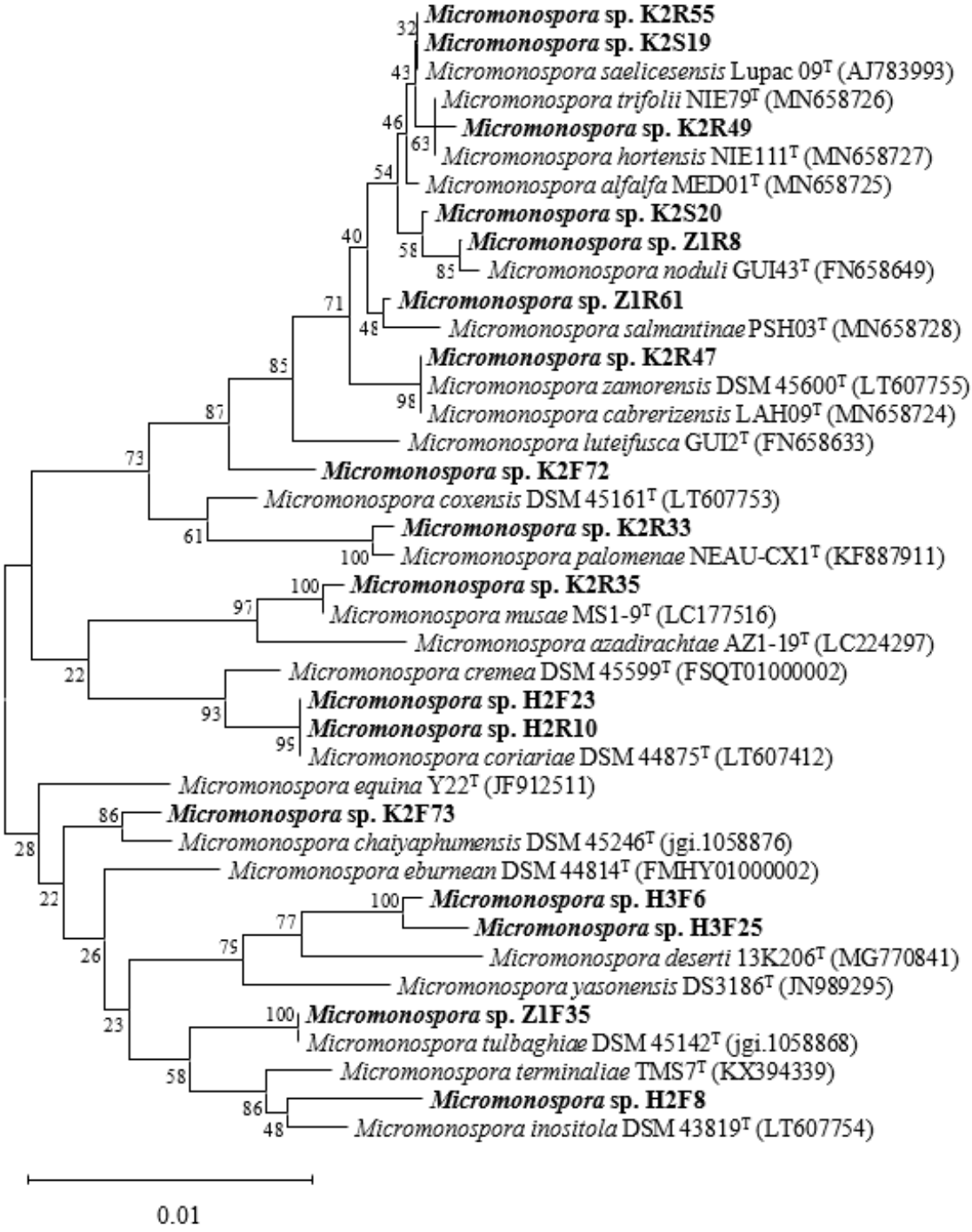


***Figure 6.*** *Neighbor-joining [25] phylogenetic tree of Micromonospora strains based on 16S rRNA gene sequence analysis using MEGA 11 software. Bootstrap values above 50% are shown in the dendrogram*

In the Neighbor-joining dendrogram created for the strains belonging to the genus *Micromonospora* (Figure 6), isolates H2F8, H3F25, H3F6 and K2F72 have the potential to be new *Actinobacteria* strains according to the tree topology they show. The 16S rRNA gene region sequences of these isolates differ 13-18 nucleotides from the closest type species. This difference supports their potential to be new species.

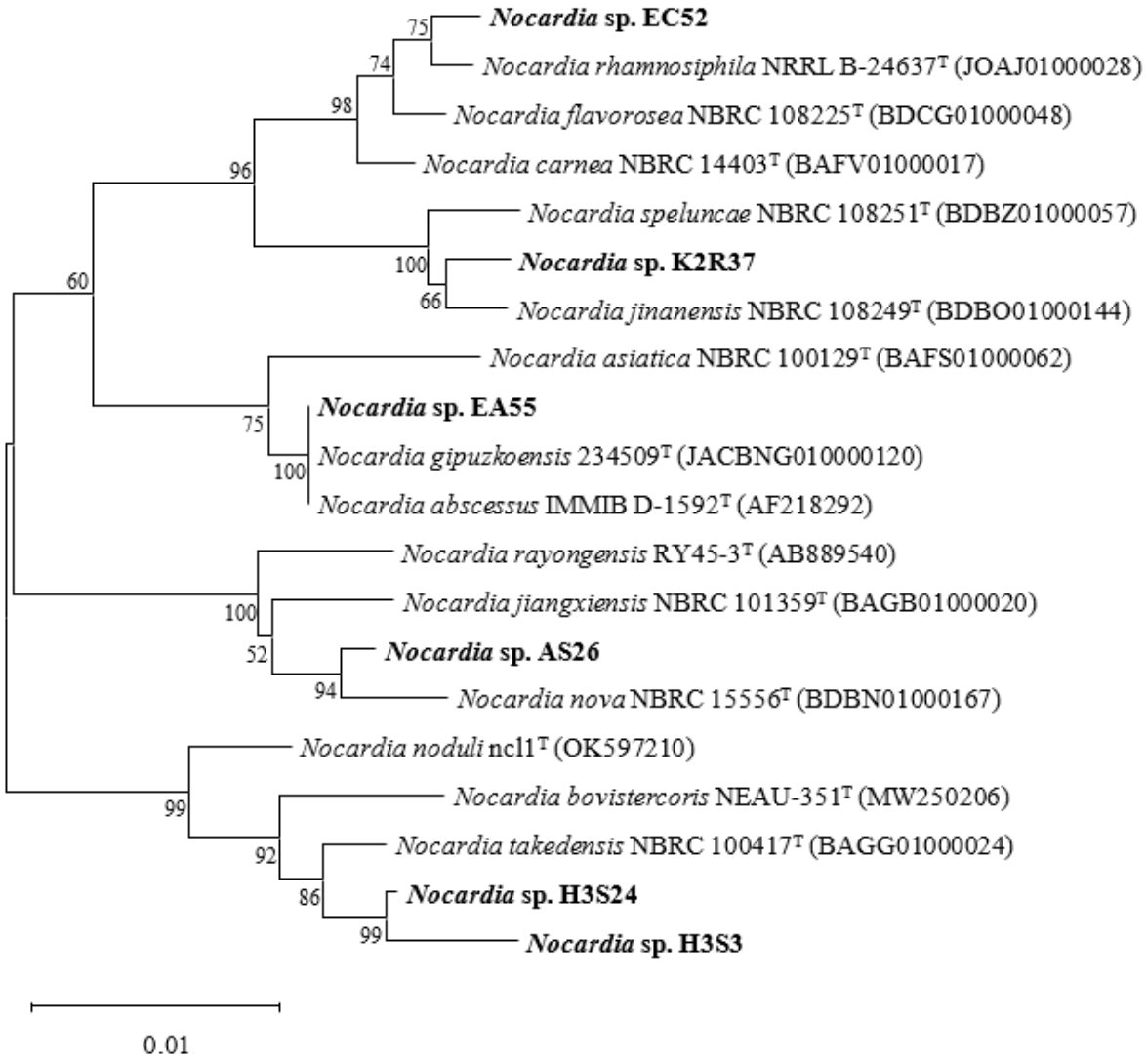


***Figure 7.*** *Neighbor-joining [25] phylogenetic tree of Nocardia strains based on 16S rRNA gene sequence analysis using MEGA 11 software. Bootstrap values above 50% are shown in the dendrogram*

In the phylogenetic dendrogram in Figure 7, it is seen that isolates H3S3 and H3S24 have a high potential to be new species considering their topological positions with *Nocardia takedensis* NBRC 100417$^{T}$, the closest type species according to 16S rRNA gene region sequence analysis.

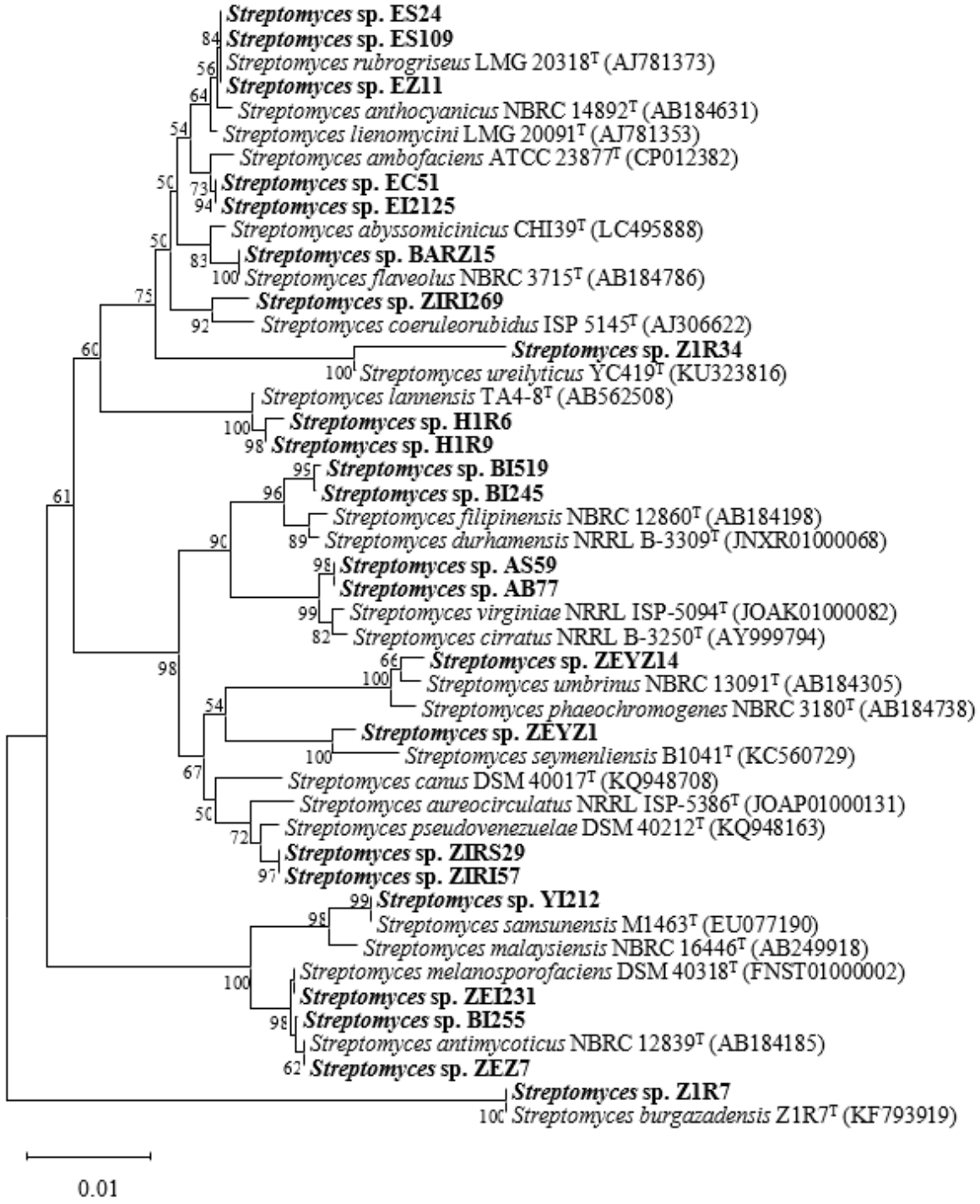


***Figure 8.*** *Neighbor-joining [25] phylogenetic tree of Streptomyces strains based on 16S rRNA gene sequence analysis using MEGA 11 software. Bootstrap values above 50% are shown in the dendrogram*

In the Neighbor-joining phylogenetic dendrogram (Figure 8) created for *Streptomyces* strains, Z1R34, ZIRI269, BI519, ZEYZ1 strains have a high potential to be new species according to their topology.

In the isolation study carried out with different isolation methods, media and number of locations from five different island soils, 103 *Actinobacteria* strains were isolated. The phylogenetic relationships of 76 of these strains with sufficient 16S rRNA gene sequences were determined. In this article, among the isolates identified by 16S rRNA gene region sequence analysis and phylogenetic relationships determined, strains with the potential to be new species belonging to different actinobacteria genera were identified. In recent studies, *Agromyces seonyunensis* MMS17-SY077$^T$ [9], *Nocardioides sambongensis* KUDC5002$^T$ [35],

*Gordonia insulae* MMS17-SY073$^T$ [36] and *Streptomyces boninensis* K11-0400$^T$ [8] were isolated from island soils and introduced to the literature as new species. *Streptomyces burgazadensis* Z1R7$^T$ [21] and *Nonomuraea insulae* H2R21$^T$ [22] isolated within the scope of this study were identified as new *Actinobacteria* species from Türkiye by completing polyphasic taxonomic studies. The strains given in Table 8 are also isolates with high potential to be new species. However, although 16S rRNA gene region sequence analyses are sufficient to determine the species to which the strains belong, they are not sufficient to provide species status [37]. Accordingly, polyphasic taxonomic studies must be completed in order to gain species status of strains with the potential to become new *Actinobacteria* in phylogenetic dendrograms, including H1R4 and Z1R34 strains with a similarity rate of less than 98.7%, proposed by Chun et al. [27] for the 16S rRNA gene region sequence similarity ratio.

In addition, actinobacteria strains isolated from soils have the ability to both synthesize bioactive molecules and promote plant growth as in many actinobacteria [38,39]. Since the actinobacteria strains isolated in this study are soil isolates, they have the potential to be both a source of new bioactive molecules and to have plant growth promoting properties. In this respect, strains that have the potential to be new species will contribute to the stock of organisms that can be used in pharmacology and agriculture.

***Table 8.*** *Isolates with the potential to be new Actinobacteria species*

| Isolate | Locality | Closest type strain | Similarity (%) | Nucleotid difference |
|---|---|---|---|---|
| AI238 | Gökçeada | *Actinomadura montaniterrae* CYP1-1B$^T$ | 98.96 | 15/1440 |
| AS21 | Gökçeada | *Actinomadura napierensis* B60$^T$ | 99.11 | 12/1344 |
| AI239 | Gökçeada | *Actinomadura napierensis* B60$^T$ | 99.18 | 11/1344 |
| AZ5 | Gökçeada | *Microbispora rosea* subsp. *rosea* ATCC 12950$^T$ | 99.10 | 13/1443 |
| ZİZ37 | Gökçeada | *Nonomuraea candida* HMC10$^T$ | 98.78 | 17/1396 |
| ZIRI269 | Gökçeada | *Streptomyces coeruleorubidus* ISP 5145$^T$ | 99.01 | 14/1412 |
| BI519 | Gökçeada | *Streptomyces filipinensis* NBRC 12860$^T$ | 99.09 | 13/1436 |
| ZEYZ1 | Gökçeada | *Streptomyces seymenliensis* B1041$^T$ | 98.76 | 18/1449 |
| B2F13 | Büyükada | *Actinomadura bangladeshensis* 3-46-b3$^T$ | 98.89 | 16/1442 |
| Z2R54 | Burgazada | *Nonomuraea lycopersici* NEAU-DE8(1)$^T$ | 99.17 | 12/1440 |
| Z1R24 | Burgazada | *Nonomuraea turkmeniaca* DSM 43926$^T$ | 98.87 | 16/1416 |
| Z1R34 | Burgazada | *Streptomyces ureilyticus* YC419$^T$ | 98.21 | 26/1449 |
| H2R22 | Heybeliada | *Kribbella turkmenica* 16K104$^T$ | 98.96 | 15/1442 |
| H2F8 | Heybeliada | *Micromonospora inositola* DSM 43819$^T$ | 98.71 | 18/1395 |
| H3F25 | Heybeliada | *Micromonospora yasonensis* DS3186$^T$ | 98.89 | 16/1437 |
| H3F6 | Heybeliada | *Micromonospora yasonensis* DS3186$^T$ | 98.96 | 15/1438 |
| H3S3 | Heybeliada | *Nocardia takedensis* NBRC 100417$^T$ | 98.85 | 16/1395 |
| H1R4 | Heybeliada | *Nonomuraea candida* HMC10$^T$ | 98.14 | 26/1396 |
| H2R15 | Heybeliada | *Nonomuraea lycopersici* NEAU-DE8(1)$^T$ | 99.03 | 14/1441 |
| K2R23 | Kınalıada | *Agromyces neolithicus* 23-23$^T$ | 98.89 | 16/1439 |
| K1R23 | Kınalıada | *Agromyces neolithicus* 23-23$^T$ | 98.75 | 18/1439 |
| K2F72 | Kınalıada | *Micromonospora luteifusca* GUI2$^T$ | 99.09 | 13/1427 |

## 4. RESULTS

In this study, culturable *Actinobacteria* strains were isolated, and the identification and phylogenetic relationships of these strains were carried out by 16S rRNA gene region sequence analysis. Among the 3 different methods used in the isolation of *Actinobacteria* with a culture-dependent approach, it has been determined that the dilution plate method gives a more successful result in determining the actinobacterial biodiversity of soils. In addition, the results obtained within the scope of the study show that increasing the selective media using the dilution plate method of more soil samples to be taken from different locations of a single island will give more successful results in determining the actinobacterial biodiversity of the islands. In addition, among the selective media used, Humic Acid-Vitamin Agar [7] was found to give more successful results in the selective isolation of *Actinobacteria* compared to other media (Figure 1).

As a result of 16S rRNA gene region sequence analysis, it was determined that the island soils have a high actinobacterial diversity. Strains belonging to the genera *Actinomadura*, *Amycolatopsis*, *Agromyces*, *Geodermatophilus*, *Geodermatophilus*, *Kribbella*, *Microbispora*, *Microbispora*, *Micromonospora*, *Nocardia*, *Nonomuraea*, *Rhodococcus* and *Saccharopolyspora*, predominantly members of the genera *Streptomyces* and *Micromonospora* with high potential for synthesis of bioactive molecules were identified. The results show that the islands, which are an important example of geographical isolation, are a valuable resource for highly new and rare *Actinobacteria* strains. In the identification using the EzBioCloud database and the phylogenetic studies carried out using the MEGA 11 program; It has been seen that the island soil hosts actinobacterial strains with high potential to become a new species. The results obtained within the scope of this study show that at least 22 isolates (Table 8) of belong to 8 different actinobacterial genera have a high potential to be new species, taking into account the similarity rates they show with their closest relatives and their topographic positions in phylogenetic dendrograms (Figures 3-8). The fact that *Actinobacteria* have the potential to produce bioactive molecules and enzymes to be used in the fields of agriculture, health and industry shows that the new species to be identified as a result of this study will make significant contributions to the stock of organisms in these areas from Türkiye. In addition, strains defined as new species are also known despite high sequence similarity rates in the 16S rRNA gene region [31, 40, 41]. According to this literature information, other strains that are isolated within the scope of the study have the potential to become new species. In conclusion, as a result of 16S rRNA gene region sequence analyzes, the taxonomic positions should be determined precisely with a polyphasic taxonomic approach in order for the isolates with both high potential to be new species and low potential to gain new species status.

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## CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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