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# BIODIVERSITY OF ACTINOBACTERIA ISOLATED FROM MARMARA AND AVŞA ISLANDS IN TÜRKİYE

**Ahmet Ridvan TOPKARA[1,2]*, Kamil ISIK[1]**

[1]*Ondokuz Mayis University, Faculty of Science, Department of Biology, 55139, Samsun, Türkiye*
[2]*Çankırı Karatekin University, Central Research Laboratory Application and Research Center, 18100, Çankırı, Türkiye*

**Abstract:** The main purpose of this study is to investigate the microbial diversity of *Actinobacteria*, living in soils in the "Marmara" and "Avşa" Islands and to identify species at genus level based on 16S rRNA gene sequences. These islands are located in the southwest of the Sea of Marmara and in the literature review, no *Actinobacteria* biodiversity studies related to Marmara and Avşa Islands were found. Such unexplored ecological habitats are potentially rich source for discovery sources of novel species and bioactive molecule. *Actinobacteria* play an important role in many natural phenomena such as nitrogen fixation, roles of these prokaryotic microorganisms. In this article, we focused on the presence and the diversity of *Actinobacteria* on the Islands by examining multiple sampling sites and using different selective isolation media. A total of 400 culturable *Actinobacteria* were isolated using ten different isolation media by dilution-plating method. Among the 400 isolates, 112 isolates were selected according to their morphology in different culture media. The isolates were characterized on the basis of 16S ribosomal RNA gene sequencing and phylogenetic analysis. The results showed a high level of actinobacterial diversity with 16 different genera. These genera obtained as a result of phylogenetic analyzes are *Streptomyces, Nonomuraea, Nocardia, Actinomadura, Micromonospora, Kribbella, Mycolicibacterium, Microbispora, Saccharopolyspora, Jiangella, Rhodococcus, Actinopolymorpha, Geodermatophilus, Dactylosporangium, Pseudonocardia* and *Nocardioides.* Many isolates are identified as new species by our current research. Findings from this study showed that the soil of Marmara and Avşa Islands can be a good source of isolation for *Actinobacteria*.

**Keywords:** *Actinobacteria*, Isolation, 16S rRNA gene, Island soil, Biodiversity

***Corresponding author:** Çankırı Karatekin University, Central Research Laboratory Application and Research Center, 18100, Çankırı, Türkiye
**E mail:** ahmetridvantopkara@karatekin.edu.tr (A. R. TOPKARA)
Ahmet Ridvan TOPKARA https://orcid.org/0000-0001-5813-1095
Kamil ISIK https://orcid.org/0000-0003-1764-8113

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## 1. Introduction

*Actinobacteria*, one of the largest bacterial phyla within the Bacteria domain; It is a group of microorganisms consisting of members widely distributed in various terrestrial and aquatic ecosystems. *Actinobacteria* (Küster, 1968), consisting of aerobic and gram-positive bacteria, help the nutrient cycle by degrading different organic compounds due to their wide distribution in nature and their saprophytic properties (Goodfellow and Williams, 1983; Hasegawa et al., 2006).

*Actinobacteria*, which are widely distributed in the soil microbiota, have been isolated from aquatic environments such as hot springs (Barabote et al., 2009; Mokrane et al., 2016; Amin et al., 2017), swamps (Suzuki et al., 1994; Tanasupawat et al., 2016), sea sponges (Kämpfer et al., 2015; Huang et al., 2016; Thawai et al., 2017), and sediments (Deng et al., 2015; Phongsopitanun et al., 2015; Veyisoglu et al., 2016) and from different habitats such as marble surface (Montero-Calasanz et al., 2014), underground caves (Groth et al., 1999; Také et al., 2018), plant rhizosphere (Zhang et al., 2011; Wang et al., 2014; Corretto et al., 2016; Sujarit et al., 2016), rocks (Trujillo et al., 2017), desert (Röttig et al., 2017; Idris et al., 2017; Saygin et al., 2019) and Island soils (Saricaoğlu et al., 2014).

The best-known feature of *Actinobacteria* is that they have the potential to produce a wide range of bioactive molecules, especially antibiotics (Lazzarini et al., 2000; Prescott et al., 2002). Investigation of these powerful potentials has allowed the discovery of more than 120 antibiotics, different enzymes, enzyme inhibitors and many useful products from actinobacterial sources (Wink et al., 2017). In addition, microorganisms such as *Actinobacteria*, which increase soil fertility and contribute to plant development, are called "biofertilizers" and are used in the preparation of microbial vaccine materials in agriculture. Most of the rare actinomycetes are used as "phytostimulators" or plant growth promoters in the soil because they produce substances (e.g., vitamins and plant hormones) that increase plant health and contribute to high yields (El-Tarabily and Sivasithamparam, 2006; Koçak, 2019). At the same time, *Actinobacteria* members are also used as biocontrol agents because they support plant growth by inactivating many plant pathogens that cause disease in plant roots with the bioactive agents they produce endophytically (El-Tarabily and Sivasithamparam, 2006; Kurtboke, 2000; Kurtboke et al., 2007).

In this study, we examined *Actinobacteria* in two regions: Marmara and Avşa Islands, which are part of the "Marmara" region. To our knowledge, the biodiversity of *Actinobacteria* of inhabiting these regions (Marmara-Avşa) has not been studied yet. The aims of the present study were to detect the diversity of *Actinobacteria* of islands (Marmara-Avşa), the presence of the secondary metabolite genes in the isolates and reveal whether soils investigated in islands represents a valuable source for new species. As a result of 16S ribosomal RNA gene analysis, A large number of isolates from two different islands were accepted as potentially new species.

## 2. Materials and Methods

### 2.1. Collection of Soil Sample and Geographical Locations

Soil samples were collected from different parts of the two islands in November 2020 as shown in Figure 1. The soil samples were randomly taken from ten different areas within Avşa and Marmara islands. Samples taken from 10 cm depth of the soil surface were placed in sterile tubes (10 g). The lands of the island of Marmara were later combined, sifted, and thoroughly mixed. Labeled samples were transferred to sterile plastic bags, transported to the laboratory, and stored at 4 °C for analysis.

### 2.2. Selective Isolation of *Actinobacteria* from Island Soils

First of all, soil samples were left to dry for 14 days at room temperature in a sterile petri dish. The dried soil samples are pulverized with a mortar so that they do not mix with each other. Each of the prepared solutions was shaken for 30 minutes. Soil samples weighing 1 g were added to 9 ml of Ringer's solution and tubes with a dilution ratio of $10^{-1}$ were obtained. Dilutions of $10^{-2}$ and $10^{-3}$ were prepared by serial dilution. For each dilution, 2 plates were prepared. The two hundred microliters solutions taken from each diluted suspensions with the help of an automatic pipette were inoculated on different media surfaces and incubated at 28 °C for 21 days. The isolation process was carried out using the dilution plate method. A total of 10 diverse media were used for the selective isolation of *Actinobacteria* members. The media commonly used for the isolation of *Actinomycetes* are shown in Table 1. Various antibiotics (Table 1) have been added to all media to eliminate unwanted gram negative and fungal growth. Inoculated plates were incubated at 28 °C for 3 weeks.

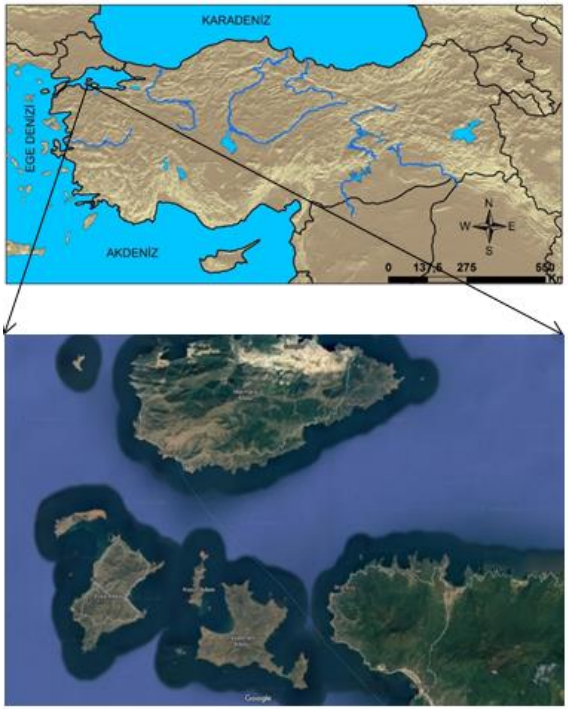


**Figure 1.** Map of the islands showing sample areas

**Table 1.** List of selective media used and antibiotics

| Number | Name of medium | Antibiotics | Reference |
|---|---|---|---|
| 1 | Czapek-Dox Agar* | Rifampicin (5 µg/ml) | Waksman, 1967 |
| 2 | Marine Agar* | Rifampicin (5 µg/ml) | ZoBell, 1941 |
| 3 | R2A Agar* | Nalidixic acid (10 µg/ml) | Reasoner and Geldreich, 1985 |
| 4 | Raffinose-Histidine Agar* | Rifampicin (5 µg/ml), Nalidixic acid (10 µg/ml) | Vickers et al.1984 |
| 5 | Humic Acid-Vitamin Agar* | Nalidixic acid (10 µg/ml) | Taechowison et al. 1993 |
| 6 | M1 Agar* | Neomycin sulphate (4 µg/ml) | Mincer et al. 2002 |
| 7 | Gause Synthetic Agar* | Neomycin (4 µg/ml), | Tan et al. 2006 |
| 8 | SM3 Agar* | Nalidixic acid (10 µg/ml), Neomycin (10 µg/ml) | Tan et al. 2006 |
| 9 | Sodium Succinate Asparagine Agar* | Neomycin sulphate (4 µg/ml) | Piao et al. 2017 |
| 10 | Starch-Casein Agar* | Rifampicin (5 µg/ml), Nalidixic acid (10 µg/ml) | Küster and Williams, 1964 |

*Cycloheximide (50 µg/ml) and nystatin (50 µg/ml) were added to each isolation medium.

**2.3. Selection, Purification and Storage of Isolates**

Colonies that were likely to be *Actinobacteria* in terms of features such as morphology and spore formation were selected from the incubated colonies. In order to obtain pure cultures of these colonies, passage was carried out by streaking on the surface of International *Streptomyces* Project Medium No.2 (ISP2; Shirling and Gottlieb, 1966) agar using sterile loop. Pure isolates were obtained from transferred plates after 14 days of incubation at 28 °C. The pure cultures obtained were transferred into glycerol stock solution (20%, v/v) and finally stored at -80 °C until the time of use.

**2.4. Genomic DNA Extraction from Pure Cultures**

PureLink® Genomic DNA Isolation Kit (Invitrogen, USA) was used to perform genomic DNA isolations of isolates for molecular identification and phylogenetic analysis (Zothanpuia et al., 2017). The presence of products obtained following DNA isolation will be checked in 1% agarose gel electrophoresis. With the Nanodrop, DNA purities and concentrations were checked using a spectrophotometer (Thermo Scientific Multiskan GO microplate reader) with an optical density ratio of 260/280.

**2.5. Identification and 16S rDNA Amplification of Actinobacterial Isolates**

The 16S rRNA gene region polymerase chain reaction (PCR) amplifications were performed on a Thermal Cycler (MyGenie-96 Gradient Thermal Cycler, Korea) using the universal primers 27F and 1525R (Table 2). A reaction mix of 50 µl was prepared for 16S rRNA PCR of each test isolate. The components of this mixture are GoTaq® Hot Start Colorless Master mix (Promega), primer 27F, primer 1525R, genomic DNA and deionized water. In the mixture, these components were added as 25 µl, 1 µl, 1 µl, 1 µl and 22 µl, respectively. PCR amplification (MyGenie-96 Gradient Thermal Cycler, Korea) contains the following parameters: Pre-denaturation at 95 °C for 2 min, denaturation at 95 °C for 1 min, consisting of 30 cycles, 1.5 min bonding at 55 °C and elongation at 72 °C for 3 min, end at 72 °C for 10 min and 4 It consists of storage stages at 4°C. Amplification products were then checked by gel electrophoresis using 4 µL of PCR product in 1.5% agarose gel (Merck) and performed using the Gel imaging system (Bio-RAD).

**Table 2.** Oligonucleotide primers used for 16S rRNA PCR amplification and sequencing

| Primer code | Sequences (5'-3') | Base length | References |
|---|---|---|---|
| 27F | AGAGTTTGATCMTGGCTCAG | 20 | Weisburg, 1991 |
| 518F | CCAGCAGCCGCGGTAAT | 17 | Buchholz-Cleven et al, 1997 |
| 800R | TACCAGGGTATCTAATCC | 18 | Chun and Goodfellow, 1995 |
| MG5F | AAACTCAAAGGAATTGACGG | 20 | Chun and Goodfellow, 1995 |
| 1525R | AAGGAGGTGWTCCARCC | 17 | Lane, 1991 |

*M; adenine or cytosine, R; adenine or guanine, W; adenine or thymine.

**2.6. 16S rRNA Gene Analysis**

Chromatogram files in ABI format obtained as a result of sequencing PCR products were checked using the Chromas version 1.7.6 (C. McCarthy, School of Health Sciences, Griffith University, Queensland, Australia) program and 16S rRNA gene nucleotide sequences were obtained in FASTA format for each organism by overlapping the reads of the primers. Sequences were analyzed and corrected manually. All sequences were compared to other sequences in the EzBioCloud database (Yoon et al., 2017) as well as to GenBank of the NCBI website by the BLAST program (Altschul et al., 1990). MEGA X program was used for phylogenetic analysis (Tamura et al., 2013). Multiple alignments were performed with the option Clustal_W (Tamura et al., 2013). Phylogenetic dendrograms were created based on neighbor-joining (Saitou and Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms. The Jukes-Cantor (Jukes and Cantor, 1969) matrix was used as the phylogenetic distance matrix. Bootstrap analyzes of the obtained phylogenetic trees were performed with 1000 repetitions (Felsenstein, 1985). The 16S rRNA gene sequences of *Actinobacteria* isolates in this study were stored in the NCBI GenBank database.

## 3. Results

**3.1. Physicochemical Properties of Soil Samples**

Soil samples were taken by GPS from different localities (considering variables such as vegetation, soil type, topography, and parent material) of two different islands (Avşa; 36 km$^2$ and Marmara; 117 km$^2$, Balıkesir-South Marmara Islands) in the Marmara region included in the study (Table 3).

**Table 3**. Locality and Geographical coordinates of soil samples

| Soil No | Locality | Geographical coordinates |
|---|---|---|
| 1 | Avşa | 40°31'52.73"K 27°30'39.60"D |
| 2 | Avşa | 40°31'39.91"K 27°31'33.27"D |
| 3 | Avşa | 40°31'12.17"K 27°32'3.82"D |
| 4 | Avşa | 40°30'49.52"K 27°32'13.12"D |
| 5 | Avşa | 40°29'26.17"K 27°32'5.83"D |
| 6 | Avşa | 40°29'11.66"K 27°30'57.66"D |
| 7 | Avşa | 40°29'38.01"K 27°30'23.03"D |
| 8 | Avşa | 40°30'18.95"K 27°30'21.38"D |
| 9 | Avşa | 40°29'29.69"K 27°29'11.73"D |
| 10 | Avşa | 40°28'22.47"K 27°29'31.80"D |
| 11 | Marmara | 40°35'31.37"K 27°33'7.013"D |
| 12 | Marmara | 40°35'44.498"K 27°32'57.156"D |
| 13 | Marmara | 40°34'42.964"K 27°34'18.318"D |
| 14 | Marmara | 40°35'42.677"K 27°35'7.879"D |
| 15 | Marmara | 40°35'13.358"K 27°36'29.398"D |
| 16 | Marmara | 40°36'32.803"K 27°41'1.835"D |
| 17 | Marmara | 40°38'21.293"K 27°41'58.909"D |
| 18 | Marmara | 40°38'59.093"K 27°41'52.127"D |

Physicochemical properties of soil samples such as electrical conductivity, amount of organic matter, moisture content, and lime content (CaCO3) were determined (Table 4-5).

Physicochemical analyzes of Marmara and Avşa soils are shown in Table 4-5. The pH values of Avşa soil samples generally vary between 4.67 and 6.90, while the pH values of Marmara soil samples generally vary between 4.98 and 7.93. Organic matter amount of Avşa soil samples varies between 0.36 and 2.74, while organic matter amount of Marmara soil samples varies between 0.44 and 1.95. These soils are also loamy-clay-sandy and have low moisture and organic matter ratios.

### 3.2. Morphological Analysis of Culturable *Actinobacteria*

As a result of soil isolation, a total of 400 isolates obtained from Marmara and Avşa Islands were divided into 40 color groups according to colony morphology and pigmentation characteristics (Kelly, 1964). After color grouping, 112 isolates were selected for 16S rRNA gene region analysis, taking into account features such as 10 different selective media, substrate mycelium, air mycelium and soil physicochemical properties, and phylogenetic analyzes were performed.

Among the actinomycete isolates, 18 strains were isolated on starch casein agar, 5 strains from Marine agar, 5 strains from R2A agar, 2 strains from SM3 agar, 2 strains from M1 agar, 15 strains from Humic acid vitamin agar, 23 strains from Gause agar, 20 strains from Czapek dox agar, 8 strains from Raffinose-Histidine agar, 14 strains from Sodium-Succinate agar and incubated at 28 °C for about 21 days (Table 6).

These results plainly showed that Gause agar was the most suitable medium for the isolation of *Actinobacteria* from islands and provided about 20% of the total isolates followed by Czapek dox agar (17%). In total, 112 culturable actinobacterial isolates were isolated from the ten different stations of the Islands: 32 isolates from the Marmara soil, 80 isolates from Avşa soil.

**Table 4.** Physicochemical properties of soil samples taken from Avşa Island

| Sample code | EC | pH | %OC | %OM | %$CaCO_3$ | %Clay | %Silt | %Sand | Texture |
|---|---|---|---|---|---|---|---|---|---|
| Avşa 1 | 445,7 | 6,90 | 0,44 | 0,76 | 1,52 | 41,30 | 18,98 | 39,72 | clay |
| Avşa 2 | 143,8 | 6,75 | 0,19 | 0,32 | 2,15 | 5,03 | 3,72 | 91,25 | sand |
| Avşa 3 | 826,5 | 4,67 | 1,07 | 1,84 | 2,15 | 11,05 | 9,81 | 79,14 | sand loamy |
| Avşa 4 | 782,3 | 6,63 | 0,87 | 1,50 | 1,43 | 19,17 | 13,94 | 66,89 | sand loamy |
| Avşa 5 | 216,7 | 5,52 | 1,59 | 2,74 | 2,15 | 15,27 | 15,92 | 68,80 | sand loamy |
| Avşa 6 | 98,1 | 6,18 | 0,23 | 0,40 | 2,15 | 7,04 | 9,86 | 83,09 | sand loamy |
| Avşa 7 | 303,2 | 5,89 | 0,44 | 0,76 | 1,46 | 11,20 | 11,77 | 77,02 | sand clay |
| Avşa 8 | 368,9 | 6,18 | 0,55 | 0,95 | 1,46 | 17,34 | 16,02 | 66,64 | sand loamy |
| Avşa 9 | 254,0 | 5,89 | 0,21 | 0,36 | 1,52 | 41,24 | 16,64 | 42,12 | clay |
| Avşa 10 | 227,9 | 6,30 | 0,94 | 1,62 | 1,44 | 6,98 | 9,70 | 83,33 | sand loamy |

EC = Electrical Conductivity, OM= Organic Matter, $CaCO_3$= Lime Quantities.

**Table 5.** Physicochemical properties of soil samples taken from Marmara Island

| Sample code | EC | pH | %OC | %OM | %CaCO3 | %Clay | %Silt | %Sand | Texture |
|---|---|---|---|---|---|---|---|---|---|
| M1 | 203,6 | 6,45 | 0,52 | 0,90 | 1,44 | 14,93 | 7,85 | 77,22 | sand loamy |
| M2 | 363,3 | 7,48 | 0,72 | 1,25 | 3,67 | 19,33 | 32,30 | 48,36 | loamy |
| M3 | 145,3 | 6,56 | 0,86 | 1,47 | 2,19 | 31,90 | 32,77 | 35,33 | clay loamy |
| M4 | 220,7 | 4,98 | 0,30 | 0,51 | 1,45 | 15,46 | 41,42 | 43,12 | loamy |
| M5 | 225,9 | 6,11 | 0,57 | 0,98 | 0,78 | 29,24 | 28,06 | 42,70 | clay loamy |
| M6 | 144,4 | 7,05 | 1,13 | 1,95 | 1,43 | 12,93 | 19,92 | 67,16 | sand loamy |
| M7 | 237,0 | 7,93 | 0,85 | 1,47 | 116,90 | 35,88 | 9,92 | 54,20 | sand clay |
| M8 | 93,8 | 7,50 | 0,25 | 0,44 | 1,43 | 8,95 | 15,80 | 75,25 | sand loamy |

*EC = Electrical Conductivity, OM= Organic Matter, $CaCO_3$= Lime Quantities.

**Table 6.** Comparison of the isolation success of each culture media in function of the number of isolates/genera obtained.

| Number | Name of medium | Isolates | Genera |
|---|---|---|---|
| 1 | Czapek-Dox Agar | 20 | 6 |
| 2 | Marine Agar | 5 | 6 |
| 3 | R2A Agar | 5 | 3 |
| 4 | Raffinose-Histidine Agar | 8 | 6 |
| 5 | Humic Acid-Vitamin Agar | 15 | 6 |
| 6 | M1 Agar | 2 | 2 |
| 7 | Gause Synthetic Agar | 23 | 12 |
| 8 | SM3 Agar | 2 | 2 |
| 9 | Sodium Succinate | 14 | 5 |
| 10 | Starch-Casein Agar | 18 | 6 |

### 3.3. Biodiversity of Culturable *Actinobacteria*

As a result of 16S rRNA gene region nucleotide sequence analysis, 112 isolates were determined to be members of the *Actinobacteria* phylum. When 112 *Actinobacteria* members were examined; It was determined that the genera *Streptomyces* (36 isolates ), *Nonomuraea* (17 isolates), *Nocardia* (11 isolates ) and *Actinomadura* (11 isolates ) were the dominant genera found in the island environments and the other strains belonged to the genera *Micromonospora* (6 isolates), *Kribbella* (6

isolates), *Mycolicibacterium* (2 isolates), *Microbispora* (7 isolates), *Saccharopolyspora* (3 isolates), *Jiangella* (1 isolates), *Rhodococcus* (2 isolates), *Actinopolymorpha* (3 isolates), *Geodermatophilus* (1 isolates), *Dactylosporangium* (3 isolates), *Nocardioides* (1 isolates) and *Pseudonocardia* (2 isolates). Finally, results obtained in this study showed a very significant level of diversity with 16 different genera of *Actinobacteria* (Figure 2).

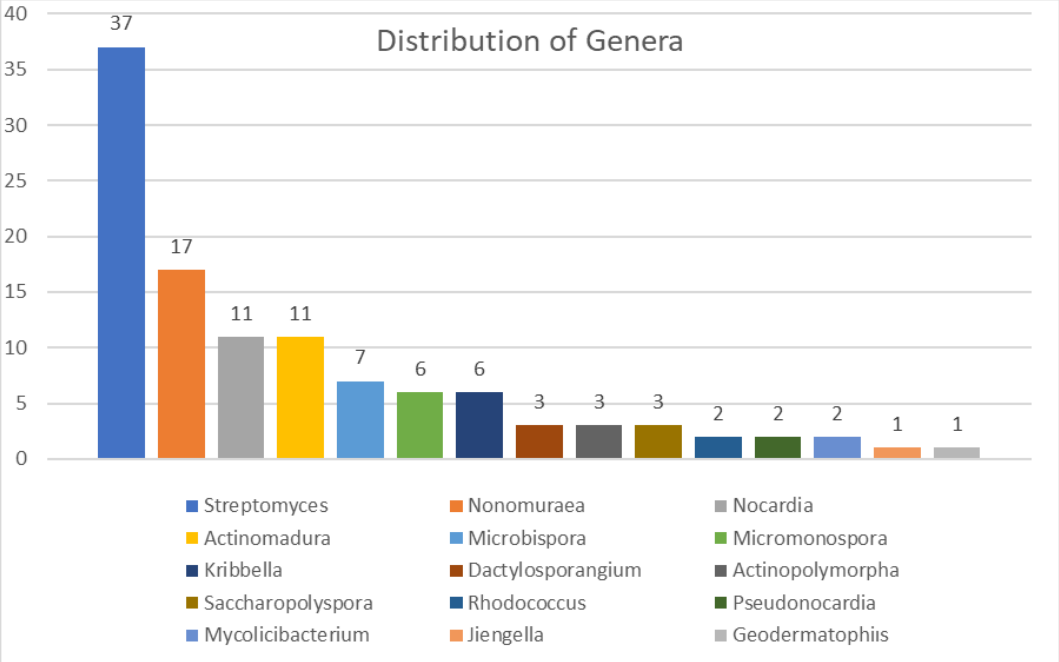


**Figure 2.** Distribution of *Actinobacteria* isolates by *genus*.

### 3.4. Phylogenetic Analysis of the Isolates

Phylogenetic trees based on 16S rRNA sequences were constructed to investigate the evolutionary relationships between the *Actinobacteria* isolates in this study and their closest taxonomic relatives. The molecular identification of isolates by amplification of 16S rRNA gene was done by using universal primers 518F, 800R and MG5F (Table 2). All the isolates of *Actinomadura* were found to be closely related to the genus *Actinomadura* and shared 16S rRNA gene similarity ranging from 98.19-100.00% (Figure 3). Isolates of the genera *Microbispora, Kribbella, Jiangella, Nocardioides Actinopolymorpha, Geodermatophilus, Micromonospora, Dactylosporangium, Nocardia, Rhodococcus, Mycolicibacterium, Saccharopolyspora* and *Pseudonocardia* were clustered together with their closest relatives (Figure 3, 4, 5, 6, 7). The phylogenetic tree, according to the neighbor-joining algorithm indicated that 17 strains were members of the genus *Nonomuraea* (Figure 8). Seventeen *Nonomuraea* isolates showed close 16S rRNA gene sequence similarity with the type strain of *Nonomuraea*, which are 97.78% and 99.79% (Table 7). Based on 16S rRNA gene sequence analysis, 37 isolates were identified as *Streptomyces* spp. The phylogenetic tree, according to the neighbor-joining algorithm, indicated that nineteen strains were members of the genus *Streptomyces* (Figure 9-10; Table 7). According to the 16S ribosomal RNA gene sequence analysis, 37 *Streptomyces* isolates showed close 16S rRNA gene sequence similarity to the *Streptomyces* type strains, which are 99.86% and 98.00%, respectively. The percentage of 16S ribosomal RNA gene sequence similarity of the isolates with their closest relatives is shown in Table 7.

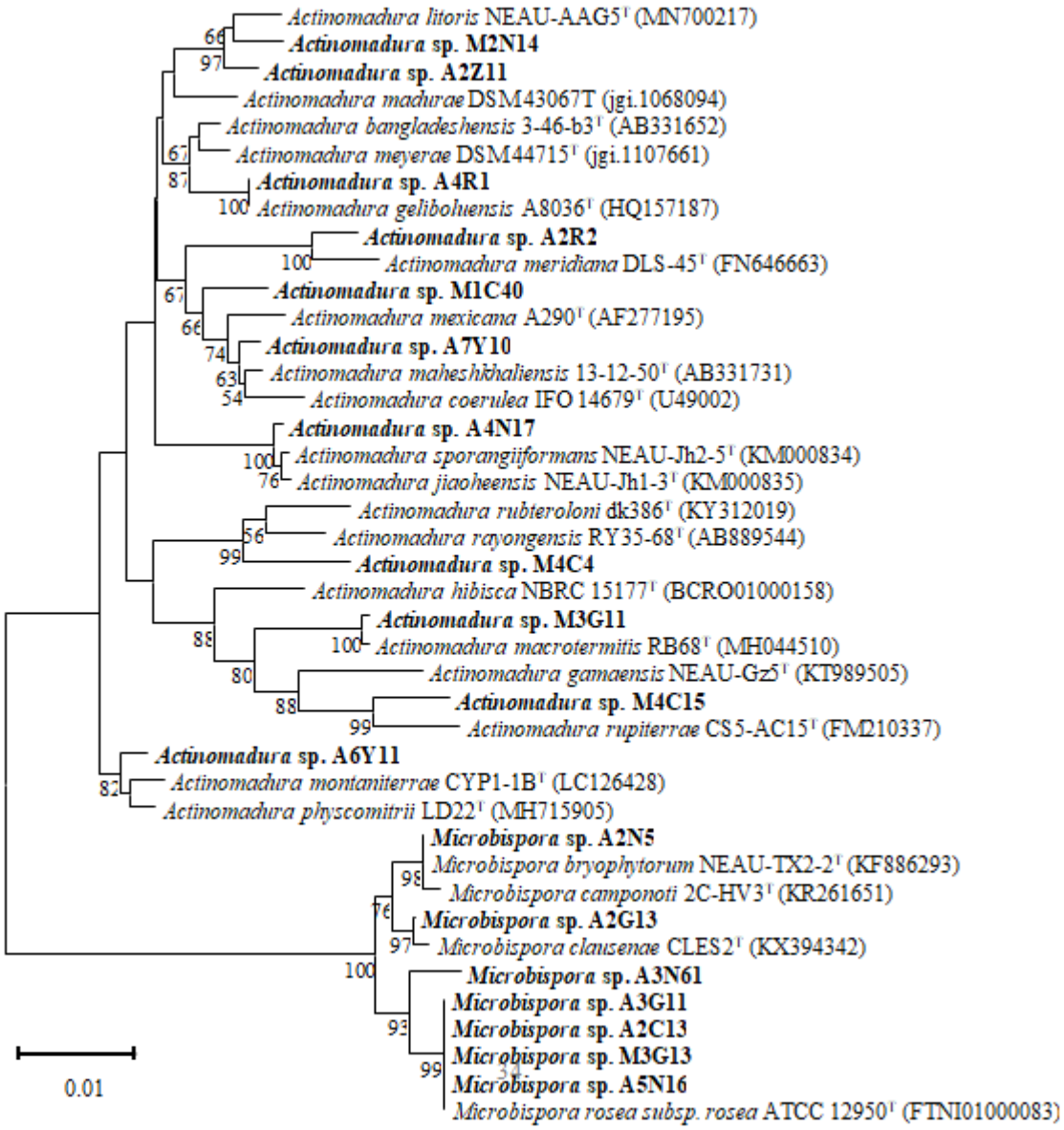


**Figure 3.** Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Actinomadura*, group *Microbispora*, and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA X software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

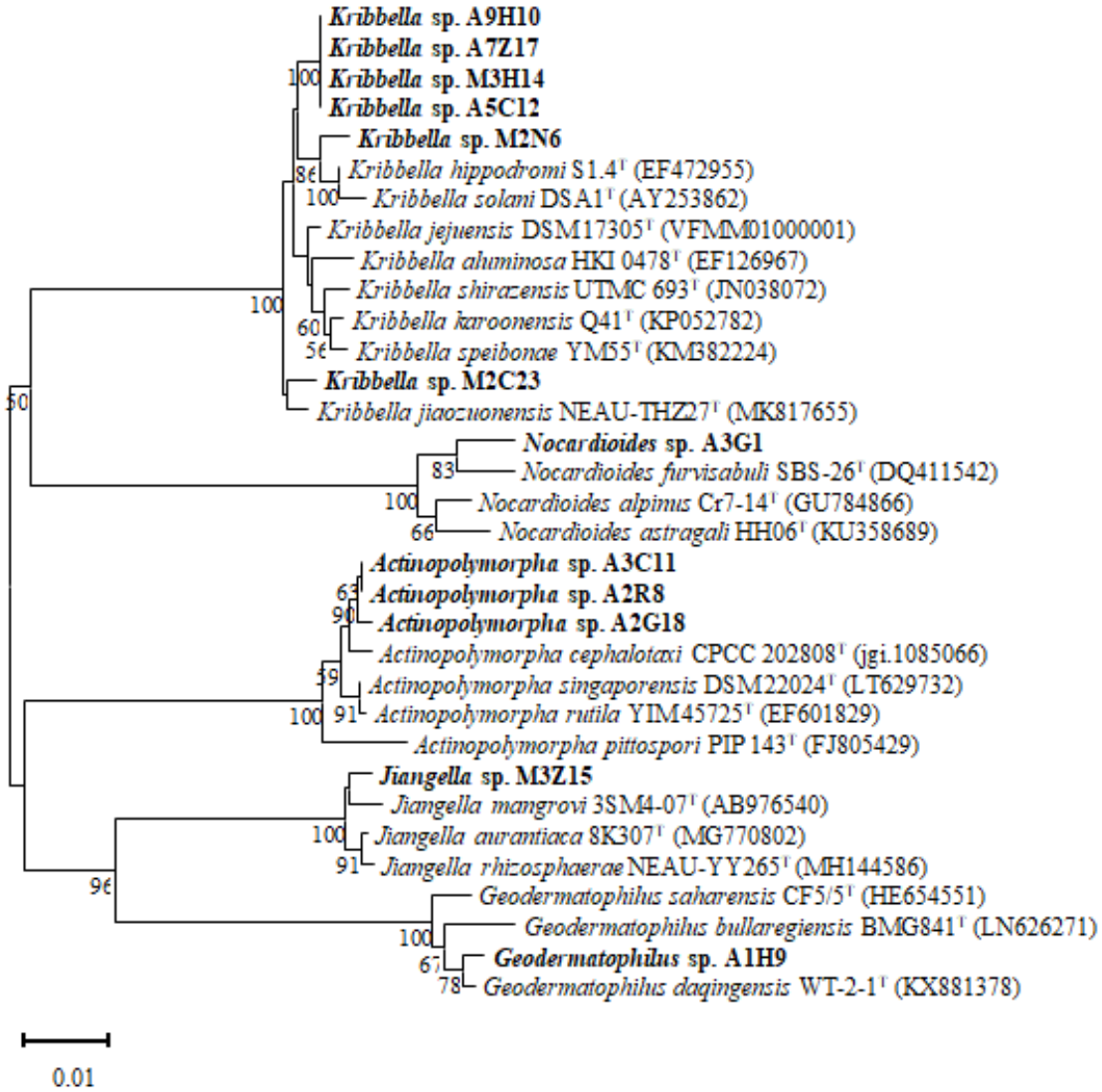


**Figure 4.** Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Kribbella*, group *Nocardioides*, group *Actinopolymorpha*, group *Jiangella*, group *Geodermatophilus*, and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA X software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

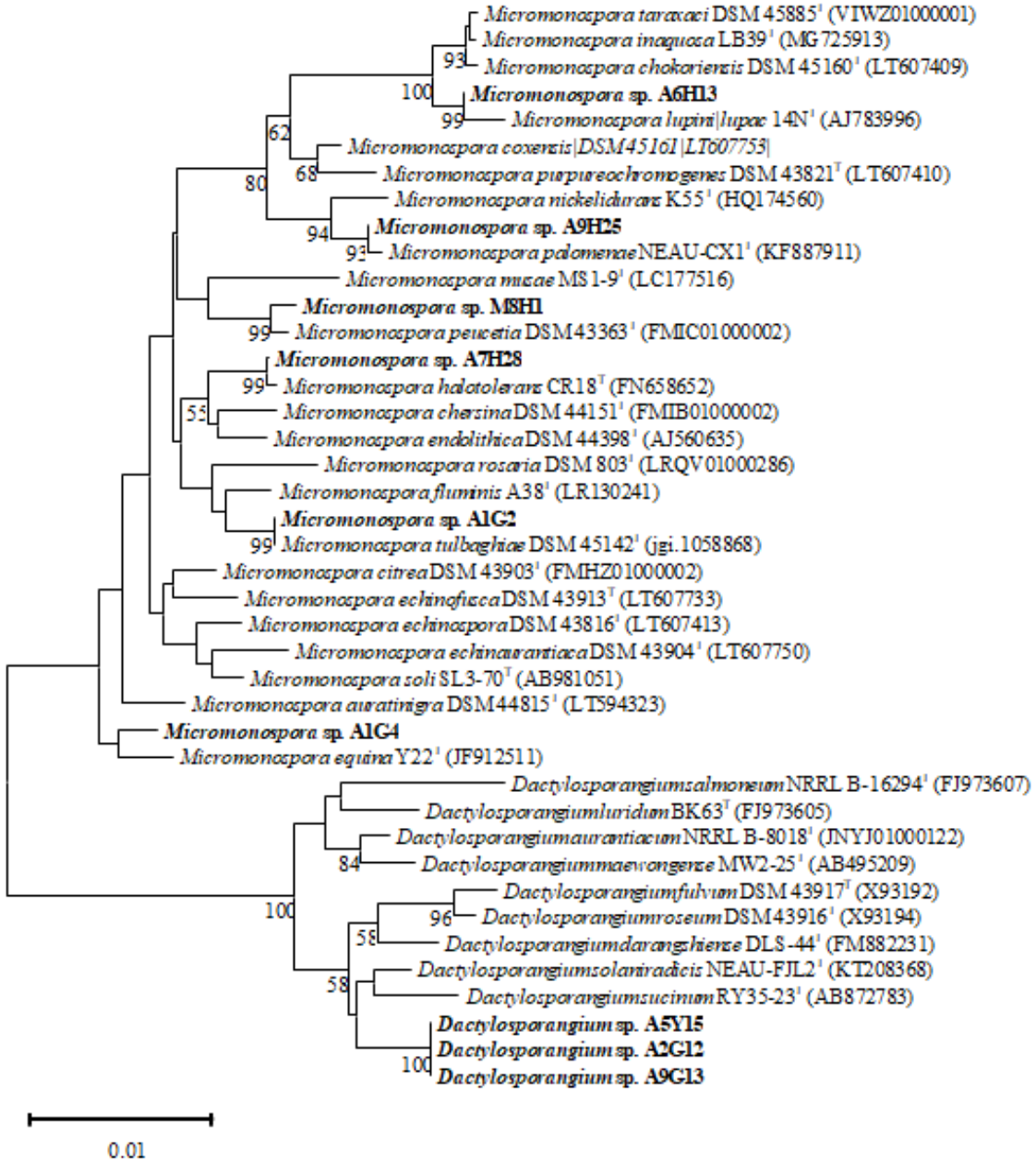


**Figure 5.** Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Micromonospora*, group *Dactylosporangium*, and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA X software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

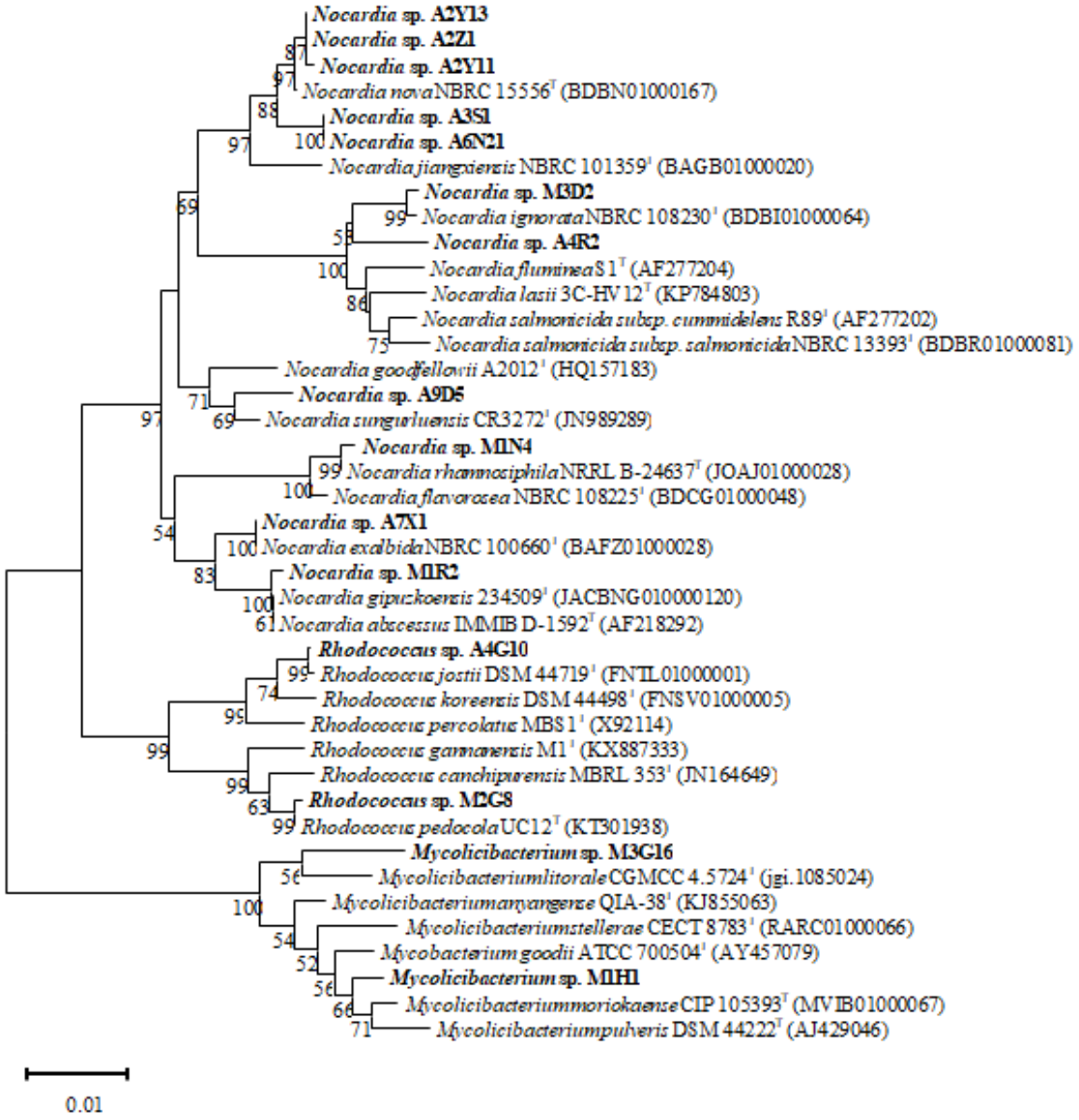


**Figure 6.** Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Nocardia*, group *Rhodococcus*, group *Mycolicibacterium*, and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA X software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

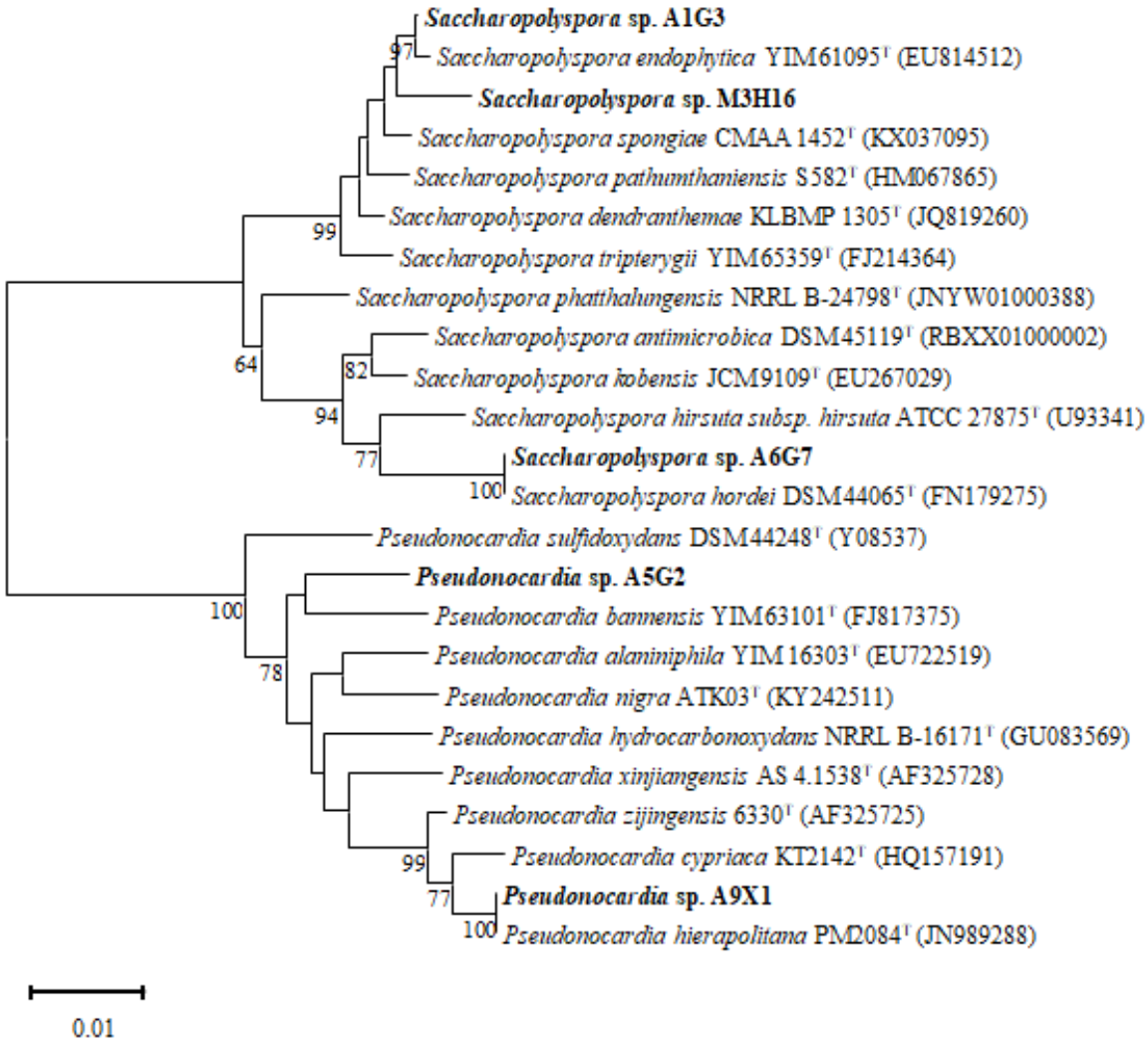


**Figure 7.** Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Saccharopolyspora*, group *Pseudonocardia*, and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA X software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

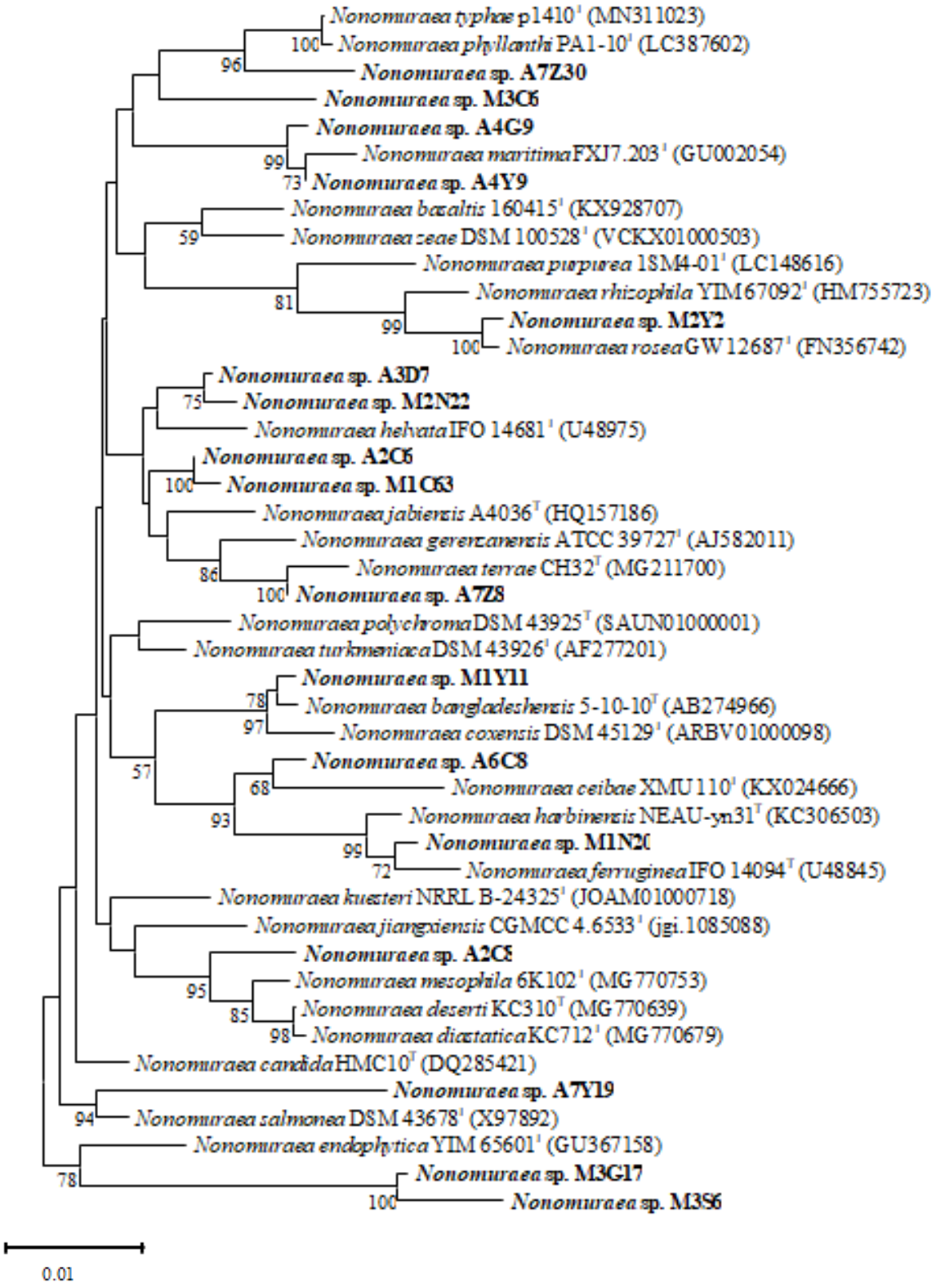


**Figure 8.** Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Nonomuraea*, and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA X software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

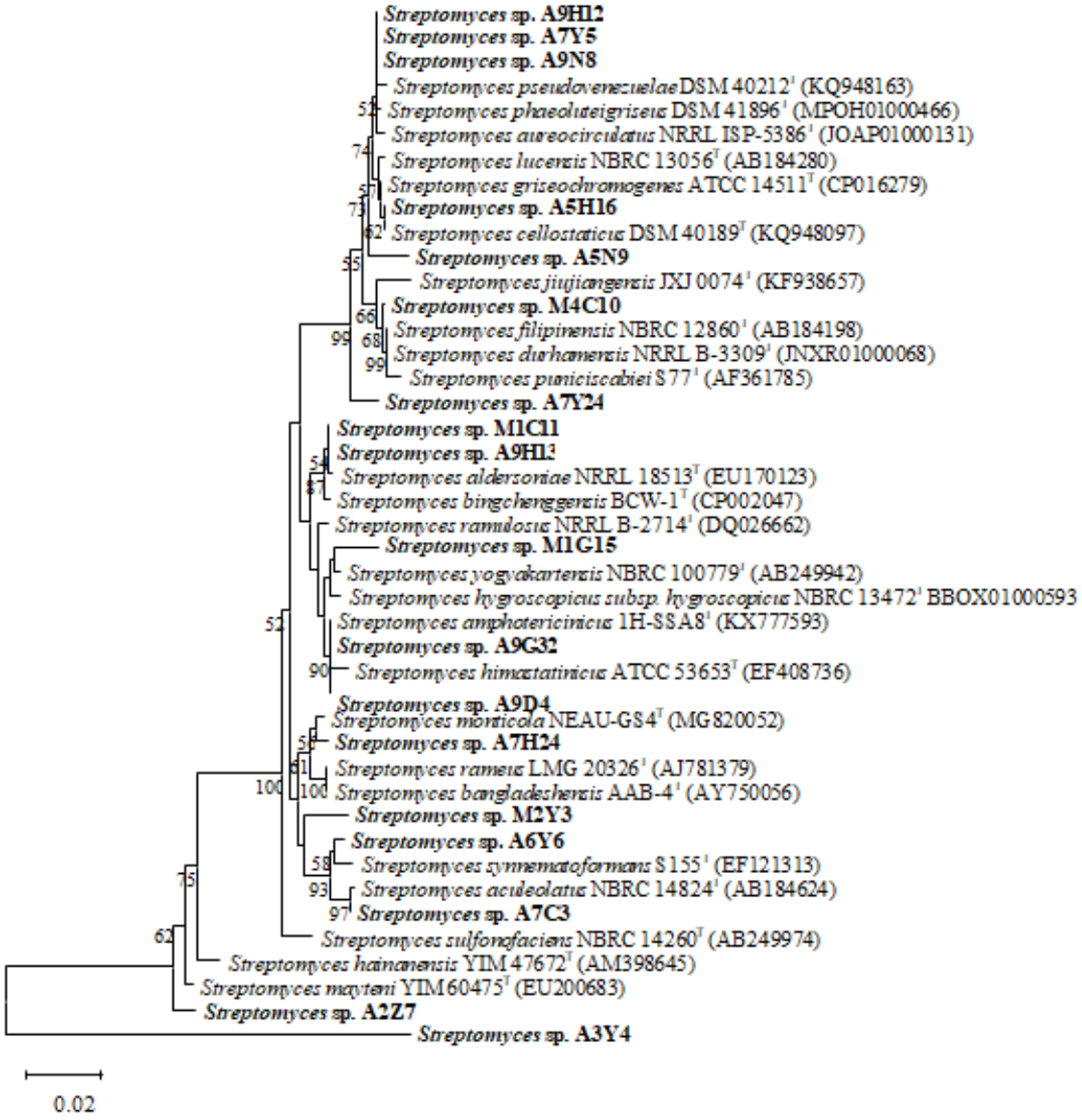


**Figure 9.** Phylogenetic relationships based on 16S rRNA sequences amongst 43 *Streptomyces* strains in relation to closely related validly described species. Phylogenetic tree was based on the Neighbor-joining method using MEGA X software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.02 substitutions per nucleotide position.

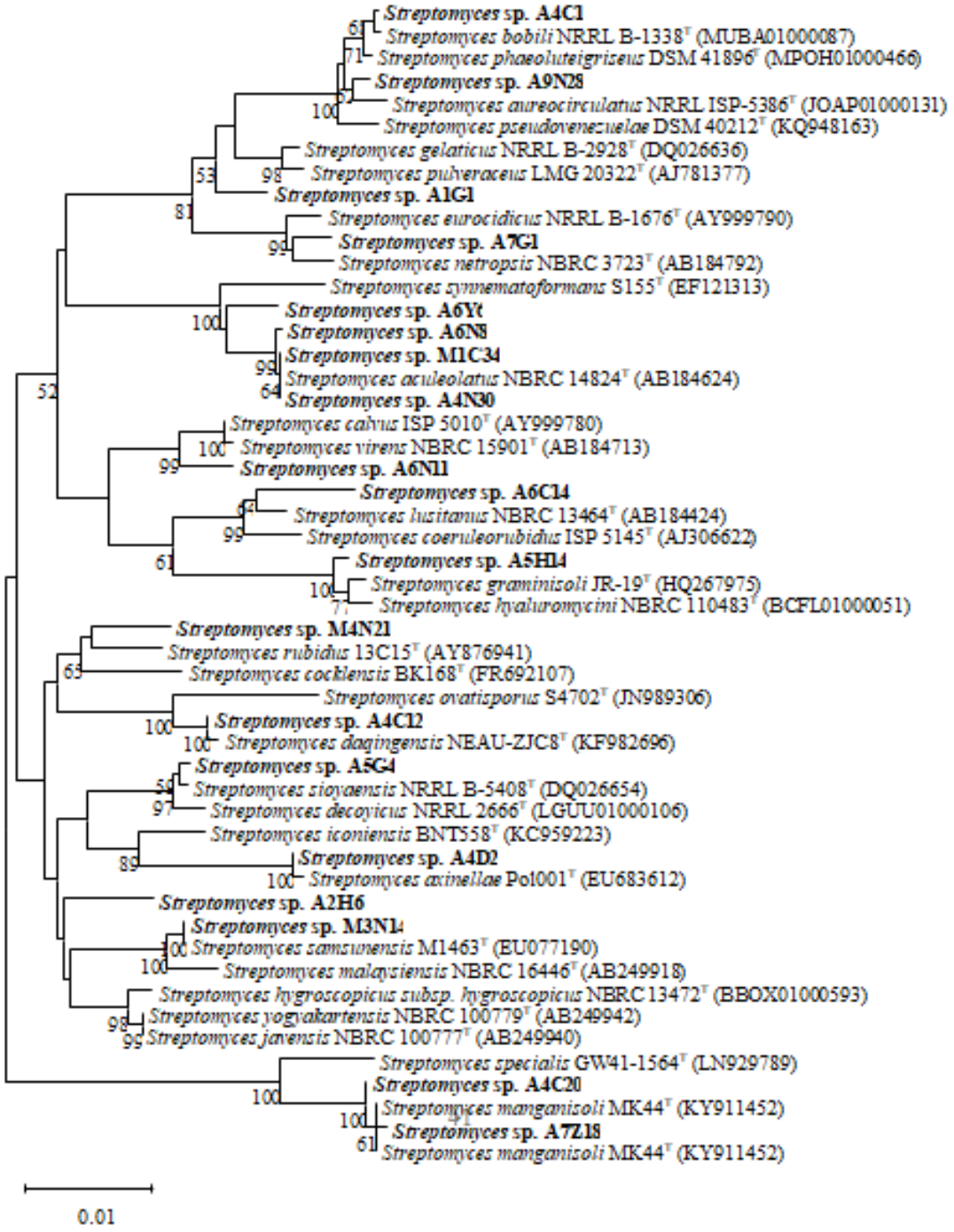


**Figure 10.** Phylogenetic relationships based on 16S rRNA sequences amongst 51 *Streptomyces* strains in relation to closely related validly described species. Phylogenetic tree was based on the Neighbor-joining method using MEGA X software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

**Table 7.** Summary of information on 16S rRNA sequence determination of isolated strains

| Number | Strain | Locality | Highest match | Similarity (%) Nucleotide difference |
|---|---|---|---|---|
| 1. | A2R2 | Avşa | *Actinomadura meridiana* DLS-45$^T$ | 98.60 - 20/1426 |
| 2. | A2Z11 | Avşa | *Actinomadura litoris* NEAU-AAG5$^T$ | 99.17 - 12/1438 |
| 3. | A4N17 | Avşa | *Actinomadura sporangiiformans* NEAU-Jh2-5$^T$ | 99.79 - 3/1440 |
| 4. | A4R1 | Avşa | *Actinomadura geliboluensis* A8036$^T$ | 100.00 - 0/1441 |
| 5. | A6Y11 | Avşa | *Actinomadura montaniterrae* CYP1-1B$^T$ | 99.31 - 10/1440 |
| 6. | A7Y10 | Avşa | *Actinomadura maheshkhaliensis* 13-12-50$^T$ | 99.58 - 6/1417 |
| 7. | M1C40 | Avşa | *Actinomadura maheshkhaliensis* 13-12-50$^T$ | 99.08 - 13/1420 |
| 8. | M2N14 | Marmara | *Actinomadura litoris* NEAU-AAG5$^T$ | 99.03 - 14/1438 |
| 9. | M3G11 | Marmara | *Actinomadura macrotermitis* RB68$^T$ | 99.86 - 2/1442 |
| 10. | M4C4 | Marmara | *Actinomadura rubteroloni* dk386$^T$ | 98.19 - 26/1438 |
| 11. | M4C15 | Marmara | *Actinomadura rupiterrae* CS5-AC15$^T$ | 98.25 - 25/1427 |
| 12. | A2G18 | Avşa | *Actinopolymorpha cephalotaxi* CPCC 202808$^T$ | 99.44 - 8/1439 |
| 13. | M3Z15 | Marmara | *Jiangella aurantiaca* 8K307$^T$ | 99.51 - 7/1441 |
| 14. | A1H9 | Avşa | *Geodermatophilus daqingensis* WT-2-1$^T$ | 99.58 - 6/1443 |
| 15. | A2G12 | Avşa | *Dactylosporangium solaniradicis* NEAU-FJL2$^T$ | 99.16 - 12/1437 |
| 16. | A5Y15 | Avşa | *Dactylosporangium solaniradicis* NEAU-FJL2$^T$ | 99.16 - 12/1437 |
| 17. | A9G13 | Avşa | *Dactylosporangium solaniradicis* NEAU-FJL2$^T$ | 99.16 - 12/1437 |
| 18. | A5G2 | Avşa | *Pseudonocardia xinjiangensis* AS 4.1538$^T$ | 97.74 - 30/1325 |
| 19. | A9X1 | Avşa | *Pseudonocardia hierapolitana* PM2084$^T$ | 99.79 - 3/1443 |
| 20. | A1G3 | Avşa | *Saccharopolyspora endophytica* YIM 61095$^T$ | 99.86 - 2/1445 |
| 21. | A6G7 | Avşa | *Saccharopolyspora hordei* DSM 44065$^T$ | 99.86 - 2/1448 |
| 22. | M3H16 | Marmara | *Saccharopolyspora spongiae* CMAA 1452$^T$ | 98.75 - 18/1443 |
| 23. | A4G10 | Avşa | *Rhodococcus jostii* DSM 44719$^T$ | 99.93 - 1/1440 |
| 24. | M2G8 | Marmara | *Rhodococcus pedocola* UC12$^T$ | 99.93 - 1/1441 |
| 25. | M1H1 | Marmara | *Mycolicibacterium moriokaense* CIP 105393$^T$ | 99.10 - 13/1444 |
| 26. | M3G16 | Marmara | *Mycolicibacterium litorale* CGMCC 4.5724$^T$ | 98.40 - 23/1442 |
| 27. | A5C12 | Avşa | *Kribbella jejuensis* DSM 17305$^T$ | 99.52 - 7/1447 |
| 28. | A7Z17 | Avşa | *Kribbella jejuensis* DSM 17305$^T$ | 99.52 - 7/1447 |
| 29. | A9H10 | Avşa | *Kribbella jejuensis* DSM 17305$^T$ | 99.52 - 7/1447 |
| 30. | M2C23 | Marmara | *Kribbella karoonensis* Q41$^T$ | 99.45 - 8/1447 |
| 31. | M2N6 | Marmara | *Kribbella hippodromi* S1.4$^T$ | 99.35 - 9/1389 |
| 32. | M3H14 | Marmara | *Kribbella jejuensis* DSM 17305$^T$ | 99.52 - 7/1445 |
| 33. | A2C13 | Avşa | *Microbispora rosea* subsp. *rosea* ATCC 12950$^T$ | 100.00 - 0/1443 |
| 34. | A2G13 | Avşa | *Microbispora clausenae* CLES2$^T$ | 99.79 - 3/1443 |
| 35. | A2N5 | Avşa | *Microbispora bryophytorum* NEAU-TX2-2$^T$ | 100.00 - 0/1443 |
| 36. | A3G11 | Avşa | *Microbispora rosea* subsp. *rosea* ATCC 12950$^T$ | 100.00 - 0/1443 |
| 37. | A3N61 | Avşa | *Microbispora rosea* subsp. *rosea* ATCC 12950$^T$ | 99.17 - 12/1443 |
| 38. | A5N16 | Avşa | *Microbispora rosea* subsp. *rosea* ATCC 12950$^T$ | 100.00 - 0/1443 |
| 39. | M3G13 | Marmara | *Microbispora rosea* subsp. *rosea* ATCC 12950$^T$ | 100.00 - 0/1443 |
| 40. | A1G4 | Avşa | *Micromonospora echinaurantiaca* DSM 43904$^T$ | 99.24 - 11/1439 |
| 41. | A1G2 | Avşa | *Micromonospora tulbaghiae* DSM 45142$^T$ | 100.00 - 0/1437 |
| 42. | A6H13 | Avşa | *Micromonospora taraxaci* DSM 45885$^T$ | 99.65 - 5/1437 |
| 43. | A7H28 | Avşa | *Micromonospora halotolerans* CR18$^T$ | 99.93 - 1/1429 |
| 44. | A9H25 | Avşa | *Micromonospora palomenae* NEAU-CX1$^T$ | 99.93 - 1/1438 |
| 45. | M4H1 | Avşa | *Streptomyces aculeolatus* NBRC 14824 T | 99.72 - 4/1442 |
| 46. | A2Y11 | Avşa | *Nocardia nova* NBRC 15556$^T$ | 99.79 - 3/1439 |
| 47. | A2Y13 | Avşa | *Nocardia nova* NBRC 15556$^T$ | 99.86 - 2/1438 |
| 48. | A2Z1 | Avşa | *Nocardia nova* NBRC 15556$^T$ | 99.86 - 2/1439 |
| 49. | A3S1 | Avşa | *Nocardia nova* NBRC 15556$^T$ | 99.37 - 9/1439 |
| 50. | A4R2 | Avşa | *Nocardia salmonicida* subsp. *cummidelens* R89$^T$ | 98.60 - 20/1425 |
| 51. | A6N21 | Avşa | *Nocardia nova* NBRC 15556$^T$ | 99.30 - 10/1438 |
| 52. | A7X1 | Avşa | *Nocardia exalbida* NBRC 100660$^T$ | 100.00 - 0/1441 |
| 53. | A9D5 | Avşa | *Nocardia sungurluensis* CR3272$^T$ | 99.24 - 11/1441 |
| 54. | M1N4 | Marmara | *Nocardia rhamnosiphila* NRRL B-24637$^T$ | 99.86 - 2/1439 |
| 55. | M1R2 | Marmara | *Nocardia gipuzkoensis* 234509$^T$ | 99.86 - 2/1441 |
| 56. | M3D2 | Marmara | *Nocardia ignorata* NBRC 108230$^T$ | 99.72 - 4/1441 |
| 57. | A2C6 | Avşa | *Nonomuraea jabiensis* A4036$^T$ | 99.31 - 10/1442 |

**Table 7.** Summary of information on 16S rRNA sequence determination of isolated strains (continuing)

| Number | Strain | Locality | Highest match | Similarity (%) Nucleotide difference |
|---|---|---|---|---|
| 58. | A2C8 | Avşa | *Nonomuraea deserti* KC310 T | 98.96 - 15/1443 |
| 59. | A3D7 | Avşa | *Nonomuraea helvata* IFO 14681 T | 98.65 - 19/1409 |
| 60. | A4G9 | Avşa | *Nonomuraea maritima* FXJ7.203 T | 99.51 - 7/1441 |
| 61. | A4Y9 | Avşa | *Nonomuraea maritima* FXJ7.203 T | 99.72 - 4/1441 |
| 62. | A6C8 | Avşa | *Nonomuraea ceibae* XMU 110 T | 98.89 - 16/1437 |
| 63. | A7Y19 | Avşa | *Nonomuraea salmonea* DSM 43678 T | 97.49 - 36/1436 |
| 64. | A7Z8 | Avşa | *Nonomuraea terrae* CH32 T | 99.65 - 5/1441 |
| 65. | A7Z30 | Avşa | *Nonomuraea nitratireducens* WYY166 T | 98.89 - 16/1444 |
| 66. | M1N20 | Marmara | *Nonomuraea harbinensis* NEAU-yn31 T | 99.65 - 5/1447 |
| 67. | M1Y11 | Marmara | *Nonomuraea bangladeshensis* 5-10-10 T | 99.79 - 3/1441 |
| 68. | M1C63 | Marmara | *Nonomuraea jabiensis* A4036 T | 99.17 - 12/1442 |
| 69. | M2Y2 | Marmara | *Nonomuraea rosea* GW 12687 T | 99.78 - 3/1375 |
| 70. | M2N22 | Marmara | *Nonomuraea jabiensis* A4036 T | 98.82 - 17/1441 |
| 71. | M3C6 | Marmara | *Nonomuraea basaltis* 160415 T | 98.68 - 19/1443 |
| 72. | M3S6 | Marmara | *Nonomuraea endophytica* YIM 65601 T | 97.28 - 39/1434 |
| 73. | M3G17 | Marmara | *Nonomuraea endophytica* YIM 65601 T | 97.78 - 32/1444 |
| 74. | A3G1 | Avşa | *Nocardioides furvisabuli* SBS-26(T) | 98.57- 20/1398 |
| 75. | A1G1 | Avşa | *Streptomyces gelaticus* NRRL B-2928 T | 99.03 - 14/1448 |
| 76. | A2H6 | Avşa | *Streptomyces hygroscopicus* subsp. NBRC 13472 T | 98.47 - 22/1442 |
| 77. | A2Z7 | Avşa | *Streptomyces mayteni* YIM 60475 T | 99.27 - 10/1378 |
| 78. | A3Y4 | Avşa | *Streptomyces mayteni* YIM 60475 T | 89.67 - 142/1374 |
| 79. | A4C1 | Avşa | *Streptomyces bobili* NRRL B-1338 T | 99.86 - 2/1448 |
| 80. | A4C12 | Avşa | *Streptomyces daqingensis* NEAU-ZJC8 T | 99.79 - 3/1450 |
| 81. | A4C20 | Avşa | *Streptomyces manganisoli* MK44 T | 99.64 - 5/1405 |
| 82. | A4D2 | Avşa | *Streptomyces axinellae* Pol001 T | 99.86 - 2/1422 |
| 83. | A4N30 | Avşa | *Streptomyces aculeolatus* NBRC 14824 T | 99.51 - 7/1440 |
| 84. | A5G4 | Avşa | *Streptomyces sioyaensis* NRRL B-5408 T | 99.79 - 3/1451 |
| 85. | A5H14 | Avşa | *Streptomyces graminisoli* JR-19 T | 99.72 - 4/1446 |
| 86. | A5H16 | Avşa | *Streptomyces cellostaticus* DSM 40189 T | 99.59 - 6/1448 |
| 87. | A5N9 | Avşa | *Streptomyces lucensis* NBRC 13056 T | 98.74 - 18/1426 |
| 88. | A6C14 | Avşa | *Streptomyces lusitanus* NBRC 13464 T | 98.67 - 19/1427 |
| 89. | A6N8 | Avşa | *Streptomyces aculeolatus* NBRC 14824 T | 99.58 - 6/1440 |
| 90. | A6N11 | Avşa | *Streptomyces calvus* ISP 5010 T | 99.10 - 13/1447 |
| 91. | A6Y6 | Avşa | *Streptomyces aculeolatus* NBRC 14824 T | 98.75 - 18/1440 |
| 92. | A7C3 | Avşa | *Streptomyces aculeolatus* NBRC 14824 T | 99.72 - 4/1442 |
| 93. | A7G1 | Avşa | *Streptomyces netropsis* NBRC 3723 T | 99.24 - 11/1445 |
| 94. | A7Y5 | Avşa | *Streptomyces aureocirculatus* NRRL ISP-5386 T | 99.52 - 7/1450 |
| 95. | A7H24 | Avşa | *Streptomyces rameus* LMG 20326 T | 99.10 - 13/1446 |
| 96. | A7Y24 | Avşa | *Streptomyces durhamensis* NRRL B-3309 T | 98.62 - 20/1448 |
| 97. | A7Z18 | Avşa | *Streptomyces manganisoli* MK44 T | 99.57 - 6/1405 |
| 98. | A9D4 | Avşa | *Streptomyces amphotericinicus* 1H-SSA8 T | 99.24 - 11/1449 |
| 99. | A9G32 | Avşa | *Streptomyces amphotericinicus* 1H-SSA8 T | 99.52 - 7/1449 |
| 100. | A9H12 | Avşa | *Streptomyces pseudovenezuelae* DSM 40212T | 99.38 - 9/1450 |
| 101. | A9H13 | Avşa | *Streptomyces aldersoniae* NRRL 18513 T | 99.79 - 3/1420 |
| 102. | A9N8 | Avşa | *Streptomyces aureocirculatus* NRRL ISP-5386 T | 99.78 - 2/911 |
| 103. | A9N28 | Avşa | *Streptomyces aureocirculatus* NRRL ISP-5386 T | 99.52 - 7/1450 |
| 104. | M1C11 | Marmara | *Streptomyces aldersoniae* NRRL 18513 T | 99.51 - 7/1420 |
| 105. | M1C34 | Marmara | *Streptomyces aculeolatus* NBRC 14824 T | 99.86 - 2/1440 |
| 106. | M2Y3 | Marmara | *Streptomyces sulfonofaciens* NBRC 14260 T | 98.00 - 29/1448 |
| 107. | M3N14 | Marmara | *Streptomyces samsunensis* M1463 T | 99.86 - 2/1425 |
| 108. | M4C10 | Marmara | *Streptomyces filipinensis* NBRC 12860 T | 99.24 - 11/1445 |
| 109. | M4N21 | Marmara | *Streptomyces rubidus* 13C15 T | 98.58 - 20/1413 |
| 110. | M8H1 | Marmara | *Micromonospora peucetia* DSM 43363T | 99.58 - 6/1437 |
| 111. | A2R8 | Avşa | *Actinopolymorpha singaporensis* DSM 22024T | 99.51 - 7/1439 |
| 112. | M1G15 | Marmara | *Streptomyces hygroscopicus* subsp. NBRC 13472T | 98.61 - 20/1442 |

## 4. Discussion

*Actinobacteria* have been isolated from many different habitats so far. Among these habitats, Island environments attract more attention in terms of their new species hosting potential. At the same time, culture media was an inflection point for the success in the number of isolates and genera. Pek Lim Chu et al identified 36 actinobacterial isolates based on 16S rRNA gene sequence analysis in a study from 15 soil samples collected at South Shetland Island. They used five different media during isolation. They concluded that 36 actinobacterial isolates were separated into a common genus (*Streptomyces*) and nine rare genera (*Micromonospora, Micrococcus, Kocuria, Dermacoccus, Brachybacterium, Brevibacterium, Rhodococcus, Microbacterium* and *Rothia*) (Lim et al., 2017).

According to a study conducted in 2021, 6 soil samples were taken from Sichang Island, Chonburi Province, Thailand and the culture-dependent method was employed to obtain *Actinobacteria* (Phongsopitanuna et al., 2021). A total of 55 culturable *actinobacteria* were isolated and 3 different genera (*Streptomyces*, *Nocardia* and *Saccharothrix*) were obtained by comparative analysis of partial 16S rRNA gene sequences. The dominant genus was *Streptomyces*, followed by *Nocardia*. Only humic acid vitamin (HV) agar was used during isolation.

In another study carried out in 2020, a total of 32 isolates were isolated from soil samples of different forest locations of Bisle Ghat and Virjapet situated in Western Ghats of Karnataka, India. The isolates were identified as species of *Streptomyces*, *Nocardiopsis*, and *Nocardioides* by cultural, morphological, and molecular studies. isolates were obtained on starch casein agar and Actinomycetes isolation agar (Siddharth et al., 2020).

In our study, 112 isolates were obtained as a result of isolation from the Islands using 10 different media. In the effort of isolating *Actinobacteria* from Islands, Gause Agar was the most efficient isolation medium and capable of recovering the highest diversity of *Streptomyces* and rare *Actinobacteria*. In this study, 20% of the *Actinobacteria* isolates were recovered from Gause Agar. Interestingly, all *Rhodococcus* and *Nocardioides* isolates were only recovered on Gause Agar, while *Streptomyces* isolates were recovered on 80 percent of the media used. This finding could relate to the natural ecological role of *Actinobacteria* in recycling and decomposition of organic materials in soils, and their prevalence distribution in humus-rich soils (Goodfellow and Williams 1983; Ventura et al., 2007).

Rare *Actinobacteria* are an important resource for the discovery of new antibiotics (Tiwari et al., 2012). Rare *Actinobacteria* are defined as certain types of *Actinobacteria* that are difficult to isolate. In our study, about 10 genera belonging to rare *Actinobacteria* members were obtained (*Micromonospora*, *Actinomadura*, *Nonomuraea*, *Nocardia*, *Pseudanocardia*, Rhodococcus, *Kribbella, Dactylosporangium, Saccharopolyspora, Microbispora*). Considering these studies, it is seen that the genus and biodiversity of *Actinobacteria* strains obtained with the use of different media in isolation studies and the increase in the number of isolated samples increased.

In 2014, Kim et al suggested that a value of 98.65% 16S rRNA gene sequence similarity could be used as the threshold for differentiating two bacterial species. Based on this data, A7Z30, A2C8, M3C6, A6C8, A3D7 and M3G17 strains may be new species belonging to the genus *Nonomuraea*. These isolates had 98.96%, 98.68%, 98.89%, 98.65% and 97.78% 16S rRNA gene sequence similarity with the closest *Nonomuraea* type species, respectively. A2R2 and M4C4 strains showed close 16S rRNA gene sequence similarity, 98.60% and 98.19%, with the type strain of *Actinomadura*, A5G2 strain showed 97.74% 16S rRNA gene sequence similarity with *Pseudanocardia* type strain, M3G16 strain showed close 16S rRNA gene sequence similarity, 98.47% with the type strain of *Mycolicibacterium,* M2Y3 strain showed close 16S rRNA gene sequence similarity, 98.00% with the type strain of *Streptomyces*. 16S rRNA gene sequence similarity ratios of other species are given in the Table 7. In this study, the 16S rRNA gene similarity of the above-mentioned isolates was lower than 98.65%, indicating that these isolates are candidates for a new species of actinobacterial taxa.

## 5. Conclusion

This research is the first to examine the diversity of *Actinobacteria* in the soils of "Marmara" and "Avşa" in Türkiye with the dilution plating method. In this study, a large number of *Actinobacteria* were effectively isolated and identified from two different islands. Our primary data based on the 16S rRNA gene revealed that the actinobacterial community is very diverse. In addition to the genera of *Actinobacteria* found in the two studied regions, some unknown members that do not belong to the class *Actinobacteria* were also identified.

According to the results of 16S rRNA gene region % similarity and nucleotide difference, *Actinomadura* sp. M4C4, *Actinomadura* sp. A2R2, *Mycolicibacterium* sp. M3G16, *Nonomuraea* sp. A7Z30, *Nonomuraea* sp. A2C8, *Nonomuraea* sp. M3C6, *Nonomuraea* sp. A6C8, *Nonomuraea* sp. A3D7, *Nonomuraea* sp. M3G17, *Saccharopolyspora* sp. M3H16, *Pseudanocardia* sp. A5G2, *Nocardia* sp. A4R2, *Streptomyces* sp. M2Y3, *Streptomyces* sp. A5N9, *Streptomyces* sp. A7Y24, *Streptomyces* sp. M4N21, *Streptomyces* sp. A6Y6, *Streptomyces* sp. A6C14 and *Streptomyces* sp. A2H6 isolates are thought to be most likely new species. In addition, all our new isolates may be important candidates for biotechnological applications.

After this work, a large number of new species were revealed, thus determining the diversity and distribution of *Actinobacteria* in island environments. Thereby,

Islands were shown to be a valuable resource for *Actinobacteria* strains with a high rate of putative new and rare species.

Two aspects appear of primary importance in regard to the high degree of novelty and diversity of *Actinobacteria* found. First, the application of various culture media significantly increased the number of species and genera obtained. Second, the geographical isolation is considered to be of importance regarding the actinobacterial novelty found.

**Author Contributions**

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

| | A.R.T. | K.I. |
|---|---|---|
| C | 50 | 50 |
| D | 50 | 50 |
| S | 50 | 50 |
| DCP | 50 | 50 |
| DAI | 50 | 50 |
| L | 50 | 50 |
| W | 50 | 50 |
| CR | 50 | 50 |
| SR | 50 | 50 |
| PM | 50 | 50 |
| FA | 50 | 50 |

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

**Conflict of Interest**

The authors declared that there is no conflict of interest.

**Ethical Consideration**

Ethics committee approval was not required for this study because of there was no study on animals or humans. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

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