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BIODIVERSITY OF *Actinobacteria* FROM KULA GEOPARK IN TÜRKİYE

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Abstract: Investigating the microbial diversity of *Actinobacteria* inhabiting the soils of the Kula-Salihli Geopark and identifying species at the genus level using 16S rRNA gene sequences are the primary goals of this work. In the literature review of this geopark located within the borders of Manisa province, no study on actinobacterial biodiversity was found. In this study, 10 different selective isolation media were used to investigate the biodiversity of *Actinobacteria* in the Geopark. A total of 469 *Actinobacteria* strains were isolated using the dilution-plate method. From these 469 strains, 34 strains were selected based on their colony morphology and pigmentation characteristics. The isolates performed phylogenetic analysis based on sequencing of the 16S ribosomal RNA gene region. The isolates were found to belong to nine different genera, including *Actinomadura*, *Amycolatopsis*, *Kribbella*, *Micromonospora, Nocardia*, *Nonomuraea*, *Pseudonocardia*, *Saccharothrix* and *Streptomyces*, according to the results of phylogenetic analysis. Five isolates have been identified as novel species as a consequence of our current study.

Keywords: *Actinobacteria*, Geopark soil, 16S rRNA gene, Biodiversity

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1. Introduction

The phylum *Actinobacteria*, currently referred to as *Actinomycetota*, is a common group of gram-positive microorganisms found in both terrestrial and aquatic environments. Although *Actinobacteria* are frequently found in both terrestrial and aquatic habitats, they can be found in a variety of harsh environments, including deep seas, deserts, hot springs, salt lakes, and caves (Barka et al., 2016; Hui et al., 2021). *Actinobacteria*, by producing a variety of compounds, play an important role in the bioremediation of pollutants, the degradation of lignocellulosic biomass, and the promotion of plant development (Saini et al., 2015; Mawang et al., 2021; Faddetta et al., 2023).

Actinobacteria's primary and secondary metabolites have been identified as significant industrial compounds. Although rare *Actinobacteria*, which are crucial for the production of novel secondary metabolites, have been discovered in a variety of soil types, volcanic regions are still a relatively unexplored resource for the detection of chemicals with industrial significance (Miao and Davies, 2010; Tiwari and Gupta, 2012).

Geographical areas referred to as UNESCO Global Geoparks are those where landscapes and places of global geological significance are managed using a comprehensive concept of conservation, education, and sustainable development. Currently, there are 213 UNESCO Global Geoparks in 48 countries. Türkiye's first and only UNESCO-registered geopark, Kula-Salihli UNESCO Global Geopark, is located in Manisa province. The geopark, which has a rich geodiversity, is one of the youngest volcanic areas in Türkiye (UNESCO, 2024).

The purpose of this study is to determine the diversity of *Actinobacteria* found in the Kula-Salihli Geopark's soils. Thus, with this study, we aim to both contribute to the biological diversity of our country and contribute to the stock of microorganisms that have the potential to be the source of various metabolites that can be used in biotechnological applications.

2. Materials and Methods

2.1. Collection of Soil Samples

In August 2023, soil samples were collected from four distinct locations inside of Kula Geopark (Figure 1). After being tagged and put in sterile plastic bags, the soil samples were transported to the actinobacterial soil research laboratory and stored there for analysis at 4 °C.

Figure 1. Location of Kula-Salihli Geopark.

2.2. Isolation of *Actinobacteria* from Geopark Soils

Ten different media were made to isolate the *Actinobacteria* members. Nalidixic acid and cycloheximide were added to each medium to prevent the growth of gram-negative and fungal species. Then, soil samples were first put on a sterile petri plate and allowed to dry at room temperature for 14 days. In a mortar, dried soil samples were pounded into a powder without being mixed. Each of the prepared solutions was then shaken for half an hour. One gram of soil sample was transferred into vials containing 9 milliliters of Ringer's solution and ten-to-one dilution ratio tubes were prepared. Dilutions of 10^{-2} and 10^{-3} were obtained via repeated dilution. 200 microliter suspensions were inoculated onto different medium surfaces using an automated pipette, and they were subsequently cultured for 21 days at 28 °C. Table 1 shows the medium used.

2.3. Selection, Purification, and Storage of Strains

Among the incubated colonies, isolates thought to be *Actinobacteria* were selected based on characteristics such as spore production and morphology. These cultures were cultivated on the surface of International *Streptomyces* Project Medium No. 2 (ISP2; Shirling and Gottlieb, 1966) agar using a sterile loop. After incubating at 28 °C for 14 days, pure isolates were obtained from transferred plates. Pure cultures were transferred to 20% v/v glycerol stock solution and stored at -80 °C until use.

2.4. Genomic Extraction of DNA and 16S rRNA Amplification

The PureLink® Genomic DNA Isolation Kit (Invitrogen, USA) was used for obtaining the isolates genomic DNA. Then DNA was detected using 1% agarose gel electrophoresis. Polymerase chain reaction (PCR) amplifications of the 16S rRNA gene region were performed on a MyGenie-96 Gradient Thermal Cycler (Korea) using universal primers 27F and 1525R (Table 2). A 50-µl reaction mixture was made for the 16S rRNA amplification of each test isolate. This mixture contains deionized water, extracted genomic DNA, 27F and 1525R primers, and Promega's GoTaq® Hot Start Colorless Master mix. There are 22 µl, 1 µl, 1 µl, 1 µl and 25 µl of these compounds in the mixture, in that order. The following parameters are used in PCR amplification (MyGenie-96 Gradient Thermal Cycler, Korea): pre-denaturation for two minutes at 95 °C; denaturation for one minute at 95 °C, comprising thirty cycles; bonding for 1.5 minutes at 55 °C and elongation for three minutes at 72 °C; final stage for ten minutes at 72 °C; and four storage stages at 4 °C. The amplification products were subsequently detected using 1.5% agarose gel electrophoresis (Merck).

2.5. 16S rRNA Gene Analysis

ABI-format chromatogram files resulting from the sequencing of PCR products were examined using the Chromas version 1.7.6 program (C. McCarthy, School of Health Sciences, Griffith University, Queensland, Australia). Each organism's 16S rRNA gene nucleotide sequence was then obtained in FASTA format by overlapping the primer reads. In the EzBioCloud database, every sequence was compared to other sequences (Yoon et al., 2017). ClustalW was used to do multiple sequence alignments in the MEGA 11 software (Tamura et al., 2021). Phylogenetic trees of the alignment sequences were constructed using the neighbor-joining method (Saitou and Nei, 1987), the Jukes-Cantor model (Jukes and Cantor, 1969) and bootstrap analyses based on 1000 resampling (Felsenstein, 1985) using MEGA 11 software. The 16S rRNA gene sequences of the *Actinobacteria* isolates used in this study were stored in the NCBI GenBank database.

Table 1. List of selective media used and antibiotics

Name of medium	Antibiotics	Reference
Actinomycete Isolation Agar	Nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml)	Kumar et al, 2012
Gause No.1 Agar	Nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml)	Tan et al., 2006
Nocardia Agar	Nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml)	Sanglier et al., 1992
Humic Acid Vitamin Agar	Nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml)	Hayakawa and Nonomuraea, 1987
R2A Agar	Nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml)	Reasoner and Geldreich, 1985
Oligotrophic Agar	Nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml)	Jiang et al., 2016
Starch-Casein Agar	Nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml)	Kuester and Williams, 1964
Hickey-Tresner Agar	Nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml)	Hickey and Tresner, 1952
Modified Soil Agar	Nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml)	Sadoway et al., 2013
ISP-2 Agar	Nalidixic acid (10 µg/ml) and cycloheximide (50 µg/ml)	Shirling and Gottlieb, 1966

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.

3. Results

3.1. Morphological Analysis of *Actinobacteria*

In the Kula-Salihli Geopark (2,320 km²), which is located inside the boundaries of the province of Manisa, soil samples were collected using a GPS device at four distinct places (Table 3).

Table 3. Locality and geographical coordinates of soil samples

Soil No.	Locality	Geographical coordinates
1	Kula Fairy Chimneys	38,60760°N 28,80798°E
2	Kula Fairy Chimneys	38,60809°N 28,80753°E
3	Kula Divlit	38,56023°N 28,66096°E
4	Kula	38,56192°N 28,66048°E

By using the dilution plate method for soil isolation, 469 *Actinobacteria* were isolated in total. A total of 152 different strains, which were selected by eliminating similar ones according to their macroscopic and microscopic images, were divided into 27 color groups according to their colony morphology and pigmentation characteristics (Kelly, 1964). Following color grouping, 34 strains were chosen for 16S rRNA gene region analysis, with consideration given to characteristics such as phylogenetic analyses, ten different selective media, substrate mycelium and air mycelium. When the distribution of actinobacteria according to the media is examined, it is seen that 8 isolates were obtained from starch casein agar, 5 isolates from R2A agar, 9 isolates from humic acid vitamin agar, 1 isolate from Gause agar and 11 isolates from ISP-2. The results showed that ISP-2, starch casein agar and humic acid vitamin agar media were the best media for isolating *Actinobacteria* from Geopark soil. Figure 2 shows the distribution of the number of isolates according to the media used.

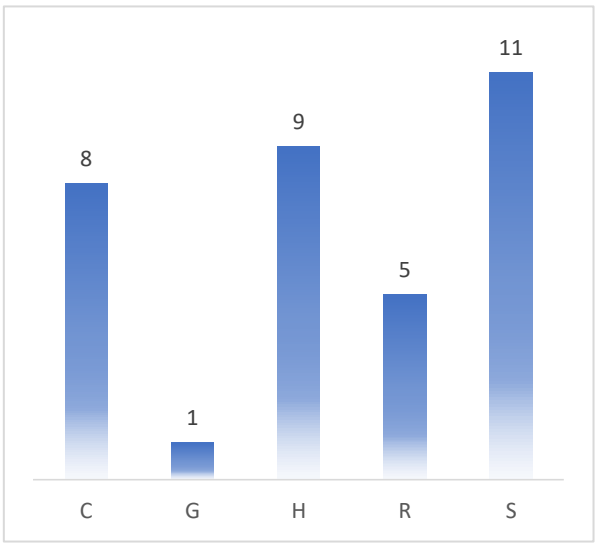

Figure 2. Media distribution of *Actinobacteria* isolates (C= Starch-Casein agar, G= Gause agar, H= Humic acid vitamin agar, R= R2A agar, S= ISP-2 Agar).

3.2. 16S rRNA Gene Sequence Analysis of *Actinobacteria*

According to the results of the nucleotide sequence analysis of the 16S rRNA gene region using universal primers (518F, 800R, and MG5F; Table 2), 34 isolates were recognized as members of the *Actinobacteria* phylum. These isolates were determined to predominantly belong to the genera *Amycolatopsis* (8 isolates), *Kribbella* (7 isolates), and *Streptomyces* (7 isolates). The remaining strains were from the genera *Nonomuraea* (4 isolates), *Micromonospora* (2 isolates), *Pseudonocardia* (2 isolates), *Nocardia* (2 isolates), *Actinomadura* (1 isolate), and *Saccharothrix* (1 isolate). In conclusion, nine different *Actinobacteria* genera were obtained from Kula-Salihli Geopark in this study. Figure 3 shows the distribution of the genus.

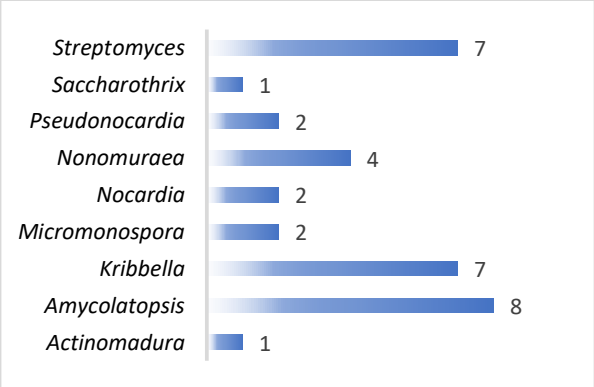

Figure 3. Genus distribution of Actinobacteria isolates.

3.3. Phylogenetic Analysis of the *Actinobacteria*

To explore the evolutionary links between the *Actinobacteria* isolates in this study and their closest taxonomic relatives, phylogenetic trees based on 16S rRNA sequences were created. The phylogenetic tree was constructed using the neighbor-joining method. After phylogenetic analysis, the most predominant *Actinobacteria* genus in this study, members of *Amycolatopsis*, have a 99.28% similarity with the closest type strain. *Kribbella*, the other major genus with seven isolates, has a similarity between 99.45% and 99.93% with its closest type strain. Seven isolates of *Streptomyces*, the other dominant genus, have similarity between 99.17% and 100.0% with their closest type strain. A similarity of the 16S ribosomal RNA gene sequences of each isolate and its closest relatives is shown in Table 4. Phylogenetic trees of the isolates based on the neighbour-joining method are also given in Figure 4-11.

Table 4. Summary of information on 16S rRNA sequence determination of isolated strains

Strain	Closest type strain	Similarity	Nucleotid difference
KC28	*Streptomyces thinghirensis* DSM 41919^T	100.00	0/1449
KC37	*Kribbella albertanoniae* BC640^T	99.79	3/1440
KC40	*Micromonospora orduensis* S2509^T	99.65	5/1437
KC48	*Streptomyces xanthophaeus* NRRL B-5414^T	99.93	1/1447
KC66	*Streptomyces cellostaticus* DSM 40189^T	99.52	7/1448
KC83	*Kribbella jejuensis* DSM 17305^T	99.45	8/1447
KC93	*Nocardia* sp.	100.00	0/1449
KC97	*Micromonospora fulviviridis* DSM 43906^T	99.44	8/1436
KG3	*Amycolatopsis lurida* DSM 43134^T	99.28	10/1386
KH2	*Nonomuraea turkmeniaca* DSM 43926^T	99.01	14/1416
KH8	*Amycolatopsis lurida* DSM 43134^T	99.28	10/1386
KH9	*Amycolatopsis lurida* DSM 43134^T	99.28	10/1386
KH16	*Nonomuraea polychroma* DSM 43925^T	98.48	22/1443
KH19	*Nonomuraea antri* NN258^T	98.75	18/1441
KH50	*Saccharothrix espanaensis* DSM 44229^T	99.72	4/1438
KH76	*Nocardia* sp.	100.00	0/1449
KH104	*Pseudonocardia cypriaca* KT2142^T	100.00	0/1442
KH114	*Pseudonocardia zijingensis* 6330^T	99.51	7/1418
KR1	*Amycolatopsis lurida* DSM 43134^T	99.28	10/1386
KR2	*Amycolatopsis lurida* DSM 43134^T	99.28	10/1386
KR3	*Amycolatopsis lurida* DSM 43134^T	99.28	10/1386
KR6	*Amycolatopsis lurida* DSM 43134^T	99.28	10/1386
KR12	*Amycolatopsis lurida* DSM 43134^T	99.28	10/1386
KS12	*Streptomyces canus* DSM 40017^T	100.00	0/1448
KS15	*Streptomyces aureocirculatus* NRRL ISP-5386^T	99.52	7/1450
KS37	*Actinomadura hibisca* NBRC 15177^T	98.96	15/1446
KS52	*Kribbella karoonensis* Q41^T	99.93	1/1447
KS86	*Kribbella karoonensis* Q41^T	99.93	1/1447
KS88	*Kribbella karoonensis* Q41^T	99.86	2/1447
KS95	*Kribbella speibonae* YM55^T	99.72	4/1447
KS96	*Kribbella karoonensis* Q41^T	99.86	2/1447
KS97	*Streptomyces canus* DSM 40017^T	100.00	0/1448
KS108	*Nonomuraea helvata* IFO 14681^T	99.43	8/1409
KS109	*Streptomyces pseudovenezuelae* DSM 40212^T	99.52	7/1450

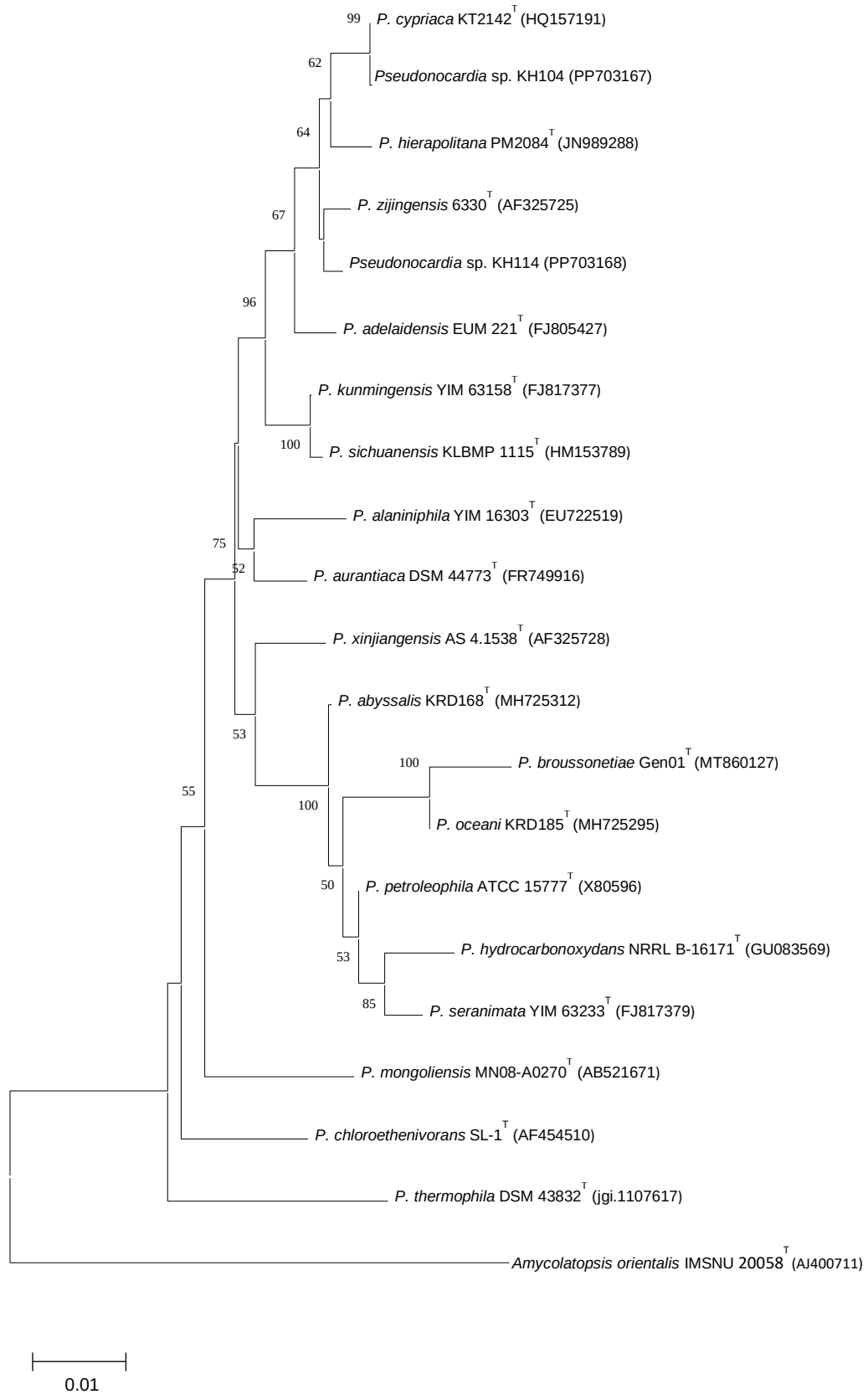

Figure 4. Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Pseudonocardia* and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA 11 software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

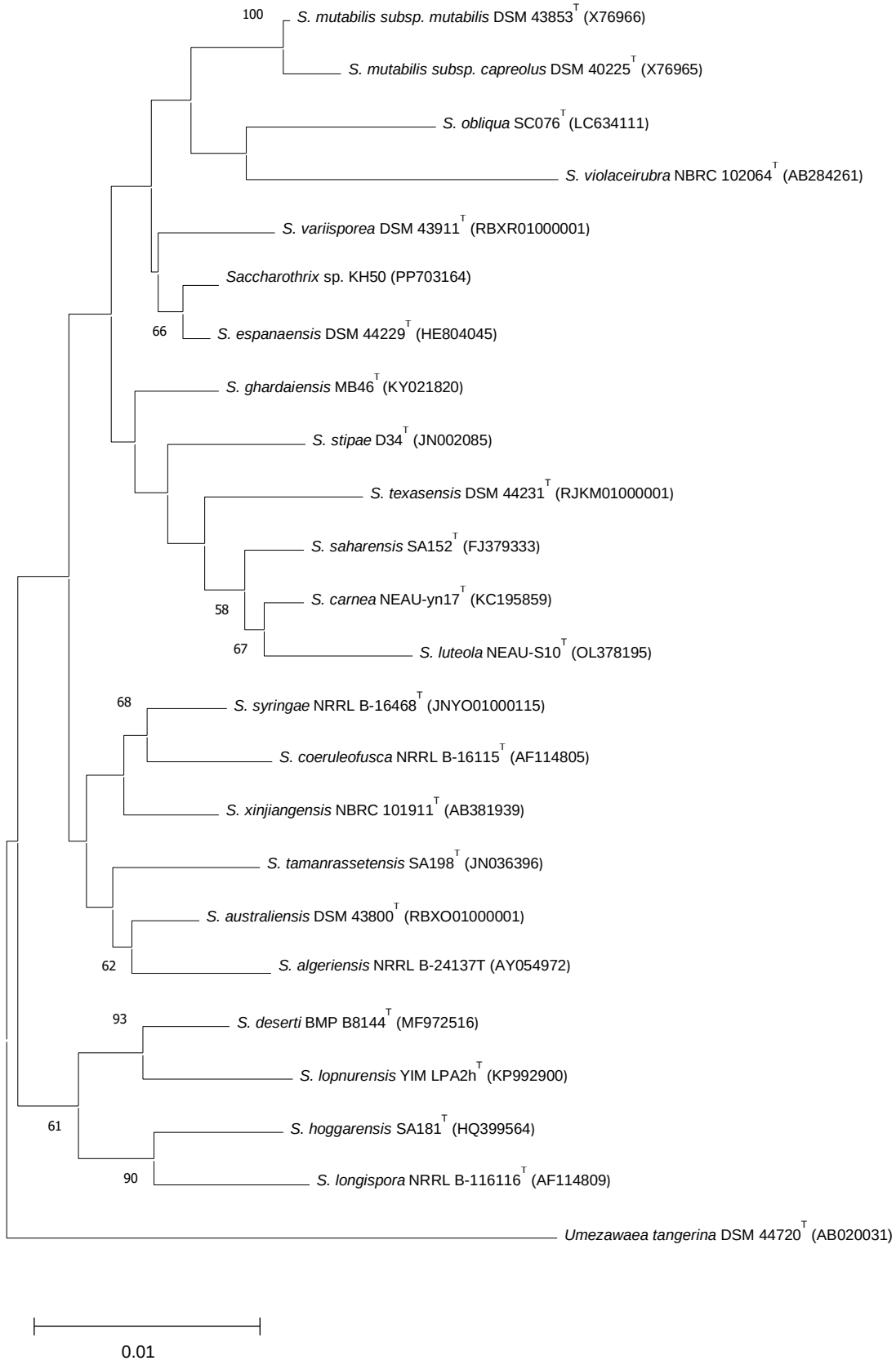

Figure 5. Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Saccharothrix* and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA 11 software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

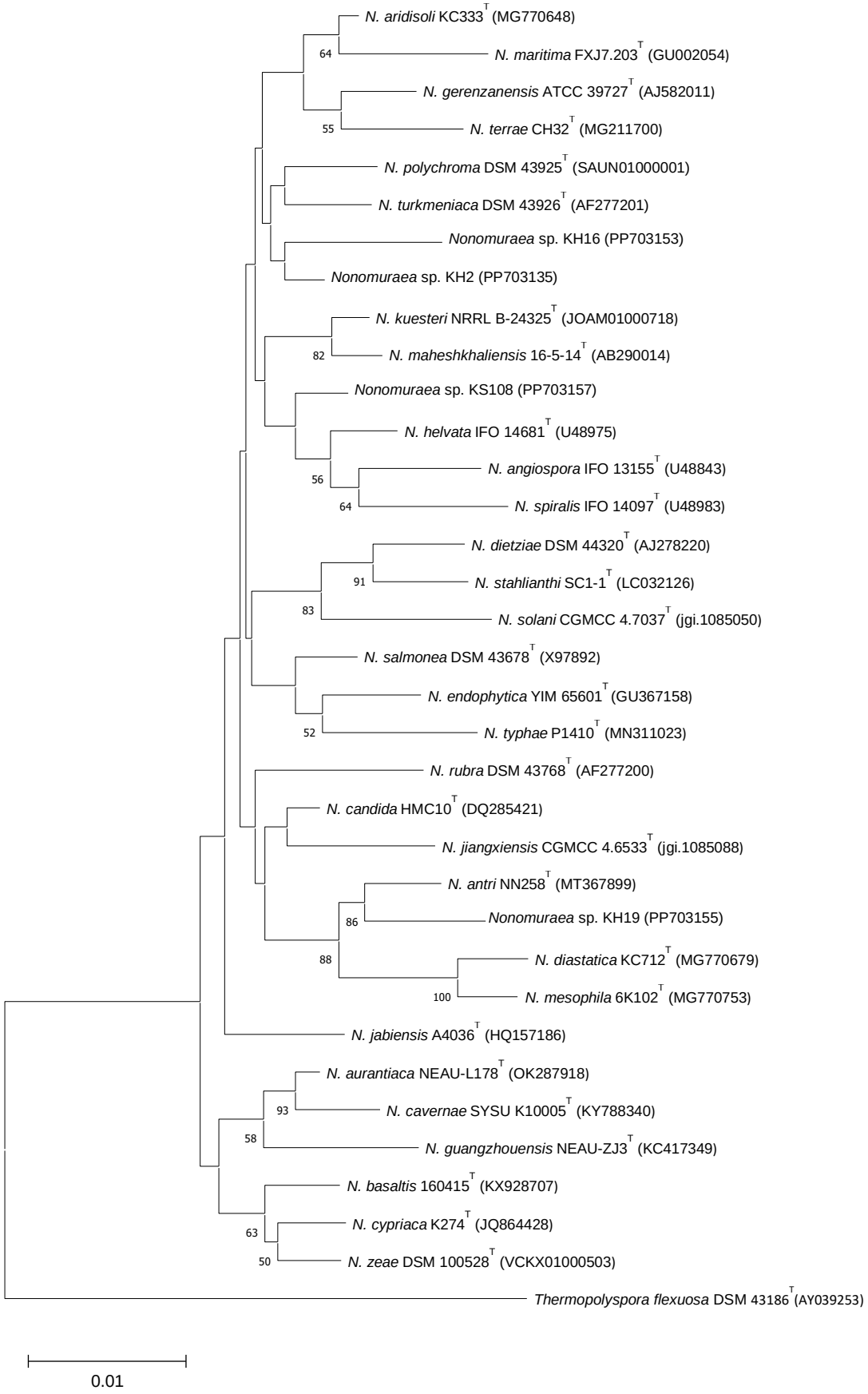

Figure 6. Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Nonomuraea* and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA 11 software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

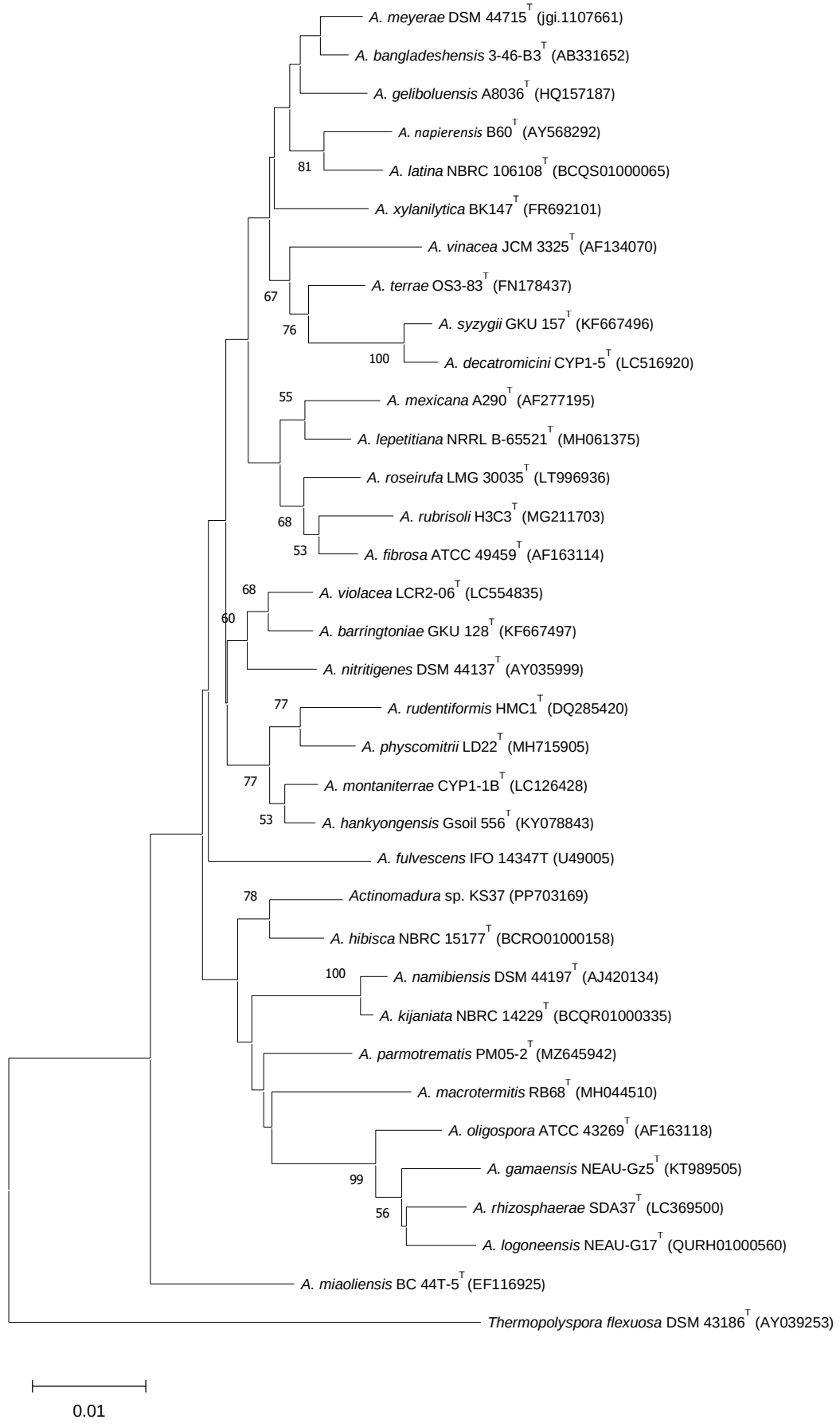

Figure 7. Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Actinomadura* and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA 11 software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

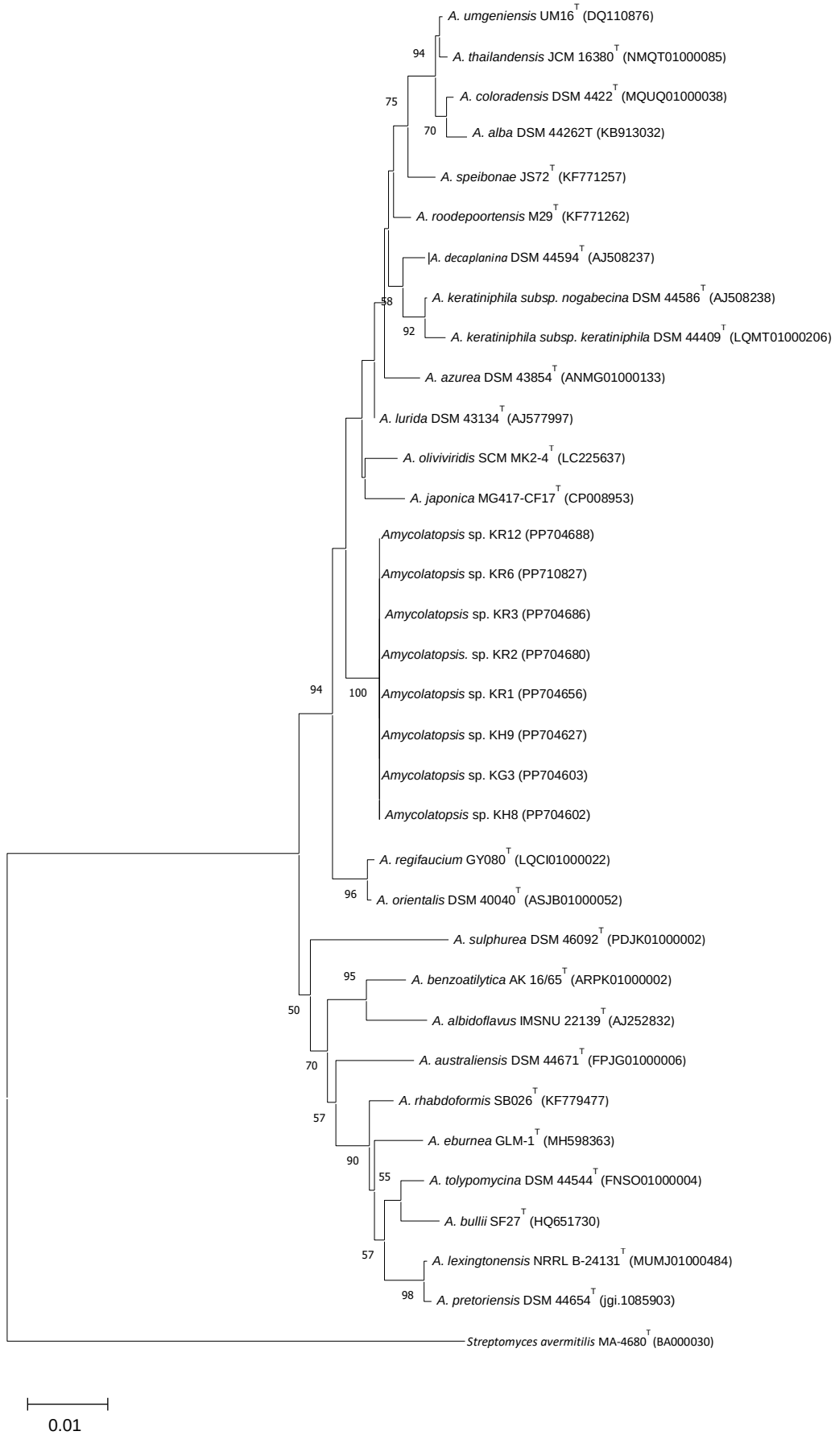

Figure 8. Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Amycolatopsis* and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA 11 software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

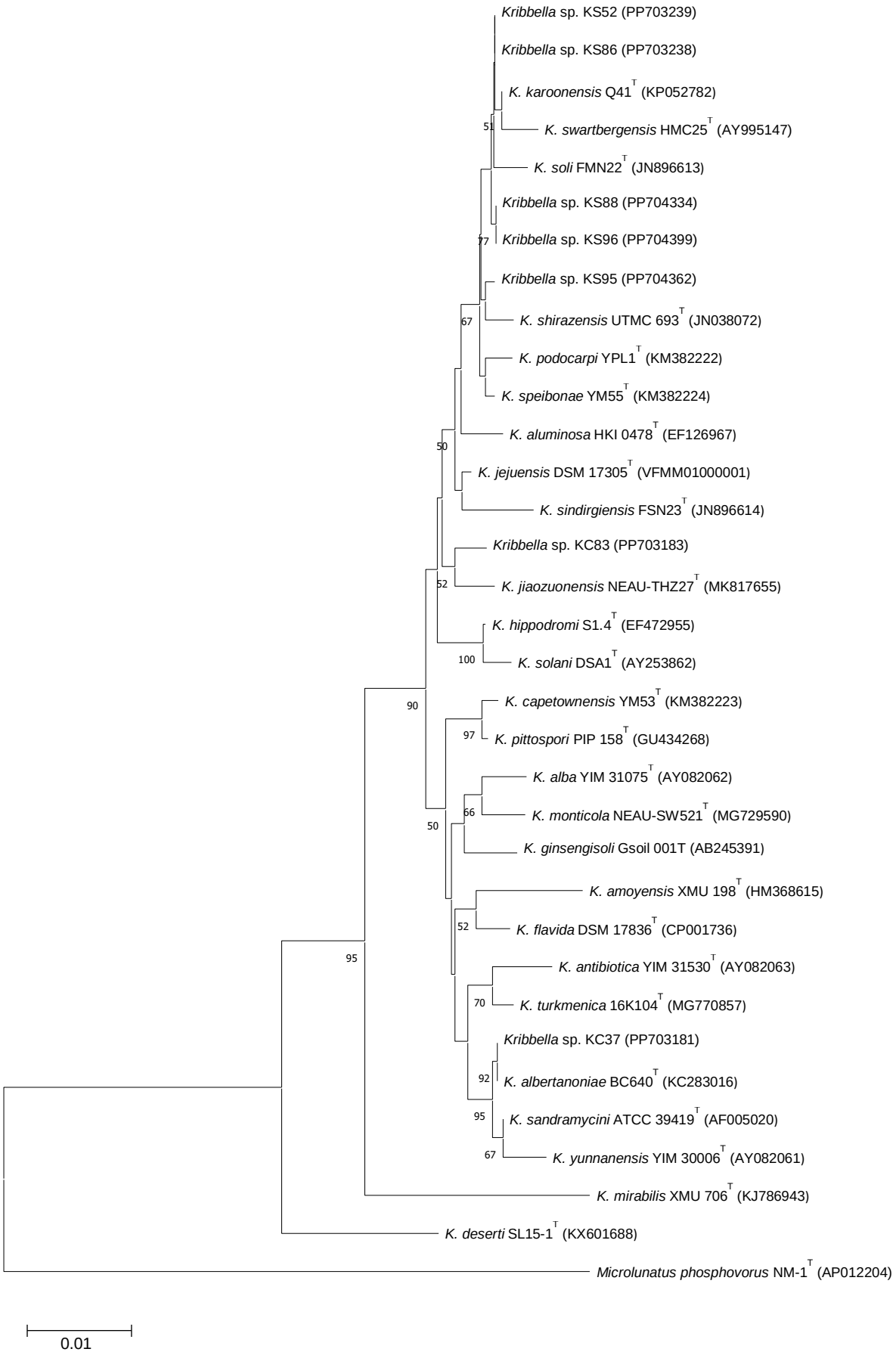

Figure 9. Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Kribbella* and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA 11 software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

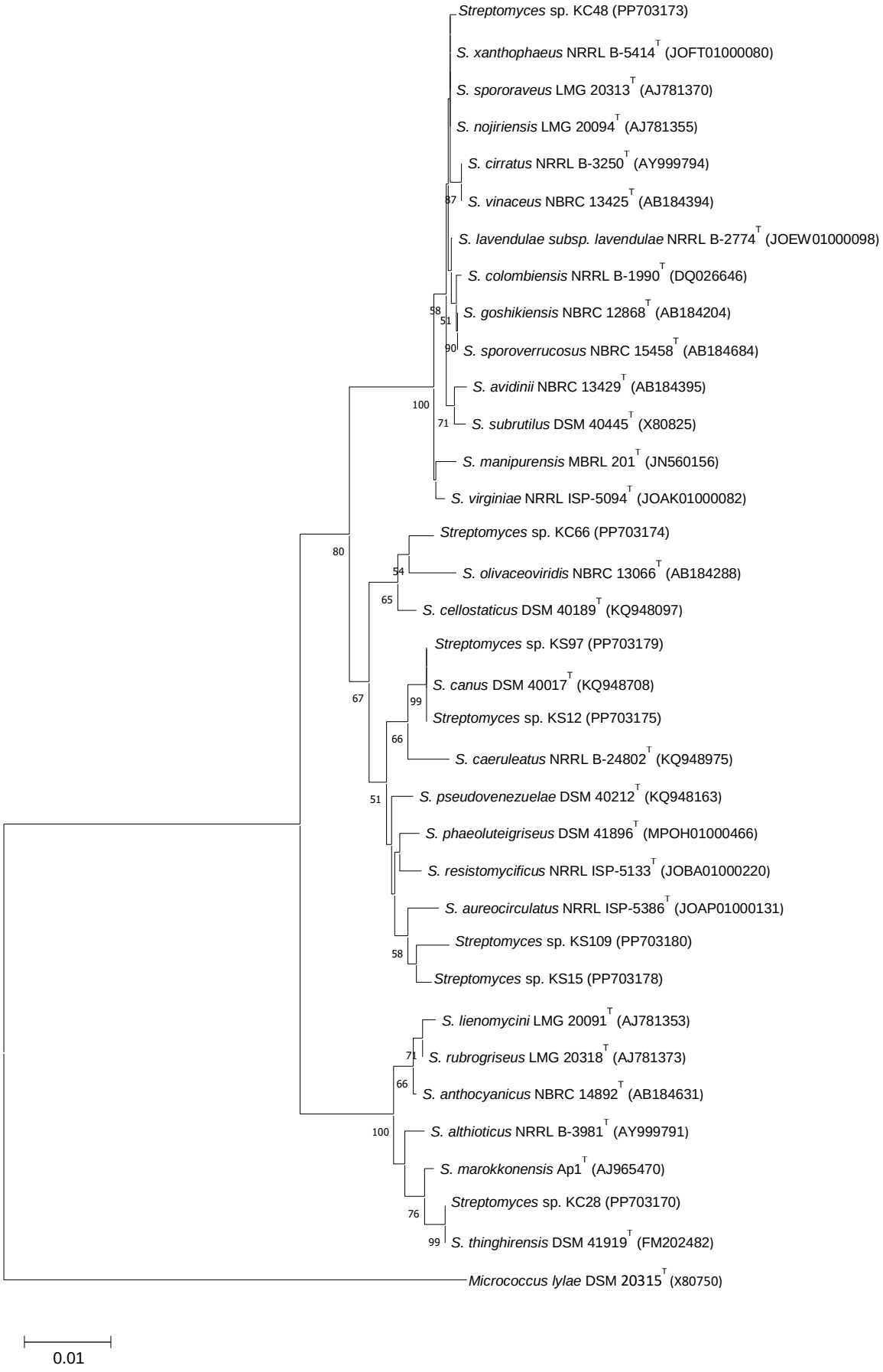

Figure 10. Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Streptomyces* and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA 11 software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

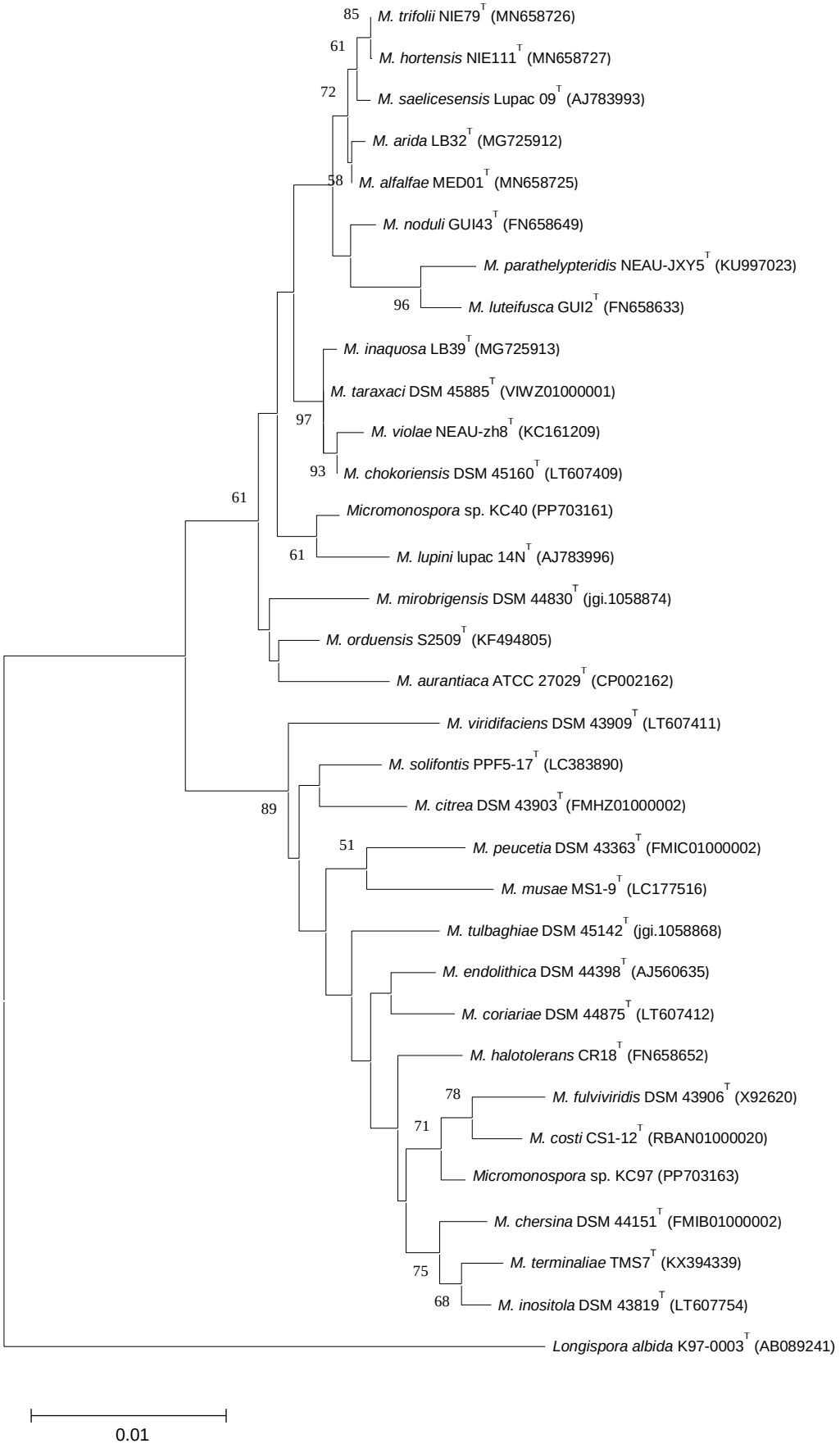

Figure 11. Phylogenetic tree showing the relationship of the actinobacterial isolates in group *Micromonospora* and related type strains. Phylogenetic tree was based on the Neighbor-joining method using MEGA 11 software. Bootstrap values above 50% (for 1000 replications) are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

4. Discussion

To date, *Actinobacteria* members have been found in a wide variety of environments. Among these environments, extreme habitats such as hot springs, volcanic areas, deserts, deep-sea sediments, caves and salt lakes are particularly important. Following a literature review, actinobacteria have also been found in different volcanic regions of the world. These include lava tubes, lakes, caves, sediments, rocks and soils. Studies in volcanic regions have shown the presence of actinobacteria belonging to the following genera: *Brevibacterium*, *Dietzia*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Salinospora*, *Streptomyces*, etc. (Riquelme et al., 2015; Meena et al, 2019; Sottorff et al., 2019).

Meena et al. reported in their study in 2019 that deep-sea sediment samples were collected from Barren Island, Andaman and Nicobar Islands. A total of 123 cultivable marine actinobacteria were isolated and identified. Isolates were categorized under 10 genera, of which *Brevibacterium, Dietzia* and *Streptomyces* are the dominant genera (Meena et al., 2019).

In another study conducted by Sottorff et al. (2019), it was determined that most of the species isolated from volcanic Easter Island were actinobacteria belonging to the *Dietzia, Micromonospora*, *Salinispora* and *Streptomyces* genera, according to the 16S ribosomal RNA gene sequence analysis.

Phylogenetic analysis of 34 isolates obtained in this study revealed that they belong to nine different genera. Five of these isolates are thought to be new species when the nucleotide similarity rates of the 16S rRNA gene region of the closest type strains are compared. The five isolates belong to *Actinomadura*, *Amycolatopsis* and *Nonomuraea* genera known as rare actinobacteria.

According to a study conducted by Penkhrue et al. in 2018, *Amycolatopsis oliviviridis* SCM_MK2-4^T strain showed the highest sequence similarity with the closest species, *Amycolatopsis azurea* JCM 3275^T with 99.4%, according to 16S rRNA gene sequence analysis results (Penkhrue et al., 2018). Strains KG3, KH8, KH9, KR1, KR2, KR3, KR6, and KR12 obtained from this study show 99.28% sequence similarity with *Amycolatopsis lurida* DSM 43134^T, the closest species according to 16S rRNA gene sequence analysis results. Based on this information, KG3, KH8, KH9, KR1, KR2, KR3, KR6, and KR12 strains are thought to be new species. Representing *Amycolatopsis lurida* strains, isolate KH8 was selected as a strain with high potential to become a novel species.

In the research of Saricaoglu et al. in 2020 based on phylogenetic analysis of 16S rRNA gene sequences revealed that *Nonomuraea basaltis* 160415^T has 99.1% similarity with the closest type species *Nonomuraea zeae* NEAU-ND5^T (Saricaoglu et al., 2020). The KH2, KH16 and KH19 strains isolated in our study have 99.01, 98.48 and 98.75 similarity with the closest type strains, respectively. According to this information, KH2, KH16 and KH19 may be new species.

In accordance with the results of phylogenetic analysis of the 16S rRNA gene sequence in the study conducted by Songsumanus et al. in 2021, *Actinomadura decatromicini* CYP1-5^T showed 99.5% similarity with *Actinomadura syzygii* GKU157^T, the closest type species (Songsumanus et al., 2021). According to the 16 rRNA sequence analysis of the KS37 strain obtained in this study, it was determined that it showed 98.96 similarity with *Actinomadura hibisca* NBRC 15177^T, the closest type species. In the light of this information, KS37 strain is thought to be a novel species.

5. Conclusion

To date, no study has been conducted on the actinobacterial diversity of Kula-Salihli Geopark, one of the youngest volcanic regions in Türkiye. Our research focused on the diversity of actinobacteria found in the soil of Kula-Salihli Geopark in Manisa. A total of 34 isolates were determined to belong to nine genus, including *Actinomadura*, *Amycolatopsis*, *Kribbella*, *Micromonospora*, *Nocardia*, *Nonomuraea*, *Pseudonocardia*, *Saccharothrix* and *Streptomyces* by phylogenetic analysis based on 16S rRNA gene sequencing. Based on the nucleotide difference and percentage similarity of the 16S rRNA gene region, it is probable that the isolates of *Actinomadura* sp. KS37, *Amycolatopsis* sp. KH8, *Nonomuraea* sp. KH2, *Nonomuraea* sp. KH16, and *Nonomuraea* sp. KH19 represent novel species. With this study, in addition to determining the new species potential of the Kula Geopark, we contribute to the biodiversity of our country and the stock of microorganisms that have the potential to be a source of various metabolites that can be used in biotechnological applications.

Author Contributions

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

	B.B.	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.

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