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# Investigation of Antimicrobial Activities and 16S rRNA Sequences of Actinomycetes Isolated from Karst Caves in the Eastern Black Sea Region of Türkiye

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## ABSTRACT

Considering that most antibiotics originate from actinomycete group bacteria, especially the *Streptomyces* genus, it is predicted that novel actinomycetes isolated from extreme environments such as caves may bring novel antibiotics to the medical world. The study aimed to screen the antimicrobial activity of actinomycetes isolated from the three karst caves in Türkiye and to identify selected isolates with antimicrobial activity by molecular methods. One hundred seventy-nine actinomycetes isolated from Akçakale, Kırklar (Altıntaş), and Köprübaşı Caves in Gümüşhane province in the Eastern Black Sea Region of Türkiye were included in the study. The antimicrobial activity of isolates was investigated using the modified cross-streak agar method against seven Gram-negative bacteria, three Gram-positive bacteria, and one yeast strain. Fifty-three isolates (29.6%) had antimicrobial activity against at least one of the tested microorganisms. The rate of isolates exhibiting antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Chromobacterium violaceum*, *Klebsiella pneumoniae*, *Salmonella* Typhimurium, *Escherichia coli*, *Acinetobacter haemolyticus*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Candida albicans* was 21.2%, 20.0%, 16.8%, 12.8%, 3.4%, 2.8%, 2.2%, 1.1%, 0.6%, 0.6%, and 0.6%, respectively. An actinomycete isolate, TRMS 124, showed antimicrobial activity against ten test microorganisms. The 16S ribosomal RNA (16S rRNA) sequencing was performed for the identification and phylogenetic analysis of 26 isolates randomly selected among actinomycetes that exhibited antimicrobial activity against at least three test microorganisms. As a result, it was determined that 24 isolates showed homology with various *Streptomyces* species and two isolates with *Embleya scabrispora* and *Couchioplanes caeruleus*, respectively. These results showed that karst caves could be good sources for isolating actinomycetes with the potential to produce antimicrobial compounds.

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# Doğu Karadeniz Bölgesindeki Karstik Mağaralardan İzole Edilen Aktinomisetlerin Antimikrobiyal Aktivitelerinin ve 16S rRNA Dizilerinin Araştırılması

## ÖZET

Günümüzde kullanılan antibiyotiklerin büyük çoğunluğunun başta *Streptomyces* cinsi olmak üzere çeşitli aktinomiset grubu bakterilerden orijin aldığı düşünüldüğünde, mağara gibi ekstrem ortamlardan izole edilecek yeni aktinomisetlerin tıp dünyasına yeni antibiyotikler kazandırabileceği öngörülmektedir. Bu çalışmada, üç farklı karstik mağaradan izole edilen aktinomiset izolatlarının antimikrobiyal aktivitelerinin araştırılması ve etkili izolatların

**Mikrobiyoloji**

**Araştırma Makalesi**

**Makale Tarihçesi**
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moleküler yöntemlerle tanımlanması amaçlanmıştır. Çalışmaya Türkiye'nin Doğu Karadeniz Bölgesinde bulunan Gümüşhane ilindeki Akçakale, Kırklar (Altıntaş) ve Köprübaşı mağaralarından izole edilen 179 aktinomiset izolatı dahil edilmiştir. İzolatların antimikrobiyal aktiviteleri yedi Gram-negatif, üç Gram-pozitif ve bir maya suşuna karşı çapraz çizgi yöntemi ile araştırılmıştır. Elli üç izolatın (%29,6) test edilen mikroorganizmalardan en az birine karşı antimikrobiyal aktiviteye sahip olduğu bulunmuştur. İzolatların *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Chromobacterium violaceum*, *Klebsiella pneumoniae*, *Salmonella* Typhimurium, *Escherichia coli*, *Acinetobacter haemolyticus*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* ve *Candida albicans*'a karşı antimikrobiyal aktivite sergileme oranı sırasıyla %21.2 %20.0, %16.8, %12.8, %3.4, %2.8, %2.2, %1.1, %0.6, %0.6, %0.6 şeklinde bulunmuştur. TRMS 124 olarak adlandırılan bir aktinomiset izolatı, 10 test mikroorganizmasına karşı antimikrobiyal aktivite sergilemiştir. En az üç test mikroorganizmasına karşı antimikrobiyal aktivite sergileyen aktinomisetler arasından randomize olarak seçilen 26 izolatın tanımlanması ve filogenetik analizi için 16S ribozomal RNA (16S rRNA) dizi analizi yapılmıştır. Buna göre 24 izolatın çeşitli *Streptomyces* türleri ile iki izolatın ise sırasıyla *Embleya scabrispora* ve *Couchioplanes caeruleus* ile homoloji gösterdiği tespit edilmiştir. Bu çalışmadan elde edilen bulgular karstik mağaraların antimikrobiyal madde üretme potansiyeline sahip aktinomisetlerin izolasyonu için doğal kaynaklar olabileceğini göstermiştir.

**Anahtar Kelimeler**
16S ribosomal RNA
Aktinomisetler
Antimikrobiyal aktivite
Mağara
*Streptomyces*

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## INTRODUCTION

Actinomycete is a common name given to members of the order *Actinomycetales* (Prudence et al., 2020). Their members are characterized by having high G+C content of DNA, a Gram-positive cell wall structure, and commonly filamentous morphology (Farda et al., 2022). They are generally found in terrestrial and aquatic environments and play an essential role in maintaining the ecological balance by producing enzymes that decompose organic materials (Devanshi et al., 2021).

Actinomycetes produce a variety of bioactive compounds with antimicrobial activity as a result of their secondary metabolism (Selim et al., 2021). In particular, the number of antibiotics brought to medical use by actinomycetes is considerable. So much so that almost two-thirds of the natural antibiotics introduced to the medical world originated from various actinomycetes, especially of the members of the *Streptomyces* genus (Procópio et al., 2012). Considering that actinomycetes are an antibiotic factory, finding a novel actinomycetes species may mean the discovery of a novel antibiotic. It is estimated that many novel actinomycetes are waiting to be discovered in habitats that have not yet been adequately researched worldwide, such as cave environments (Cheeptham et al., 2013).

Cave environments are aphotic and oligotrophic habitats with high humidity (Kováč, 2018). In such specialized environments, most microorganisms can produce various biomolecules for nutrient competition. Therefore, microorganisms adapted to the cave conditions have the potential to be a source of novel bioactive products, including antimicrobial metabolites (Cheeptham et al., 2013). Actinomycetes are the dominant members of the microbial flora in the cave ecosystem. Therefore, it is highly probable that cave actinomycetes are significant sources for the discovery of novel antibiotics (Rangseekaew & Pathom-Aree, 2019). The best example of this is cervimycin, an aromatic polyketide derivative. Cervimycin was obtained from an actinomycete isolate identified as *Streptomyces tendae*, isolated from the Grotta dei Cervi (Italy) cave (Herold et al., 2005).

Recently, there has been an increase in research on the isolation of actinomycetes from various caves and their potential to produce antimicrobial agents (Belyagoubi et al., 2018; Long et al., 2019; Hamedi et al., 2019;

Syiemiong & Jha, 2019; Jaroszewicz et al., 2021). There are also studies on the isolation and identification of bacteria that have the potential to produce antimicrobial substances from caves in Türkiye. For instance, Yücel & Yamaç (2010) stated that 290 *Streptomyces* spp. isolates were obtained from 19 different karst caves in western Türkiye, and 180 of these isolates were found to exhibit antimicrobial activity against at least one of the tested microorganisms. Yamaç et al. (2011) investigated the potential of these isolates to become novel *Streptomyces* species. For this reason, the biochemical, physiological, nutritional, and morphological characters of the isolates were evaluated, and most of them formed different clusters from the reference *Streptomyces* strains. Consequently, the researchers stated that caves are potential sources for the isolation of novel *Streptomyces* species. Doğruöz-Güngör et al. (2020) investigated the antimicrobial activity of bacteria isolated from Kadıini Cave in Antalya, Türkiye. The researchers reported that the isolates of *Brevibacterium* spp., *Bacillus* spp., and *Pseudomonas* spp. showed antibacterial activity against tested Gram-positive bacteria. In another study, the antimicrobial activity of actinomycetes isolated from various habitats, including cave water, was investigated in Burdur province, Türkiye. As a result, an isolate identified as *Microbiospora* spp. exhibited antimicrobial activity against the tested microorganisms (Bedel, 2020). However, we think that research on the antimicrobial activity of cave microorganisms should increase in Türkiye, which is believed to have more than 40,000 caves and is defined as a "cave paradise country" by many researchers. This study scoped actinomycetes isolated from three different karst caves that have not been opened to tourism and have not been investigated in terms of microflora before, located in the province of Gümüşhane in the Eastern Black Sea Region of Türkiye. The antimicrobial activity of actinomycetes isolates was screened against some laboratory microorganisms, and phylogenetic analyses of selected isolates were determined using the 16S ribosomal RNA (rRNA) sequencing.

## MATERIALS and METHODS

### Sampling Area and Sample Collection

Between 14-16 October 2016, a total of 71 samples (soil, sand, rock soil, mud, guano, lichen, and water) were collected from the entrance, twilight, and dark zones of Akçakale (40º26'03"N; 39º31'54"E, 1585 m altitude, 290 m length), Kırklar (Altıntaş) (40°18'16.5"N; 39°47'32.09"E, 2160 m altitude, 295 m length), and Köprübaşı Caves (40°31'14.67"N; 39°24'55.96"E, 1050 m altitude, 249 m length) in Gümüşhane province, Türkiye (Figure 1). Of 71 samples, 31 were collected from Akçakale Cave ( Figure 2), 32 from Kırklar (Altıntaş) Cave (Figure 3), and eight from Köprübaşı Cave (Figure 4). The samples were taken aseptically and randomly from different regions along the visitable parts of the caves. About 50-100 g samples were transferred into sterile containers using sterile spatulas and forceps (for solid samples) or sterile serological pipettes and injectors (for liquid samples), and transferred to the laboratory in a cooler with ice packs and stored at 4 ºC until processing.


Figure 1. Location of the Köprübaşı Cave (A), Akçakale Cave (B), and Kırklar Cave (C)
*Şekil 1. Köprübaşı mağarası (A), Akçakale mağarası (B) ve Kırklar (Altıntaş) Mağarasının (C) konumu*

### Actinomycetes Isolation

For solid samples, a 5 g sample was weighed under aseptic conditions and mixed in 50 mL sterile phosphate buffer solution (PBS). For liquid samples, about 50 mL sample was centrifuged at room temperature at 10,000 rpm for 5 min. The pellet was then resuspended with 1 mL PBS. The mixtures were then vigorously vortexed at room temperature. Then, serial dilutions of up to $10^{-4}$ were prepared for each sample, and 100 µL of each dilution was spread on Actinomycetes Isolation agar (AIA, Sigma-Aldrich Co., St. Louis, MO, USA) with supplemented nystatin (40 µg $mL^{-1}$) as an antifungal agent. The cultures were incubated in the dark at 28 °C for 28 days and examined daily. Subcultures of isolates resembling colony formation of actinomycetes were prepared, and Gram reactions were examined. Actinomycete colonies classically have well-developed radial mycelium, and cells are Gram-positive and filamentous morphology. All actinomycetes with different colony formations

were coded as TRMS [Türkiye-Mağara (cave)-*Streptomyces*] with a number and stored at -80 ºC in nutrient broth, including 20% glycerol.

## Screening of Antimicrobial Activity

The antimicrobial activity of actinomycetes isolates was investigated against three Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633), seven Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter haemolyticus* ATCC 19002, *Chromobacterium violaceum* ATCC 12472, *Escherichia coli* ATCC 25922, *Enterobacter aerogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC 13883, and *Salmonella* Typhimurium ATCC 14028), and one yeast (*Candida albicans* ATCC 10231) strain as representing pathogens. All strains were American Type Culture Collection (ATCC) standard microorganisms and were obtained from the culture collection of the Department of Medical Microbiology, Medicine Faculty, Karadeniz Technical University.

The antimicrobial activity was screened using the modified cross-streak agar method as described previously (Velho-Pereira & Kamat, 2011). This method is commonly used to investigate the antagonism between microorganisms. Briefly, fresh cultures of actinomycetes were inoculated in a straight line on Tryptic Soy agar (TSA, Lab M, Lancashire, UK) plates and incubated at 28 ºC for one week. After the incubation, test microorganisms were adjusted to 0.5 McFarland turbidity standards and inoculated in duplicate perpendicular to the actinomycetes. The plates were incubated at 37 ºC for 24 hours. It was investigated whether the test microorganisms' growth was inhibited on the actinomycetes-facing side.

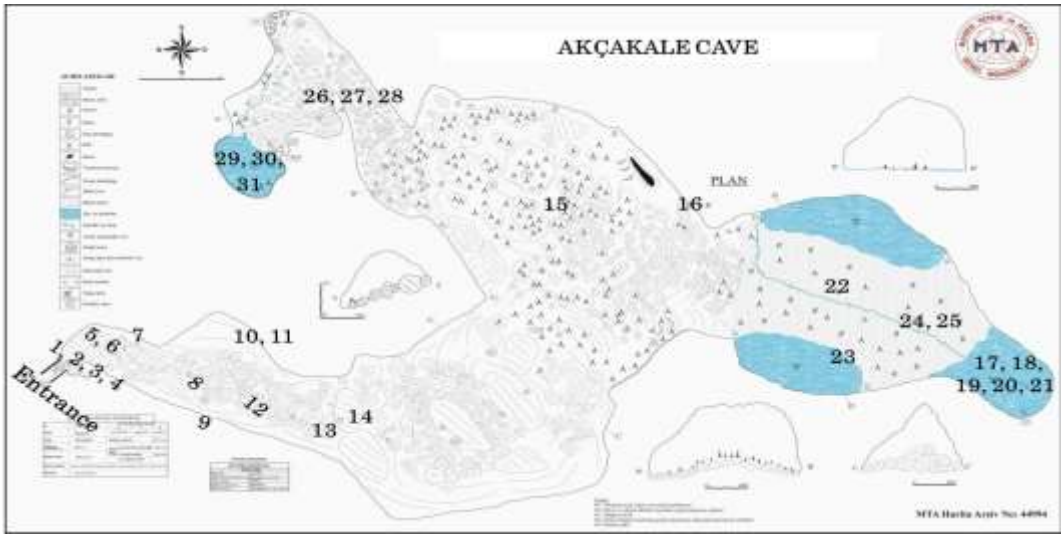


Figure 2. Sampling sites of Akçakale Cave
*Şekil 2. Akçakale mağarası örnekleme alanları*

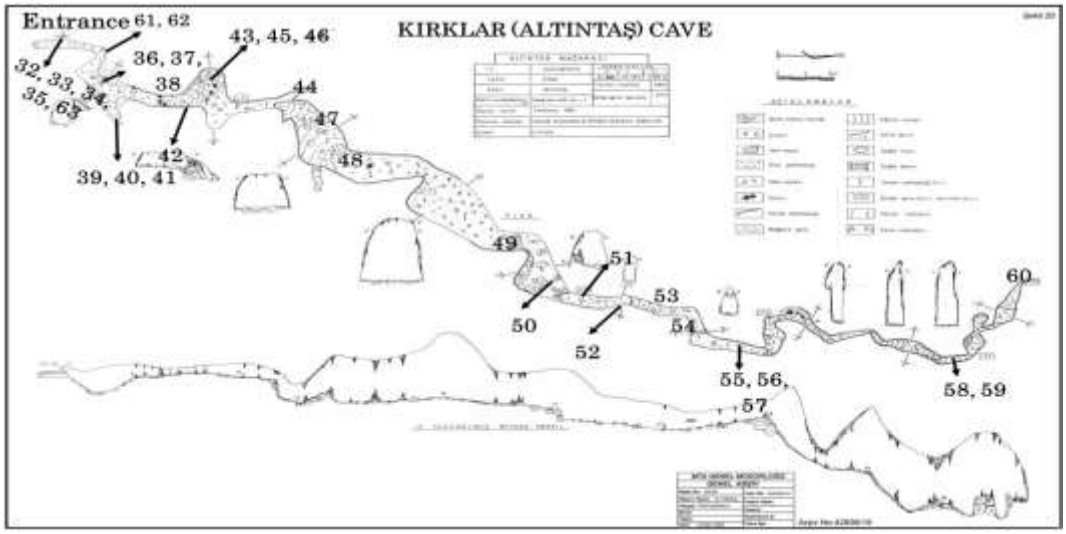


Figure 3. Sampling sites of Kırklar (Altıntaş) Cave
*Şekil 3. Kırklar (Altıntaş) mağarası örnekleme alanları*

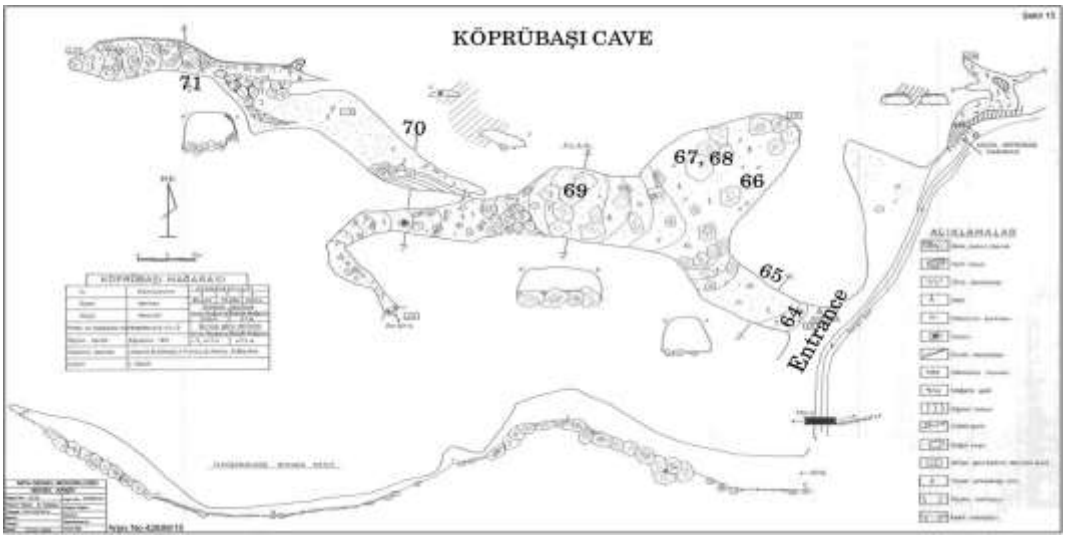


Figure 4. Sampling sites of Köprübaşı Cave
*Şekil 4. Köprübaşı mağarası örnekleme alanları*

### 16S rRNA Gene Sequencing and Phylogenetic Analysis

The 16S rRNA sequence analysis of 26 isolates among the actinomycetes exhibiting antimicrobial activity against at least three test microorganisms was performed. Firstly, genomic DNA isolation of the isolates was performed as described by Chen & Kuo (1993). In addition, lysozyme (1 µg $mL^{-1}$) was added to the lysis buffer of the mentioned protocol and the cells were bead-beating using 1 mm diameter glass beads (Marienfeld, Lauda-Koenigshofen, Germany).

The 16S rRNA gene was amplified by PCR method using the conserved primers as 27F (5'-AGAGTTTGGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR reactions were prepared to contain the following ingredients; 2 µL DNA template, 0.8 µL each primer (10 pmol $µL^{-1}$), 8 µL 5x FIREPol® Master Mix (SolisBioDyne, Tartu, Estonia), and ultra-pure water up to 40 µL. The thermocycler conditions were as follows: initial denaturation at 94 °C for 2 min; 35 cycles of 94 °C for 45 s, 55 °C for 60 s, 72 °C for 60 s; final extension at 72 °C for 10 min (Tufekci et al., 2019). The amplicons (about 1500 bp) were purified before sequencing using the PureLinkTM Quick PCR Purification Kit (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions.

The purified amplicons were sequenced using BigDye terminator chemistry (Applied Biosystems, Foster City, CA, USA) on the ABI 3130 capillary DNA sequencer (Applied Biosystems). The sequencing was performed using primers 27F and 1492R. The results were compared for similarity with sequences in the National Center of Biotechnology Information (NCBI) rRNA/ITS databases using the Basic Alignment Search Tool (BLAST). The partial 16S rRNA sequences were submitted to GenBank with accession numbers OP781986-OP7820011.

A phylogenetic tree was constructed based on the partial 16S rRNA sequences using the Neighbor-Joining (NJ) method (Saitou & Nei, 1987). The evolutionary distances were calculated using the Kimura 2-parameter method (Kimura, 1980). The robustness of the phylogenetic tree was determined with 1.000 bootstrap replicates, using the Molecular Evolutionary Genetic Analysis (MEGA) 11.0 program package (http://www.megasoftware.net) (Tamura et al., 2021). The bootstrap values above 50% were indicated at the nodes of the phylogenetic tree. *Escherichia coli* was used as the outgroup in the phylogenetic tree.

### Statistical Analysis

The data were analyzed using the Pearson chi-square test in SPSS 23.0 for Windows (IBM Inc., Armonk, NY, USA) and the statistical significance was taken as $p<0.05$.

## RESULTS

A total of 179 actinomycetes were isolated from three caves based on the morphological appearance of the colonies (glabrous or chalky, heaped, folded), and the colors of the aerial and substrate micelles (gray, brown, white, yellow, beige, and orange) (Figure 5). Of them, 31 were from Akçakale Cave, 61 were from Köprübaşı Cave, and 87 were from Kırklar Cave.

The antimicrobial activity of all isolates was screened. Of these, 53 isolates (29.6%) showed antimicrobial activity against at least one of the tested microorganisms. The results are shown in Table 1.

Actinomycetes mainly exhibited antimicrobial activity against Gram-positive bacteria. For example, 21.2% (n=38) of the isolates showed antimicrobial activity against *S. aureus*, 20.0% (n=35) against *B. subtilis*, and 16.8% (n=30) against *E. faecalis*. However, there was no significant difference ($p>0.05$) in susceptibility

among Gram-positive bacteria. On the other hand, 12.8% (n=23) of the isolates had antimicrobial activity against *C. violaceum*, 3.4% (n=6) against *K. pneumoniae*, 2.8% (n=5) against *S.* Typhimurium, 2.2% (n=4) against *E. coli*, 1.1% (n=2) against *A. haemolyticus*, and 0.6% (n=1) against *P. aeruginosa* and *E. aerogenes*. *C. violaceum* was the most susceptible Gram-negative bacteria to actinomycetes ($p<0.05$).

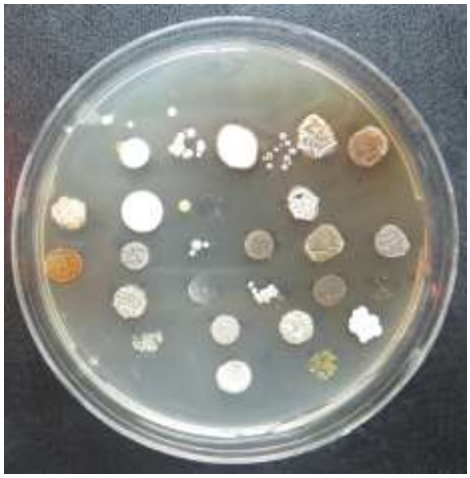

Figure 5. The representative image of subcultured actinomycete isolates

*Şekil 5. Alt kültürleri yapılmış aktinomiset izolatlarının temsili görüntüsü*

One isolate, designated TRMS 124, exhibited antimicrobial activity against *C. albicans*. TRMS 124 was also the most potent isolate by showing antimicrobial activity against ten microorganisms (*S. aureus*, *E. faecalis*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *E. aerogenes*, *K. pneumoniae*, *S.* Typhimurium, *C. violaceum*, and *C. albicans*). The representative images of the modified cross-streak agar test result are represented in Figure 6.

The 16S rRNA genes (1222 to 1412 bp long) of 26 isolates were sequenced and compared with the sequences deposited in the NCBI rRNA/ITS databases. Twenty-four of the 26 isolates belonged to the *Streptomyces* genus. Moreover, 22 different *Streptomyces* species were identified as closely related species of the isolates. The others were closely related to *Embleya scabrispora* and *Couchioplanes caeruleus* species. The similarity of the isolates to their closest strains was more than 99% (Table 2).

Based on the tree topology, we grouped 24 *Streptomyces* isolates into two main clusters (Figure 7). Cluster I was the largest cluster in the phylogenetic tree and consisted of the TRMS 88, TRMS 117, TRMS 124, TRMS 539, TRMS 543, TRMS 609, TRMS 673, TRMS 713, TRMS 714, TRMS 3120, TRMS 5515, TRMS 5517, TRMS 5814, TRMS 6015, TRMS 6027, TRMS 6124, and TRMS 6127 isolates related to 20 different *Streptomyces* species. Cluster II consisted of seven isolates closely related to *Streptomyces zagrosensis* and *Streptomyces niveus*: TRMS 6025, TRMS 6330, TRMS 6344, TRMS 6712, TRMS 6726, TRMS 6734, and TRMS 6736.

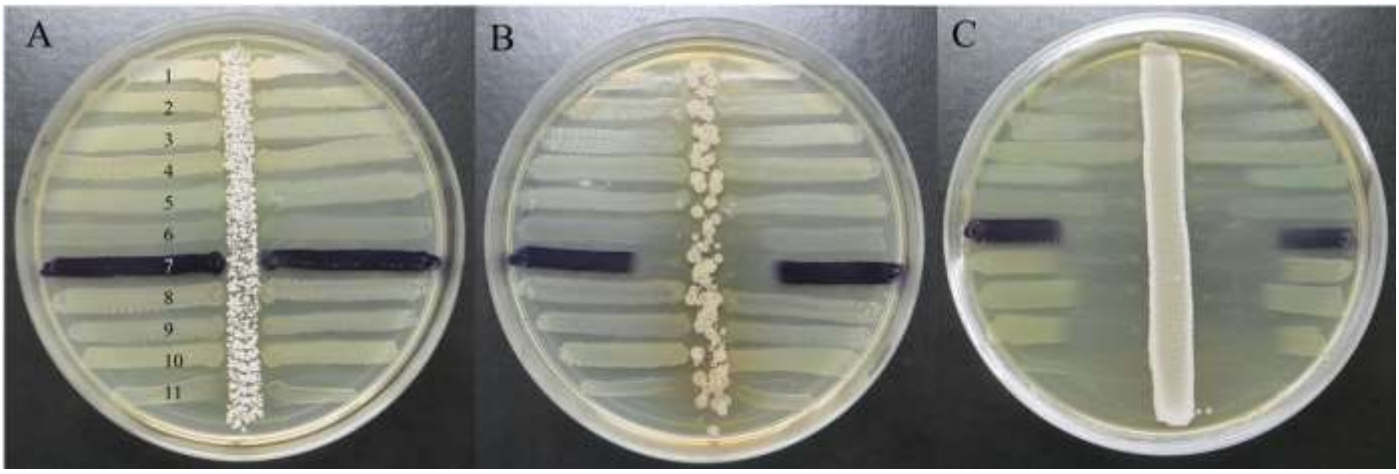


Figure 6. The representative images of the modified cross-streak agar test result. A, TRMS 6615 (no antimicrobial activity); B, TRMS 3120; C, TRMS 124 (1: *S. aureus*, 2: *E. coli*, 3: *B. subtilis*, 4: *A. haemolyticus*, 5: *P. aeruginosa*, 6: *E. faecalis*, 7: *C. violaceum*, 8: *K. pneumoniae*, 9: *S.* Typhimurium, 10: *E. aerogenes*, 11: *C. albicans*)

*Şekil 6. Çapraz çizgi yöntemi test sonucunun temsili görüntüleri. A, TRMS 6615 (antimikrobiyal aktivite mevcut değil); B, TRMS 3120; C, TRMS 124 (1: S. aureus, 2: E. coli, 3: B. subtilis, 4: A. haemolyticus, 5: P. aeruginosa, 6: E. faecalis, 7: C. violaceum, 8: K. pneumoniae, 9: S. Typhimurium, 10: E. aerogenes, 11: C. albicans)*

Table 1. The antimicrobial activity of the actinomycetes isolates against test microorganisms (+, positive; -, negative)
*Çizelge 1. Aktinomiset izolatlarının test mikroorganizmalarına karşı antimikrobiyal aktivitesi (+, pozitif; -, negatif)*

| Isolate name | Cave | Isolation Source | Test microorganisms | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *S. aureus*[1] | *E. faecalis*[1] | *B. subtilis*[1] | *A. haemolyticus*[2] | *E. coli*[2] | *P. aeruginosa*[2] | *E. aerogene*[2] | *K. pneumoniae*[2] | *S.* Typhimurium[2] | *C. violaceum*[2] | *C. albicar*[3] |
| TRMS 59 | Akcakale | Aqueous sample (Sampling site 5) | - | - | - | - | - | - | - | - | - | + | - |
| TRMS 88* | Akçakale | Rock soil (Sampling site 8) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 117* | Akçakale | Rock soil (Sampling site 1) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 124* | Akçakale | Rock soil (Sampling site 1) | + | + | + | - | + | + | + | + | + | + | + |
| TRMS 185 | Akçakale | Guano (Sampling site 18) | - | - | - | - | - | - | - | - | - | + | - |
| TRMS 512 | Akçakale | Aqueous sample (Sampling site 5) | - | - | - | - | - | - | - | - | - | + | - |
| TRMS 515 | Akçakale | Aqueous sample (Sampling site 5) | - | - | - | - | - | - | - | - | - | + | - |
| TRMS 539* | Kırklar | Mud (Sampling site 53) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 543* | Kırklar | Mud (Sampling site 54) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 553 | Kırklar | Soil (Sampling site 55) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 609* | Kırklar | Sand (Sampling site 60) | + | + | + | - | - | - | - | - | - | + | - |
| TRMS 636 | Kırklar | Lichen (Sampling site 63) | + | - | - | - | - | - | - | - | - | - | - |
| TRMS 639 | Kırklar | Lichen (Sampling site 63) | + | - | + | - | - | - | - | - | - | - | - |
| TRMS 673* | Köprübaşı | Soil (Sampling site 67) | + | + | + | - | - | - | - | - | - | + | - |
| TRMS 713* | Akçakale | Rock soil (Sampling site 7) | + | + | + | + | + | - | - | + | + | + | - |
| TRMS 714* | Köprübaşı | Soil (Sampling site 71) | + | + | + | + | + | - | - | + | + | + | - |
| TRMS 814 | Akçakale | Rock soil (Sampling site 8) | - | + | - | - | - | - | - | - | - | - | - |
| TRMS 2224 | Akçakale | Rock soil (Sampling site 22) | - | - | - | - | - | - | - | - | - | + | - |
| TRME 2510* | Akçakale | Rock soil (Sampling site 25) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 2514 | Akçakale | Rock soil (Sampling site 25) | - | - | - | - | - | - | - | - | - | + | - |
| TRMS 3120* | Akçakale | Water (Sampling site 31) | + | + | - | - | - | - | - | - | - | + | - |
| TRMC 3225* | Kırklar | Lichen (Sampling site 32) | + | - | - | - | - | - | - | + | - | + | - |
| TRMS 4012 | Kırklar | Mud (Sampling site 40) | - | - | - | - | - | - | - | - | - | + | - |
| TRMS 5515* | Kırklar | Soil (Sampling site 55) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 5517* | Kırklar | Soil (Sampling site 55) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 5611 | Kırklar | Soil (Sampling site 56) | + | - | - | - | - | - | - | - | - | - | - |
| TRMS 5814* | Kırklar | Mud (Sampling site 58) | - | + | + | - | - | - | - | - | - | + | - |
| TRMS 5818 | Kırklar | Mud (Sampling site 58) | + | - | + | - | - | - | - | - | - | - | - |
| TRMS 6015* | Kırklar | Sand (Sampling site 60) | + | + | + | - | - | - | - | + | - | + | - |
| TRMS 6025* | Kırklar | Sand (Sampling site 60) | + | + | + | - | - | - | - | - | + | + | - |
| TRMS 6027* | Kırklar | Sand (Sampling site 60) | + | + | + | - | + | - | - | - | - | + | - |
| TRMS 6124* | Kırklar | Soil (Sampling site 61) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 6127* | Kırklar | Soil (Sampling site 61) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 6131 | Kırklar | Soil (Sampling site 61) | + | - | - | - | - | - | - | - | - | - | - |
| TRMS 6133 | Kırklar | Soil (Sampling site 61) | + | - | + | - | - | - | - | - | - | - | - |
| TRMS 6135 | Kırklar | Soil (Sampling site 61) | - | - | - | - | - | - | - | - | - | + | - |
| TRMS 6316 | Kırklar | Lichen (Sampling site 63) | - | - | - | - | - | - | - | + | + | + | - |
| TRMS 6323 | Kırklar | Rock soil (Sampling site 63) | + | - | - | - | - | - | - | - | - | - | - |
| TRMS 6326 | Kırklar | Rock soil (Sampling site 63) | + | - | - | - | - | - | - | - | - | - | - |
| TRMS 6327 | Kırklar | Rock soil (Sampling site 63) | - | - | + | - | - | - | - | - | - | + | - |
| TRMS 6330* | Kırklar | Rock soil (Sampling site 63) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 6342 | Kırklar | Rock soil (Sampling site 63) | + | - | + | - | - | - | - | - | - | - | - |
| TRMS 6344* | Kırklar | Rock soil (Sampling site 63) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 6712* | Köprübaşı | Soil (Sampling site 67) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 6721 | Köprübaşı | Soil (Sampling site 67) | - | - | + | - | - | - | - | - | - | - | - |
| TRMS 6726* | Köprübaşı | Soil (Sampling site 67) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 6732 | Köprübaşı | Soil (Sampling site 67) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 6734* | Köprübaşı | Soil (Sampling site 67) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 6735 | Köprübaşı | Soil (Sampling site 67) | - | - | + | - | - | - | - | - | - | - | - |
| TRMS 6736* | Köprübaşı | Soil (Sampling site 67) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 6738 | Köprübaşı | Soil (Sampling site 67) | + | + | + | - | - | - | - | - | - | - | - |
| TRMS 6739 | Köprübaşı | Soil (Sampling site 67) | + | + | + | - | - | - | - | - | - | + | - |
| TRMS 6913 | Köprübaşı | Soil (Sampling site 69) | - | - | - | - | - | - | - | - | - | + | - |

*The 16S rRNA sequence analysis was performed, [1]Gram-positive bacteria, [2]Gram-negative bacteria, [3]Yeast

Table 2. The results of 16S rRNA sequencing analysis
*Çizelge 2. 16S rRNA dizi analizinin sonuçları*

| **Isolate** | | **Closest Strain(s) in Gene Bank** | | | |
|---|---|---|---|---|---|
| **Code** | **Accession Number** | **Strain** | **Accession Number** | **Identity (%)** | **Query Cover (%)** |
| TRMS 88 | OP781989 | *Streptomyces spororaveus*<br>*Streptomyces nojiriensis*<br>*Streptomyces cirratus*<br>*Streptomyces subrutilus*<br>*Streptomyces xanthophaeus*<br>*Streptomyces avidinii*<br>*Streptomyces vinaceus* | NR_112469<br>NR_112414<br>NR_112388<br>NR_112385<br>NR_112294<br>NR_041132<br>NR_041131 | 99.63 | 99 |
| TRMS 117 | OP781986 | *Streptomyces cyaneofuscatus* | NR_115383 | 100 | 100 |
| TRMS 124 | OP781987 | *Streptomyces anulatus*<br>*Streptomyces baarnensis*<br>*Streptomyces praecox*<br>*Streptomyces fimicarius*<br>*Streptomyces caviscabies*<br>*Streptomyces pratensis* | NR_112527<br>NR_112440<br>NR_112358<br>NR_112347<br>NR_114493<br>NR_125619 | 99.93 | 94 |
| TRMS 539 | OP781993 | *Streptomyces spororaveus*<br>*Streptomyces nojiriensis*<br>*Streptomyces cirratus*<br>*Streptomyces subrutilus*<br>*Streptomyces xanthophaeus*<br>*Streptomyces avidinii* | NR_112469<br>NR_112414<br>NR_112388<br>NR_112385<br>NR_112294<br>NR_041132 | 99.70 | 100 |
| TRMS 543 | OP781994 | *Streptomyces vinaceus*<br>*Streptomyces cirratus* | NR_041131<br>NR_112388 | 99.49 | 100 |
| TRMS 609 | OP781998 | *Streptomyces microflavus*<br>*Streptomyces alboviridis*<br>*Streptomyces griseus*<br>*Streptomyces erumpens* | NR_103947<br>NR_112340<br>NR_112475<br>NR_112455 | 99.42 | 100 |
| TRMS 673 | OP782006 | *Streptomyces exfoliatus* | NR_041117 | 99.85 | 100 |
| TRMS 713 | OP781988 | *Streptomyces anulatus*<br>*Streptomyces baarnensis*<br>*Streptomyces praecox*<br>*Streptomyces fimicarius*<br>*Streptomyces caviscabies*<br>*Streptomyces pratensis* | NR_112527<br>NR_112440<br>NR_112358<br>NR_112347<br>NR_114493<br>NR_125619 | 99.86 | 100 |
| TRMS 714 | OP782011 | *Streptomyces anulatus*<br>*Streptomyces praecox* | NR_112527<br>NR_112358 | 100 | 99 |
| TRME 2510 | OP781990 | *Embleya scabrispora* | NR_112597 | 100 | 100 |
| TRMS 3120 | OP781991 | *Streptomyces vinaceus*<br>*Streptomyces cirratus* | NR_041131<br>NR_112388 | 99.54 | 100 |
| TRMC 3225 | OP781992 | *Couchioplanes caeruleus* | NR_037054 | 99.35 | 100 |
| TRMS 5515 | OP781995 | *Streptomyces cirratus*<br>*Streptomyces vinaceus* | NR_112388<br>NR_041131 | 99.36 | 100 |
| TRMS 5517 | OP781996 | *Streptomyces vinaceus*<br>*Streptomyces cirratus* | NR_041131<br>NR_112388 | 99.77 | 100 |
| TRMS 5814 | OP781997 | *Streptomyces cyaneofuscatus* | NR_115383 | 99.85 | 100 |
| TRMS 6015 | OP781999 | *Streptomyces microflavus*<br>*Streptomyces alboviridis*<br>*Streptomyces griseus*<br>*Streptomyces erumpens* | NR_103947<br>NR_112340<br>NR_112475<br>NR_112455 | 99.64 | 99 |
| TRMS 6025 | OP782000 | *Streptomyces zagrosensis* | NR_134202 | 99.85 | 100 |
| TRMS 6027 | OP782001 | *Streptomyces lunaelactis* | NR_134822 | 99.09 | 100 |
| TRMS 6124 | OP782002 | *Streptomyces spororaveus*<br>*Streptomyces nojiriensis*<br>*Streptomyces cirratus*<br>*Streptomyces subrutilus* | NR_112469<br>NR_112414<br>NR_112388<br>NR_112385 | 99.69 | 100 |

| | | | | | |
|---|---|---|---|---|---|
| | | *Streptomyces xanthophaeus* | NR_112294 | | |
| | | *Streptomyces avidinii* | NR_041132 | | |
| | | *Streptomyces vinaceus* | NR_041131 | | |
| | | *Streptomyces spororaveus* | NR_112469 | | |
| | | *Streptomyces nojiriensis* | NR_112414 | | |
| | | *Streptomyces cirratus* | NR_112388 | | |
| TRMS 6127 | OP782003 | *Streptomyces subrutilus* | NR_112385 | 99.70 | 100 |
| | | *Streptomyces xanthophaeus* | NR_112294 | | |
| | | *Streptomyces avidinii* | NR_041132 | | |
| | | *Streptomyces vinaceus* | NR_041131 | | |
| TRMS 6330 | OP782004 | *Streptomyces niveus* | NR_115784 | 99.54 | 100 |
| TRMS 6344 | OP782005 | *Streptomyces niveus* | NR_115784 | 99.62 | 100 |
| TRMS 6712 | OP782007 | *Streptomyces zagrosensis* | NR_134202 | 99.76 | 100 |
| TRMS 6726 | OP782008 | *Streptomyces zagrosensis* | NR_134202 | 99.69 | 100 |
| TRMS 6734 | OP782009 | *Streptomyces zagrosensis* | NR_134202 | 99.69 | 100 |
| TRMS 6736 | OP782010 | *Streptomyces zagrosensis* | NR_134202 | 99.92 | 100 |

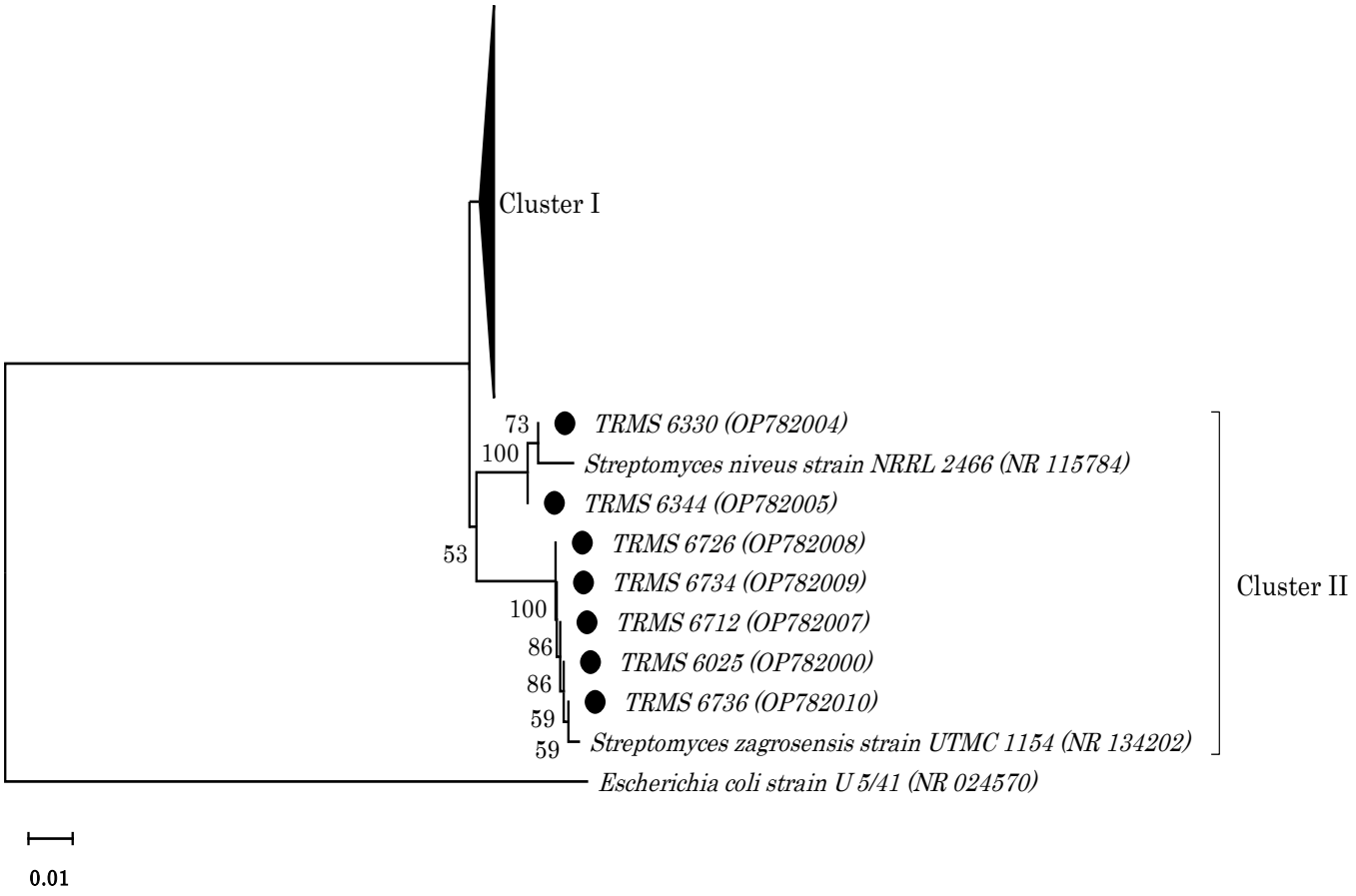


Figure 7. The phylogenetic tree based on the partial 16S rRNA gene sequences of the *Streptomyces* isolates and related species. The phylogenetic tree was constructed using the Neighbor-Joining method with 1.000 bootstrap replicates in the MEGA 11.0 program. The isolates were highlighted with a sircular sign. The accession numbers were demonstrated in parentheses to the right of the isolate name. *Escherichia coli* was used as an outgroup. The scale bar represents 0.01 substitutions per nucleotide position

*Şekil 7. Streptomyces türleri ile yakın akrabalık gösteren izolatların ve ilgili türlerin kısmi 16S rRNA gen dizilerine dayanan filogenetik ağaç görüntüsü. Filogenetik ağaç, MEGA 11.0 programında 1.000 bootstrap tekrarı ile Neighbor-Joining yöntemi kullanılarak oluşturulmuştur. İzolatlar siyah daire ile işaretlenmiştir. Erişim numaraları, izolat adının sağında parantez içerisinde gösterilmiştir. Escherichia coli grup dışı kontrol olarak kullanılmıştır. Ölçek çubuğu, nükleotid pozisyonu başına 0.01 baz ikameyi temsil etmektedir*

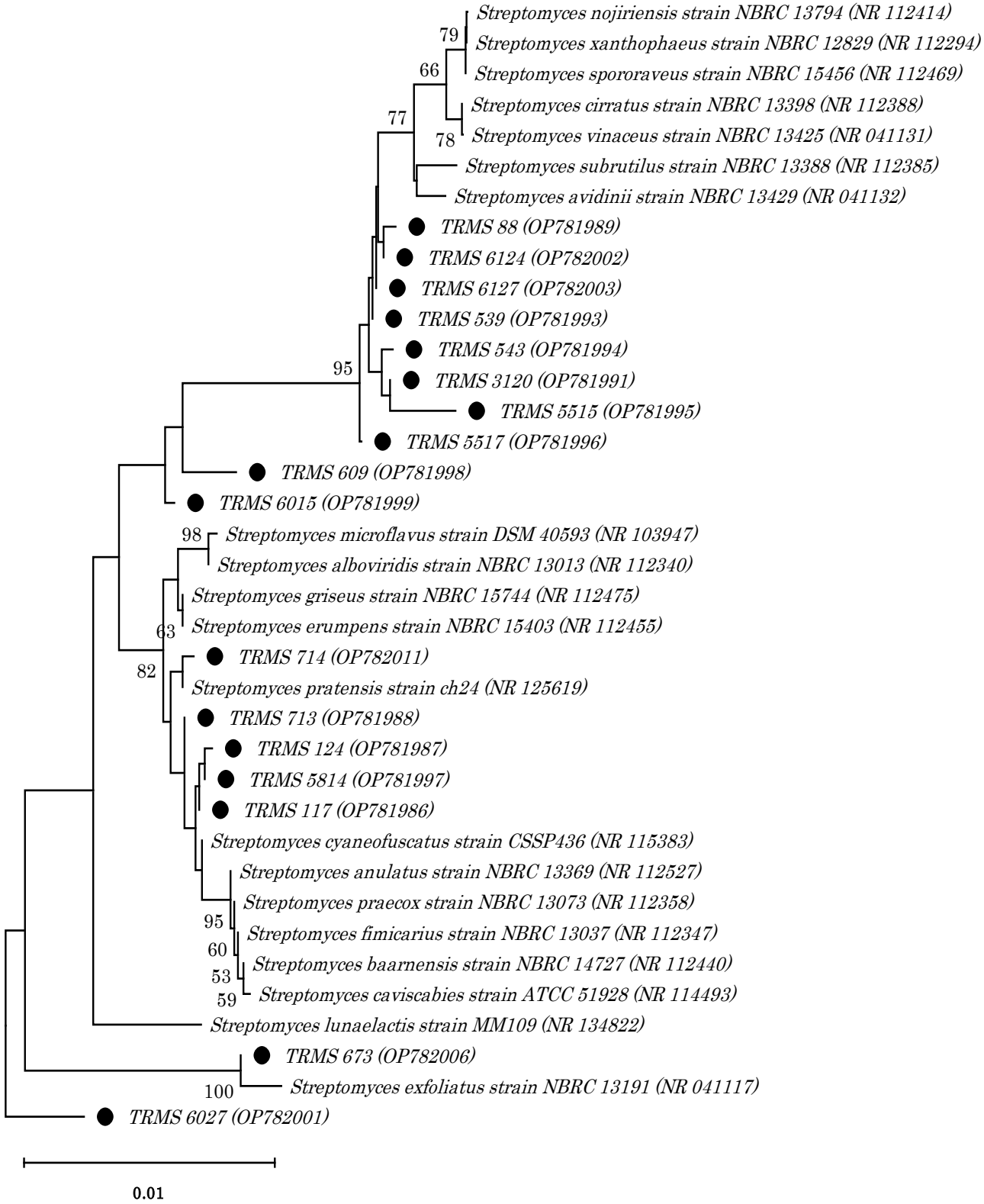


Figure 7 (Continued). Cluster I
*Şekil 7 (Devam). Küme I*

## DISCUSSION and CONCLUSION

The rapid spread of antibiotic resistance among bacteria causes severe problems in the treatment of infectious diseases, leading to economic losses and increased death rates. Therefore, there is a need for the discovery or development of novel antimicrobial agents (Miethke et al., 2021). Considering that two-thirds of the natural antibiotics used today originate from

various actinomycetes members, novel actinomycete strains emerge as a strategic method for finding novel antibiotics (Cheeptham et al., 2013).

Caves have harsh living conditions with a dark and low-nutrient environment. Low nutrient levels can encourage microorganisms to produce various antimicrobial compounds to survive and grow (Hibbing et al., 2010). Therefore, it is predicted that caves may be the natural sources of microorganisms synthesizing effective and new bioactive compounds. Studies on the microbial diversity of caves have reported that the microbial flora of each cave is unique and diverse. Actinomycetes, especially the *Streptomyces* genus, were nevertheless stated as predominant members of the cave flora (Jaroszewicz et al., 2021). In the present study, only the isolation of actinomycetes in the culture of cave samples was focused on, and total aerobic mesophilic microorganism counts were dismissed. As a result, the study continued with 179 actinomycetes isolates thought to have different colony morphology.

There are several studies investigating the antimicrobial activities of actinomycetes isolated from volcanic (Cheeptham et al., 2013) and karst caves (Yücel & Yamaç, 2010; Nimaichand et al., 2015; Maciejewska et al., 2016; Belyagoubi et al., 2018; Hamedi et al., 2019; Syiemiong & Jha, 2019; Jaroszewics et al., 2021; Pradana et al., 2022) in the literature. Furthermore, Yücel & Yamaç (2010), Cheeptham et al. (2013), and Jaroszewics et al. (2021) reported that *Streptomyces* strains isolated from the cave could exhibit antibacterial activity even against resistant strains such as methicillin-resistant *S. aureus*, vancomycin-resistant enterococci, and extended-spectrum beta-lactamases producing *E. coli*. In this study, 53 actinomycetes isolates showed antimicrobial activity against at least one of the tested standard microorganism strains. Besides, the antimicrobial activity of TRMS 124, TRMS 713, and TRMS 714 isolates against at least eight microorganisms suggest that these isolates may be potential sources of broad-spectrum antibiotics. Our findings support that cave actinomycetes can be potential sources of effective antimicrobial agents in line with the literature.

In the current study, the number of isolates exhibiting antimicrobial activity against Gram-positive bacteria was higher. Some reports have also stated that actinomycetes exhibit more antimicrobial activity against Gram-positive bacteria, as in this study (Maciejewska et al., 2016; Belyagoubi et al., 2018). This might be due to the difference in the cell wall structures of Gram-positive and Gram-negative bacteria. Because the cell wall of Gram-negative bacteria contains a more complex structure than Gram-positive bacteria. Lipopolysaccharides (LPS) are the main components found in the outer membrane of Gram-negative bacteria. Moreover, LPS could protect bacterial cells against harmful molecules such as antimicrobial compounds or toxins by providing a structurally effective permeability barrier (Farhana & Khan, 2022). On the other hand, actinomycetes had the most antimicrobial activity against *C. violaceum* among Gram-negative bacteria. Because *C. violaceum* is one of the dominant members of the soil flora and the competition between bacteria, it can be expected that most actinomycetes have an antagonistic effect against *C. violaceum* (Alisjahbana et al., 2021). Also, the isolates other than TRMS 124 had no antimicrobial activity against *C. albicans*.

The 16S rRNA sequence analysis of 26 randomly selected isolates among the actinomycetes exhibiting antimicrobial activity against at least three test microorganisms was performed, and 24 isolates were identified as *Streptomyces* spp. However, previous studies reported that 16S rRNA sequencing alone could be insufficient to distinguish closely related species of the *Streptomyces* genus. Thus, based on the 16S rRNA sequence analysis, some isolates were found to be closely related to more than one *Streptomyces* species at the same score and percentages in this study. Therefore, more research is needed to identify these isolates at the species level. The researchers recommend performing multilocus sequence analysis (MLSA), including housekeeping genes (*atpD*, *gyrB*, *recA*, *rpoB*, *trpB*) for species-level identification of *Streptomyces* spp. (Guo et al., 2008; Labeda, 2011; Rong & Huang, 2012).

The isolates found in cluster II exhibited antimicrobial activity against Gram-positive bacteria, while TRMS 6025 additionally showed antimicrobial activity against *S.* Typhimurium and *C. violaceum*. TRMS 6025, TRMS 6712, TRMS 6726, TRMS 6734, and TRMS 6736 were closely related to *S. zagrosensis*. In addition, since the antimicrobial activity spectrum of these isolates (except TRMS 6025) against the tested microorganisms was the same, we might name TRMS 6712, TRMS 6726, TRMS 6734, and TRMS 6736 as different strains of the same species. Moreover, the fact that TRMS 6025 exhibited antimicrobial activity against more test microorganisms than other isolates with which it is closely related might suggest that TRMS 6025 might be a different species. On the other hand, TRMS 6330 and TRMS 6344 were closely related to *S. niveus*, and these two isolates are likely to be *S. niveus* strains. In addition, *S. niveus* is the source of the novobiocin antibiotic which is an inhibitor of bacterial DNA gyrase (Procópio et al., 2012). Cluster I was a more heterogeneous group in terms of the antimicrobial activity spectrum. This cluster also harbored isolates with the broadest antimicrobial activity spectrum against test microorganisms such as TRMS 124, TRMS 713, and TRMS 714. Furthermore, cluster I contained *S. subrutilus*, *S. vinaceus*, *S. nojiriensis*, *S. xhantophaeus*, *S. anulatus*, and *S.*

*griseus* which are the producers of antibacterials such as hydroxystreptomycin, viomycin, nojirimycin, geomycin, actinomycin, and streptomycin, respectively (Selim et al., 2021). On the other hand, the antimicrobial effects observed in this study may be attributed to bioactive compounds such as cervimycin A-D, undecylprodigiosin, xiakemycin A, chaxalactin B, as well as the antibiotics mentioned above. Because these bioactive compounds have been purified from some *Streptomyces* spp. isolates with antimicrobial activity isolated from various caves until now and have been held responsible for the antimicrobial effect (Rangseekaew & Pathom-Aree, 2019).

Several considerations limit this study. For example, biochemical tests were not performed for the characterization of actinomycetes. Antimicrobial activity was determined using the modified cross-streak test. Therefore, the results were presented only as the presence (+) or absence (-) of antimicrobial activity. Any quantitative data on antimicrobial activity is not available. Also, antibiotic-resistant strains were not used as the test microorganism.

This study provides preliminary data that actinomycetes isolated from Akçakale, Kırklar, and Köprübaşı Caves may be species with the potential to produce novel antimicrobial compounds. The results supported that caves could be the source of actinomycetes that produce bioactive compounds, as described previously. The antimicrobial activities of these isolates will be further investigated in a follow-up study using the disk diffusion and broth microdilution methods. In addition, the secondary metabolites of these isolates should be determined in detail.

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### Author's Contributions

Authors declare the contribution of the authors is equal.

### Statement of Conflict of Interest

The authors have declared no conflict of interest.

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