Journal of Animal and Veterinary Advances 13 (16): 978-988, 2014
ISSN: 1680-5593


# Identification of Biodiversity of Some *Streptomyces* Species and Determination of a Restriction Fragment Length Polymorphism (RFLP) Profile of *16S rDNA* Gene Region

[1]K. Ozdemir, [1]E. Ogun, [1]M. Ertas, [3]S. Acar and [2]E. Atalan
[1]Department of Molecular Biology and Genetics, Faculty of Science,
Yuzuncu Yil University, 65080 Van, Turkey
[2]Department of Biology, Faculty of Arts and Sciences, Inonu University, 44100 Malatya, Turkey
[3]Turkey National Hygiene Center,
National Molecular Microbiology Research and Application Laboratory, Ankara, Turkey

**Abstract:** In this study, soil samples from the roots and peripheral regions of some agricultural plant species growing in the Van region (such as sainfoin, bean, malus, wheat and sugar beet) were collected between May and June 2004 and 139 strains of Streptomyces bacteria were isolated from these samples. Moreover, researchers determined the physicochemical characteristics of these soil samples. Streptomyces bacteria were color-grouped and the Diaminopimelic Acid (DAP) forms were detected followed by the determination of then phenotypic characteristics. When these isolates were divided into 39 color groups, 20 strains were found to contain L-DAP and therefore were confirmed as members of the genus *Streptomyces*. The species were identified using the TAXON Software and similarity dendrograms were constructed by the Unweighted Pair-Group Method with an Arithmetic average (UPGMA) cluster analysis using the Multi-Variate Statistical Package (MVSP 3.1). Genomic DNA was isolated from these bacteria and *16S rDNA* gene was amplified by PCR followed by restriction digestion with Bsp143I, HaeIII, MnlI and SphI for Restriction Fragment Length Polymorphism (RFLP) analysis. In the dendrogram obtained at the end of the RFLP analysis, four groups of two or more members and six 1-member groups were established according to the similarity ratio criterion.

**Key words:** Streptomyces, isolation, 16S rDNA, RFLP, ratio criterion

## INTRODUCTION

Due to seasonal and climatic changes and human intervention, there is little information on geographical distribution of the *Streptomyces* sp. (Sembiring *et al*., 2000; Saintpierre *et al*., 2003). *Streptomyces* are a genus of bacteria that live for a long time in the soil as arthrospores and germinate under appropriate conditions. It is known that the spore form survive longer than the hyphae phase and are scattered in the soil by arthropods and the flow of water through soil and in the atmosphere by wind and rain. Basic factors that affect the activity and scattering of Streptomyces include nutrients, heat, pH, moisture, a soil type and seasonal changes (Upton, 1994; Atalan, 1993; Leiminger *et al*., 2013). Similarly, physiological processes are also important factors that determine the activity and scatter of the Streptomyces bacteria. Under *in situ* conditions, most *Streptomyces* sp. grow at an optimum temperature. Mud and humic acid content of the soil affects activity of Streptomyces. These bacteria live in the soil as Gram-positive filaments and undergo morphological differentiation at different life stages. Under normal conditions they do not form spores. If sufficient moisture and nutrients are available then they germinate and form vegetative mycelia. However, the lack of nutrients or water deficiency halts mycelium formation and Streptomyces survive such environmental stresses through the formation of spores. Moreover, these bacteria simultaneously initiate the production pigments, antibiotics and other secondary metabolites (Kutzner, 1986).

Soil is the most frequent habitat of Streptomyces bacteria and they are present in all kinds of soil (Williams *et al*., 1989). These bacteria generally play a substantial ecological role in decomposing different components in soil in addition to plant waste and fungus mycelia on the surface (Samson *et al*., 1994). Streptomyces are present in the habitats that are rich in organic matter (Hagedorn, 1976). The 1-20% of all live microorganisms present in the soil are generally

**Corresponding Author:** Kerem Ozdemir, Department of Molecular Biology and Genetics, Faculty of Science,
Yuzuncu Yil University, 65080 Van, Turkey

Streptomyces (Xu *et al*., 1996), in addition, 64-97% of all Actinomycetes that can be isolated are Streptomycetes (Wang *et al*., 1999; Chronakova *et al*., 2010; Demain, 1999).

Streptomyces which are considered to be indicators of soil moisture that triggers microbial development are known to be producers of secondary metabolites that have various antibacterial, antifungal, antiparasitic, antitumor and immunostimulatory properties (Mishra *et al*., 1980). Some species cause infections but most are non-pathogenic (Williams *et al*., 1983a). It is not generally accepted that Actinomycetes play a sufficient role in plant root systems (Sardi *et al*., 1992) nevertheless, it has been reported that *Streptomyces* species are generally scattered throughout plant root systems (Atalan *et al*., 2000; Jussila *et al*., 2003). However, data on the activity, microbial interspecific interactions and biodiversity of Streptomyces in root systems are scarce. It is reported that Streptomyces positively affects root systems of wheat, tomato, soy, barley and perennial grasses (Trejo-Estrada *et al*., 1998). There are numerous studies analyzing the mutualistic relationships between *Streptomyces* sp. and roots for example these bacteria suppress fungal pathogen growth in roots which affects the development and growth of plants (Adams, 1990). Moreover, the use of Streptomyces bacteria as biocontrol agents for inhibiting the growth of fungal pathogens has been recommended. Streptomyces antibiosis helps rhizosphere development in the presence of one or more diffusible components, antibiosis inhibits the development and growth of pathogens and prevents colony formation thereby preventing the development of diseases as well. Microparasitic biocontrol is quantified as the physical scatter of the fungal cell wall under the influence of a hydrolytic enzyme produced by the biocontrol agent (Satheeja and Jebakumar, 2011; Pissowotzki *et al*., 1991).

The genes encoding proteins that are responsible for synthesis and regulation of secondary metabolites which confer antibiotic resistance are occasionally present as groups (Egan *et al*., 1998). Secondary metabolite genes are sometimes available in plasmids and participate in horizontal gene transfer in the soil as well (Omura *et al*., 2001; Williams *et al*., 1984).

Numerous nonselective media have been used in the past for isolation of Streptomyces bacteria that were known to have high activity and significant presence in the soil (Labeda and Lyons, 1991). However, selective media are currently used to eliminate undesirable microorganisms and therefore, it is important that antibiotics be added to the medium (Da Rosa *et al*., 2013; Williams *et al*., 1983b).

Molecular systematic methods were used by Williams *et al*. (1983a) and Crameri *et al*. (1983) to characterize some taxa of *Streptomyces* species. Some nucleic acid fingerprinting studies have been performed in order to differentiate similar genera and species. Crameri *et al*. (1983) and Beyazova and Lechevalier (1993) digested genomic DNA with restriction enzymes to separate *Streptomyces* species thereby producing many more short segmented bands. Based on the results of their analysis in terms of distinguishing species from one another, it was found that these methods are not sensitive enough.

Beyazova and Lechevalier (1993), Labeda (1992) and (Doering-Saad *et al*., 1992) applied Low-Frequency Restriction fragment Analysis (LFRA) in order to show the relationship among the strains of eight species which included *S. albus*, *S. ipomoea* and *S. somaliensis* that cause infections in plants and humans. Based on their results, *S. ipomoea* was divided into two subgroups. However, other test organisms were not sufficiently classified. Doering-Saad *et al*. (1992) and Kim *et al*. (1999) performed ribotyping on 40 Streptomyces strains which both caused and inhibited *Synchytrium endobioticum* growth. Although, the results demonstrated the variability among pathogenic strains at the high level, the results were concordant with Restriction Fragment Length Polymorphism (RFLP) numeric and phenotypic data. In conclusion, it was reported that genes responsible for pathogenicity are scattered in mobile elements among the different Streptomyces strains.

It was also reported that ribotyping data are useful for differentiating between isolates such as those in the *S. thermodiasicus* group (Kim *et al*., 1998). This result is concordant with Random Amplified Polymorphic DNA PCR (RAPD-PCR) data (Clarke *et al*., 1993). Clarke *et al*. (1993) and Steingrube *et al*. (1997) showed that 14 Streptomyces strains demonstrated rather strong variation by an RFLP analysis on *rDNA* genes and reported that these genes were present in all or some bands during the gel analysis, thereby showing that the analysis of *rDNA* genes by RFLP techniques are accurate as well as rapid. Fifteen clinical Streptomyces isolates were subjected to PCR-RFLP analysis of a gene (439 bp) that encodes a 65 kDa heat shock protein (Reed and Cummings, 1945). Test organisms were divided into five groups and a group was established with reference bacteria accurately. Therefore, it is apparent that RFLP analysis is a rapid method for identification of the clinical Streptomyces bacteria as well.

## MATERIALS AND METHODS

Soil samples were collected from the roots and peripheral regions of the plants such as such as sainfoin, bean, malus, wheat and sugar beet grown in the seeding areas of Central County, Ercig, Ahlat, Edremit, Ozalp,

Gevag, Tatvan and Muradiye over a month as representative samples from the Van Lake basin (Fig. 1). Moisture, organic matter and pH values were determined and compared between the different localities (Sveshnikova *et al*., 1976).

For isolation of Streptomyces bacteria, starch-casein (Vickers *et al*., 1984) and raffinose-histidine (Wellington and Williams, 1978) agar were used with the addition of 50 μg $mL^{-1}$ cycloheximide and 50 μg $mL^{-1}$ nystatin. Petri dishes were incubated for 14 days at 25°C and the colonies were subsequently examined both with the naked eye and under a stereoscope microscope. On each petri dish, Streptomyces and total Actinomycetes colonies and were counted and colony numbers in 1 g of dried soil (g/dried soil) were expressed as colony-forming units (cfu).

Streptomyces colonies were transferred to Bennet's agar petri plates and pure colonies were isolated. In total, 139 Streptomyces colonies were purified. These isolates were resuspended in 2 mL of glycerol (20% v/v) (Kuster, 1959) and stored at -20°C. All these colonies were injected onto the surface of oatmeal (Shirling and Gottlieb, 1966) and peptone iron agar and they were incubated at 25°C for 14 days (Jones, 1949). Next, they were examined for the color of airy and substrate mycelia, diffused pigment production and melanin pigmentation. Based on these results, color-grouping was performed and sample strains were selected for identification studies; L-Diaminopimelic Acid (L-DAP) presence was tested to see whether the 20 Streptomyces isolates belonged to that genus and all strains were confirmed to be Streptomyces bacteria (Jones, 1949). Additionally, five reference strains of bacteria were selected (50366, 52331, 51291, N7363 and 51311A) and all isolates were tested for 45 diagnostic characteristics as described previously by Williams *et al*. (1983a).

Morphological and pigmentation properties were tested based on the medium and according to the method described by Shirling and Gottlieb (1966) which has been used for separation of test strains. Spore chain morphology and pigmentation characterization were performed in the medium containing inorganic salt starch agar (ISP4; International Streptomyces Project 4; Difco). The isolates examined were added to the ISP4 medium and were incubated for 14 days at 25°C. After determination of the airy spore and substrate mycelium color, red/orange and yellow/brown pigments were separated from the isolates and glycerol-asparagine was added into the agar medium (ISP5; Difco) and it was incubated for 7 days at 25°C.


Fig. 1: Region surrounding the Lake Van basin from where the soil samples were collected

pH-sensitive pigmentation analysis was performed as described previously by Shirling and Gottlieb (1966). For melanin production, peptone-yeast extract iron (ISP6; Difco) and tyrosine agar (ISP7; Difco) were added to the medium and the mixture was incubated for 7 days at 25°C the microbes showing brownish black coloring were identified as positive. The spore chains examined were categorized into four groups: flat, spindle, knot, spiral and opened spiral. Researchers analyzed the use of carbon sources such as L-cysteine, L-valine, L-rhamnose, D-melositosis, sucrosis, mannitol, adonitol, xylitol, raffinose and D-melibiose. The use of nitrogenous sources such as aminobutyric acid, L-phenylalanine, L-histidine, L-hydroxyproline, threonine and L-asparagine and degradation of substances such as phenol (0.1%), sodium azide (0.01%), crystal violet (0.0001%), xanthine (0.4%), guanine (0.05% w/v), sodium chloride (7%), arbutin, elastin, guanine, pectin, xanthine and xylan was also analyzed. Moreover, the resistance of the bacteria to antibiotics such as oleandomycin (100 μg $mL^{-1}$), penicillin G (50 μg $mL^{-1}$), rifampicin (50 μg $mL^{-1}$) and neomycin (50 μg $mL^{-1}$) was also determined.

All the test results were analyzed in TAXON Software and the identification of species was performed in the software MVSP 3.1, researchers performed the Unweighted Pair-Group Method with an Arithmetic average (UPGMA) cluster analysis of similarity dendrograms as described by Sneath (1980).

For genomic DNA isolation from Streptomyces bacteria, the method developed by Ausubel *et al*. (1994) was used with some modifications. DNA samples were visualized using a UV transilluminator, after 20 min of agarose gel electrophoresis at 80 V. The concentrations of DNA were measured at 260 nm spectrophotometrically. The bacterial genomic DNA samples were used as a template in PCR with the 27F (5'AAGGAGGTGA TCCATCC3') and R1492 (5'TACGGCTACCTTGTTA GCACTT'3) primers and *16S rDNA* gene site was amplified (on a Perkin Elmer DNA Thermal Cycler). The amplification was carried out in a 50 μL volume. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles at 95°C for 30 sec, 55°C for 90 sec and 72°C for 120 sec; final extension at 72°C for 5 min. The amplified products were examined using 1% agarose gel electrophoresis. The PCR products were purified on Nucleotrap columns and those products were incubated with Bsp143I, HaeIII, MnlI and SphI (Fermentas) enzymes at 37°C for 2 h. DNA bands were excised out and resolved further at 100 V for 20 min via horizontal electrophoresis in a 4% NuSieve agarose gel (FMC Bioproducts, Rockland, ME, USA). The bands were visualized using a Gel BIO-PRINT 0028A imaging system and the band patterns were analyzed using BioRad quantity one software and the results were presented as a dendogram using UPGMA coefficients (Kim *et al*., 1996; Rintala *et al*., 2002; Lanoot *et al*., 2005; Guo *et al*., 2008).

## RESULTS AND DISCUSSION

The pH of soil samples acquired from roots and peripheral regions of plants ranged between 7.6-8.55 indicating neutral soil type. Moisture ratios varied between 1.31 and 20.9% and most samples had low moisture content. Organic matter content had very low ratios in all soil samples and generally varied between 0.1 and 4.4%.

The quantities of Streptomyces and Actinomycetes bacteria isolated from all soil samples are summarized in Table 1. Streptomyces colonies growing on starch-casein agar and raffinose-histidine agar were isolated from the others based on the characteristic micelle and pigment patterns (Fig. 2). In soil samples collected from the root section of the malus plant, the highest count of Streptomyces bacteria was found to be $48\times10^5$ cfu $g^{-1}$ of dried soil in the starch-casein agar medium.

On starch-casein agar, the total bacterial count was found to be $80.19\times10^7$ and $70.3\times10^7$ cfu g/dried soil from the soil sample that was acquired from the root and periphery of wheat and beet, respectively. The lowest bacterial count was found to be $0.032\times10^7$ cfu g/dried soil in soil sample number 38. The highest count of Streptomyces colonies was $77.5\times10^5$ cfu g/dried soil in the soil sample collected from the root of the malus tree and cultured in raffinose-histidine agar medium. The total microorganism count for the soil sample collected from the peripheral regions of wheat was determined to be $9.62\times10^7$ cfu $g^{-1}$ dried soil.

Based on the morphological and culture characteristics, the 139 isolated Streptomyces bacteria were divided into 39 color groups. Some groups consisted of more than five test organisms and the others contained less than five. In the 1st color group, there were a total of 16 isolates while in the other groups there were fewer. Those isolates were obtained from various habitat types and in different periods when they were isolated these isolates were generally present in different groups.

Using the phenotypic and chemical test results, the identification of isolates was performed in the TAXON Software (Table 2). In order to perform the diagnosis, researchers used the characteristics described in the study by Williams *et al*. (1983b) and in the 4th volume of

Table 1: The results of the total Actinomycetes and Streptomyces bacterial counts isolated from the soil (cfu: colony-forming units)

| | Starch-casein agar (cfu $g^{-1}$) | | Raffinose-histidine agar (cfu $g^{-1}$) | |
|---|---|---|---|---|
| Isolates | Streptomyces ($\times10^5$) | Total Actinomycetes ($\times10^7$) | Streptomyces ($\times10^5$) | Total Actinomycetes ($\times10^7$) |
| AA | 3.04 | 15.700 | - | 114.485 |
| AB | 2.02 | 3.500 | - | 695.051 |
| BA | 1.01 | 3.900 | 2.148 | 305.048 |
| BB | 2.02 | 7.660 | - | 696.035 |
| DA | 1.00 | 10.490 | - | 625.393 |
| DB | 2.01 | 13.420 | - | 548.523 |
| B1B | 4.04 | 6.830 | - | 868.391 |
| B2B | 4.03 | 3.820 | - | 335.164 |
| B2A | 1.00 | 4.430 | 1.046 | 27.196 |
| B4B | 1.02 | 3.710 | - | 399.092 |
| C1 | 2.03 | 80.190 | - | 912.887 |
| C2 | 2.01 | 1.720 | 3.282 | 707.877 |
| C3 | 2.01 | 7.930 | 4.255 | 897.872 |
| C4 | 3.04 | 4.440 | 1.191 | 331.346 |
| C5 | 2.01 | 70.300 | 6.593 | 316.483 |
| C6 | 2.02 | 8.330 | 2.301 | 163.406 |
| C7 | 3.02 | 2.850 | 1.086 | 852.173 |
| C8 | 2.01 | 4.100 | 1.081 | 800.010 |
| C9 | 6.06 | 1.940 | 2.244 | 20.202 |
| C10 | 2.01 | 6.000 | 5.500 | 853.685 |
| C11 | 1.01 | 5.750 | 1.106 | 977.876 |
| C12 | 1.00 | 4.670 | 1.062 | 845.908 |
| C13 | 1.01 | 12.190 | 3.671 | 621.787 |
| C14 | 1.00 | 11.220 | - | 153.508 |
| C15 | 1.01 | 10.650 | 1.114 | 178.372 |
| C16 | 5.05 | 11.830 | 2.265 | 348.810 |
| C17 | 20.24 | 8.490 | 2.155 | 281.250 |
| C18 | - | 8.900 | - | 131.090 |
| C19 | 2.02 | 3.630 | - | 141.906 |
| C20 | 30.30 | 4.890 | 3.325 | 360.801 |
| C21 | - | 1.940 | 2.214 | 236.987 |
| C22 | 2.02 | 4.950 | - | 527.027 |
| C23 | 2.02 | 7.290 | 1.140 | 342.075 |
| C24 | 1.01 | 2.180 | 77.448 | 583.143 |
| C25 | 1.01 | 13.300 | 3.412 | 286.689 |
| D1 | 2.02 | 0.470 | 3.325 | 47.671 |
| D2 | 3.04 | 0.820 | 2.159 | 56.155 |
| D3 | 1.01 | 0.032 | 1.094 | 107.221 |
| D4 | 4.04 | 0.620 | 1.096 | 154.605 |
| D5 | 1.02 | 1.560 | 2.173 | 81.521 |

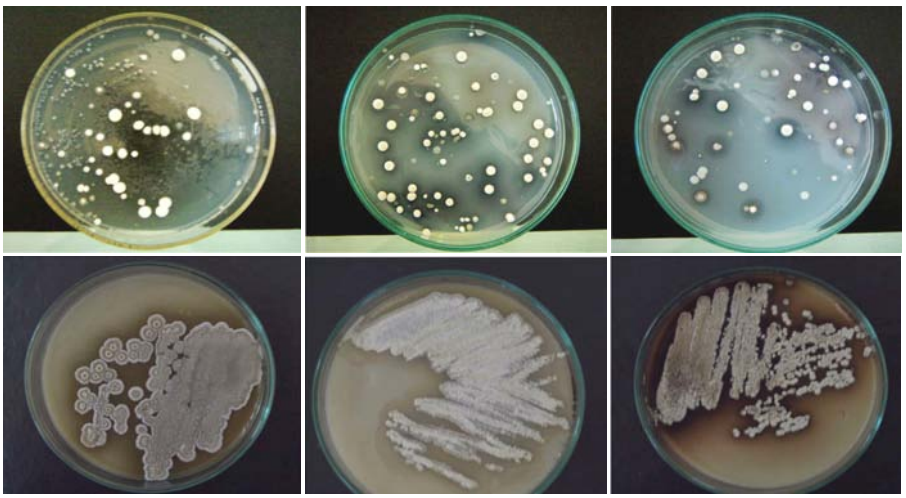

Fig. 2: Isolation and color group photos of Streptomyces

Table 2: Identification of Streptomyces isolates according to the frequency matrix

| Isolates No. | Taxa | Willcox probability | Range of taxa | Diameter of taxa | Taxa group | The most likely taxa, name of species | Result |
|---|---|---|---|---|---|---|---|
| *5131A 1A | *S. albidoflavus* | 0.997745 | 0.5715 | 0.4533 | 1A | *S. albidoflavus* | ✓ |
| *50366 42 | *S. rimosus* | 0.857452 | 0.5285 | 0.3993 | 42 | *S. rimosus* | ✓ |
| *52331 1A | *S. albidoflavus* | 0.922476 | 0.5294 | 0.3442 | 1A | *S. albidoflavus* | ✓ |
| *51291 12 | *S. rochei* | 0.883699 | 0.6047 | 0.4533 | 12 | *S. rochei* | ✓ |
| *N7363 32 | *S. violaceusniger* | 0.999918 | 0.5074 | 0.3442 | 32 | *S. violaceusniger* | ✓ |
| A2B3C | New | 0.997424 | 0.5529 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| A1A4C | New | 0.838470 | 0.6329 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| E1505 | New | 0.942642 | 0.6070 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| F1705 | New | 0.991716 | 0.4952 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| E1205 | New | 0.999790 | 0.5401 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| F1105 | New | 0.925791 | 0.5529 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| B2A5B | New | 0.984550 | 0.5226 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| F145B | New | 0.999506 | 0.5296 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| A1A3F | New | 0.975026 | 0.5226 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| C904H | New | 0.999608 | 0.5775 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| C204C | New | 0.871774 | 0.5653 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| D155C | New | 0.998395 | 0.5049 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| F1805 | New | 0.963340 | 0.5136 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| C204B | New | 0.975939 | 0.4952 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| C904I | New | 0.995725 | 0.5649 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| C804I | New | 0.995984 | 0.5503 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| C164I | New | 0.987070 | 0.5244 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| D905C | New | 0.919288 | 0.5524 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| B2B5C | New | 0.970809 | 0.5181 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| F205A | New | 0.946165 | 0.5444 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| F705A | New | 0.999676 | 0.5379 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| A2B3E | New | 0.999140 | 0.4445 | 0.4044 | 37 | *S. griseoflavus* | ✓ |
| D205D | New | 0.954224 | 0.5086 | 0.4044 | 37 | *S. griseoflavus* | ✓ |
| A2A3B | New | 0.826566 | 0.5520 | 0.4044 | 37 | *S. griseoflavus* | ✓ |
| E195A | New | 0.999849 | 0.4695 | 0.4044 | 37 | *S. griseoflavus* | ✓ |
| F405A | New | 0.995939 | 0.5504 | 0.3993 | 1C | *S. albidoflavus* | ✓ |
| F205I | New | 0.830739 | 0.5549 | 0.3993 | 1C | *S. albidoflavus* | ✓ |
| C1304 | New | 0.941998 | 0.4827 | 0.3919 | 3 | *S. atroolivaceus* | ✓ |
| D1005 | New | 0.890337 | 0.6032 | 0.3919 | 3 | *S. atroolivaceus* | ✓ |
| E3105 | New | 0.915418 | 0.5258 | 0.3919 | 3 | *S. atroolivaceus* | ✓ |
| B2B4B | New | 0.997783 | 0.5062 | 0.3919 | 3 | *S. atroolivaceus* | ✓ |
| E105B | New | 0.988081 | 0.4990 | 0.3742 | 10 | *S. fulvissimus* | ✓ |
| C204A | New | 0.957113 | 0.5138 | 0.3742 | 10 | *S. fulvissimus* | ✓ |
| D3105 | New | 0.859470 | 0.4859 | 0.3742 | 10 | *S. fulvissimus* | ✓ |
| A4B3G | New | 0.964454 | 0.4899 | 0.4485 | 5 | *S. exfoliatus* | ✓ |
| C804B | New | 0.921145 | 0.5444 | 0.4533 | 19 | *S. lavendulae* | ✓ |
| D155D | New | 0.986255 | 0.5164 | 0.3919 | 3 | *S. rochei* | ✓ |
| C204I | New | 0.741117 | 0.5138 | 0.3742 | 10 | *S. fulvissimus* | ✓ |
| D905I | New | 0.617339 | 0.5181 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| C804A | New | 0.729232 | 0.5756 | 0.4143 | 1B | *S. albidoflavus* | ✓ |
| D165B | New | 0.727986 | 0.5653 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| C904A | New | 0.566634 | 0.5378 | 0.4313 | 18 | *S. cyaneus* | ✓ |
| E805A | New | 0.792110 | 0.5322 | 0.3864 | 12 | *S. rochei* | ✓ |
| D1705 | New | 0.531830 | 0.5405 | 0.4313 | 18 | *S. cyaneus* | ✓ |
| D905D | New | 0.694308 | 0.5113 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| E1105 | New | 0.349345 | 0.5801 | 0.3839 | 29 | *S. iydicus* | ✓ |
| A1A3A | New | 0.679325 | 0.5715 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| A104B | New | 0.483349 | 0.5206 | 0.4485 | 5 | *S. exfoliatus* | ✓ |
| D905B | New | 0.664212 | 0.4923 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| F205B | New | 0.730062 | 0.5385 | 0.3993 | 1C | *S. albidoflavus* | ✓ |
| C204D | New | 0.502546 | 0.5591 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| C904B | New | 0.457328 | 0.5568 | 0.3864 | 12 | *S. rochei* | ✓ |
| C164B | New | 0.553678 | 0.4904 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| B2B3A | New | 0.486684 | 0.6218 | 0.4533 | 19 | *S. diastaticus* | ✓ |
| B2B3L | New | 0.759959 | 0.5336 | 0.3919 | 3 | *S. atroolivaceus* | ✓ |

Bergey's manual. In the case of isolates with high Willcox probability, the low taxonomic interval value, taxon diameter and the diagnosis were determined. Furthermore, the data were analyzed in the MVSP 3.1 Software and similarity dendrograms were constructed using cluster analysis (Fig. 3). The genomic DNA isolated from Streptomyces bacteria's *16S rDNA* gene region was amplified and digested with the MnlI, HaeIII, Bsp143I

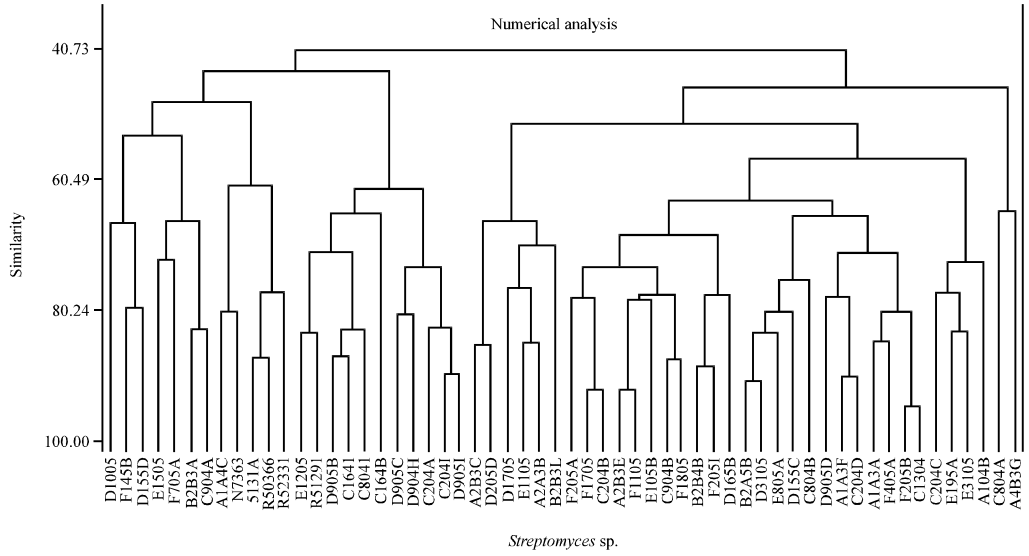


Fig. 3: The similarity dendrogram of phenotypic and chemical test results of Streptomyces bacteria. The data were analyzed in the Multi-Variate Statistical Package (MVSP) Software

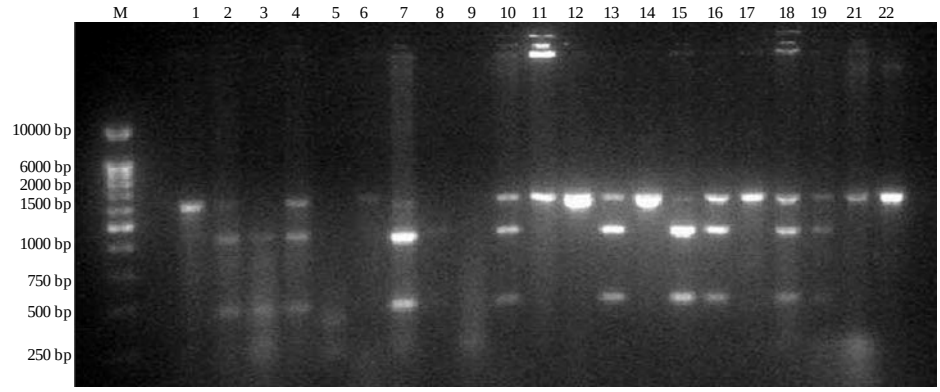


Fig. 4: Restriction Fragment Length Polymorphism (RFLP) band profiles of the *16S rDNA* gene

and SphI enzymes individually and restriction maps were generated (Fig. 4) digesting the *16S rDNA* gene region generally resulted in two or three bands. Those bands contained 1000 and 500 bp DNA fragments. The results of UPGMA analyses were presented as a dendrogram and interspecies similarity ratios and RFLP profiles were determined.

Based on the dendrogram with the ratio of 75% similarity, D3105 and E105B were in the same group whereas C145B and E805A in the second group. Similarly, A1A3F, A2B3E and C904H test organisms formed another group and the rest of the test organisms all formed only one group (Fig. 5).

In this study, the biodiversity of Streptomyces bacteria isolated from the soil samples was determined following which an RFLP profile of *16S rDNA* gene was constructed. The 139 isolated Streptomyces bacteria were divided into 39 color groups based on spore surface and melanin pigment production. Sixteen isolates formed a brown substrate mycelium and a white airy mycelium while others generally were assigned to the cream and yellow color group. The results demonstrated that various isolates exist in the soil samples collected from the roots and peripheral region of agricultural plant species.

Colony isolation was performed and the numbers of Streptomyces bacteria from different plant roots were determined. The largest count of Actinomycetes bacteria was found to be $977.876\times10^7$ cfu g/dried soil in the culture of the soil sample from root and periphery of *Phaseolus vulgaris* in raffinose-histidine agar medium. The bacterial count of *Streptomyces* sp. found to be $77.448\times10^5$ cfu g/dried soil in the soil sample taken from the root and periphery of the malus and cultured on the raffinose-histidine agar medium.

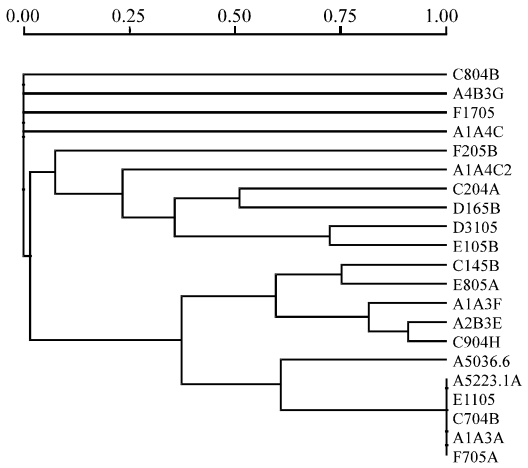


Fig. 5: An Unweighted Pair Group Method with an Arithmetic average (UPGMA) dendrogram. The data are based on Restriction Fragment Length Polymorphism (RFLP) analysis of the *16S rRNA* gene

In general, Actinomycetes and Streptomyces bacterial counts were the highest in the raffinose-histidine agar medium. Raffinose-histidine agar was found to be especially suitable for Streptomyces isolation in the numerical taxonomic studies and the data are concordant with those previous studies (Vickers *et al*., 1984). In the soil types collected from different plant roots and peripheral regions, no significant differences were detected.

Annaliesa reported that in Brazil, researchers selected soy bean from two distinct localities and isolated microbes from the soil and Streptomyces bacteria were not associated directly with a plant type and some of them synthesized the streptomycin antibiotic (Huddleston *et al*., 1997). It could be said that this plant had a negligible impact on the growth or development of Streptomyces bacteria. However, it was apparent that within various periods, small numerical differences were present.

The content ratio for the Actinomycetes count was found to be the highest in soil samples with an organic content of 3.1%. Moreover, in concordance with the study conducted by Hagedorn (1976), the results confirm that the largest numbers of microorganisms tend to live in the habitats that are rich in organic matter. In the other soil samples, the Actinomycetes bacterial count was generally found to be correlated with organic matter content. In summary, a higher bacterial count might be detected in soils with higher content of organic matter. The results suggest that the percentage of organic matter is an important environmental factor that determines the variability and development of the bacteria. In addition, it is also known that Streptomyces bacteria are isolated in defined nutrient environments formulated to isolate nonselective and other bacterial types. Indeed, Streptomyces bacteria were detected in the highest numbers in the starch-casein agar medium to which the antibiotic novobiocin was added this formulation has also been suggested for isolation of Actinomadurae bacteria (Satheeja and Jebakumar, 2011; Kim *et al*., 1998; Sveshnikova *et al*., 1976).

The isolates chosen for the final diagnosis were analyzed in the TAXON Software and it was found that five Streptomyces isolates selected as reference strains were identified accurately (Williams *et al*., 1983b). The Streptomyces major group frequency matrix showed that 80% of Streptomyces were identified accurately (Atalan, 1994, 1995). In a diagnosis study, the Willcox probability should be analyzed by considering the standard error ratio criteria. In the present study, Willcox probability over 80% and use of TAXON diameter criteria were considered significant. Nevertheless as stated above, the criteria can be partially changed.

Using frequency matrixes, Williams *et al*. (1983a) performed the diagnosis of neutrophilic Streptomyces isolates that had been isolated from different habitats (Chronakova *et al*., 2010; Goodfellow and O'Donnell, 1989; Stanton, 1984). Alkalotolerant and thermophilic Streptomyces isolates were successfully detected (Sardi *et al*., 1992). In the study, the characterization of all Streptomyces isolates was performed in the same way. In addition, the data were analyzed in the MVSP 3.1 Software and similarity dendrograms were constructed using UPGMA cluster analysis.

It is estimated that only 0.001-15% of all microorganisms present in the natural habitats have been isolated and characterized. Although, Streptomyces bacteria are generally cultured easily, selectivity of the medium is important (Goodfellow and Simpson, 1987).

Streptomyces bacteria were isolated from soil samples collected from roots and peripheral regions of plants after their identification for phenotypic characterization, the results should be supported by genetic, serological and chemotaxonomic results. For this purpose, after amplification of the 16S rDNA region with appropriate primers from Streptomyces test isolates, RFLP analysis was performed. A major part of the *16S rDNA* gene is conserved but some parts are variable and RFLP is effective in the diagnosis of the species within Streptomyces (Metsa-Ketela *et al*., 2002).

The use of macromolecules in the phylogenetic analysis has generated substantial amounts of reliable data for characterization of identified bacteria. It is known that rDNA (5S, 16S and 23S) is helpful, particularly for

demonstrating the phylogenetic relationships based on the rRNA of bacteria. Because in the structure of 16S rRNA is highly conserved, the results obtained are reliable for determining the phylogenetic relationships among species. Although, it has been established that the similarity between *16S rRNA* genes is <97% (Stackebrandt and Embley, 2000; Stackebrandt and Goebel, 1994; Beffa *et al*., 1996), researchers report a technique for RFLP analysis that produces the similar results.

## CONCLUSION

In the study, based on a RFLP analysis some strains were found to be present in the isolated soil samples in different groups but others are detected only in the one group. Based upon the similarity ratio of the dendrogram constructed as a result of the RFLP analysis, four multi-member groups (two or more members) and six 1-member groups were compiled.

## ACKNOWLEDGEMENT

This research was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) Grant Number TBAG-2344(103T156).

## REFERENCES

Adams, P.B., 1990. The potential of mycoparasites for biological control of plant diseases. Ann. Rev. Phytopathol., 28: 59-72.

Atalan, E., 1993. Selective isolation of actinomycetes species from soil samples. J. Sci., 4: 21-29.

Atalan, E., 1994. The identification of *Streptomyces* isolated from environmental soil samples with hyphal mycelium inoculum on computer assisted identification. J. Environ. Sci. Health Part A: Environ. Sci. Eng., 29: 2127-2138.

Atalan, E., 1995. Identification of *Streptomyces* isolated from environmental soil samples using rapid enzyme data. J. Environ. Sci. Health Part A: Environ. Sci. Eng. Toxicol., 30: 1133-1143.

Atalan, E., G.P. Manfio, A.C. Ward, R.M. Kroppenstedt and M. Goodfellow, 2000. Biosystematic studies on novel *Streptomycetes* from soil. Ann. Rev. Microbiol., 77: 337-353.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl, 1994. Current Protocols in Molecular Biology. John Wiley and Sons, New York.

Beffa, T., M. Blanc, P.F. Lyon, G. Vogt, M. Marchiani, J.L. Fischer and M. Aragno, 1996. Isolation of Thermus strains from hot composts (60-80°C). Applied Environ. Microbiol., 62: 1723-1727.

Beyazova, M. and M.P. Lechevalier, 1993. Taxonomic utility of restriction endonuclease fingerprinting of large DNA fragments from *Streptomyces* strains. Int. J. Syst. Evol. Microbiol., 43: 674-682.

Chronakova, A., V. Kristufek, M. Tichy and D. Elhottova, 2010. Biodiversity of streptomycetes isolated from a succession sequence at a post-mining site and their evidence in Miocene lacustrine sediment. Microbiol. Res., 165: 594-608.

Clarke, S.D., D.A. Ritchie and S.T. Williams, 1993. Ribosomal DNA restriction fragment analysis of some closely related *Streptomyces* species. Syst. Applied Microbiol., 16: 256-260.

Crameri, R., G. Hintermann and R. Hutter, 1983. Deoxyribonucleic acid restriction endonuclease fingerprint characterization of actinomycete strains. Int. J. Syst. Bacteriol., 33: 652-655.

Da Rosa, J.P., E. Korenblum, M.N. Franco-Cirigliano, F. Abreu and U. Lins *et al*., 2013. *Streptomyces lunalinharesii* strain 235 shows the potential to inhibit bacteria involved in biocorrosion processes. BioMed. Res. Int. 10.1155/2013/309769.

Demain, A.L., 1999. Pharmaceutically active secondary metabolites of microorganisms. Applied Microbiol. Biotechnol., 52: 455-463.

Doering-Saad, C., P. Kampfer, S. Manulis, G. Kritzman and J. Schneider *et al*., 1992. Diversity among Streptomyces strains causing potato scab. Applied Environ. Microbiol., 58: 3932-3940.

Egan, S., P. Wiener, D. Kallifidas and E.M.H. Wellington, 1998. Transfer of streptomycin biosynthesis gene clusters within *Streptomycetes* isolated from soil. Applied Environ. Microbial., 64: 5061-5063.

Goodfellow, M. and A.G. O'Donnell, 1989. Search and Discovery of Industrially Significant Actinomycetes. In: Microbial Products: New Approaches, Baumberg, S., M. Rhodes and I. Hunter (Eds.). Cambridge University Press, London, pp: 343-383.

Goodfellow, M. and K.E. Simpson, 1987. Ecology of *Streptomyces*. Front Applied Microbiol., 2: 97-125.

Guo, Y., W. Zheng, X. Rong and Y. Huang, 2008. A multilocus phylogeny of the Streptomyces griseus 16S rRNA gene clade: Use of multilocus sequence analysis for streptomycete systematics. Int. J. Syst. Evol. Microbiol., 58: 149-159.

Hagedorn, C., 1976. Influences of soil acidity on *Streptomyces* populations inhabiting forest soils. Applied Environ. Microbiol., 32: 368-375.

Huddleston, A.S., N. Cresswell, M.C. Neves, J.E. Beringer, S. Baumberg, D.I. Thomas and E.M. Wellington, 1997. Molecular detection of streptomycin-producing streptomycetes in Brazilian soils. Applied Environ. Microbiol., 63: 1288-1297.

Jones, K.L., 1949. Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic. J. Bacteriol., 57: 141-145.

Jussila, J., J. Pelkonen, V.M. Kosma, J. Maki-Paakkanen, H. Komulainen and M.R. Hirvonen, 2003. Systemic immunoresponses in mice after repeated exposure of lungs to spores of *Streptomyces californicus*. Clin. Vaccine Immun., 10: 30-37.

Kim, B., N. Sahin, D.E. Minnikin, J. Zakrzewska-Czerwinska, M. Mordarski and M. Goodfellow, 1999. Classification of thermophilic *Streptomycetes* including the description of *Streptomyces thermoalcalitolerans* sp. nov. Int. J. Syst. Bacteriol., 49: 7-17.

Kim, B.S., J.Y. Lee and B.K. Hwang, 1998. Diversity of actinomycetes antagonistic to plant pathogenic fungi in cave and sea-mud soils of Korea. J. Microbiol., 36: 86-92.

Kim, D., J. Chun, N. Sahin, Y.C. Hah and M. Goodfellow, 1996. Analysis of thermophilic clades within the genus *Streptomyces* by 16S ribosomal DNA sequence comparisons. Int. J. Syst. Bacteriol., 46: 581-587.

Kuster, E., 1959. Outline of a comparative study of criteria used in characterization of the actinomycetes. Int. Bull. Bacteriol. Nomencl. Taxonomy, 9: 97-104.

Kutzner, K.J., 1986. The Family *Streptomycetaceae*. In: The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria, Starr, M.P., H. Stolp, H.G. Truper, A. Balows and H.G. Schlegel (Eds.). Springer-Verlag, New York, USA., pp: 2028-2090.

Labeda, D.P. and A.J. Lyons, 1991. The *Streptomyces violaceusniger* cluster is heterogenous in DNA relatedness among strains: Emendation of the descriptions of *S. violaceusniger* and *Streptomyces hygroscopicus*. Int. J. Syst. Bacteriol., 41: 398-401.

Labeda, D.P., 1992. DNA-DNA hybridization in the systematics of *Streptomyces*. Gene, 115: 249-253.

Lanoot, B., M. Vancanneyt, B. Hoste, K. Vandemeulebroecke and M.C. Cnockaert *et al*., 2005. Grouping of streptomycetes using 16S-ITS RFLP fingerprinting. Res. Microbiol., 156: 755-762.

Leiminger, J., M. Frank, C. Wenk, G. Poschenrieder, A. Kellermann and A. Schwarzfischer, 2013. Distribution and characterization of *Streptomyces* species causing potato common scab in Germany. Plant Path., 62: 611-623.

Metsa-Ketela, M., L. Halo, E. Munukka, J. Hakala, P. Mantsala and K. Ylihonko, 2002. Molecular evolution of aromatic polyketides and comparative sequence analysis of polyketide ketosynthase and 16S ribosomal *DNA* genes from various *Streptomyces* species. Applied Environ. Microbiol., 68: 4472-4479.

Mishra, S.K., R.E. Gordon and D.A. Barnett, 1980. Identification of nocardiae and Streptomycetes of medical importance. J. Clin. Microbiol., 11: 728-736.

Omura, S., H. Ikeda, J. Ishikawa, A. Hanamoto and C. Takahashi *et al*., 2001. Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deducing the ability of producing secondary metabolites. Proc. Natl. Acad. Sci., 98: 12215-12220.

Pissowotzki, K., K. Mansouri and W. Piepersberg, 1991. Genetics of streptomycin production in *Streptomyces griseus*: Molecular structure and putative function of genes *strELMB2N*. Mol. General Genet., 231: 113-123.

Reed, J.F. and R.W. Cummings, 1945. Soil reaction-glass electrode and colorimetric methods for determining pH values of soils. Soil Sci., 59: 97-104.

Rintala, H., A. Nevalainen and M. Suutari, 2002. Diversity of streptomycetes in water-damaged building materials based on 16S rDNA sequences. Lett. Applied Microbiol., 34: 439-443.

Saintpierre, D., H. Amir, R. Pineau, L. Sembiring and M. Goodfellow, 2003. *Streptomyces yatensis* sp. nov., a novel bioactive streptomycete isolated from a New-Caledonian ultramafic soil. Antonie van Leeuwenhoek, 83: 21-26.

Samson, R.A., B. Flannigan, M.E. Flannigan, A.P. Verhoeff, O.C.G. Adan and E.S. Hoekstra, 1994. Recommendations. In: Health Implications of Fungi in Indoor Environments: Air Quality Monographs, Samson, R.A., B. Flannigan, M.E. Flannigan, A.P. Verhoeff, O.C.G. Adan and E.S. Hoekstra (Eds.)., Vol. 2. Elsevier, Amsterdam, pp: 529-538.

Sardi, P., M. Saracchi, S. Quaroni, B. Petrolini, G.E. Borgonovi and S. Merli, 1992. Isolation of endophytic *Streptomyces* from surface-sterilized roots. Applied Environ. Microbiol., 58: 2691-2693.

Satheeja, S.V. and S.R.D. Jebakumar, 2011. Phylogenetic analysis and antimicrobial activities of *Streptomyces* isolates from mangrove sediment. J Basic Microbiol., 51: 71-79.

Sembiring, L., A.C. Ward and M. Goodfellow, 2000. Selective isolation and characterisation of members of the *Streptomyces violaceusniger* clade associated with the roots of *Paraserianthes falcataria*. Antonie Van Leeuwenhoek, 78: 353-366.

Shirling, E.B. and D. Gottlieb, 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Evol. Microbiol., 16: 313-340.

Sneath, P.H.A., 1980. Basic program for the most diagnostic properties of groups from an identification matrix of percent positive characters. Comput. Geosci., 6: 21-26.

Stackebrandt, E. and B.M. Goebel, 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in-bacteriology. Int. J. Syst. Bacteriol., 44: 846-849.

Stackebrandt, E. and T.M. Embley, 2000. Diversity of Uncultured Microorganisms in the Environment. In: Nonculturable Microorganisms in the Environment, Colwell, R.R. and D.J. Grimes (Eds.). ASM Press, Washington, DC., pp: 57-75.

Stanton, L.J., 1984. Actinomycetes associated with freshwater habitats. Ph.D. Thesis, Newcastle Upon Tyne: Newcastle University.

Steingrube, V.A., R.W. Wilson, B.A. Brown, K.C. Jost Jr., Z. Blacklock, J.L. Gibson and R.J. Wallace, 1997. Rapid identification of clinically significant species and taxa of aerobic actinomycetes including *Actinomadura*, *Gordona*, *Nocardia*, *Rhodococcus*, *Streptomyces* and *Tsukamurella* isolates by DNA amplification and restriction endonuclease analysis. J. Clin. Microbiol., 35: 817-822.

Sveshnikova, M.A., N.T. Chormonova, N.V. Lavrova, L.P. Terekhova and T.P. Preobrazhenskaia, 1976. Isolation of soil actinomycetes on selective media with novobiocin. Antibiotiki, 21: 784-787.

Trejo-Estrada, S.R., A. Paszczynski and D.L. Crawford, 1998. Antibiotics and enzymes produced by the biocontrol agent *Streptomyces violaceusniger* YCED-9. J. Ind. Microbiol. Biotechnol., 21: 81-90.

Upton, M., 1994. Ecological approaches to selective isolation of actinomyctes for bioactivity screening. Ph.D. Thesis, Newcastle University, Newcastle Upon Tyne, England.

Vickers, J.C., S.T. Williams and G.W. Ross, 1984. A Taxonomic Approach to Selective Isolation of *Streptomyces*. In: Biological, Biochemical and Biomedical Aspects of Actinomycetes, Ortiz-Ortiz, L., L.F. Bojalil and V. Yakoleff (Eds.). Academic Press, Orlando, pp: 553-561.

Wang, Y., Z.S. Zhang, J.S. Ruan, Y.M. Wang and S.M. Ali, 1999. Investigation of actinomycete diversity in the tropical rainforests of Singapore. J. Ind. Microbiol. Biotechnol., 23: 178-187.

Wellington, E.M.H. and S.T. Williams, 1978. Preservation of actinomycete inoculum in frozen glycerol. Microbios Lett., 6: 151-157.

Williams, S.T., M. Goodfellow and G. Alderson, 1989. Genus *Streptomyces* Waksman and Henrici 1943. In: Bergey's Manual of Systematic Bacteriology, Williams, S.T., M.E. Sharpe, J.G. Holt (Eds.). Springer-Verlag, New York, USA., pp: 2452-2492.

Williams, S.T., M. Goodfellow and J.C. Vickers, 1984. New Microbes from Old Habitats? In: The Microbes 1984: II: Prokaryotes and Eukaryotes, Kelly, D.P. and N.G. Carr (Eds.). Cambridge University Press, Cambridge, UK., ISBN-13: 978-0521260572, pp: 219-256.

Williams, S.T., M. Goodfellow, G. Alderson, E.M.H. Wellington, P.H.A. Sneath and M.J. Sackin, 1983a. Numerical classification of *Streptomyces* and related genera. J. Gen. Microbiol., 129: 1743-1813.

Williams, S.T., M. Goodfellow, E.M.H. Wellington, J.C. Vickers and G. Alderson *et al*., 1983b. A probability matrix for identification of some *Streptomycetes*. J. Gen. Microbiol., 129: 1815-1830.

Xu, L., Q. Li and C. Jiang, 1996. Diversity of soil actinomycetes in Yunnan, China. Applied Environ. Microbiol., 62: 244-248.