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Research article/Araştırma makalesi

API-ZYM and numerical analysis of 16S rRNA gene ıdendified *micromonospora* ısolates from the Black Sea region

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Abstract

In this study, molecular and numerical methods were applied to identify possible novel *Micromonospora* isolates. The isolation study of different actinomycetes obtained from the Black Sea Region soil samples were carried out by modifying different isolation techniques. Genomic DNA of bacteria is obtained by using DNA isolation kit. PCR-mediated amplification of the 16S rRNA gene region of test isolates were performed by using 27F and 1525R universal primers. Almost complete 16S rRNA gene sequences of the isolates were determined using automatic sequencer with 800R, MG3F and MG5F primers. Phylogenetic trees based on 16S rRNA gene were constructed with the neighbour-joining, maximum likelihood and maximum parsimony algorithms by using MEGA6 software. In this study, we obtained one hundred fifty-one isolates in total, and it was determined that these microorganims belong to different groups of actinomycetes. Eleven possible novel strains which belong to the genus *Micromonospora* isolates were subjected to numerical and biochemical analysis. Putative novel *Micromonospora* soil isolates are expected to contribute to taxonomy of actinomycetes and also many novel species are sure to emerge.

Key words: actinomycetes, *Micromonospora*, 16S rRNA gene sequencing, numeric taxonomy

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Karadeniz bölgesinden izole edilen 16S rRNA gen bölgesi ile tanımlanan *Micromonospora* izolatlarının API-ZYM ve nümerik analizleri

Özet

Çalışmamızda yeni tür olması muhtemel *Micromonospora* izolatlarına moleküler ve nümerik metodlar uygulanmıştır. Karadeniz bölgesinden alınan toprak örneklerinden izole edilen farklı aktinomisetlerin izolasyon çalışması farklı izolasyon teknikleri kullanılarak gerçekleştirilmiştir. Bakterilerin genomic DNA'sı DNA izolasyon kiti kullanılarak elde edilmiştir. Test izolatlarına ait 16S rRNA gen bölgesi çoğaltımı 27F ve 1525R primerleri ile gerçekleştirilmiştir. İzolatlara ait gen dizileri 800R, MG3F ve MG5F evrensel primerleri ile otomatik dizileme cihazı kullanılarak belirlenmiştir. 16S rRNA gen bölgesi temelli filogenetik ağaçlar MEGA6 programında neighbour-joining, maximum likelihood ve maximum parsimony algorithmaları kullanılarak oluşturulmuştur. Bu çalışmada, toplamda yüz elli bir aktinomiset izolatı elde edilmiş ve bunların farklı aktinomiset gruplarına dahil oldukları belirlenmiştir. Bu izolatlardan *Micromonospora* cinsine ait olan yeni tür olması muhtemel 11 tanesine nümerik ve biyokimyasal testler uygulanmıştır. Yeni olduğu varsayılan *Micromonospora* toprak izolatlarının aktinomiset taksonomisine katkı sağlayacağı ve aynı zamanda birçok yeni türün ortaya çıkacağı düşünülmektedir.

Anahtar kelimeler: aktinomiset, *Micromonospora*, 16S rRNA gen sekansı, nümerik taksonomi

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1. Introduction

Actinomycetaceae are a group of gram-positive bacteria which have high G+C content, branching, and fragmenting filaments without aerial hyphae, and spores commonly found in nature, especially in soil (Slack and Gerencser, 1976).

Members of *Micromonospora* genus produce many useful bioactive compounds, such as antibiotics, anti-cancer agents and immunosuppressant agents (Wanbanjob, 2008; Hassan and Wellington, 2009).

The genus *Micromonospora* has three types of sporulating structures which are single spores, spore chains, and sporangia. Spores may be nonmotile or motile with tufts of polar flagella. This genus is aerobic, non-acid fast and mesophilic microorganisms. Many of them produce carotenoid mycelial pigments, giving the colonies an orange to red appearance; furthermore blue-green, brown or purple pigments are also produced (Carro et al., 2012).

Members of the genus *Micromonospora* found in soil, lake water and insects in the intestines. In addition, this genus has been isolated from leaves and nitrogen-fixing root nodules such as the actinorhizal plants *Casuarina equisetifolia* and *Coriaria myrtifolia* and root nodules of the leguminous plants *Lupinus angustifolius* and *Pisum sativum* (Hirsch and Valdés, 2010).

Since the middle of 2008, the genomes of three *Micromonospora* have been sequenced and are nowadays being annotated. Several more *micromonosporas* are also in the pipeline for the genome sequencing. *Micromonospora* species are known as antibiotics productor, particularly aminoglycoside, enediyne and oligosaccharide antibiotics. As a result, their impact on medicine is notable, and *Streptomyces* and *Micromonospora* species produce many of the best-known antibiotics. For example; the aminoglycoside, gentamicin and netamicin antibiotics are mainly obtained from *Micromonospora* species (Bérdy, 2005).

In this study, we aimed to identify *Micromonospora* strains isolated from Black Sea Region soil using molecular and numerical methods.

2. Materials and methods

2.1. Selection of soil samples and isolation of microorganisms

For this study, soil samples obtained from seven different localities of the Eastern Black Sea Region was put in sterile container sand sterile plastic bags (Figure 1). Collected soil samples were labelled by laboratory number and stored at 4° C. Each soil sample, which weighed 20-25g was added to100 ml beaker. After sufficient amount of distilled water was added and samples were held for 24h, pH was determined with pH meter for each soil sample. Values are also shown in Table 1.

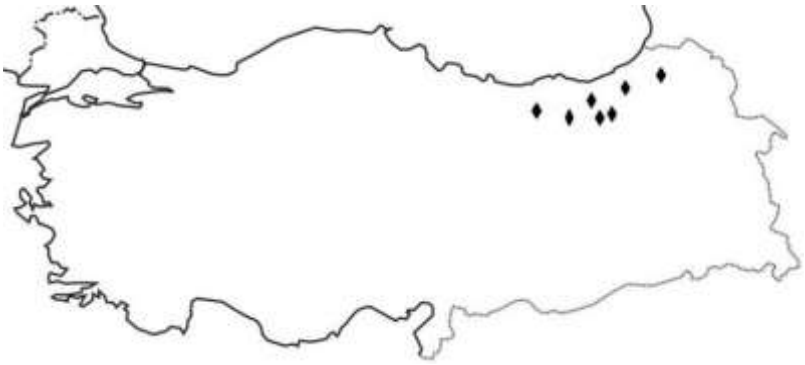

Figure 1: The sampling in the the Black Sea region in Turkey

Sucrose gradient method was applied as a selective isolation method with utilization of 20% solution of sucrose. The solution was prepared in a screw cap centrifuge tube (105 mm) and 1 ml of spore suspensions of the test actinomycete strains prepared by conventional technique was added to the solution (Yamamura et al., 2003).

Aliquots (200 µl) of this diluted suspensions were inoculated on agar plates containing antibiotics with filter sterilised cycloheximide (50 µg ml^{-1}), nalidixic acid (10 µg ml^{-1}) and rifampicin (0,5 µg ml^{-1}), (Abbas, 2006). Humic acid-vitamin (HV, Hayakawa and Nonomura, 1987), tryptone-yeast glucose extract (TYG, Blackall et al., 1989), TYG with vitamin agar, glucose-yeast extract agar (GYEA, Gordon and Mihm, 1962), GYM with vitamin agar and oatmeal agar (Küster, 1959), which were used as selective mediums to collect desired rare actinomycetes. Plates incubated at 28 °C for 14-21 days.

Morphological characteristics such as colony morphology, aerial spore mass colour, substrate mycelial pigmentation and the colour of diffusible pigments of microorganisms which were sub-cultured on glucose yeast extract agar, glucose yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3) and tryptone-yeast extract agar incubated at 28°C for 10 days were observed by light microscopy (Zeiss Axio Lab A1) (Hayakawa, 2008). Spore suspensions and mycelial fragments of the isolates were preserved in 20% glycerol (v/v) at –20 °C until required.

Table 1. Sources and strain histories of the test microorganism sand pH of soil samples

Number	Source of soil	Location	pH
1	Plateau soil	Plateau soil, Meşeli, Şavşat, Artvin	6.3
2	Plateau soil	Plateau soil, Aydıntepe, Bayburt	6.4
3	Plateau soil	Plateau soil, Kop dağı, Bayburt	6.5
4	Plateau soil	Plateau soil, Durundas, Çamoluk, Giresun	6.4
5	Plateau soil	Plateau soil, Çevrepınarı, Şiran, Gümüşhane	6.3
6	Plateau soil	Plateau soil, Şemsahat, Çayeli, Rize	6.5
7	Plateau soil	Plateau soil, Kaskar, Maçka, Trabzon	6.2

2.2. Culture conditions and DNA extraction

Test strains, stored as glycerol suspensions (20%, v/v) at -20° C, were maintained on glucose-yeast extract agar (GYEA, Gordon and Mihm, 1962) slopes. Biomass of microorganisms was grown on modified tryptone-yeast glucose extract broth (TYG, Blackall et al., 1989); these cultures were incubated for 6 to 8 days at 28°C for DNA extraction. Chromosomal DNA was isolated by using PureLink® Genomic DNA İzolation Kit (Invitrogen, USA).

2.3. 16S rDNA sequence analysis

The 16S rRNA genes (rDNA) were amplified by using universal primers 27f (5'-AGA GTT TGA TCM TGG CTC AG-3'; positions 8 to 27; (Lane, 1991)) and 1525r (5'-AAG GAG GTG WTC CAR CC-3'; (Lane 1991)). Each PCR reaction mixture (50 µl) was prepared as described by Chun and Goodfellow (1995). The DNA thermal cycler (PCR Express, ThermoHybaid, Middlesex, UK) used for thermal amplification was programmed as follows in Table 2.

The nearly full-length sequences of the 16S rRNA gene were analysed to assess the identities of the strains isolated. PCR product of DNA (QIAquick purification kits, Qiagen, Hilden, Germany) sequencing of selected isolates was performed by ABI PRISM Big Dye Terminator Cycle Sequencing kits (Macrogen, Netherland) (Table 2). The results obtained from 16S rRNA gene sequences (1.325-1.435 nucleotides) were compared with GenBank/EMBL/DDBJ database using BLAST program (http://www.ncbi.nlm.nih.gov/) and relative phylogenetic positions of the isolates were determined. Phylogenetic analysis was conducted by utilization of MEGA 6.0 (Tamura et al., 2013) in order to generate a complete alignment of 16S rRNA gene sequences of the isolates and type strains of all valid species.

Table 2. Oligonucleotide primers used in the PCR amplification and sequencing of 16S rRNA.

Target	Usage	Primer name (position)[b]	Forward primer (5`to 3`)[a]	Source	PCR cycling conditions
16S	PCR	27f (bp 8-27)	AGAGTTTGATCTGGCTCAG	Lane, 1991	5 min 95°C; 1 min 95°C; 2 min 55°C; 3 min 72°C; 10min 72°C
		1525r (bp 1544-1525)	AAGGAGGTGWTCCARCC	Lane, 1991	
	Seq	MG3f (bp 520-536)	CAGCAGCCGCGGTAATAC	Kagayema et al., 2004	
		MG5f (bp 907-926)	AAACTCAAAGGAATTGACGG	Chun, 1995	
		800r (bp 800-782)	TACCAGGGTATCTAATCC	Chun, 1995	
		1115r (bp 1115-1131)	AGGGTTGCGCTCGTTG	Gyobu and Miyadoh, 2001	
		1492r (bp 1492-1474)	TACGGYTACCTTGTTACGACT	Gyobu and Miyadoh, 2001	

[a] Degeneracies according to Lane (1991): Y=C:T; R=A:G; W=A:T.

[b] Binding site on the 16S rRNA molecule: Numbering according to *Escherichia coli* numbering system (Brosius et al., 1978).

2.4. Phylogenetic analysis

The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012). Phylogenetic analysis was conducted using MEGA 6.0 (Tamura et al., 2013) by generating a complete alignment of 16S rRNA gene

sequences of the isolates and type strains of all valid species. A phylogenetic tree was constructed with neighbour-joining tree (Jukes and Cantor, 1969), maximum parsimony (Kluge and Farris 1969) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA 6.0 (Tamura et al., 2013). Evolutionary distances were calculated using the model of Jukes and Cantor (1969). The resultant tree topology was evaluated by a bootstrap analysis (Felsenstein, 1985) with 1000 resamplings from the neighbour-joining dataset using MEGA 6.0. Only nodes with bootstrap values over 50% were considered to be significant.

2.5. Numerical analysis

Each of the selected *Micromonospora* isolate was tested for 70 unit characters, namely cultural, morphological, pigmentation, physiological, nutritional, and biochemical. The characteristics of the isolates were determined according to the methods described by Shirling and Gottlieb (1966). Eleven duplicated test strains were also used for the reproducibility of the results.

2.6. API ZYM

API test strips consists of microtubes (cupules) containing dehydrated substrates to detect the enzymatic activity or the assimilation / fermentation of sugars by the inoculated test microorganisms. The API ZYM system is a semiquantitative micromethod consisting of 20 microcupules, 19 of which contain dehydrated chromogenic substrates for detecting 19 preformed enzyme activities. The test strips are inoculated and incubated aerobically at 37°C for 4 h, and then two reagents are added to develop the chromogenic substrates. The resultant colorimetric reactions are indicative of the degree of enzyme activity and are graded on a scale of 0 to 5 in comparison with the control well and a color chart (Stoyanovski et al., 2013).

3. Results

In this study, sucrose gradient method and selective media have been used for *Actinomycetes* isolation from Balck Sea region plateau soil samples. One hundred fifty-one *Actinobacteria* were isolated after incubation at 28 °C for 14-21 days on HV, TYGA with and without vitamin agars supplemented with nalidixic acid, rifampicin and cycloheximide.

Organisms used in this study were cultivated TYGA and oatmeal agar. All the organisms were identified as formation of orange and black spores which were observed at TYGE. BY707, BY300 and BY700 isolates were observed to show more intensive development than the other eight isolates. BY300 and BY368 colony morphology have also a spiral structure. ART34, BY707, BY700, BY368 and GS150 isolates formed dark brown spores on oatmeal agar. Spore formation was not observed in other isolates on oatmeal agar, and colony morphology of all isolates were Spherical-Puffy. All comparisons were carried out using the reference type strains and National Bureau of Standards (NBS) Colour Name Charts (Kelly, 1964) were also used for determining colour designation and names. Cultural and growth characteristics of the test strains are shown in Table 3.

Indication of different pigmentation and substrate mycelium colour of eleven *Micromonospora* colonies were selected to study further moleculer techniques. All of the strains were maintained on glucose-yeast extract agar (GYEA; Gordon and Mihm, 1962) at 28 °C and stored as glycerol suspensions (20%, v/v) at -20 °C for future work. Genomic DNA of chosen microorganisms is obtained by DNA isolation kit. The 16S ribosomal RNA gene was amplified by using the PCR method with Taq DNA polymerase and primers forward and reverses. During 16S rRNA gene sequencing, 800R, MG3F, MG5F, 1115R and 1492R primers were also used to get a nearly complete sequence data of test strains.

16S rRNA gene sequence data was determined for eleven test strains according to Blast analysis. Phylogenetic analysis was conducted by utilization of 1410 nucleotide base pair. The results obtained from 16S rRNA sequencing were compared against 16S rRNA gene sequences of tree representatives of closely related type strains of *Micromonospora* (Figure 3). ART42, ART47, BY300, BY700 and BY351 have differences less than 10 nucleotide differences to the nearest type strain. BY707 and BY298 are similar, 98.1-98%, to the closest type of species and have a 23-24 nucleotide differences. BY368, GR19 and GS162 are similar 99% to the nearest the type strain of *M. chokoriensis* and have the 12-10-12 nucleotide differences, respectively. Most of the isolates are closely related to *M. endolithica* and *M. chokoriensis.* Obtained results of nucleotide similarities (%) and differences based on 16S rRNA gene sequences data are given in Table 4.

Table 3. Growth and cultural characteristics of the test microorganisms in different culture medium

	Microorganism Number	**1.**	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	
Medium	**Isolate no**	**ART34**	**ART47**	**BY707**	**BY300**	**BY700**	**BY351**	**BY368**	**Gl**
TYGA	**Growth**	++	++	+++	+++	+++	++	++	+
	Cultural characteristics								
	Spore colour	Black	Black	Black	Black	Black	Black	Black	Bl
	Substrate mycelium colour	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Ora
	Colony morphology	Spherical - Puffy	Spherical - Puffy	Spherical - Puffy	Spherical	Spherical - Puffy	Spherical - Puffy	Spherical	Sphe Pu
OATMEAL AGAR	**Growth**	+	+	+	+	+	+	+	
	Cultural characteristics								
	Spore colour	Dark brown	None	Dark brown	None	None	None	Dark brown	D bro
	Substrate mycelium colour	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Ora
	Colony morphology	Spherical - Puffy	Spherical - Puffy	Spherical - Puffy	Spherical - Puffy	Spherical - Puffy	Spherical - Puffy	Spherical - Puffy	Sphe Pu

Key: +++, abundant growth; ++, moderate growth; +, poor growth; -, no growth

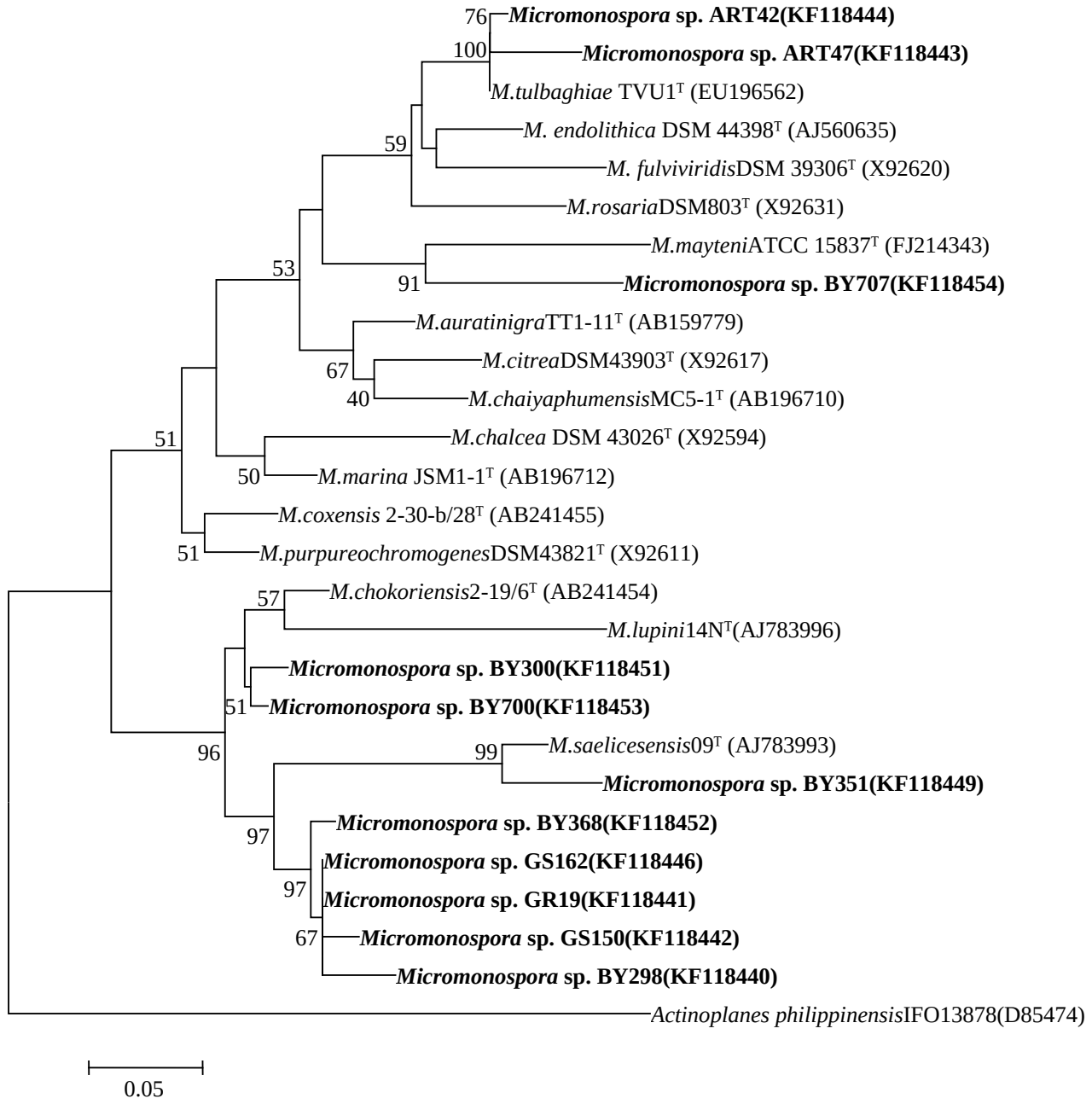

Figure 3. Neighbour-joining tree (Lane, 1991) based on the16S rRNA gene sequences (1410 bp length) showing the phylogentic relationships between test soil isolates and closely related type strains of the genus *Micromonospora*. Numbers on branch nodes are bootstrap values (1000 resamplings; only values over 50% are given). Bar 0.005 substitutions per nucleotide position.

Eleven *Micromonospora* isolates from plateau soil identified in 16S rRNA gene were analyzed for 70 unit characters using numerical methods. All test microorganisms for some numerical tests gave positive and negative results; positive results in L-Arabinose, Suctose, D-Fructose, L-Proline, L-Serine, L-Methionine, L-Ile, Tween 20 and negative results in D-Trehalose, Palatinose, Indol, MR, Citrate, Nitrate reduction, Gelatine and Adenine. In nutritional tests, Paatinose (%1, w/v) and D-Trehalose (%1, w/v) are negative (-) for all isolates of the carbon source, but D-raffinose (%1, w/v), D – Mannose (%1, w/v), L – Arabinose (%1, w/v), Sucrose(%1, w/v) and D – Fructose (%1, w/v) gave positive (+) results in all isolates. In growth on nitrogen sources, Copper II sulfate is negative for all isolates, additionally except L-Valine, L-lle, L-Histidine, Potassium nitrate 0.005 gr and Copper II sulfategave positive (+) results for all isolates. Urea, one of the biochemical tests gave negative (-) results for isolates BY351, GR19 and the test are positive (+) for the rest of isolates. Indol, MR, Citrate, Nitrate reduction and Hydrogen sulphite production tests are negative (-) for all of isolates. In physiological tests pH 9 and 28°C is positive for all isolates, but 40°C, 50°C and 55°C are negative (-). Gelatin, Adenine, Tyrosine and Xanthine was not degraded by all organisms, but Tween 20 degraded by all organisms (Table 5).

Table 4. Nucleotide similarities (%) and differences based on the 16S rRNA sequences of the test soil isolates and closely related valid strains of *Micromonospora*.

	Isolate no:	Accessione number	Closest to two types of species	Percent similarity	Nucleotide differences	Source
1.	ART42	**KF118444**	*M. endolithica,* *M. tulbaghiae*	%**99.2** %**99.9**	10 1	**This study**
2.	ART47	**KF118443**	*M. endolithica,* *M. tulbaghiae*	%**98.8** %**99.6**	14 5	**This study**
3.	BY707	**KF118454**	*M. endolithica,* *M. mayteni*	%**98.0** %**98.1**	25 23	Isik et al., 2014
4.	BY300	**KF118451**	*M. purpureochromogenes,* *M. chokoriensis*	%**98.6** %**99.4**	17 7	Isik et al., 2014
5.	BY700	**KF118453**	*M. purpureochromogenes,* *M. chokoriensis*	%**98.7** %**99.6**	16 4	Isik et al., 2014
6.	BY351	**KF118449**	*M. chokoriensis,* *M. saelicesensis*	%**97.7** %**99.0**	28 8	Isik et al., 2014
7.	BY368	**KF118452**	*M. purpureochromogenes,* *M. chokoriensis*	%**98.4** %**99.0**	22 12	Isik et al., 2014
8.	GR19	**KF118441**	*M. purpureochromogenes,* *M. chokoriensis*	%**98.4** %**99.0**	22 10	**This study**
9.	GS150	**KF118442**	*M. coxensis,* *M. chokoriensis*	%**98.1,** %**98.8**	23 14	**This study**
10.	GS162	**KF118446**	*M. purpureochromogenes,* *M. chokoriensis*	%**98.4** %**99.0**	22 12	**This study**
11.	BY298	**KF118440**	*M. saelicesensis,* *M. purpureochromogenes*	%**97.9** %**98.0**	26 24	Isik et al., 2014

Within the framework of these data, error rate was calculated for each test isolate. 1.1% test error was detected in the final data matrix. Positive and negative results of all strains were excluded from these calculations. In the statistics program of Statistic PASW Data Editor 22.0, used to Ward's method and dissimilarity matrix was obtained using Squared Euclidiean Distance of test isolates.When assessing some new typeof API-ZYM application test data, after adding liquids using the appropriate media tools, the kits were exposed to strong light for 30 seconds for the better activity observation **(**Figure 4). Obtained API ZYM data showed that the type of organism and some test isolates gave positive results to Lipase (C 14), Trypsin and Chymotryp, but BY700, GR19 and GS150 were negative for them. At the same time, Acid phosphatase, β-Galactosidase and α-GlucosidaseAPI-ZYM tests have been revealed as positive results for all test microorganisms. BY700 and GR19 gave pozitif results to α-Galactosidase, β-Galactosidase, β-Glucuronidase, α-Glucosidase, β-Glucosidase, N-acetyl-β-glucosaminidase and α-Mannosidase. All obtained API ZYM results are shown in the Table 6.

Figure 4. Samples of API ZYM test a) *M. chokoriensis,* b) BY298, c) BY352

Table 5. Results of numerical analysis applied to the test microorganisms

Isolate number	1	2	3	4	5	6	7	8	9
A. NUTRITIONAL TESTS									
Growth on carbon sources									
(+) control D-Glikoz									
D-Mannitole	+	+	+	-	-	-	+	-	+
D-Sorbitole	-	-	-	-	-	-	-	+	-
D-Galactose	-	+	-	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	-	-
D – Mannose	+	+	+	+	+	+	+	+	+
D-Raffinose	-	-	+	-	+	-	+	+	+
D-Trehalose	-	-	-	-	-	-	-	-	-
meso – Inositol	-	-	-	-	-	-	-	-	+
Alfa L-rhamnoz	+	-	+	-	-	-	+	-	-
D – Xylose	+	+	+	+	+	+	+	+	-
Inulin	-	-	-	-	-	-	-	+	+
L – Arabinose	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+
D – Lactose	+	+	+	-	+	+	+	+	+
D – Fructose	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	-	+	+	+	+	+
Palatinose	-	-	-	-	-	-	-	-	-
Growth on nitrogen sources									
N(+) control									
L – Arginine									
L-Proline	+	+	+	+	+	+	+	+	+
L-Serine	+	+	+	+	+	+	+	+	+
L-Valine	-	+	-	-	-	-	-	+	-
L-Methionine	+	+	+	+	+	+	+	+	+
L-Ile	+	+	+	+	+	+	+	+	-
L-Histidine	+	+	-	-	+	+	-	+	+
L-Alanine	+	+	+	+	+	+	+	+	+
L - Hydroxyproline	+	+	+	+	+	+	+	+	+
L-Triptofane	+	+	+	+	+	+	+	+	+
L-Glisine	+	+	+	+	+	+	+	+	+
Growth in the presence of:									
Potassium nitrate 0.001gr	+	+	+	+	+	+	+	+	+
Potassium nitrate 0.005gr	+	-	+	+	+	+	+	+	+
Zinc chloride	+	+	+	+	+	+	+	+	+
Zinc sulfate	+	+	+	+	+	+	+	+	+
Iron II sulfate 0.01gr	+	+	+	+	+	+	+	+	+
Iron II sulfate 0.05gr	+	+	+	+	+	+	+	+	+
Copper II sulfate	-	-	-	-	-	+	-	-	-

Table5. (contiuned)

Isolate number	1	2	3	4	5	6	7	8	9
B.BIOCHEMICAL TESTS									
Indol	-	-	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-	-	-
Allantoin	-	+	-	-	-	-	-	-	-
VP	-	-	-	-	+	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-
Urea hydrolysis	+	+	+	+	+	-	+	-	+
Nitrate reduction	-	-	-	-	-	-	-	-	-
Hydrogen sulphite production	-	-	-	-	-	-	-	-	-
C.PHYSIOLOGICAL TESTS									
pH 4	+	+	+	-	+	+	-	-	-
pH 9	+	+	+	+	+	+	+	+	+
pH 10	-	-	-	-	-	-	-	-	-
4°C	-	-	+	-	+	-	-	-	+
28°C	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+
40°C	-	-	-	-	-	-	-	-	-
50°C	-	-	-	-	-	-	-	-	-
55°C	-	-	-	-	-	-	-	-	-
%7 NaCI	-	-	-	-	-	-	-	-	-
%14 NaCI	-	-	-	-	-	-	-	-	-
D.DEGRADATION TESTS									
Gelatin	-	-	-	-	-	-	-	-	-
DNA	-	+	-	-	-	-	-	-	-
Starch	-	-	+	-	-	-	-	-	-
Adenine	-	-	-	-	-	-	-	-	-
Tyrosine	-	-	-	-	-	-	-	-	-
Xylan	+	+	-	+	+	+	+	+	+
Xanthine	-	-	-	-	-	-	-	-	-
Hypoxanthine	-	-	-	-	-	-	-	-	-
Aesculin	+	+	+	+	+	+	+	-	+
Arbutin	+	+	+	+	+	-	+	+	+
Guanine	+	-	-	-	-	-	-	+	+
Tween 20	+	+	+	+	+	+	+	+	+
Tween 40	+	-	+	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+	-	+	+

Table 6. API ZYM data to the test microorganisms and the closest type strain of *Micromonospora*

Test No	Enzyme	1	2	3	4	5	6	7	8	9	10	11	12	13
1.	Alkaline phosphatase	−	+	−	+	−	−	+	−	+	−	−	−	−
2.	Esterase (C4)	+	−	−	−	+	−	−	−	+	+	−	−	−
3.	Esterase lipase (C8)	−	+	−	+	−	+	−	−	−	+	−	+	+
4.	Lipase (C14)	−	+	−	+	−	−	+	−	−	−	−	+	+
5.	Leucine arylamidase	+	−	+	−	+	+	+	+	+	+	+	+	+
6.	Valine arylamidase	+	−	−	−	+	−	+	−	+	+	+	−	−
7.	Cystine arylamidase	+	−	−	+	+	−	+	−	+	+	+	−	−
8.	Trypsin	+	+	−	+	−	−	+	−	−	+	+	+	+
9.	Chymotrypsin	+	+	−	+	−	+	+	−	−	+	+	+	+
10.	Acid phosphatase	+	+	+	+	+	+	+	+	+	+	+	+	+
11.	Naphthol-AS-BI-phosphohydrolase	+	−	+	−	+	+	+	+	+	+	+	+	+
12.	α-Galactosidase	−	−	+	+	−	−	−	+	−	−	+	−	−
13.	β-Galactosidase	+	+	+	+	+	+	+	+	+	+	+	+	+
14.	β-Glucuronidase	−	−	+	−	−	−	−	+	−	−	−	−	−
15.	α-Glucosidase	+	+	+	+	+	+	+	+	+	+	+	+	+
16.	β-Glucosidase	+	+	+	+	+	+	−	+	+	+	+	+	+
17.	N-acetyl-β-glucosaminidase	−	+	+	+	−	+	−	+	+	−	+	+	+
18.	α-Mannosidase	−	−	+	−	−	+	−	+	−	+	−	+	+
19.	**α-Fucosidase**	−	−	−	−	−	−	−	−	−	−	−	−	−

Key: +, positive; -, negative

*****1:ART42, 2:ART47, 3:BY707, 4:BY300, 5:BY700, 6:BY351, 7:BY368, 8:GR19, 9:GS150, 10:GS162, 11:BY298, 12:*M. endolithica*, 13:*M. chokoriensis*

4. Conclusions and discussion

Actinomycetes have been used to synthesize many active secondary metabolites such as cosmetics, vitamins, nutritional materials, herbicides, antibiotics, pesticides, anti-parasitic and enzymes. They are free and saprophytic bacteria and these microorganisms are also major source for the production of different antibiotics.

In agreement with the wide distribution of Micromonosporaceae strains in nature, samples from various habitats such as soil, rhizosphere soil, sediment, mud, water, plant material, and invertebrates are used as good sources of inoculum. Although we have learned a great deal about Micromonospora, we need more studies to classify the different ecological habitats and distinguishing characteristic genotypes of the species that locate in these environments. These microorganisms play an important role in soil ecology, biodegradation, biocontrol and plant growth promotion. Usage possibilities of Micromonospora sp. as a biocontrol agent and biofuels was determined by Hirsch and Valdés (2010). In a study conducted by de Menezes et al., 2008 identification of Micromonospora sp. and cellulose degradation levels were also determined. Micromonospora genus is important as a significant source of secondary metabolites for biomedicine, especially Micromonospora species produce a lot of antibiotics, such as the aminoglycoside antibiotics (gentamicin and netamicin), anti-tumor antibiotics (lomaiviticins A and B,tetrocarcin A, LL-E33288 complex, etc.) and anthracycline antibiotics. Novel actinomycete species also have potential to produce new bioactive secondary metabolites. Therefore, many research projects undertaken in this field continue identifying many new types and screening of secondary metabolites.

Micromonospora sediminis as a novel actinomycete was idendified and isolated from mangrove sediment sampled from Thailand (Phongsopitanun et al., 2016). Micromonospora jinlongensis grown on humic acid-vitamin agar (HV) (Hayakawa and Nonomura 1987), was isolated from muddy soil collected from a stream of Jinlong Mountain in Harbin, using the standard dilution plate method (Gao et al., 2014). Micromonospora *maritima* was isolated from mangrove soil from Thailand by using starch casein nitrate agar (Tanasupawat et al., 2010) supplemented with nystatin and nalidixic acid and incubated at 30 °C for 14 days.

In this study we had a total of 151 strains of *Actinobacteria* which were isolated from 7 different soil samples collected from different locations of northeast Turkey. This research are presented heterogeneous environments for actinobacterial biodiversity. The methods used for isolation of actinomycete strains were similar to earlier studies (Yamamura et al., 2003), but this method were modified by using different medium and antibiotic (Ozdemir Kocak et al., 2014; Ozdemir Kocak and Isik., 2015). According to the results of 16S rRNA gene analysis, 11 isolates were identified as *Micromonospora*. Numerical analysis and APYZYM tests were also carried out to check their biochemical and nutritional sources using capacity related to two *Micromonospora* type strains (Table 5 and 6).The focus of this study *Micromonospora* isolates based on 16S rRNA gene sequencing analysis, and *Micromonospora* sp. ART42 and ART47 showed close relatedness to type strains of *M. endolithica* having 99,2 % and

98,8% nucleotide similarities and 10 and 14 nucleotide differences, respectively. *Micromonospora* sp. GR19 and GS162 shared same nucleotide similarities and differences (98,4% and 22 nt) whereas *Micromonospora* sp. GS150 showed relatedness to type strains of *M. coxensis* having 98,1% nucleotide similarities and 23 nucleotide differences. Acccording to up-to-date literature that the 16S rRNA genes sequence threshold value between 98.2 and 99.0% appears reasonable and can be used for the present species denifinition of bacteriology (Meier-Kolthoff et al., 2013)**.**

Althouh members of the *Micromonospora* genus can be distinguished from strains classified in other genera of the family Micromonosporaceae using morphological and 16S rRNA gene region analysis, for a novel *Micromonospora* species is necessary for *gyrB* multilocus sequence analyses, DNA-DNA homology analysis and chemotaxonomical analysis. So, deposit of the sequence data of the strains to Genbank identified as likely to be new species, DNA-DNA homology analysis, in addition to *gyrB* gene, *atpD, recA, rpoB* multilocus sequence analyses, whole cell assays, analyses of fatty acids and menaquinons, spore morphology analysis are being planned to be done in near future to add to microbiota literature from northeast Turkish plateu soil.

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