JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2014. Vol. **8**(5), p. 3781-3788

Selective Isolation and Molecular Identification of Different Actinomycetes from Various Habitats

Fadime Ozdemir Kocak[1]*, Talha Gencbay[2] and Kamil Isik[2]*

[1]Department of Nursing, School of Health, Bilecik Seyh Edebali University, Bilecik,Turkey.
[2]Department of Biology, Faculty of Science and Arts, Ondokuz Mayis University, Samsun, Turkey.

(Received: 29 September 2013; accepted: 11 December 2013)

Isolation of actinomycetes is still valuable from different habitats because these organisms are vital groups for clinical, ecological and agricultural applications. This study was aimed to identify the possible novel actinomycetes that are potentially secondary metabolite producers, and to gain novel species for literature. In this study, different soil samples were used for isolation of different actinomycete strains, and totally 2169 isolates were obtained with selective and conventional isolation methods. The isolates are selected according to the results of chemotaxonomy tests and various morphological characteristics for further molecular tests. The PCR-based molecular method, which is the commonly used in identification of bacterial isolates and in determination of phylogenetic relationships, was used for obtaining 16S rRNA gene sequences. A total of 107 isolates, 41 of which contain mycolic acids, were analysed in terms of 16S rRNA gene sequences. Taxonomic positions of actinomycete isolates were determined by analysing 16S rRNA data sets. Accession numbers from NCBIand culture collection numbers from the culture collections (DSMZ and KCTC) were provided for the possible novel actinomycete isolates of this study.

Key words: Selective isolation, actinomycete diversity, 16S rRNA gene sequencing.

Actinomycetes are widely distributed in terrestrial and aquatic ecosystems, especially in soil, where they play a crucial role in the recycling of refractory biomaterials by decomposition and humus formation[1]. They are also of industrial importance for the formation of a wide variety of secondary metabolites, which notably potent antibiotics. Furthermore, various different lifestyles are encountered among *Actinobacteria*, and the phylum includes pathogens (e.g., *Mycobacterium* spp., *Nocardia* spp., *Tropherymas*pp., *Corynebacterium* spp., and *Propionibacterium* spp)., soil inhabitants (*Streptomyces* spp.), plant commensals (*Leifsonia*spp.), nitrogen-fixing symbionts (*Frankia*), and gastrointestinal tract (GIT) inhabitants (*Bifidobacterium* spp.). Therefore, the isolation and subsequent characterization of pure cultures of these organisms from natural habitats are important in order to understand their ecological role, to assess health hazards that they may present, and to identify useful strains that produce novel bioactive metabolites[2].

The genus *Streptomyces*, of which members are the most dominant actinomycete species in soil samples, is considered one of the most important genera of actinomycetes because of its capacity for natural bioactive compound production. However, the number of reports revealing new bioactive metabolites from the streptomycetes has reduced. In order to isolate uncommon actinomycete species other than *Streptomyces* spp. and to use untapped potential of these microorganisms, different isolation strategies are required. On the other hand, different

* To whom all correspondence should be addressed.
Tel.: +90 228 214 1388; Fax: +90 228 214 1017;
E mail: fadime.ozdemirkocak@bilecik.edu.tr;

actinomycetes have usually been regarded as strains of actinomycetes whose isolation frequency by conventional methods is much lower than that of streptomycete strains[3]. When conventional isolation techniques were applied, most of the isolates recovered on agar plates have been identified as genus *Streptomyces* because of the selective agar media were primarily designed to isolate *Streptomyces* spp.[4]. Hayakawa and Nonomura formulated a new selective agar medium named humic acid-vitamin (HV) agar. This new medium containing soil humic acid as the sole carbon and nitrogen source[5-6] supports sporulation of differentactinomycetes[7].

Several methods have been developed to facilitate efficient isolation of different actinomycetes. One of them is pretreatment of soil by drying and heating to reduce the number of nonfilamentous bacteria. On the other hand, the aerial spores of most actinomycete genera were found to resist desiccation and show a slightly higher resistance to wet or dry heat.An alternative pretreatment procedure is required to add chemicals such as 1.5% phenol to the soil suspensions. Chemical pretreatment procedure is more effective than drying and heating when used in combination with selective agar medium [humic acid–vitamin (HV) agar]supplemented with appropriate antibiotics, such as chlortetracycline[2].

In this study, we compared the efficiency of sucrose gradient centrifugation and conventional methods for selective isolation of different actinomycete genera such as *Micromonospora, Kribbella, Actinomadura* and *Nocardioides* etc. The isolates were characterized by analysing 16S rRNA gene sequences.

MATERIALS AND METHODS

Collection of samples

Seventeen soil samples were collected from tree different country France (Gap), Russia (Moscow) and Turkey (Artvin, Balikesir, Bayburt, Giresun, Gümüþhane, Kütahya, Rize, Trabzon). Soil types and habitats have been shown in Table 1. A clean sampling scoop was used to take about 500 g samples from 5- to 20-cm depths (A or A1-horizon of the soil) and the foam sample, about 200 ml was taken from dam water. A portion of the soil samples was passed through a 2 mm mesh sieve, air-dried at room temperature for 14 days and triturated by using a sterile pestle. These samples were subsequently used for actinomycete isolation.

Isolation of organisms from soil samples

Seventeen soil samples were processed by using two different methods described below.

In conventional method, one gram of soil was suspended in 9 ml sterile strength Ringer's solution (Merck, Darmstadt, Germany) and shaken for 30 min on a tumble shaker. The suspensions were then treated in a pre-warmed water bath at 60 °C for 20 min. These 10^{-1} dilutions were serially diluted down to 10^{-4}. From each dilution, 0.2 ml was taken and spread evenly over the plates by using swab stick and incubated at 28 °C for 14-21 days. Selective isolation media used in this study are as follows: HV agar [humic acid–vitamin agar (5)], GYME agar [glucose-yeast extract-malt extract agar with $CaCO_3$, ISP 2(8)], GYME with vitamin agar, TYG agar [tryptone-yeast glucose extract (9)], TYG with vitamin agar plates supplemented with filter sterilised cycloheximide (50 µg ml^{-1}), nalidixic acid (10 µg ml^{-1}) and rifampicin (0,5 µg ml^{-1}). Plated dilutions that gave 20–200 colonies were chosen for further isolation. Single colonies were successively transferred on to tryptone yeast glucose (TYG) agar and incubated until pure isolates were obtained.

In sucrose gradient centrifugation method, 20% solution of sucrose that was prepared in a screw cap centrifuge tube (105 mm) was added 1 ml of purified spore suspensions of the test actinomycete strains prepared by using conventional technique and the tube was centrifuged (room temperature, 30 min, 240 g) in a swinging bucket rotor. After centrifugation, each sucrose layer (1 ml) was transferred sequentially from the top of the gradient using a different sterile pipette for each layer, and then diluted in a 10-fold series in sterile Ringer's solution (Merck, Darmstadt, Germany) (2, 10).

Aliquots (200 µl) of this diluted suspensions were plated in triplicate on the surface of five solid media, i.e. HV agar, GYME (ISP 2), GYM with vitamin agar, TYG agar, TYG with vitamin agar plates supplemented with filter sterilised cycloheximide (50 µg ml^{-1}), nalidixic acid (10 µg ml^{-1}) and rifampicin (0,5 µg ml^{-1}), and incubated at 28 °C for 14-21 days. As a control, purified spore suspensions were directly diluted

with sterile Ringer's solution and plated in the same way. Experiments were performed in triplicate in order to obtain mean colony counts.

Isolates putatively assigned to the different actinomycetes on the basis of colony morphology, notably aerial spore mass colour, substrate mycelial pigmentation and the colour of diffusible pigments, 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 and incubated at 28°C for 10 days. Observed morphological characteristics were compared with those of type strains by light microscopy under described conditions[7].

Spore suspensions and mycelial fragments of the isolates were preserved in 20% glycerol (v/v) at – 20 °C until required.

Identification of isolates

Chemotaxonomy

Pure cultures were inoculated on tryptone-yeast extract agar plates, on which sterile filter was placed, and incubated at 28°C for 10 days. Wet biomass of strains was degraded by acid methanolysis and hexane extracts were examined for mycolic acid[11, 12].

DNA extraction, sequencing, and analysis

Selected 107 isolates were subjected to 16S rRNA gene sequence analysis for precise genus and species identification. The identities of the organisms were determined based on partial or nearly full length 16S rRNA gene sequence analysis. The genomic DNA of each isolate was extracted using the modified guanidine thiocyanate DNA extraction procedure (13). The 16S rRNA genes (rDNA) were amplified by using universal primers 27f [5'-AGA GTT TGA TCM TGG CTC AG-3'; (14)] and 1525r [5'-AAG GAG GTG WTC CAR CC-3';(14)]. Each 50 µl PCR mixture contained primers (each at a concentration of 20 ìM, Invitrogen), a mixture of deoxynucleoside triphosphates (Promega) (each at a concentration of 25 mM),*Taq* polymerase buffer (HotStarTaq®, QIAGEN),chromosomal DNA (50-300 ng) and *Taq* polymerase (2.5 U, HotStarTaq®, QIAGEN). PCR amplifications were performed in the DNA thermal cycler (PCR Express, ThermoHybaid, Middlesex, UK) under following conditions: an initial denaturation step at 95°C for 5 min; 35 cycles at 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min; and a final extension step at 72°C for 10 min. The PCR products were electrophoresed on 1% agarose gel (Amresco, Solon, Ohio) in 1×TBE buffer, stained with ethidium bromide and were visualized with the Gene Genius Bio imaging system.

16S rRNA sequence analysis

In order to assess the identities of isolated strains, nearly full-length sequences of the 16S rRNA genes were analysed. The DNA sequencing of the selected isolates was performed by Macrogen, Korea, using ABI 3730 XL DNA sequencer. The resulting 16S rRNA gene sequences (1,227-1,515 nucleotides) were used to search the GenBank database with the BLASTN program to determine relative phylogenetic positions. Phylogenetic analysis was conducted using MEGA5[15] by first generating a complete alignment of 16S rRNA gene sequences of the isolates and type strains of all valid species. The 16S rRNA sequences obtained in the present study were manually aligned with the published sequences of the validly described species available from the GenBank / EMBM/ DDBJ databases. A phylogenetic tree was inferred using neighbour-joining tree algorithms[16]. 16S rRNA datasets were cooperatively analysed using MEGA5 and PHYLIP[17], which was used to calculate evolutionary distances and similarity values. Topography of the constructed tree was evaluated by bootstrap analysis with 1000 replicates[17]. Only nodes with bootstrap values over 50% were considered to be significant.

Nucleotide sequence accession numbers

The 16S rRNA sequences which we determined in this study have been deposited in the NCBI (GenBank) database under the accession numbers listed in Table 2.

RESULTS AND DISCUSSION

Selective isolation of different actinomycetes from soil

By conventional method, prepared dilutions were inoculated over the surface of five solid media and incubated at 28 °C for 14-21 days (Fig. 1 a-c).

Dilutions of decontaminated soil suspensions containing several actinomycetes strains were centrifuged through layers of 20% sucrose at 240 x g for 30 min. After centrifugation, the number and type of actinomycetes present in

sucrose layer was determined by the plate culture technique using HV agar, TYG, TYG with vitamin, ISP2 and ISP2 with vitamin agar with the antifungal antibiotic cycloheximide (Fig. 1 d-f). Use of the layer of 20% sucrose was effective in separation of different actinomycetes. However, *Streptomyces* spp., which constitute the major group of actinomycetes, were also recovered from the agar plates. To reduce the number of colonies in isolation plates belonging to these most-abundant soil actinomycetes, we sought to use sucrose-gradient centrifugation method in combination with selective agar media for less-abundant actinomycete groups. Use of 20% sucrose solution and HV agar with cycloheximide resulted in a significant decrease in the number of *Streptomyces* spp. and also suppressed nonfilamentous bacteria on the plate (Fig. 1. d-k).

A total of 2169 actinomycete isolates were recovered from all plates prepared by either conventional or sucrose-gradient centrifugation method. Of the all isolates, 1536 recovered from

Table 1. Soil types and habitats used in actinomycete isolation

Code	Type of Soil	Habitat
1	Soil of forest	Ozdemir-Koçak, F., Moscow (Yuga Zapadnaya, southwest park forest)
2	Soil of field	Ozdemir-Koçak, F., Gap-Veynes Town-Saint Aubant
3	Soil of Pinus rizosfere	Ozdemir-Koçak, F., Kütahya-Simav-gölcük crater lake
4	Soil of forest	Ozdemir-Koçak, F., Kütahya-Simav-gölcük crater lake
5	Soil of border lake	Ozdemir-Koçak, F., Kütahya-Simav- gölcük crater lake
6	Soil of Oak rizosfere	Ozdemir-Koçak, F., Kütahya-Simav- gölcük crater lake
7	Soil of swamp (soil of trough water)	Ozdemir-Koçak, F., Kütahya-Simav- gölcük crater lake
8	Flora of foam	Ozdemir-Koçak, F., Balikesir-Sindirgi dam lake
9	Hardpan of border lake	Ozdemir-Koçak, F., Balikesir-Sindirgi dam lake
10	Soil of forest	Ozdemir-Koçak, F., Balikesir-Sindirgi dam lake
11	Plateau soil	Gençbay, T., Artvin- Meseli, Savsat
12	Plateau soil	Gençbay, T., Bayburt-Aydintepe
13	Plateau soil	Gençbay, T., Bayburt- Kop dagi
14	Plateau soil	Gençbay, T., Giresun- Durundas, Çamoluk
15	Plateau soil	Gençbay, T., Gümüþhane- Çevrepinari, Siran
16	Plateau soil	Gençbay, T., Rize- Semsahat, Çayeli
17	Plateau soil	Gençbay, T., Trabzon- Kaskar, Maçka

Table 2. Accession numbers of representative different actinomycetes isolated from various soil samples

Isolate Number	Organism	Accession number	Isolate No	Organism	Accession number
ART34	*Actinomadura sp.*	KF118447	ART64	*Actinomadura sp.*	KF118448
GR10	*Kribbella sp.*	KC767543	BY909	*Kribbella sp.*	KF118456
BY298	*Micromonospora sp.*	KF118450	BY803	*Microbacterium*sp.	KF118455
BY368	*Micromonospora sp.*	KF118452	BY707	*Micromonospora sp.*	KF118454
BY351	*Micromonospora sp.*	KF118449	BY300	*Micromonospora sp.*	KF118451
FSN13	*Nocardia sp.*	KC993082	BY700	*Micromonospora sp.*	KF118453
FSN14	*Nocardia sp.*	KC993083	NEA44	*Nocardia sp.*	KC993089
FSN34	*Nocardia sp.*	KC993084	FMN06	*Nocardia sp.*	JN896620
FSN35	*Nocardia sp.*	KC993081	FMN15	*Nocardia sp.*	JN896621
FSN37	*Nocardia sp.*	KC993085	FGN17	*Nocardia sp.*	KC993086
GR130	*Nocardia sp.*	KF118457	FGN19	*Nocardia sp.*	KC993087
GR14	*Nocardia sp.*	KC767546	FGN39	*Nocardia sp.*	KC993079
FGN46	*Nocardia sp.*	KC993088	FGN43	*Nocardia sp.*	KC993080

TYG and ISP2 agar media plated by conventional method while 226 were recovered from TYG, TYG with vitamin, ISP2 and ISP2 with vitamin agar media plated and 407 were from HV agar by selective isolation method. A total of 479 isolates were found to be non-streptomycete actinomycetes recovered from all media inoculated with samples processed by conventional dilution plate technique or sucrose-gradient centrifugation technique.

Selected 214 non-streptomycete isolates were degraded by acid methanolysis and hexane extracts were examined for mycolic acids by TLC as described by Minnikin et al.[11, 12]. Consistent with the literature, obtained Rf values (0.40 - 0.53) of 41 isolates confirmed that 39 isolates belong to *Nocardia* and 2 belong to *Gordonia*. Mycolic acid containing isolates were amplified by PCR and identified on genus level.

PCR amplification of different actinomycete isolates estimated to be non-streptomycetes performed by using universal primers 27f and 1525r generated a fragment of ~1500 bp long for each isolate. Sequences of these isolates were aligned with those of other known different actinomycetes genera from the GenBank / EMBM/ DDBJ(Table 2) and phylogenetic tree was constructed on the basis of distance and neighbour-joininganalyses(Fig. 2).

All representative isolates (different actinomycete-like isolates (non-streptomyces) together with mycolic acid containing ones) were characterized by 16S rRNA sequence analysis to establish their phylogenetic positions. For the construction of phylogenetic tree, eight genera to which 16 isolates belong were used. Test organisms were supported by the highest bootstrap value according to phylogenetic analysis (Fig. 2).

The actinomycetes isolated in this study displayed considerable diversity.They were distributed among six subordos *Streptomycineae* (*Streptomyces* two strains), *Micromonosporineae* (*Micromonospora* three strains), *Micrococcineae* (*Microbacterium* one strain), *Streptosporangineae* (*Actinomadura* two strains), *Propionibacterineae* (*Kribbella* one strains), *Corynebacterineae* (*Gordonia* two strainsand *Nocardia* five strains)and —within the class *Actinobacteria* on the phylogenetic tree including more than 16 species (Fig. 2).

Soil isolates *Actinomadura* sp. ART34 and ART64 share 99.2% and 99.6% of 16S rRNA gene sequence similarity with the type strains of

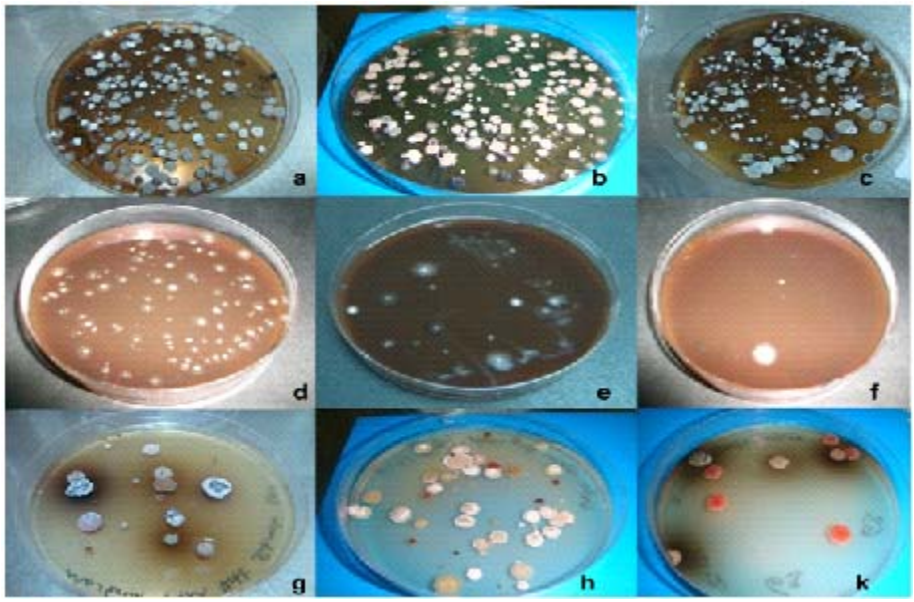

Fig. 1. Isolation plates HV agar (d,e,f), TYG Agar (a,b,c) and ISP2 (g,h,k) of different genera of actinomycetes. Conventional plates (TYG agar) of dominant streptomycetes and different genera of actinomycetes(a) 10^{-2}, b) 10^{-2} and c) 10^{-3} plate respectively). Selective isolation plates HV agar (d) 10^{-2}, e) 10^{-3}, and f)10^{-4} plate respectively). Selective isolation plates TYG Agar, TYG Agar with vitamin and ISP2 Agar with vitamin (g) 10^{-3}, h) 10^{-2}, and k) 10^{-4} plate respectively).

A. citrea and *A. citrea*, values corresponding to 5 and 10nt differences, respectively. An isolate of *Microbacterium* sp. BY 803 shares 99.8% 16S rRNA gene sequence similarity with the type strain of *M. paraoxydans*, value corresponding to 2 nt difference.

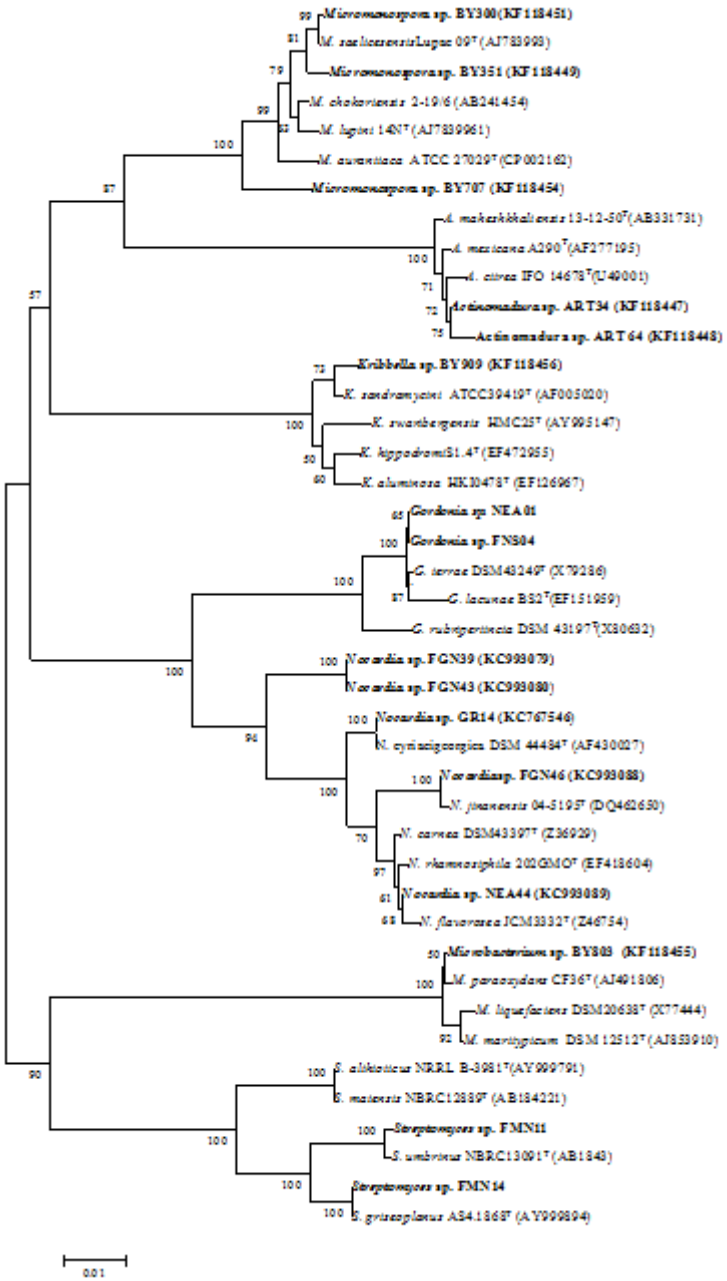

Fig. 2. Neighbour-joining tree (14) based on nearly complete 16S rRNA gene sequences (1313bp length) showing relationships between test isolates and closely related type strains of the genera *Actinomadura*, *Gordonia*, *Kribbella*, *Micromonospora*, *Microbacterium*, *Nocardia*, *Nocardioides*, *Streptomyces*. Numbers on branch nodes are bootstrap values (1000 resamplings; only values over 50% are given). Bar 0.01 substitutions per nucleotide position. GenBank accession numbers are also given in parenthesis

Nocardia sp. FGN39 and FGN43 isolates were shared 99.5% 16S rRNA gene sequence similarity with the type strain of *N. takedensis*,values corresponding to 7 nt differences. *Nocardia* sp. FSN14, FSN13, GR14 isolates share 16S rRNA gene sequence similarity with the type strain of *N. abscessus* with the ratios of 100, 99.8, 98.9%,values corresponding to 0, 2 and 12 nt differences, respectively. *Nocardia* sp. FGN46 isolate share 16S rRNA gene sequence similarity with the type strain of *N. jinanensis* with the ratios of100%, while NEA44 isolate share 99.7% similarity with the type strain of *N. rhamnosiphila,* values corresponding to 3nt differences, respectively. An isolate of *Gordonia* sp. NEA01 and *Gordonia* sp. FSN04 share 99.8% 16S rRNA gene sequence similarity with the type strain of *Gordonia terrae,* value corresponding to 2 nt difference.

Kribbella sp. BY909 shares 99.4% and 99.0% of 16S rRNA gene sequence similarity with the type strains of *K. sandramycini* and *K.hippodromi,* values corresponding to 7 and 12nt differences, respectively.

Soil isolates *Micromonospora* sp. BY707, BY300 and BY351 showed close sequence similarity with the type strain of *M. chokoriensis* with the ratios of 97.6, 99.3 and 98.9%, values corresponding to 31, 8 and 14nt differences, respectively. *Streptomyces* sp. FMN11 and FMN14 share 99.6% of 16S rRNA gene sequence similarity with *S. umbrinus* and *S. griseoplanus*.

According to 16S rRNA sequence analysis, it was determined that of 107 isolates, 39 belong to *Nocardia*, 2 *Gordonia*, 1 *Nocardioides*, 7 *Kribbella*, 23 *Micromonospora*, 5 *Actinomadura*, 1 *Promicromonospora*, 2 *Nonomuraeae*, 1 *Microbacterium* and 26 *Streptomyces*. Isolates sharing 97.2 -100% gene sequence similarity with closely related type strains are phylogenetically presented in fig. 2.

One of the important results of the study is pathogenic strains isolated from different localities in Turkey. FSN13, FSN14, FSN34 and FSN37 isolated from Sindirgi dam lake were found to be closely related to pathogenic *Nocardia abcessus* while GR14 and GR130 isolated from Giresun (Turkey) have similarity with *N.cyriacigeorgica*. In addition, soil isolates NEA01 and FSN04 were found to be related to clinically important strain *G. terrae*.

The twenty of 107 isolates that taxonomic positions were determined by analysing 16S rRNA which are prosperous novel species and accession numbers from NCBI and culture collection numbers from the culture collections (DSMZ and KCTC) were also provided for the possible novel nineactinomycete isolates including 2 *Kribbella*, 1*Micromonospora*, 2 *Nonomuraea*, 1 *Promicromonospora*, 1 *Nocardioides*, 2 *Nocardia*.

DISCUSSION

For the selective isolation of different actinomycetes, various methods such as cesium chloride density gradient ultracentrifugation[18], modified paraffin bait method[19] and sucrose-gradient centrifugation method have been developed. Sucrose-gradient centrifugation method followed by use of selective medium that HV, TYG and ISP 2 added vitamine is proven as an effective method to recover non-streptomycete actinomycetes. Compared to conventional dilution plate technique, sucrose-gradient centrifugation method is highly effective in isolation of different actinomycete strains. In addition, sucrose-gradient centrifugation method is determined to useful for eliminating undesired species while favouring isolation of different actinomycetes from soil[2,7].

This study determined that 39 isolates belong to *Nocardia* and 2 belong to *Gordonia*. Mycolic acid containing *Nocardia* and *Gordonia* isolates were identified on genus level by 16S rRNA gene sequence analysis. It is important that *Nocardia* isolates of FSN13, FSN14, FSN34, FSN37, GR14 and GR130 were found to be associated with clinical strains of *Nocardia abcessus* and *N. cyriacigeorgica* while NEA01 and FSN04 were identified to be related to *G. terrae*. Isolation of these pathogenic *Nocardia* and *Gordonia* strains, which is the first indication report from Turkey, is also important[20, 21].

Taxonomic characterization of isolates based on 16S rRNA gene sequence analysis showed that 107 isolates are representatives of different actinomycete genera, of which *Actinomadura*, *Kribbella*, *Microbacterium*, *Nocardioides*, *Nonomuraea* and *Promicromonospora* isolates are rare Actinomycetes that obtained in this study.

The isolates having 97.2 – 99.5% gene sequence similarity to closely related type strains, which are estimated to be novel species, will be further studied for numerical and chemotaxonomic characterization and DNA-DNA homology analysis. Novel actinomycete species have also potential to produce new bioactive secondary metabolites. Isolates of the genera *Nocardia*, *Kribbella*, *Micromonospora*, *Nonomuraea*, *Nocardioides* and *Streptomyces* are worth further investigation as potential producers of valuable antibiotics and/or other bioactive secondary metabolites.

ACKNOWLEDGEMENTS

This research was supported by the Ondokuz Mayis University Research Program of Turkey (Project No PYO.FEN.1904.09.009 - PYO. FEN.1901.11.006 - F-473).

REFERENCES

1. Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G. F., Chater, K. F., Sinderen, D. Genomics of *Actinobacteria*: Tracing the evolutionary history of an ancient phylum. *Microbiol and Mol Biology Reviews*., 2007; **71**: 495–548.
2. Yamamura, H., Hayakawa, M., Iimura, Y. Application of sucrose-gradient centrifugation for selective isolation of *Nocardia* spp. from soil. *J of Appl Microbiol*., 2003; **95**: 677–685
3. Seong, C.N., Choi, J.H., Baik, K-S.An improved selective isolation of rare actinomycetes from forest soil. *The J Microbiol*., 2001; **39**:17-23.
4. Hayakawa, M., Yoshida, Y., Iimura, Y. Selective isolation of bioactive soil actinomycetes belonging to the *Streptomyces violaceusniger* phenotypic cluster. *J ApplMicrobiol*., 2004; **96**: 973–981.
5. Hayakawa, M., Nonomura, H.Humicacid-vitamin agar, a new medium for the selective isolation of soilactinomycetes. *J Ferment Technol*., 1987a; **65**: 501–509.
6. Hayakawa, M., Nonomura, H.Efficacy of artificialhumicacid as a selectivenutrient in HV agarusedfortheisolation of soilactinomycetes. *J Ferment Technol*., 1987b; **65**: 609– 616.
7. Hayakawa, M.Studies on the isolation and distribution of rare actinomycetes in soil. *Actinomy*., 2008; **22**:12–19.
8. Shirling, E. B., Gottlieb, D. Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol*., 1966; **16**: 313-340.
9. Bowers., Hucker.Tech. Bull. 228. NY State Agar. Exp. Sta.1935.
10. Hayakawa. M., Nonomura, H. A new method for the intensive isolation of actinomycetes from soil. *Actinomycetol*., 1989; **3**:95–104.
11. Minnikin, D.E., Alshamaony, L., Goodfellow, M.Differention of *Mycobacterium, Nocardia* and related taxa by thin-layer chromatographic analysis of whole organism methanolysates. *J Gen Microbiol*., 1975; **88**: 200-204.
12. Minnikin, D.E., Hutchinson, I.G., Caicott, A.B., Goodfellow, M. Thin-layer chromatography of methanolysates of mycolic acid-containing bacteria. *J Chromatogr*., 1980; **188**: 221-233.
13. Pitcher, D.G., Saunders, N.A., Owen, R.J. Rapid extraction of bacterial genomic DNA with guanidiumthiocyanate. *Lett Appl Microbiol*., 1989; **8**: 151-156.
14. Stackebrandt, E., Goodfellow, M., Wieley, J.(ed): 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematic. New York, Lane, D.J., 1991; 115-175.
15. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsinomy Methods. *Mol Bio Evol*.,2011; **28**: 2731–2739.
16. Saitou, N., Nei, M. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*., 1987; **4**: 406-425.
17. Felsenstein, J. Confidence limits on phylogeny: an appropriate use of the bootstrap. *Evolution*.,1985; **39**: 783-791.
18. Karwowski, J.P. The selective isolation of *Micromonospora* from soil by cesium chloride density gradient ultracentrifugation. *J of Indust Microbiol*.,1986; **1**: 181–186.
19. Kurup, P.V., Schmitt, J.A. Isolation of *Nocardia* from soil by a modified parafin bait method. *Mycologia*., 1971; **63**: 175–177.
20. Ozdemir-Kocak, F. Moleculer systematic of some Nocardioform isolates by 16S rRNA, *rpoB* and *gyrB* gene sequencing analyses. 2011; Phd, Ondkuz Mayis University, Samsun, Turkey.
21. Gencbay, T. Phylogenetic analysis of 16S rRNA gene region of different actinomycetes strains isolated from East Black Sea region plateau soil. MSc, Ondokuz Mayis University, Samsun, Turkey, 2012.