www.biodicon.com Biological Diversity and Conservation

ISSN 1308-8084 Online; ISSN 1308-5301 Print 8/2 (2015) 122-133

Research article/Araştırma makalesi

Molecular ıdentification of Nocardia Diversity in soil by multilocus sequence analysis

Fadime ÖZDEMİR-KOÇAK [*1], Kamil IŞIK [2]

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

Abstract

Identification of *Nocardia* strains is difficult due to the failure of 16S rRNA sequence analysis, chemotaxonomical and biochemical tests. In this study, identification of the possible novel *Nocardia* isolates was made by Multilocus Sequence Analysis. Four multilocus gene regions, gyrase B, β subunit of DNA topoisomerase (*gyrB*), 16S rRNA (16S rDNA), subunit A of SecA preprotein translocase (*secA1*), and RNA polymerase (*rpoB*), were used to identify ten *Nocardia* strains isolated from different soil samples. The region of *gyrB*, 16S rRNA, *secA1* and *rpoB* genes of ten isolates were compared with related type strains of *Nocardia*. Concatenate sequence analysis of 2.061 bp allowed differentiation of all isolates and type strains, with a range of interspecies similarity of 91.4-99.8%. *Nocardia* sp. FSN13, FSN14, FSN34 and FSN37 isolates shared 95.4- 96.4% of the concatenated *gyrB*-16S rRNA-*secA1-rpoB* sequences similarity with the type strain *N. abscessus*. FSN35, FMN06 and FMN15 *Nocardia* isolates were determined to be related with *N. rhamnosiphila* 97.9, 95.3 and 98.6% sequences similarity, respectively. It was determined that FSN27 isolate was closely related with *N. takedensis* and had 96.7% similarity, FGN17 and FGN19 isolates were determined to be related with *N. speluncae* the similarity of 95.5 and 95.3%., respectively.

Key words: 16S rRNA, *gyrB*, *rpoB*, *secA1*, *Nocardia*

---------- * ----------

Multilokus dizi analizleri ile topraktaki *Nocardia* çeşitliliğinin moleküler tanımlaması

Özet

Nocardia suşlarının tanımlanması 16S rRNA dizi analizleri, kemotaksonomik ve biyokimyasal testlerdeki hatalar nedeniyle zordur. Bu çalışmada, olası yeni *Nocardia* izolatlarının tanımlaması Multilokus Dizi Analizleri kullanılarak yapıldı. Dört multilokus gen bölgesi, giraz B, DNA topoisomerazın β altünitesi (*gyrB*), 16S rRNA (16S rDNA), SecA preprotein translokazın A altünitesi (*secA1*) ve RNA polymeraz β altünitesi (*rpoB*), farklı toprak örneklerinden izole edilen on *Nocardia* suşunun tanımlanmasında kullanıldı. On izolatın *gyrB*, 16S rRNA, *secA1* ve *rpoB* gen bölgeleri ilişkili *Nocardia* tip suşları ile karşılaştırıldı. 2.061 bp'in birleştirilmiş dizi analizleri tüm izolat ve tip suşlarının 91.4-99.8%. tür içi benzerlik oranı ile farklılaşmasını sağladı. *Nocardia* sp. FSN13, FSN14, FSN34 ve FSN37 isolatları *N. abscessus* tip suşu ile birleştirilmiş *gyrB*-16S rRNA-*secA1-rpoB* dizi benzerliği 95.4- 96.4% olduğu belirlendi. FSN35, FMN06 ve FMN15 *Nocardia* isolatlarının 97.9, 95.3 ve 98.6% dizi benzerliği ile *N. rhamnosiphila'*a akraba olduğu belirlendi. FSN27 izolatı *N. takedensis* ile yakın ilişkili olduğu belirlendi ve FGN17 ve FGN19 izolatlarınında *N. speluncae* ile 95.5 ve 95.3% benzerlik oranı ile ilişkili olduğu belirlendi.

Anahtar kelimeler: 16S rRNA, *gyrB*, *rpoB*, *secA1*, *Nocardia*

1. Introduction

The members of the genus *Nocardia* among aerobic actinomycetes, which are Gram-positive and partially acid-alcohol fast at some stage of the growth cycle, can be found as worldwide microorganisms in fresh and salt water, soil, dust, decaying vegetation and fecal deposits from animals (Brown-Elliott et al., 2006). While some strains of

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +902282141388; Fax.: +902282141382; E-mail: ozdemirkocak@bilecik.edu.tr

Nocardia can cause primary pulmonary or cutaneous diseases or central nervous system and disseminated diseases in human and animals, some members of the genus are industrially important for the production of bioactive secondary metabolites such as monobactam antibiotic called as nocardiocin. Up to date, approximately 100 species have been described for *Nocardia* (Euzéby, 2012).

Due to the difficulty of identifying *Nocardia* isolates by standard phenotypically based methods, sequence analysis of 16S rRNA gene has become the 'gold standard' for bacterial identification. Because 16S rRNA gene has a low mutation rate in evolution, 16S rRNA gene sequence cannot be distinctive. For instance, there were 99,8% 16S rRNA gene sequence similarity between *N. veterana* and *N. Kruczakiae*, but these *Nocardia* strains were identified as different species (Harayama and Kasai, 2006).

Currently, multilocus sequence analysis (MLSA) is used to determine the taxonomic positions in prokaryotes. Moreover, MLSA has been a valuable method for the taxonomic investigation due to ease of use, accuracy, and discriminatory power (Mctaggart et al., 2010). Fortunately, The evolution of MLSA genes occur slowly by the random accumulation of neutral mutations. To determine phylogenetic relation within different bacterial genera, MLSA has been used with a number of protein coding genes called *gyrB*, *secA1*, *rpoB*, *recA* and *hsp65* (Kim et al., 2003; Kang et al., 2009; Mctaggart et al., 2010).

The *gyrB* gene codes for the β-subunit of DNA gyrase, a type II DNA topoisomerase, is chosen for phylogenetic studies, because horizontal gene transfer (HGT) occurs rarely in informational genes involved in transcription and translation (Harayama and Kasai 2006). To understand inter- and intra-species relationships, *gyrB* gene has been researched within different actinobacterial genera including *Nocardia* (Takeda et al., 2010), *Amycolatopsis* (Everest and Meyers, 2009), *Gordonia* (Kang et al., 2009), *Kribbella* (Kirby et al., 2010), *Microbacterium* (Richert et al., 2007), *Mycobacterium* (Devulder et al., 2005) and the whorl-forming *Streptomyces* species (Hatano et al., 2003).

The *rpoB* gene encoding the subunit of DNA-dependent RNA polymerase has been successfully used to determine phylogenetic relationships among strains in the genus *Amycolaptosis*, *Corynebacterium*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Streptomyces* and *Tsukamurella* (Kim et al., 2003). The *rpoB* gene is made up of a one-copy and a functional gene. Therefore, the amino acid sequence with DNA sequence information can also be used for genus separation (Kim et al., 2003).

The *SecA1* gene is an essential component of the major pathway of protein secretion across the bacterial cytoplasmic membrane. The discrimination of 29 *Mycobacterium* species has been done by the *secA1* gene (Zelazny *et al*., 2005). The type strain and isolates of *Nocardia* have been identified using sequence analysis of both a portion of *secA1* gene and the amino acid sequence (Kong et al., 2010).

The aim of this study was to analyse four-locus (*gyrB*-16S-*secA1*-*rpoB*) gene regions for the identification of *Nocardia* soil isolates. Ten Nocardia species isolated from various soil samples were characterized in this study through phylogenetic analysis of concatenated sequences consisting of partial fragments of *gyrB*, 16S rRNA, *secA1* and *rpoB* genes.

2. Materials and methods

2.1. Strains

Ten soil isolates and five type species of *Nocardia* were used in the study (Table 1). Ten soil samples were collected from three different countries, France (Gap), Russia (Moscow) and Turkey (Balıkesir, Kütahya). For the selective isolation of *Nocardia*, we used sucrose-gradient centrifugation method with humic acid-vitamin (HV) agar (Hayakawa and Nonomura, 1987; Yamamura et al., 2003). Mycolic acid test was applied for the selection of *Nocardia* isolates and mycolic acid methyl esters were determined by thin layer chromatography (Minnikin et al., 1980). *Nocardia* type strains were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). The bacterial type strains and soil isolates used in this study were presented in Table 1. All tested strains were incubated at 30°C for 3-7 days on TYG medium (0.3% tryptone, 0.5% yeast extract, 0.5% glucose, 1.5% agar) or GYME medium (0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.2% CaCO3, 1.2% agar).

2.2. DNA extraction, PCR and sequencing

The guanidine thiocyanate DNA extraction procedure of Pitcher et al. (1989) was used with modifications (pretreatment with proteinase K, 100µg/ml and sodium dodecyl sulphate SDS, final concentration 2%, w/v) to obtain genomic DNA. Full 16S rRNA and partial *secA1*, *rpoB*, and *gyrB* sequences were amplified using a HotStarTaq® (Qiagen) in Thermal Cycler (PCR Express, ThermoHybaid, Middlesex, UK). The primers and PCR cycling conditions were listed in Table 2-3. PCR conditions were carried out in 50 µl reaction volumes of 16S rRNA and *gyrB* gene regions. Each PCR mixture of 16S and *gyrB* contains 20 µM of each primer (Invitrogen and IDT, respectively), a mixture of 25 mM of each deoxynucleoside triphosphates (Promega) and Taq polymerase buffer (Qiagen).

Chromosomal DNA (50-300 ng) and Taq polymerase (2.5 U, HotStarTaq®, Qiagen) were also added to the solution. The amplification conditions are presented in Table 3. Similar PCR conditions were carried out in 25 µl reaction volumes of *rpoB* and *secA1* gene regions. The sequence analysis of obtained PCR product was performed by Macrogen (Seoul, Korea and Amsterdam the Netherland) using ABI 3730 XL DNA sequencer.

Table 1. GenBank accession numbers of the *gyrB*-16S-*secA1*-*rpoB* sequences for *Nocardia* type species and soil isolates used in this study

		Accession No			
İsolate No:	**İsolate Name:**	**16S**	***gyrB***	***rpoB***	***secA1***
FMN06*	*Nocardia sp.*	JN896620	JQ040832	JQ040839	JX982582
FMN15*	*Nocardia sp*	JN896621	JQ040834	JQ040840	JX982587
FGN17*	*Nocardia sp*	KC993086	JX982575	JQ349533	JX982581
FGN19*	*Nocardia sp*	KC993087	JX982576	JQ349534	JX982584
FSN13*	*Nocardia sp*	KC993082	JQ349548	JQ349540	JX982590
FSN14*	*Nocardia sp*	KC993083	KF723322	JQ349541	JX982591
FSN27*	*Nocardia sp*	KC993078	KF835566	KF835567	KF835565
FSN34*	*Nocardia sp.*	KC993084	JX982574	JQ349543	JX982592
FSN35*	*Nocardia sp.*	KC993081	JX982572	JQ349544	JX982594
FSN37*	*Nocardia sp.*	KC993085	JQ349547	JQ349545	JX982593
45078*	*N. speluncae* T	AM422449	HM856186	JQ349524*	JX982578*
N1373*	*N. takedensis* T	AB158277	AB450809	JQ349525*	JX982577*
N1187*	*N. cyriacigeorgica* T	AF430027	AB450784	JQ349527*	ABD37096
202GMO	*N. rhamnosiphila* T	EF418604	HM856180	JN215693	ADN88953
DSM 43397	*N. carnea* T	Z36929	AB075569	JN215703	ABD37101
JCM 3332	*N. flavorosea* T	Z46754	AB450787	DQ085118	AEH95186
IMMIB D-1592	*N. abscessus* T	AF218292	GQ496132	AAS48628	DQ360260
JCM 12235	*N. testacea* T	AB192415	GQ496091	DQ085134	DQ360286
IFM 10088	*N. sienata* T	AB121770	GQ496094	JN215698	ABD37115
IFM 10035	*N. arthritidis* T	AB108781	AB450769	DQ085141	ABD37092
IFM 0245T	*N. asiatica* T	AB092566	AB450770	DQ085139	ABD37093
IFM 10311	*N. shimofusensis* T	AB108775	AB450806	DQ085143	EU178751

T: Type strain *Multilocus gene regions have been studied and deposited to GenBank-NCBI database in this study.

Table 2. Primers used in PCR and sequencing for 16S rRNA, *rpoB*, *gyrB* and *secA1*

Gene	**Primer name**	**Sequence (5'-3')**	**Size**	**Region**		**Uses**		**Source**
						PCR	**Sequences**	
16S rRNA	27f	AGAGTTTGATCTGGCTCAG	20	8	27	√	√	Lane, 1991
	MG3f	CAGCAGCCGCGGTAATAC	18	520	536		√	Kagayma et al., 2004
	MG5f	AAACTCAAAGGAATTGACGG	20	907	926		√	Chun, 1995
	1525r	AAGGAGGTGWTCCARCC	17	1544	1525	√	√	Lane, 1991
	1115r	AGGGTTGCGCTCGTTG	16	1115	1131		√	Gyobu and Miyadoh, 2001
	1492r	TACGGYTACCTTGTTACGACT	21	1492	1474		√	Gyobu and Miyadoh, 2001
rpoB	MF	CGACCACTTCGGCAACCG	18	203	221	√	√	Kim et al., 2003
	MR	TCGATCGGGCACATCCGG	18	554	536	√		Kim et al., 2003
gyrB	Noc-gyrB-F	CTTCGCCAACACCATCAACAC	21	972	992	√	√	McTaggart et al.,2010
	Noc-gyrB-R	TGATGATCGACTGGACCTCG	20	1563	1582	√	√	McTaggart et al.,2010
SecA1	secA1-F47	GCGACGCCGAGTGGATGG	18	413	430	√	√	McTaggart et al.,2010
	secA1-ConR2	TTGGCCTTGATGGCGTTGTTC	21	876	896	√		McTaggart et al.,2010

Table 3. PCR amplification conditions for 16S rRNA, *rpoB*, *gyrB* and *secA1*

Condition	**16S rRNA**	***gyrB***	***rpoB***	***secA1***
Denaturation	5 min 95°C; 1 cycle	5 min 95°C; 1 cycle	3 min 95°C; 1 cycle	5 min 95°C; 1 cycle
Annealing	95°C, 1 min ; 2 min 55°C, 3 min 72°C; 35 cycles	95°C, 1 min ; 1 min 55-65°C, 1 min 72°C; 35 cycles	95°C, 20 s; 20 s 58-60°C, 20 s 68°C; 30 cycles	30 s 95°C; 45 s 65°C, 50 s 72°C; 30 cycles
Final extension	10 min 72° C; 1 cycle	5 min 72° C; 1 cycle	5 min 68° C; 1 cycle	5 min 72° C; 1 cycle

2.3. Cluster analysis

The 16S rRNA, *gyrB*, *rpoB* and *secA1* gene sequences of strains were determined and compared with the corresponding sequences in the GenBank database using the BLAST program and the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) following the description presented by Kim and colleagues (Kim et al., 2012). Phylogenetic analysis was conducted using MEGA 5.0 (Tamura et al., 2011) by first generating a complete alignment of 16S rRNA, *gyrB*, *rpoB* and *secA1* gene sequences of the isolates and type strains of valid species. For the analysis, sequences were aligned and trimmed with start and end positions to yield fragments of the following sizes: 1.300-1.500 bp for 16S, 500-600 bp for *gyrB*, 240-300 bp for *secA1* and 300 bp for *rpoB*. The trimmed sequences were concatenated in the order *gyrB*-16S-*secA1*-*rpoB* to generate a 2.061 bp sequence. The 16S rRNA, *gyrB*, *rpoB* and *secA1* gene sequences obtained in this study were manually aligned with the published sequences of the species available from the NCBI-EMBL and DDBJ databases. The individual and concatenated sequence alignments were performed by using the program CLUSTAL W in MEGA5.0 and BioEdit 7.2.0, which were used to calculate evolutionary distances and similarity values (Hall 1999; Tamura et al., 2011). Phylogenetic trees were constructed using the neighbour-joining (Saitou and Nei, 1987), maximum parsimony (Kluge and Farris, 1969) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA5.0 (Tamura et al., 2011). Evolutionary distances were calculated using the model of Kimura 2-parameter (K2P) (Saitou and Nei 1987). Topologies of the resultant trees were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings.

2. 4. Nucleotide sequence accession numbers

The 16S rRNA, *gyrB*, *rpoB* and *secA1* sequences of some type strains and test samples, which we primarily determined in this study, have also been deposited in the NCBI database under the accession numbers listed in Table 1.

3. Results

In this study, selective isolation technique and media have been used for *Nocardia* isolation from various soil samples. Totally four hundred seventy-six actinomycetes were isolated after incubation at 28 °C for 14-21 days on HV, TYG with and without vitamin, GYM medium with and without vitamin supplemented with nalidixic acid, rifampicin and cycloheximide.

Selected 125 *Nocardia*-like isolates were degraded by acid methanolysis, and hexane extracts were examined for mycolic acids by TLC. Obtained Rf values (0.47) for *Nocardia* isolates were similar with the literature, except for *N. abscessus* having the value of 0.91. In addition, mycolic acid containing isolates were amplified by PCR for the identification of genus level (Ozdemir-Kocak, 2011).

PCR amplification of mycolic acid containing isolates was performed for 16S rRNA gene region using universal primers 27f and 1525r generating a fragment of ~1500 bp long for each isolate. While all test isolates yielded ~1500 bp sequence, except one test isolate, FGN19 produced 1300 nt sequence. *gyrB* gene amplification of soil isolates was achieved by primers Noc-gyrB-F and Noc-gyrB-F generating a partial fragment of ~500-600 bp for each isolate. For *rpoB* gene amplification of these organisms, MF and MR primers were used, and a fragment of 300 bp was generated. PCR amplifications of other gene region *secA1* were performed by using primers secA1-F47 and secA1-ConR2, and a fragment of 450 bp long for each soil isolate was created.

gyrB-16S rRNA-*secA1*-*rpoB* sequences of soil isolates were aligned with those of other known closely related *Nocardia* type species obtained from the GenBank/EMBM/DDBJ (Table 1) and phylogenetic tree was constructed on the basis of distance and neighbour-joining analyses (Figure 1).

Each of the gene regions was individually analysed and all isolates shared 95.4% and 100% of 16S rRNA gene sequence similarity with the type strain value, corresponding to 54 and 0 nt differences, respectively, while *Nocardia* soil isolates were found to be 84% and 100% of *gyrB* gene sequence similarity with the type strain values,

corresponding to 68 and 0 nt differences, respectively. For all type strains and soil isolates, similarity for the *rpoB* gene ranged from 78.1% to 100% and nt differences were found as 58 to 0, and also these organism shared 81% and 100% of *secA1* gene sequence similarity, 33 to 0 nt differences, respectively (Supplementary Figure 1-4).

Nocardia soil isolates were also characterized by *gyrB*-16S-*secA1-rpoB* sequence analysis to establish their phylogenetic positions. The neighbour-joining, maximum-parsimony and maximum-likelihood methods were implemented to each of the four gene regions in order to identify sequence diversity within *Nocardia*. In this study, four gene regions were used to compare *Nocardia* soil isolates and type strains and it was found that the similarity of the concatenated *gyrB*-16S rRNA-*secA1-rpoB* sequences ranged from 91.4-100%, with 0 to 176 nt differences occurring between strains (Table 4).

The concatenated *gyrB*-16S rRNA-*secA1-rpoB* sequences analysis was described as a powerful taxonomic tool, which were useful for identification of novel species. In this study, FSN27 isolate was determined, which was closely related with *N. takedensis* and had 99.3% of 16S rRNA gene sequence similarity with 8 nt difference, and also the similarity of the concatenated *gyrB*-16S rRNA-*secA1-rpoB* sequences ranged from 96.7%, with 66 nt difference (Table 4).

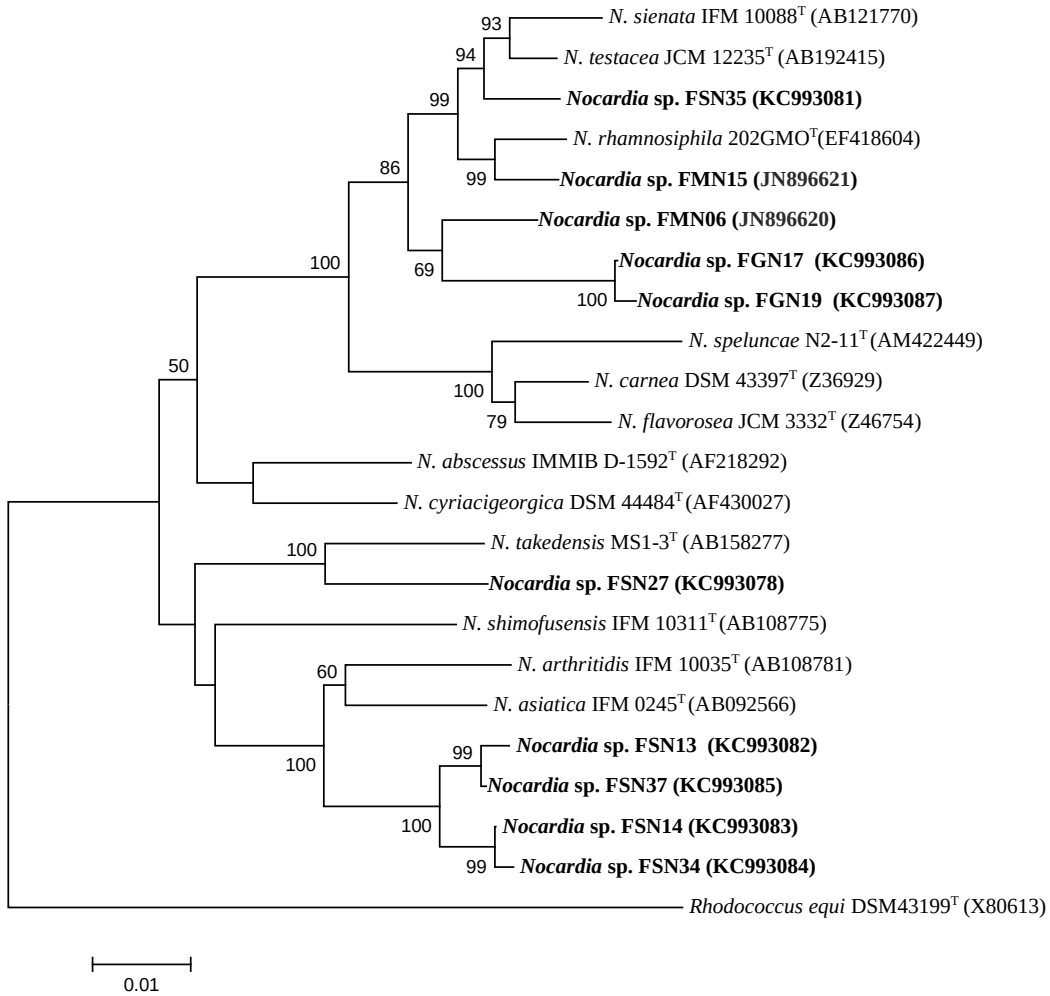


Figure 1. Neighbour-joining tree (Lane, 1991) based on the concatenated *gyrB*-16S rRNA-*secA1-rpoB* sequences (2061 bp length) showing the relationships between test soil isolates and closely related type strains of the genus *Nocardia*. Numbers on branch nodes are bootstrap values (1000 resamplings; only values over 50% are given). Bar 0.01 substitutions per nucleotide position. 16S rRNA GenBank accession numbers are given in parenthesis.

Nocardia sp. FSN13, FSN14, FSN34 and FSN37 shared 96.0, 95.6, 95.4 and 96.4% the concatenated *gyrB*-16S rRNA-*secA1-rpoB* sequences similarity with the type strain *N. abscessus*, with 81, 89, 93 and 74 nt differences occurring between strains while *Nocardia* sp. FSN13, FSN14, FSN34 and FSN37 shared 99.8 and 100% the next three of 16S rRNA gene sequence similarity with the type strain *N. abscessus* values, corresponding to 2 and 0 nt differences, respectively (Table 4).

FSN35 and FMN15 *Nocardia* isolates were determined to be closely related with *N. rhamnosiphila* 99.5, 99.6% of 16S rRNA gene sequence similarity with 6 and 5 nt difference, and also the similarity of the concatenated *gyrB*-16S rRNA-*secA1-rpoB* sequences ranged from 97.9, 98.6%, with 43 and 28 nt difference, respectively (Table 4).

There were 95.3% similarity in the concatenated *gyrB*-16S rRNA-*secA1-rpoB* sequences between *Nocardia* FMN06 and *N. flavorosea*, *N. carnea*, with 96 nt difference while FMN06 shared 99.5 of 16S rRNA gene sequence similarity with the type strain of *N. flavorosea* and *N. carnea*, with 7 nt difference (Table 4).

It was determined that FGN17 and FGN19 *Nocardia* isolates were closely related with *N. jinanensis* and *N. speluncae* 99.6, 99.3 and 99.6, 99.1 % of 16S rRNA gene sequence similarity with 6, 9 and 4, 10 nt difference. FGN17 and FGN19 isolates were closely related with *N. speluncae* and also the similarity of the concatenated *gyrB*-16S rRNA-*secA1-rpoB* sequences ranged from 95.5, 95.3%, with 91 and 95 nt difference, respectively (Table 4).

The concatenated *gyrB*-16S rRNA-*secA1-rpoB* sequence analysis result in 10 isolates, sharing 91.4-99.8% gene sequence similarity with related type strains which were presented in Figure 1. More importantly, *Nocardia* FSN13, FSN14, FSN34 and FSN37 isolated from Sındırgı Dam Lake were found to be related to pathogenic *Nocardia abcessus*..

4. Conclusions

Many studies have been performed using several selective medium and isolation techniques in order to achive isolation of *Nocardia* from natural habitats and clinical samples. The recent researchers screened marine and soil actinomycetes intensively to elucidate the structures of bioactive molecules produced by them (Yamamura et al., 2003; Goodfellow and Fiedler 2010). Potentially ten mycolic acid containing novel *Nocardia* soil isolates were identified on the genus level by 16S rRNA gene sequence analysis in this study.

Recently, numerous taxonomic studies of soil and clinical *Nocardia* isolates have also been performed using MLSA for taxonomic position determination. Three- (*gyrB*-16S-*secA1*), four- (*gyrB*-16S-*secA1-hsp65*) or five-locus (*gyrB*-16S-*secA1-hsp65-rpoB*) for MLSA were found to be reliable methods to explain the identification of taxonomic positions of *Nocardia* species (Mctaggart et al., 2010). Thus, *secA1*, *gyrB* and *rpoB* genes analyses are more distinctive than data obtained by 16S rRNA gene sequence analysis. While examining individually, the distinction of each isolates was found to be more efficient by *secA1*, *gyrB* and *rpoB* gene analysis than 16S rRNA gene sequence analysis. In this study, *Nocardia* sp. FSN13, FSN14, FSN34 and FSN37 shared 99.8 and 100% 16S rRNA gene sequence similarity with the type strain *N. abscessus*, in contrast, according to four-locus (*gyrB*-16S-*secA1-rpoB*) of MLSA analysis of this soil isolates, they showed that these isolates were significantly different from *N. abscessus*. Isolates of FSN13, FSN14, FSN34 and FSN37 were found to be pathogenic *Nocardia* strains, which will be the first report from Turkey.

In conclusion, using of *secA1*, *gyrB* and *rpoB* genes in MLSA with 16S rRNA gene analysis may finally be most useful as part of a multigene approach for the identification of *Nocardia* isolates. *Nocardia* isolates have 91.4-99.8% gene sequence similarity to closely related type strains, which are estimated to be potentially novel species. The identified *Nocardia* isolates in this study will be further searched for numerical and chemotaxonomic characterization and also DNA-DNA homology analysis is needed to accommodate as a new species in the genus *Nocardia*..

Acknowledgements

We would like to thank Ondokuz Mayıs University Research Program for supporting this Project (Project No PYO. FEN.1904.09.009) financially.

References

Brown-Elliot, B.A., Brown, J.M., Conville, P.S., Wallace, R.J. 2006. Clinical and Laboratory features of the *Nocardia* spp. Based on current molecular taksonomy. Clin Microbiol Rev 19: 259-282.

Devulder, G., Pe´rouse de Montclos, M., Flandrois, J.P. 2005. A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. Int J Syst Evol Micr 55: 293–302.

Everest, G.J., Meyers, P.R. 2009. The use of *gyrB* sequence analysis in the phylogeny of the genus *Amycolatopsis*. A van Leeuw J Microb 95: 1–11.

Euzéby, J. 2012. Notification that new names and new combinations have appeared in volume 62, part 3, of the IJSEM. Int J Syst Evol Micr 62: 1221–1222.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17: 368-376.

Felsenstein, J. 1985. Confidence limits on phylogeny: an appropriate use of the bootstrap. Evolution 39: 783-791.

Goodfellow, M., Fiedler, H.P. 2010. A guide to succesful bioprospecting: informed by actinobacterial systematics. A van Leeuw J Microb 98: 119-142.

Hall, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis. http.//www.mbio.ncsu.edu/BioEdit/bioedit.html.

Harayama, S., Kasai, H. 2006. Bacterial phylogeny reconstruction from molecular sequences. *In*: Stackebrandt, E. (ed). Molecular identification, systematics, and population structure of prokaryotes. Springer, Berlin, Germany, 105-139.

Hatano, K., Nishii, T., Kasai, H. 2003. Taxonomic re-evaluation of whorl-forming *Streptomyces* (formerly *Streptoverticillium*) species by using phenotypes, DNA–DNA hybridization and sequences of *gyrB*, and proposal of *Streptomyces luteireticuli* (ex Katoh and Arai 1957) corrig., sp. nov., nom. rev. Int J Syst Evol Micr 53: 1519–1529.

Hayakawa, M., Nonomura, H. 1987. Humic acid-vitamin agar, a new medium for selective isolation of soil actinomycetes. J Ferment Technol 65: 501–509.

Kang, Y., Takeda, K., Yazawa, K., Mikami, Y. 2009. Phylogenetic studies of *Gordonia* species based on *gyrB* and *secA1* gene analyses. Mycopathologia 167: 95-105.

Kim, B-J., Koh, Y.H., Chun, J., Kim, C.J., Lee, S.H., Cho, M., Hyun, J.W., Lee, K.H., Cha, Y.C., Kook, Y.H. 2003. Differentiation of Actinomycete genera based on partial *rpoB* gene sequences. J Microbiol Biotechn13: 846-852.

Kim, O.S., Cho, Y-J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi, H., et al. 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Micr 62: 716–721.

Kirby, B.M., Everest, G.J., Meyers, P.R. 2010. Phylogenetic analysis of the genus *Kribbella* based on the *gyrB* gene: proposal of a *gyrB*-sequence threshold for species delineation in the genus *Kribbella*. A van Leeuw J Microb 97: 131–142

Kluge, A.G., Farris, F.S. 1969. Quantitative phyletics and the evolution of anurans. Syst Zool 18: 1–32.

Kong, F., Wang, H., Zhang, E., Sintchenko, V., Xiao, M., Sorrell, T.C., Chen, X., Chen, S.C-A. 2010. *SecA1* Gene Sequence Polymorphisms for Species Identification of *Nocardia* and Recognition of Intraspecies Genetic Diversity. J Clin Microbiol 48: 3928-34.

McTaggart, L.R,, Richardson, S.E., Witkowska, M., Zhang, S.X. 2010. Phylogeny and identification of *Nocardia* species on the basis of multilocus sequence analysis. J Clin Microbiol 48: 4525–4533.

Minnikin, D.E., Hutchinson, I.G., Caicott, A.B., Goodfellow, M. 1980. Thin-layer chromatography of methanolysates of mycolic acid-containing bacteria. J Chromatogr 188: 221-233.

Ozdemir-Kocak, F. 2011. Moleculer systematic of some Nocardioform isolates by 16S rRNA, *rpoB* and *gryB* gene sequences analyses. Samsun, Turkey, 187 p (Ph.D. Dissertation. Institute faculty of science. OMU).

Pitcher, D.G., Saunders, N.A., Owen, R.J. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 8: 151-156.

Richert, K., Brambilla, E., Stackebrandt, E. 2007. The phylogenetic significance of peptidoglycan types: molecular analysis of the genera *Microbacterium* and *Aureobacterium* based upon sequence comparison of *gyrB*, *rpoB*, *recA* and *ppk* and 16S rRNA genes. Syst Appl Microbio 30: 102–108.

Saitou, N., Nei, M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.

Takeda, K., Kang, Y., Yazawa, K., Gonoi, T., Mikami, Y. 2010. Phylogenetic studies of *Nocardia* species based on *gyrB* gene analyses. J Med Microbiol 59: 165-171.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsinomy Methods. Mol Bio Evol 28: 2731–2739.

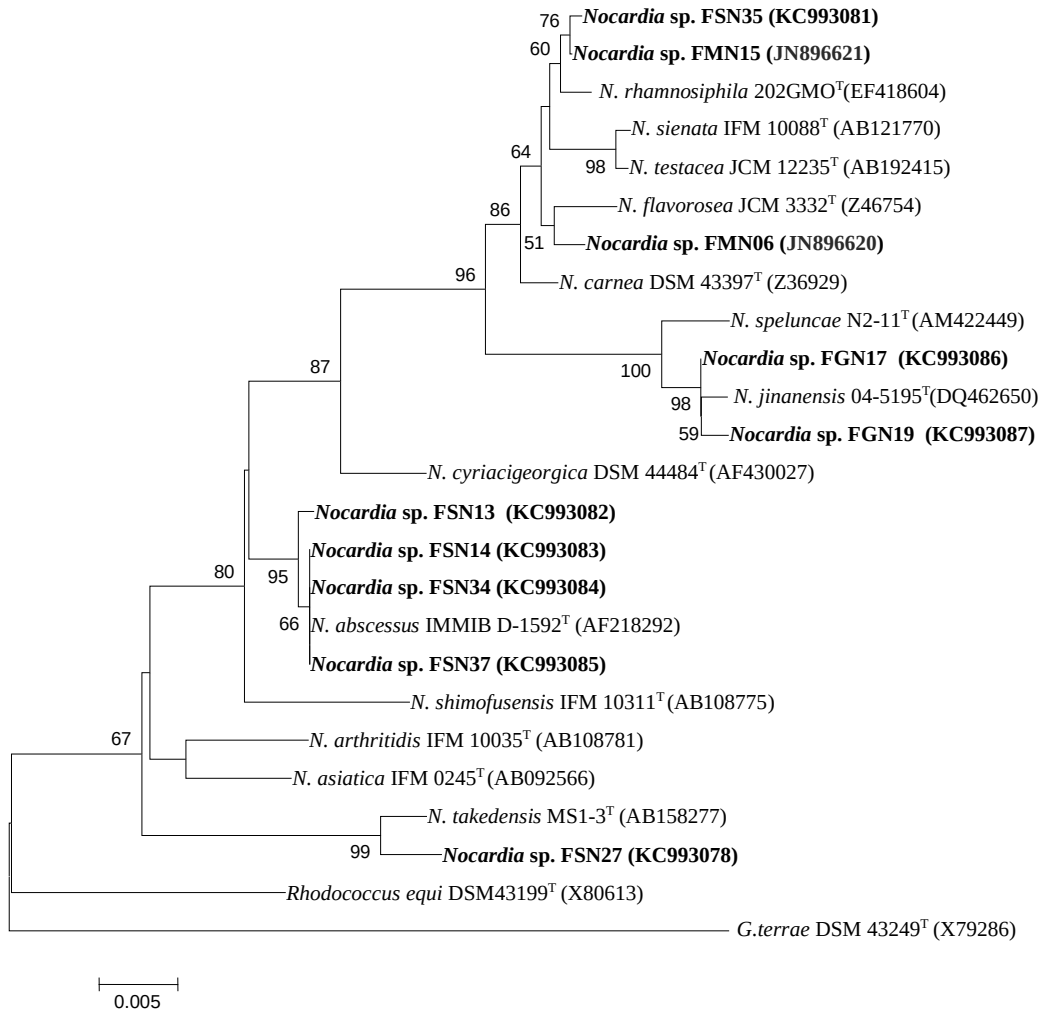
Yamamura, H., Hayakawa, M., Iimura, Y. 2003. Application of sucrose-gradient centrifugation for selective isolation of *Nocardia* spp. from soil. J App Microbiol 95: 677–685.

Zelazny, A.M., Calhoun, L.B., Li, L., Shea, Y.R., Fischer, S.H. 2005. Identification of *Mycobacterium* species by *secA1* sequences. J Clin Microbiol 43: 1051–58.

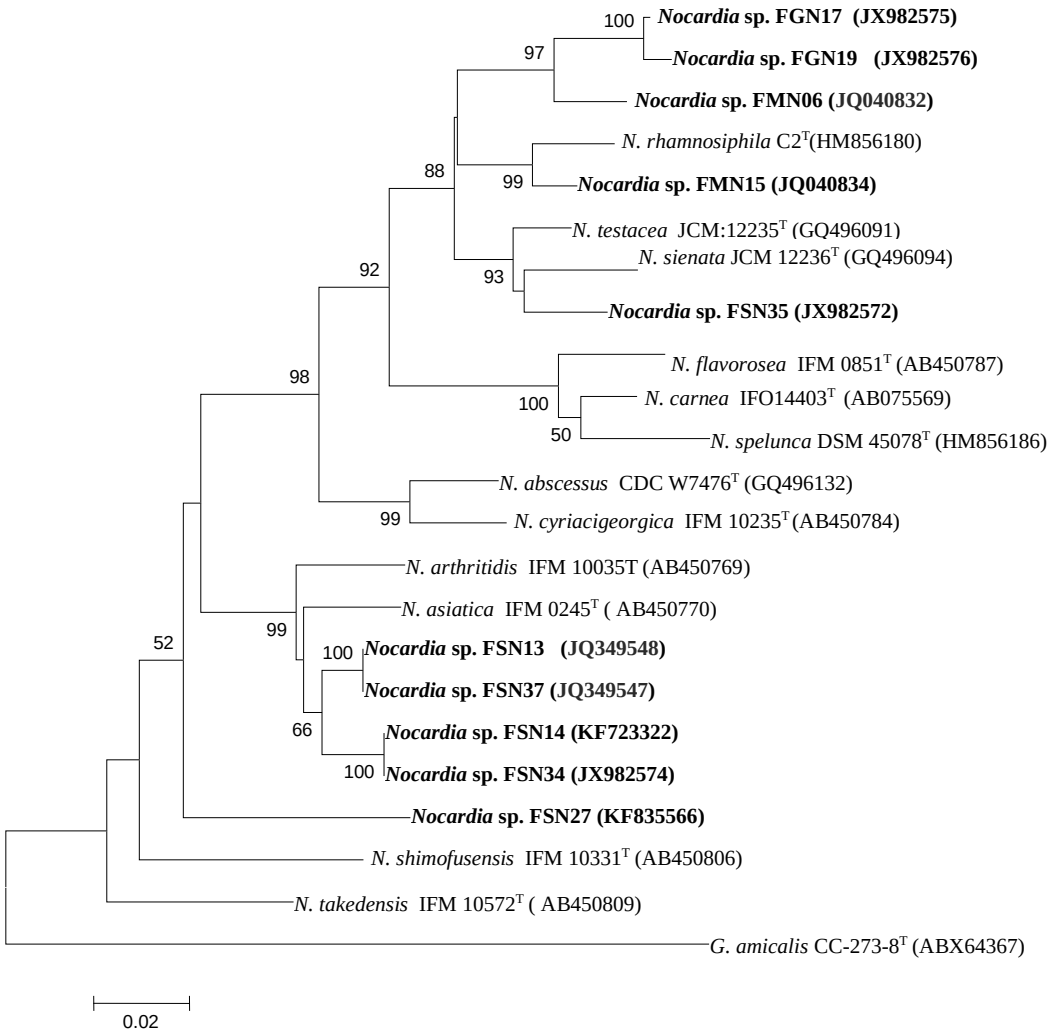
Table 4. Nucleotide similarities (%) and differences based on the concatenated *gyrB*-16S rRNA-*secA1-rpoB* sequences (2061 bp length) of the test soil isolates and closely related valid strains of *Nocardia*

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
1. *Rhodococcus equi*	-	211	225	206	243	222	248	237	202	241	256	230	233	244	247	239	238	235	235	232	239	228	230
2. *N. abscessus*	89.7	-	92	88	120	62	126	119	90	124	136	108	120	124	128	123	121	81	89	107	93	117	74
3. *N. arthritidis*	89.0	95.5	-	63	165	116	169	157	107	163	175	96	153	160	164	161	156	72	69	107	73	153	65
4. *N. asiatica*	90.0	95.7	96.9	-	160	110	164	148	100	157	176	108	148	160	164	153	149	77	73	113	77	147	70
5. *N. carnea*	88.2	94.1	91.9	92.2	-	110	35	93	148	96	56	169	91	99	103	93	87	156	158	164	160	92	155
6. *N. cyriacigeorgica*	89.2	96.9	94.3	94.6	94.6	-	117	114	109	110	140	114	102	133	137	117	115	113	109	116	113	106	108
7. *N. flavorosea*	87.9	93.8	91.8	92.0	98.3	94.3	-	92	154	98	66	170	94	107	111	96	90	159	160	161	163	99	157
8. *N. rhamnosiphila*	88.5	94.2	92.3	92.8	95.4	94.4	95.5	-	140	52	118	147	42	80	84	57	28	154	149	145	153	43	149
9. *N. shimofusensis*	90.1	95.6	94.8	95.1	92.8	94.7	92.5	93.2	-	151	166	112	139	154	157	146	141	112	108	120	112	141	105
10. *N. sienata*	88.3	93.9	92.0	92.3	95.3	94.6	95.2	97.4	92.6	-	121	144	29	81	84	64	56	165	159	147	163	40	160
11. *N. speluncae*	87.5	93.4	91.5	91.4	97.2	93.2	96.7	94.2	91.9	94.1	-	172	117	91	95	115	114	168	168	157	171	119	166
12. *N. takedensis*	88.8	94.7	95.3	94.7	91.8	94.4	91.7	92.8	94.5	93.0	91.6	-	137	153	156	145	146	127	122	66	126	139	120
13. *N. testacea*	88.6	94.1	92.5	92.8	95.5	95.0	95.4	97.9	93.2	98.5	94.3	93.3	-	81	84	56	38	156	148	143	152	31	151
14. *Nocardia* sp.FGN17	88.1	93.9	92.2	92.2	95.1	93.5	94.8	96.1	92.5	96.0	95.5	92.5	96.0	-	5	56	70	161	153	152	157	75	158
15. *Nocardia* sp.FGN19	88.0	93.7	92.0	92.0	95.0	93.3	94.6	95.9	92.3	95.9	95.3	92.4	95.9	99.7	-	59	74	165	157	155	161	78	162
16. *Nocardia* sp.FMN06	88.4	94.0	92.1	92.5	95.4	94.3	95.3	97.2	92.9	96.8	94.4	92.9	97.2	97.2	97.1	-	54	152	152	150	155	59	148
17. *Nocardia* sp.FMN15	88.4	94.1	92.4	92.7	95.7	94.4	95.6	98.6	93.1	97.2	94.4	92.9	98.1	96.6	96.4	97.3	-	152	146	144	150	41	148
18. *Nocardia* sp.FSN13	88.5	96.0	96.5	96.2	92.4	94.5	92.2	92.5	94.5	91.9	91.8	93.8	92.4	92.1	91.9	92.6	92.6	-	28	126	24	153	7
19. *Nocardia* sp.FSN14	88.5	95.6	96.6	96.4	92.3	94.7	92.2	92.7	94.7	92.2	91.8	94.0	92.8	92.5	92.3	92.6	92.9	98.6	-	126	4	148	23
20. *Nocardia* sp.FSN27	88.7	94.8	94.8	94.5	92.0	94.3	92.1	92.9	94.1	92.8	92.3	96.7	93.0	92.6	92.4	92.7	93.0	93.8	93.8	-	130	144	121
21. *Nocardia* sp.FSN34	88.4	95.4	96.4	96.2	92.2	94.5	92.0	92.5	94.5	92.0	91.7	93.8	92.6	92.3	92.1	92.4	92.7	98.8	99.8	93.6	-	152	27
22. *Nocardia* sp.FSN35	88.9	94.3	92.5	92.8	95.5	94.8	95.1	97.9	93.1	98.0	94.2	93.2	98.4	96.3	96.2	97.1	98.0	92.5	92.8	93.0	92.6	-	148
23. *Nocardia* sp.FSN37	88.8	96.4	96.8	96.6	92.4	94.7	92.3	92.7	94.9	92.2	91.9	94.1	92.6	92.3	92.1	92.8	92.8	99.6	98.8	94.1	98.6	92.8	-

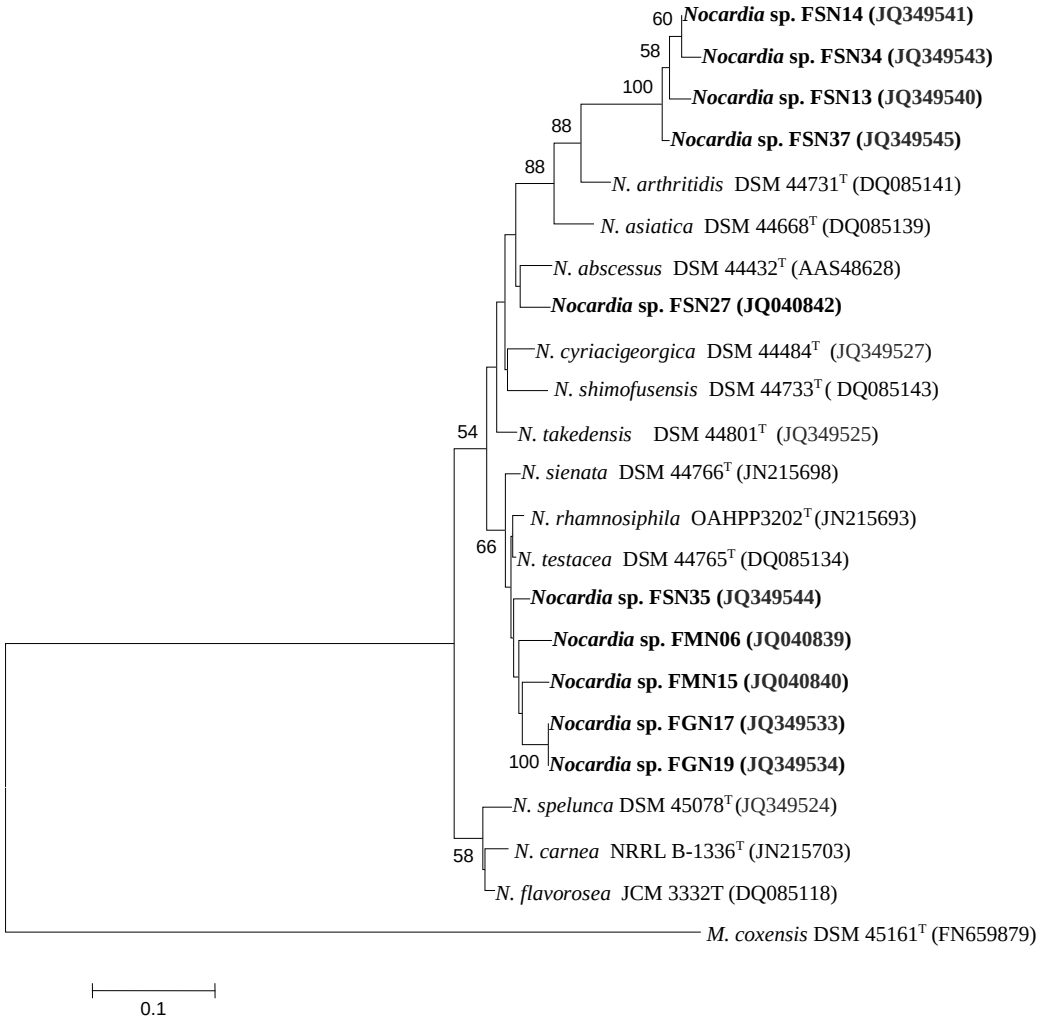
Appendices



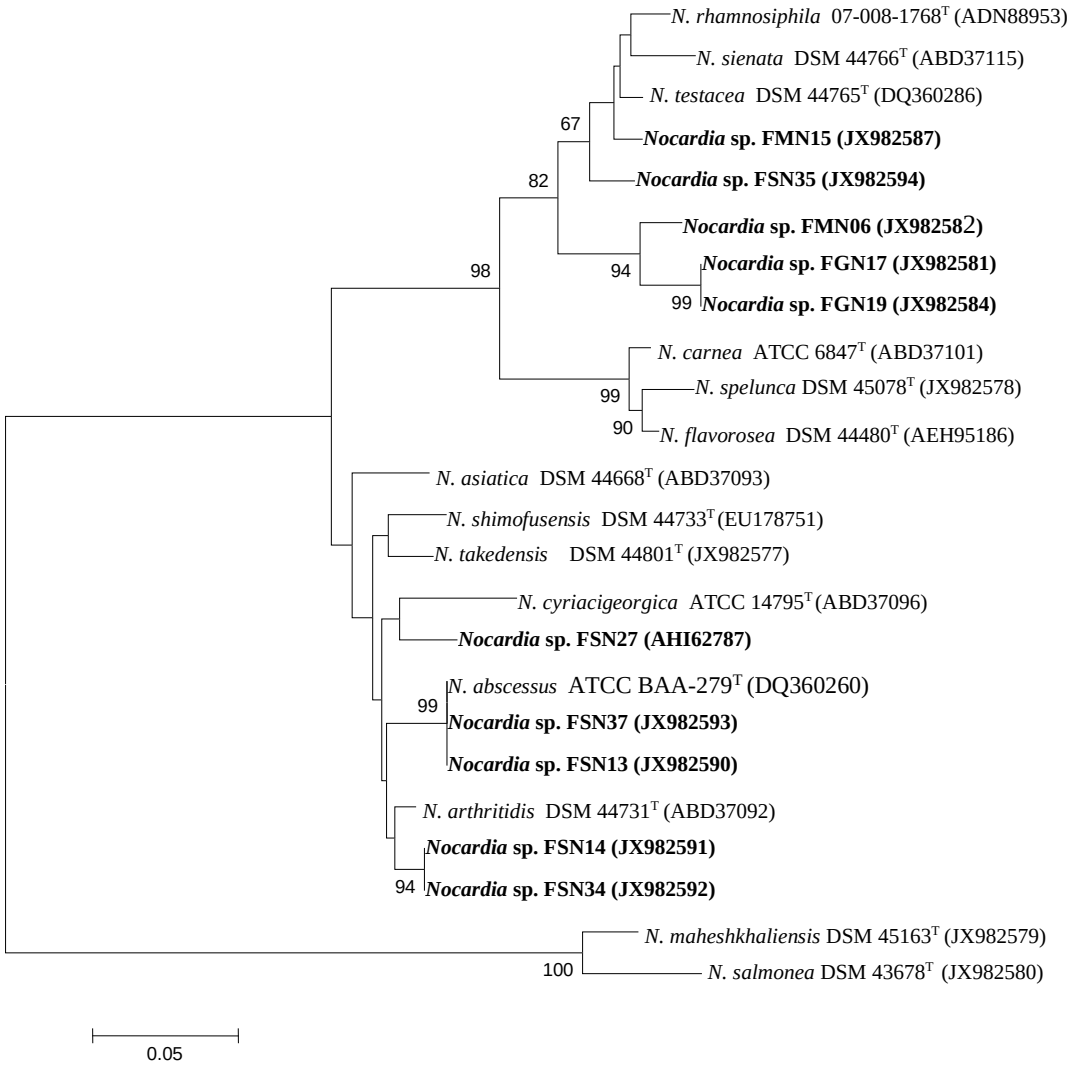
Supplementary Figure 1. Neighbour-joining tree based on the 16S rRNA sequences showing the relationships between test soil isolates and closely related type strains of the genus *Nocardia*. 16S rRNA GenBank accession numbers are given in parenthesis.



Supplementary Figure 2. Neighbour-joining treebased on the *gyrB* sequences showing the relationships between test soil isolates and closely related type strains of the genus *Nocardia*. *gyrB* GenBank accession numbers are given in parenthesis.



Supplementary Figure 3. Neighbour-joining tree based on the *rpoB* sequences showing the relationships between test soil isolates and closely related type strains of the genus *Nocardia*. *rpoB* GenBank accession numbers are given in parenthesis.



Supplementary Figure 4. Neighbour-joining tree based on the *secA1* sequences showing the relationships between test soil isolates and closely related type strains of the genus *Nocardia*. *secA1*GenBank accession numbers are given in parenthesis.

(Received for publication 28 February 2015; The date of publication 15 August 2015)