Turk J Biol
34 (2010) 355-365

doi:10.3906/biy-0903-7

Molecular fingerprinting of some clinically significant *Nocardia* and related strains by restriction polymorphism ribosomal RNA analyses

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Received: 10.03.2009

Abstract: Fifty-three *Nocardia* strains were the subject of a restriction polymorphic ribosomal RNA analysis (ribotyping) designed to distinguish between representatives of clinically significant species and related strains. The organisms were assigned to 19 groups using a combination of *Eco*RV gene restriction endonuclease patterns and a digoxigenin-labelled *Streptomyces violaceoruber* TK21 rDNA probe. Each ribotype group contained 4 to 13 restriction fragments that ranged in size from 20.7 to 0.9 kb. The *N. brasiliensis*, *N. crassostreae*, *N. farcinica*, *N. otitidiscaviarum*, and *N. seriolea* strains showed distinct ribotype patterns. Unique banding patterns were also seen for the type strains of *N. brevicatena*, *N. carnea*, *N. salmonicida*, *N. uniformis*, and *N. vaccinii*, and for the single representatives of *"N. fusca"*, *"N. pseudosporangifera"*, and *"N. violaceofusca"*. More than one banding pattern was detected for the *N. asteroides*, *N. flavorosea*, *N. nova*, *N. pseudobrasiliensis*, and *N. transvalensis* strains. The results are in line with current trends in nocardial systematics thereby indicating that restriction polymorphism ribosomal RNA analyses provide valuable data for the classification and identification of novel and pathogenic nocardiae at the species level.

Key words: *Nocardia,* ribotyping, taxonomy

Klinik önemli bazı *Nocardia* ve ilgili suşların restriksiyon polimorfik ribozomal RNA analiz yöntemiyle moleküler parmakizleri

Özet: Klinik açıdan önemli olan toplamda 53 *Nocardia* örneğini ve ilgili suşları birbirinden ayırt etmek için ribosomal tiplendirme (restriksiyon polimorfizim ribozomal analiz) yöntemi uygulandı. *Streptomyces violaceoruber* TK21 suşunun digoksigeninle işaretlenen rDNA probu ve *Eco*RV restriksiyon endonükleaz enziminin birlikte uygulanmasıyla, mikroorganizlar 19 ribotip olarak belirlendi. Herbir ribotip, 20,7 ile 0,9 kb ölçüm değerinde 4 ile 13 fragment içermektedir. *N. brasiliensis*, *N. crassostreae*, *N. farcinica*, *N. otitidiscaviarum* ve *N. seriolea* türleri ribotip bandları göstermiştir. Tek suşla temsil edilen *"N. fusca"*, *"N. pseudosporangifera"* ,*"N. violaceofusca"* örnekleri ve *N. brevicatena*, *N. carnea*, *N. salmonicida*, *N. uniformis* ve *N. vaccinii* tip suşları içinde tektip ribotip bandları gözlenmiştir. *N. asteroides*, *N. flavorosea*, *N. nova*, *N. pseudobrasiliensis* ve *N. transvalensis* örnekleri için birden fazla ribotip bandı belirlenmiştir. Sonuçlar güncel nokardial sistematikle uyum içinde olup, aynı zamanda restriksiyon polimorfik ribosomal RNA analiz yöntemi, yeni ve patojenik *Nocardia*'ların tür seviyesinde tanımlanma ve sınıflandırılmasında oldukça değerli veriler sağlamaktadır.

Anahtar sözcükler: *Nocardia*, ribosomal tiplendirme, taksonomi

Introduction

Members of the genus *Nocardia* belongs to the suborder *Corynebacterineae* (1), which also encompasses the genera *Corynebacterium*, *Dietzia*, *Gordonia*, *Millisia, Mycobacterium*, *Nocardia, Rhodococcus, Segniliparus*, *Smaragdicoccus*, *Skermania*, *Tsukamurella*, and *Williamsia* (2,3). Members of these taxa can be distinguished from one another using a combination of biochemical, chemical, and morphological criteria (4-6). The taxonomic status of most of the validly described species of *Nocardia* is supported by genotypic and phenotypic data (7-9), though improved procedures are needed for the identification of unknown nocardiae to the species level. It is important that nocardiae are correctly identified for biotechnological, clinical, and ecological purposes. Indeed, identification of clinically significant nocardiae to the species level is important for establishing the spectrum of diseases caused by members of each pathogenic species (8,9) and for predicting their antimicrobial susceptibility (6,10).

The standard phenotypic tests recommended for the identification of *Nocardia* species (4,5,11) are unsatisfactory as they only give presumptive identifications, but there is some evidence that molecular fingerprinting procedures may prove to be useful diagnostic tools (12-16). More recently, rapid and reliable typing techniques have been used to differentiate diverse and clinically significant bacteria (14,17). In particular, ribotyping has been used to distinguish between *Actinomyces* (18), *Corynebacterium* (19-21). *Mycobacterium* (22-24), *Rhodococcus* (25,26), *Streptomyces* (27,28), and *Tsukamurella* (29). The method has also been used to clarify taxonomic relationships between *Nocardia asteroides* complex and related species (13,30-33).

The present investigation was designed to evaluate ribotyping as a tool for the taxonomy and identification of clinically significant representatives of *Nocardia* and related strains.

Materials and methods

Strains, maintenance, and cultivation. The strain histories of the tested organisms are shown in the Table. All of the strains were maintained as glycerol suspensions (20%, v/v) at –20 °C. Single colonies of all but 2 of the organisms were grown on glucose-yeast extract agar plates (GYEA) (34) for 7 days at 30 °C and the resultant biomass used to inoculate 50 mL amounts of GYE broth in 100 mL conical flasks. The exceptions, the *N. brevicatena* and *N. crassostreae* strains, were grown on brain heart infusion agar plates (BHI) (35) at 25 °C for 15 days and single colonies used to inoculate 100 mL amounts of BHI broth in 100 mL conical flasks. All of the inoculated flasks were shaken at 150 revolutions per minute (rpm) at 25 or 30 °C, as appropriate, for 7 days and growth subcultured onto BHI or GYEA plates to check that the cultures were pure. Biomass was harvested by centrifugation at 10,000 rpm for 10 min, washed twice with sterile TE buffer (Tris – HCl, pH 8.0; 10 mM; EDTA, 1 mM) (36) and stored at –20 °C. Approximately 100 mg of wet weight biomass of each organism was used for DNA extraction.

Extraction and purification of DNA. The guanidine thiocyanate DNA extraction procedure of Pitcher et al. (37) was used to isolate DNA from each of the tested strains. Pretreatment of cells with proteinase K (100 μg mL^{-1}) and sodium dodecyl sulphate (SDS; 2%, w/v) greatly facilitated the susceptibility of cells to the extraction procedure.

Ribotyping of ribosomal RNA genes. Initially, 3 restriction enzymes, *EcoR* V, *Nco* I, and *Pst* I, were tested for ribotyping using 17 representative nocardiae. Genomic DNA (ca. 2-3 μg) was digested with *EcoR* V (C↓AATTC) restriction endonuclease using 10 units of enzyme per μg of DNA in a 20 μL volume reaction, as recommended by the manufacturer (New England Biolabs). Southern transfer and hybridisation were performed as outlined previously (38,39). The rDNA probe for ribotyping was labelled by random-primer labelling by using a DNA label commercial kit, according to the recommendations of the manufacturer (Boehringer Mannheim, Biochemica). A lambda *Hind* III digest (Boehringer) was applied twice in each gel to allow normalisation between the different electrophoresis runs.

Evaluation of ribotyping data. Membranes were scanned by using a Hewlett Packard PSC 1315 scanner. Numerical analyses of the banding patterns were compared using GelComparV4.0 software (Applied Maths). The similarities between each of the organisms was evaluated using the simple matching coefficient

(S_{SM}) from the X-TAXON program (40), and clustering achieved using the unweighted pair group method using the arithmetic averages algorithm (UPGMA) from the NTSys-pc program (41).

Results and discussion

DNA samples prepared from the test strains were checked to ensure the absence of extensive shearing and degradation prior to digestion with the industrial restriction enzymes and after electrophoresis of the genomic digests. All of the strains gave chromosomal DNA pure enough for ribotyping; the required amount of DNA needed for each test strain was obtained from about 100 mg of biomass.

The pilot study was carried out to evaluate the ribotype patterns obtained with 3 restriction endonuclease enzymes; that is, *Eco*R V, *Nco* I, and *Pst* I, were used to cut the genomic DNA of the 17 test strains. Ideally, ribotype patterns should consist of several discrete bands spread across gels. It can be seen from Figure 1 that *Eco*R V was the most suitable of the 3 enzymes as distinct hybridisation fragments were obtained for most of the test strains. This enzyme, which has 1 or 2 restriction sites in the16S rRNA gene of members of *Nocardia* species, was chosen to examine all 53 strains. In contrast, DNA extracted from the strains digested with *Nco* I and *Pst* I was either too little to be separated into discrete bands or showed incomplete or no digestion.

The digoxigenin-labelled rDNA probe, which contained fragments of the 5S, 16S, and 23S rRNA genes of *Streptomyces* (*lividans*) *violaceoruber* strain TK21, hybridised with 4 to 8 fragments of the *EcoR* V-cleaved chromosomal DNA of the test strains (Figure 1). The hybridised fragments, which ranged from around 20.7 to 0.9 kb in size, were well resolved and found to give the same patterns in the reproducibility studies. Each unique ribosomal DNA restriction profile was designated a ribotype.

The representative strains from the remaining validly described species, namely *N. asteroides*, *N. flavorosea*, *N. nova*, *N. pseudobrasiliensis*, and *N. transvalensis*, gave more than one banding pattern (Figures 2-4), though it is evident from the

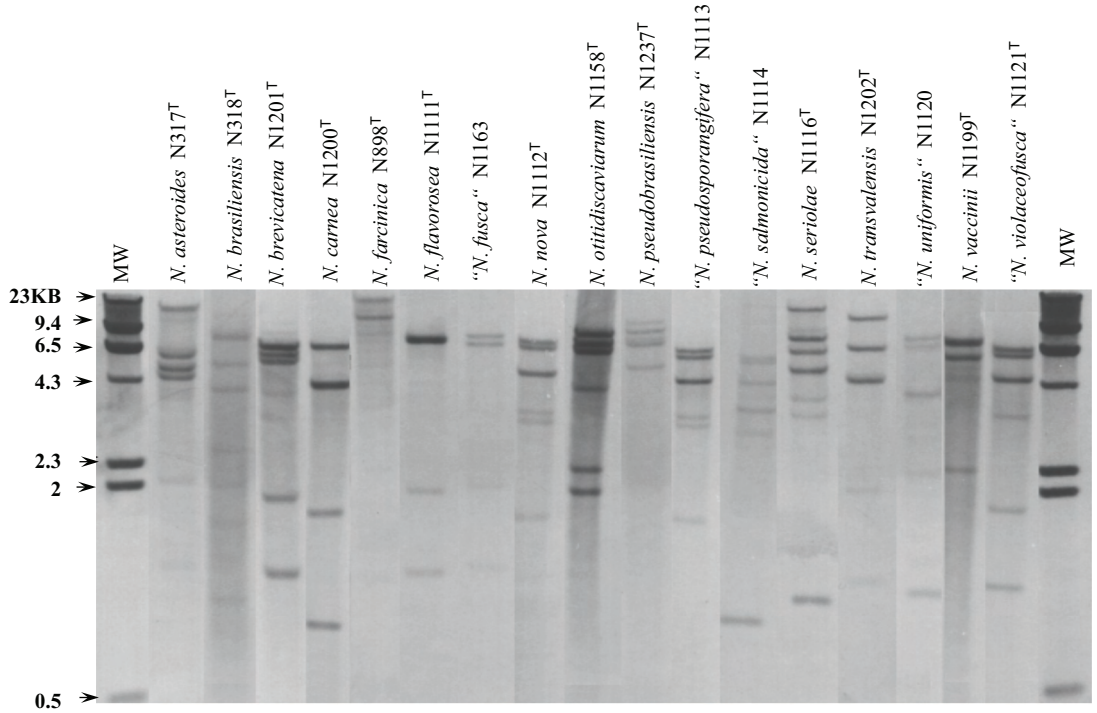

Figure 1. Ribotype patterns of representative nocardiae. The patterns were obtained by electrophoretic separation of *Eco*RV digests of genomic DNA and hybridisation with the digoxigenin-labelled rDNA probe (colorimetric detection). MW, the molecular weight marker was lambda DNA cleaved with *Hind* III.

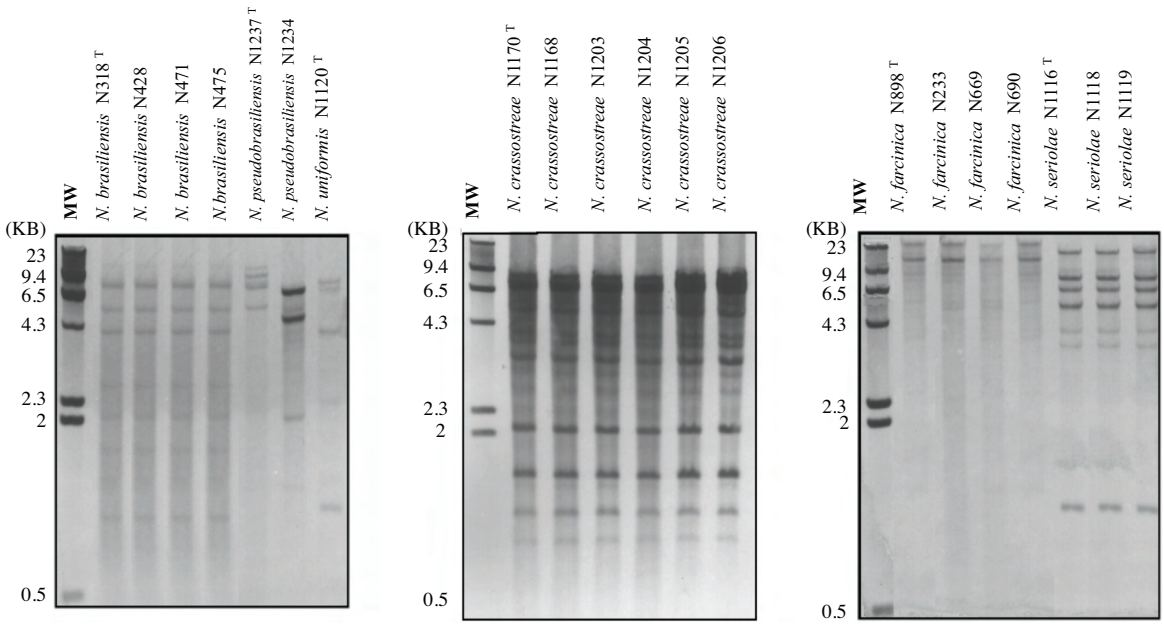

Figure 2. Ribotype patterns of representative strains of *N. brasiliensis, N. crassostreae, N. farcinica, N. pseudobrasiliensis, N. seriolae*, and *N. uniformis*. The patterns were obtained following electrophoretic separation of *EcoRV* digests of genomic DNA and hybridisation with a digoxigenin-labelled probe (colorimetric detection). MW, the molecular weight marker was lambda DNA cleaved with *HindIII*.

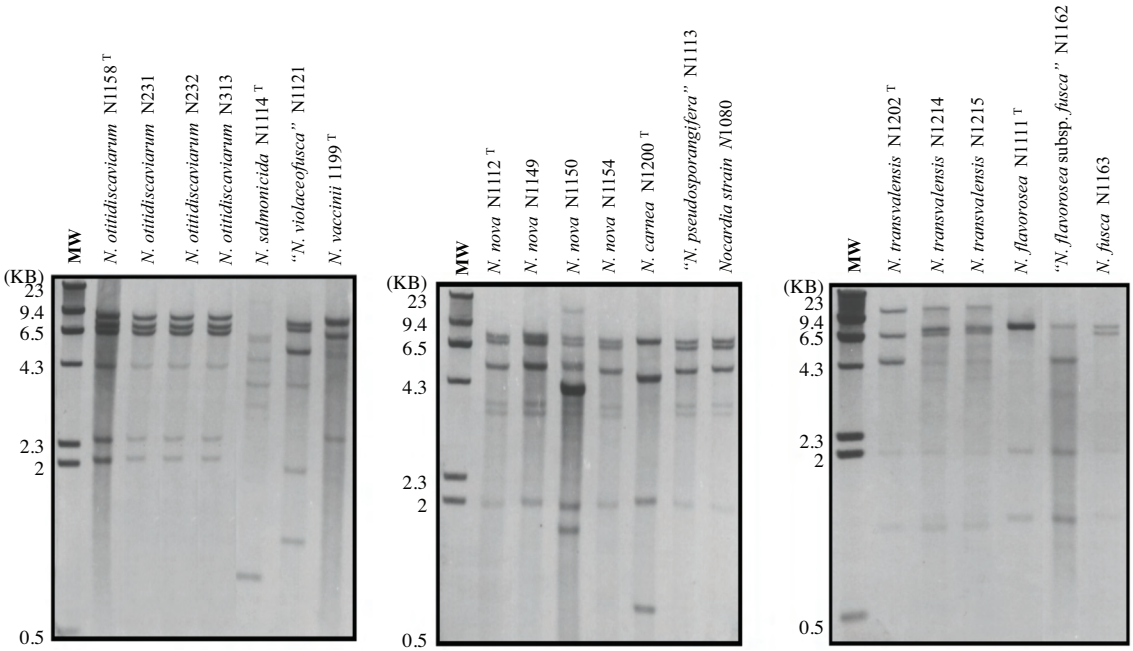

Figure 3. Ribotype patterns of representative strains of *N. carnea, N. flavorosea, "N. flavorosea* subsp. *fusca"*, "*N. fusca", N. nova, N. otitidiscaviarum, "N. pseudosporangifera", N. salmonicida, N. transvalensis, N. vaccinii, "N. violaceofusca",* and *Nocardia* strain N1080. The patterns were obtained following electrophoretic separation of *EcoRV* digests of genomic DNA and hybridisation with a digoxigenin-labelled probe (colorimetric detection). MW, the molecular weight marker was lambda DNA cleaved with *Hind III*.

dendrogram that the *N. nova* and *N. transvalensis* strains formed distinct, homogeneous groups (Figure 5). It can also be seen from the dendrogram that the *N. asteroides* strains form a heterogeneous group with the type strain forming a distinct ribotype pattern, a result in line with previous studies (13,30-33).

The results obtained for the *N. asteroides* strains are encouraging as they are in agreement with a host of other studies showing that *N. asteroides* is a markedly heterogeneous species (4,5,42). Indeed, the *N. asteroides* strains included in the present study were drawn from a number of clusters delineated in numerical taxonomic surveys (Table). The congruence between the molecular fingerprinting and the numerical phenetic data indicates that the restriction polymorphism ribosomal RNA analyses procedure might be used to help unravel the taxonomy *N. asteroides* and closely related strains. Similarly, further fingerprinting studies on additional strains of *N. flavorosea* and *N. pseudobrasiliensis* are needed to establish the extent of the taxonomic variation encompassed by these taxa.

"*Nocardia fusca*" (43), "*N. pseudosporangifera*" (44), and "*N. violaceofusca*" (45) have been described but not validated and hence do not have any nomenclatural standing (Figure 3). Nevertheless, it was interesting that the representatives of these taxa formed distinct banding patterns, as did the 2 organisms labelled "*N. pseudosporangifera*" and *Nocardia* sp. N1080 (Figure 3). Further comparative studies are needed to establish the taxonomic status of these organisms, especially since the "*N. pseudosporangifera*" strain has an almost identical 16S rDNA sequence with the type strain of *N. nova* (4). "*Nocardia fusca*" strain N1163 and "*N. flavorosea* subsp. *fusca*" strain N1162 also show a close phylogenetic relationship to one another and to a lesser extent with the type strain of *N. carnea* (Figure 3). "*Nocardia violaceofusea*" strain N1121 formed a distinct cluster with a *Nocardia* strain in a numerical taxonomic study that included nearly all of the organisms included in the present investigation (4).

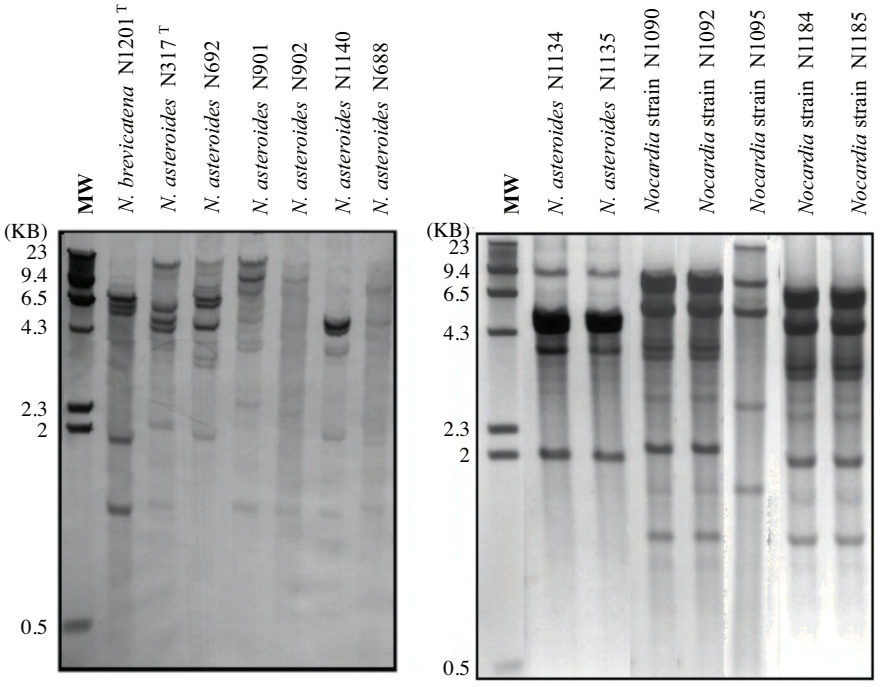

Figure 4. Ribotype patterns of representative strains of *Nocardia asteroides, Nocardia* strains, and *Nocardia brevicatena*. The patterns were obtained following electrophoretic separation of *EcoRV* digests of genomic DNA and hybridisation with a digoxigenin-labelled probe (colorimetric detection). MW, the molecular weight marker was lambda DNA cleaved with *HindIII*.

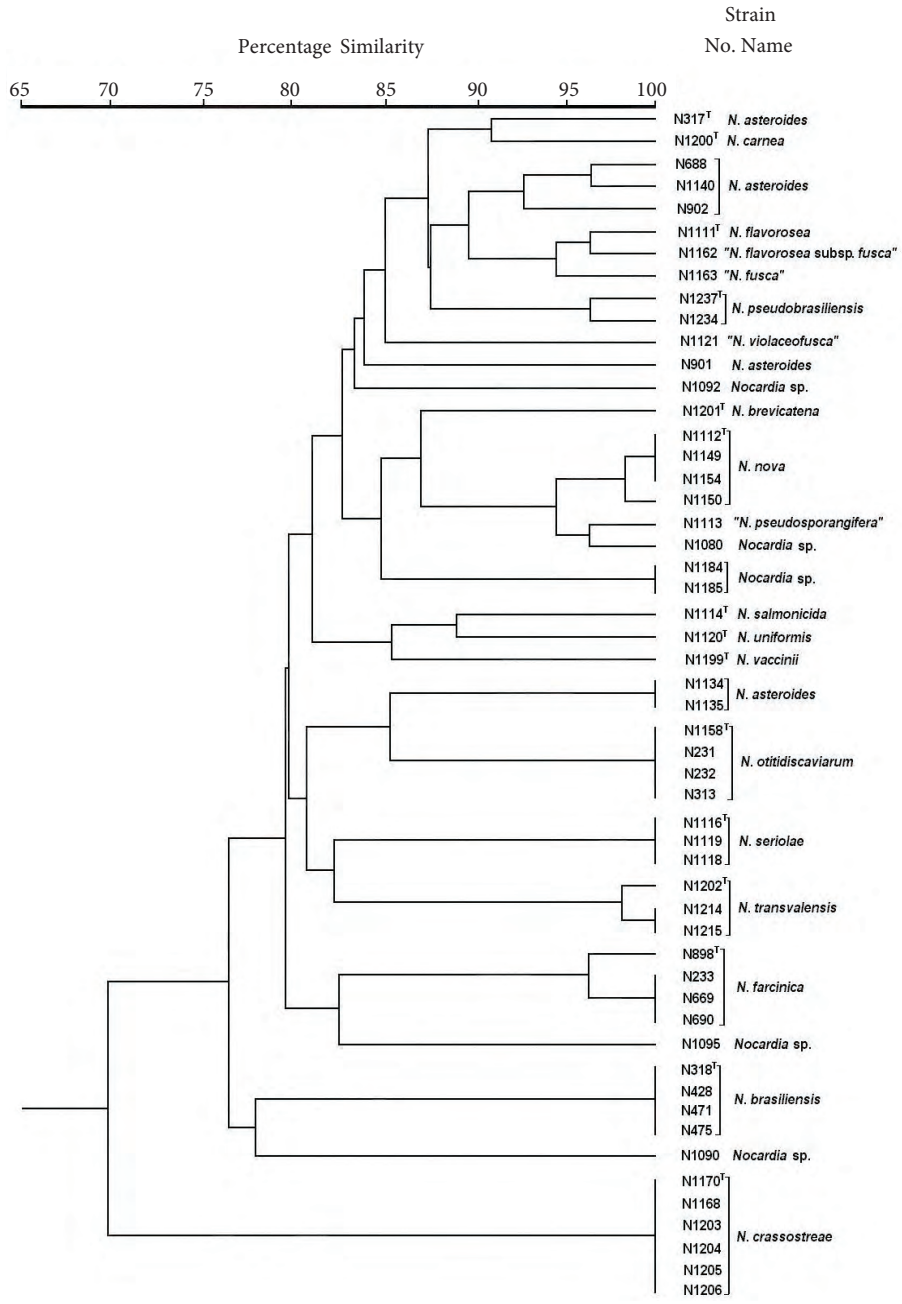

Figure 5. Dendrogram showing relationships between *Nocardia* strains based on rDNA ribotype data. The similarity between the strains was calculated by comparing the bands derived from the electrophoretic DNA profiles using the similarity coefficient (S_{SM}) with clustering achieved using the unweighted pair group method with arithmetic averages (UPGMA). ^{T}, Type strains.

Table. Sources and histories of the test strains.

Laboratory number	Species	Source
N 317^{T}	*N. asteroides*	Gordon RE, Rutgers University, New Brunswick, USA., IMRU 727; L. Ajello, M 170-6; W.M. Bowman, PSA 165 [Goodfellow (1971), subgroup 1D; Orchard & Goodfellow (1980), subcluster 1A; Hookey (1983), cluster 27 (*N. asteroides*)]
N 688	*N. asteroides*	Schaal KP, Institute of Medical Microbiology and Immunology, University of Bonn, Bonn, Germany, N2; (strain Gelsenkirchen), clinical isolate [Schaal & Reutersberg (1978), cluster III A_1; Orchard & Goodfellow (1980), cluster 5; Hookey (1983), cluster 29 (*N. asteroides*)]
N 692	*N. asteroides*	Schaal KP, N23, strain Copenhagen; H. Mordarska, [Schaal & Reutersberg (1978), cluster $IIIA_2$; Orchard & Goodfellow (1980), cluster 9; Hookey (1983), cluster 32 (*N. asteroides*)]
N 901	*N. asteroides*	Stanford JL, N37; pulmonary nocardiosis [Orchard & Goodfellow (1980), subcluster 8A; Hookey (1983), cluster 28 (*N. farcinica*)]
N 902	*N. asteroides*	Stanford JL, N38, pulmonary nocardiosis [Hookey (1983), cluster 28]
N 1134	*N. asteroides*	Schaal KP, N11 [Schaal & Reutersberg (1978), subgroup III A_1]
N 1135	*N. asteroides*	Schaal KP, N19; S.A. Waksman; ATCC 3306
N 1140	*N. asteroides*	Schaal KP, N129 [Schaal & Reutersberg (1978), subgroup III A_1] soil isolate
N 318^{T}	***N. brasiliensis***	Gordon RE, IMRU 845; Schneidau JD, Jr 381; Batista A, 631; IP 337 [Goodfellow (1971), cluster 5; Orchard & Goodfellow (1980), subcluster 4B; Hookey (1983), cluster 33 (*N. brasiliensis*)]
N 428	*N. brasiliensis*	Gordon RE, IMRU 1336; Lechevalier MP, L-36 (*Nocardia* sp.), soil
N 471	*N. brasiliensis*	González-Ochoa A, Instituto de Salubridad y Enfermedades Tropicales, Mexico; 4115; mycetoma, lower leg
N 475	*N. brasiliensis*	González-Ochoa A, 4023; mycetoma, forearm
N 1201^{T}	***N. brevicatena***	DSM 43024; Lechevalier HA; sputum
N 1200^{T}	***N. carnea***	DSM 43397; Gordon RE, IMRU 3419
N 1170^{T}	***N. crassostreae***	Friedman CS, Bodega Marine Laboratory, Bodega Bay, California, U.S.A., NB4H; nocardiosis in *Crassostrea gigas* (ATCC 700418)
N 1168	*N. crassostreae*	Friedman CS, CB 29; nocardiosis in *Crassostrea gigas*
N 1203	*N. crassostreae*	Friedman CS, RB1; nocardiosis in *Crassostrea gigas*
N 1204	*N. crassostreae*	Friedman CS, OB5; nocardiosis in *Crassostrea gigas*
N 1205	*N. crassostreae*	Friedman CS, RB29; nocardiosis in *Crassostrea gigas*
N 1206	*N. crassostreae*	Friedman CS, OB3P; nocardiosis in *Crassostrea gigas*
N 898^{T}	***N. farcinica***	Tsukamura M, Chubu Chest Hospital, Obu, Aichi-chen 474, Japan, 23102 (R-3318); ATCC 3318; R.E. Gordon [Orchard & Goodfellow (1980), subcluster 1A; Hookey (1983), cluster 28 (*N. farcinica*)]

Table. (Continued).

Laboratory number	Species	Source
N 233	*N. farcinica*	Olds RJ, Department of Pathology, University of Cambridge, Cambridge, UK, CN 470; *(N. asteroides)*; cow's milk [Goodfellow (1971), subgroup 1B; Orchard & Goodfellow (1980), cluster 11)
N 669	*N. farcinica*	Bradley SG, MAC 300 [Orchard & Goodfellow (1980), cluster 9 (*N. asteroides*); Hookey (1983), cluster 28 (*N. farcinica*)]
N 690	*N. farcinica*	Schaal KP, N5; strain Karlsruhe [Orchard & Goodfellow (1980), cluster 11]
N 1111^{T}	***N. flavorosea***	JCM 3332; KCC A0332; J. Ruan 10.268-1 soil, Yunnan Province, People's Republic of China. (IFO 14341= NRRLB-16176)
N 1162	*"N. flavorosea* subsp. *fusca"*	Liu ZH, Institute of Microbiology, Academia Sinica, Beijing, Yunnan Province, People's Republic of China, IFO 14342; soil
N 1163	*"N. fusca"*	Liu ZH, IFO 14340; soil
N 1112^{T}	***N. nova***	JCM 6044; Tsukamura M. 23095; Gordon RE, R 443; I.B. Christison; NF, Conant 2338 (ATCC 33726)
N 1149	*N. nova*	Schaal KP, N395; Tsukamura M, 23006, M93, N.M. McClung; I. Uesaka
N 1150	*N. nova*	Schaal KP, N396; Tsukamura M, 23019, (M78); N. M. McClung; I. Uesaka
N 1154	*N. nova*	Schaal KP, N400; Tsukamura M, 23096, 443(2), R.E.Gordon
N 1158^{T}	***N. otitidiscaviarum***	NCTC 19349; ATCC 14629; Gordon RE; infected middle ear of guinea pig
N 231	*N. otitidiscaviarum*	Olds RJ, CN 749; isolated from a dachshund (Schaal KP, N 206)
N 232	*N. otitidiscaviarum*	Olds RJ, CN 751, isolated from a corgi (Schaal KP, N 207)
N 313	*N. otitidiscaviarum*	I.P 751: Schaal KP, N 208
N 1237^{T}	***N. pseudobrasiliensis***	Boiron P, Unite de Mycologie, Institut Pasteur, Paris, France, CIP 104600; leg abscess (ATCC 51512)
N 1234	*N. pseudobrasiliensis*	Boiron P, N 249; brain abscess
N 1113	***"N. pseudosporangifera"***	JCM 3288; KCC A-0288; IAM 0501; Seto N and Izuka H 2-40; soil, Nishiyama oil field, Niigata, Japan (Putative type strain).
N 1114^{T}	***N. salmonicida***	JCM 4826; KCC S-0826; IFO 13393; ISP 5472; Lederle Laboratory. A-7604; Rucker R, ATCC 27463; blueback salmon (*Oncorhynchus nerka*) (putative type strain).
N 1116^{T}	***N. seriolae***	JCM 3360; K. Hatai; NA 8191; spleen of a yellowtail (*Seriola quinqueradiata*), Nagasaki, Japan
N 1118	*N. seriolae*	JCM 5849; Hatai K, NA 8231; spleen of a yellowtail (*Seriola quinqueradiata*), Nagasaki, Japan
N 1119	*N. seriolae*	JCM 5850; Hatai K, N.Matsumoto KRN 8403; kidney of a japanese flounder (*Paralichthys olivaceus*), Kagawa, Japan

Table. (Continued).

Laboratory number	Species	Source
N 1202[T]	***N. transvalensis***	DSM 43405; Gordon RE, IMRU 3426; mycetoma pedis
N 1214	*N. transvalensis*	Poonwan N, 34-104-3 (10-7-91); pus from eye
N 1215	*N. transvalensis*	Poonwan N,34-43-6 (16-1-91); bronchial washing
N 1120[T]	***N. uniformis***	JCM 3224; KCC a-0224; DSM 43136; Ettlinger L, A-3004; ETH 26650; I. Szabo; salty soil (putative type strain).
N 1199[T]	***N. vaccinii***	DSM 43285; Kieslich K, Schering 245; ATCC 111092; stem galls on blueberry
N 1121	***"N. violaceofusca"***	JCM 3343; Ruan JS, 78-N26; soil, People's Republic of China (putative type strain).
N 1080	*Nocardia* sp.	Beaman BL, UC-67; Department of Microbiology, University of California, Davis, U.S.A.
N 1090	*Nocardia* sp.	Beaman BL, UC-83
N 1092	*Nocardia* sp.	Beaman BL, UC-85
N 1095	*Nocardia* sp.	Beaman BL, UC-89
N 1184	*Nocardia* sp.	Beaman BL, UC-103
N 1185	*Nocardia* sp.	Beaman BL, UC-109

[T],Type strains. Abbreviations: **ATCC**, American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA; **CIP**, Collection National de Culture de Microorganisms, Paris, France; **DSM**, Deutsche Sammlung von Microorganismen und Zellkulturen, Mascheroder Weg 1b, D-38124 Braunschweig, Germany; **IFO**, Institute for Fermentation, Osaka, Japan; **IMRU**, Institute of Microbiology, Rutgers State University, New Brunswick, NJ, USA.; **IP**, Institut Pasteur, Rue du Dr. Roux, Paris, France; **JCM**, Japan Collection of Microorganisms, Saitama, Japan; **NCIMB**, National Collection of Industrial and Marine Bacteria, St. Machar Drive, Aberdeen, Scotland, UK; **NCTC**, National Collection of Type Cultures, Central Public Health Laboratory, London, UK; and **UQM**, Center for Bacterial Diversity and Identification, University of Queensland, Brisbane, Australia.

The ribotype patterns obtained for the representative test strains are in good agreement with corresponding albeit preliminary results from earlier studies that suggested that ribotyping might be of value in the classification and identification of nocardiae (13,30-33). Similar conclusions have been drawn from studies on members of other actinomycete taxa, including representatives of the genera *Corynebacterium* (19-21,46), *Mycobacterium* (22-24), *Rhodococcus* (25,26), *Streptomyces* (27,28), and *Tsukamurella* (29).

It can be concluded from the results of this study that ribotyping provides a reliable and accurate way of distinguishing between representatives of clinically significant species of *Nocardia* and related strains.

Acknowledgements

Dr. Kamil IŞIK is grateful to the University of Ondokuz Mayıs, Samsun, and the Council of Higher Education of Turkey for supporting him with a scholarship.

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