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Identification and Evaluation of Clinically Significant Nocardia brasiliensis, Nocardia farcinica and Nocardia otitidiscaviarum Strains Using Pyrolysis Mass Spectrometry (PyMS)

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Abstract: Out of a total of thirty-nine *Nocardia* strains, eight species of *N. brasiliensis*, seventeen species of *N. farcinica* and fourteen species of *N. otitidiscaviarum* were identified using Pyrolysis mass spectrometry. N428, N477 *N. brasiliensis*; N669, N233 *N. farcinica*; and N231, N232 *N. otitidiscaviarum* duplicated strains were clustered in their own groups. Strains belonging to *Nocardia brasiliensis, Nocardia farcinica* and *Nocardia otitidiscaviarum* formed distinct pyrogroups corresponding to clusters defined in the numerical study. It can be concluded that PyMS provides a rapid and reproducible method of evaluating the taxonomic significance of *Nocadia* species.

Key Words: *Nocardia,* PyMS, Taxonomy

Klinik Önemi Olan *Nocardia brasiliensis, Nocardia farcinica* ve *Nocardia otitidiscaviarum*'un Pirolizis Kütle Spektrometrisi Kullanılmasıyla İdentifikasyonu ve Değerlendirilmesi

Özet: Sekiz *Nocardia brasiliensis*, onyedi *Nocardia farcinica* ve ondört *Nocardia otitidiscaviarum* olmak üzere toplam otuzdokuz *Nocardia* suşu Pirolizis kütle spektrometri metodu kullanılarak tanımlandı. *Nocardia brasiliensis*'den N428, N477; *Nocardia farcinica*'dan N669, N223 ve *Nocardia otitidiscaviarum*'dan N231 ve N232 nolu test edilen duplikat suşlar ilgili gruplarıyla kümelendiler. *Nocardia brasiliensis, Nocardia farcinica* ve *Nocardia otitidiscaviarum* ait suşlar, numerik çalışmada belirlenen kümelere benzer, açık bir şekilde pyrogroup oluşturdular. Buradan *Nocardia* örneklerinin taksonomik önemini değerlendirmede PyMS'in hızlı ve üretken bir teknik olduğu neticesine varılmaktadır.

Anahtar Sözcükler: *Nocardia,* PyMS, Taksonomi

Introduction

Nocardia are aerobic, catalase-positive actinomycetes that form extensively branched rudimentary, substrate hyphea often fragmented *in situ* or formed into a rod-like to coccoid shape with the effect of mechanical disruption and non-motile elements. Members of the genus have a peptidoglycan composed of N-acetylglucosamine, D-alanine, L-alanine and D-glutamic acid with *meso* A_2pm as the diamino acid, muramic acid in the N-glycolated form, fatty acid profiles showing major amounts of straight chain, unsaturated and tuberculostic acids (1) and mycolic acids with 40 to 60 carbons and up to three double bonds (2). *Nocardiae* are also responsible for a wide range of animal diseases (3). *Nocardia asteroides* is the most common causal agent, followed by *Nocardia farcinica*, *Nocardia brasiliensis* and *Nocardia otitidiscaviarum* (4). These may cause abortion, mycetoma, as well as pulmonary and systemic nocardiosis (5).

Numerous taxonomic studies have established the heterogeneity of *Nocardia* through a variety of methods, including numerical taxonomy (6, 7), DNA relatedness (8), phage sensitivity (9), immunology (10), antimicrobial susceptibility (11) and molecular fingerprinting (12, 13).

There is a need for an extensive polyphasic taxonomy study of the genus *Nocardia* to determine the relationship between newer species and the more established taxa. An improved classification of the genus should help highlight characters that might be used in the identification of clinically significant *Nocardiae*.

Materials and Methods

Test strains: Thirty-nine representatives of *N. brasiliensis*, *N. farcinica* and *N. otitidiscaviarum* were examined. The list and histories of test strains used in this study are in Table 1. Six duplicate samples from N428, N477 (*N. brasiliensis*); N669, N233 (*N. farcinica*); and N231, N232 (*N. otitidiscaviarum*) were used to determination the reproducibility of the system. All of the strains were maintained as frozen glycerol stock solution.

Growth conditions: Glycerol stock cultures were used to inoculate sterile polyvinyl membrane filters (0.45µm, 47 mm diameter; Millipore) placed over dried GYEA (Glucose Yeast Extract Agar) plates (14). Duplicated preparations were incubated for 5 days at 30 °C and the growth obtained was used to inoculate a further set of plates which were incubated under the same conditions.

Pyrolysis mass spectrometry: Pyrolysis foils and tubes (Horizon Instruments Ltd.) were put in acetone and then dried overnight. Single foils were inserted with flamed forceps or sterile plastic loops into pyrolysis tubes so that the foil protruded about 6 mm from the mouth. For each strain, small amounts of biomass (*ca.* 50 µg wet weight) were scraped from the growth on the Millipore filters and uniformly smeared onto the protruding foils. The inoculated foils were placed in an 80 °C oven for 15 minutes to dry the biomass. For analysis, the foils were tamped into tubes with a flamed stainless steel tool (gagua) to 10 mm from the mouth. Viton

Table 1. Source and histories of the test strains examined using pyrolysis mass spectrometry.

Lab. number	Species	Source
N 318^T	***N.brasiliensis***	R.E. Gordon, IMRU 845; J.D. Schneidau Jr.,381; A. Batista,631; IP 337 [Goodfellow (1971), cluster 5; Orchard & Goodfellow (1980), subcluster 4B; Hookey (1983), cluster 33 (*N.brasiliensis*)]
N 1159^T	***N.brasiliensis***	NCTC 11294; ATCC 19296
N 14	*N.brasiliensis*	R.E. Gordon, IMRU 744; A. Gonzáles-Ochoa, 409; ATCC 19295; NCTC 10300 [Goodfellow (1971) cluster 5; Hookey (1983) single member cluster]
N 428 ^{d}	*N.brasiliensis*	R.E. Gordon, IMRU 1336; M.P. Lechevalier,L-36 (*Nocardia* sp.), soil
N 467	*N.brasiliensis*	M. Magnusson, Farcha Laboratory, Fort Lamy, Chad, IP 708; bovine farcy
N 474	*N.brasiliensis*	A Gonzáles-Ochoa, 4212; mycetoma, ankle
N 477 ^{d}	*N.brasiliensis*	A. Gonzáles-Ochoa, 4204; mycetoma heel; K.P. Schaal, N219
N 1148	*N.brasiliensis*	K.P. Schaal, N224; M. Goodfellow, N530; L. Ajello 45-247-71
N 898^T	***N.farcinica***	M. Tsukamura, 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*)]
N 233 ^{d}	*N.farcinica*	R.J. Olds, Department of Pathology, University of Cambridge, CN 470; *N.asteroides*; cow's milk [(Goodfellow (1971), subgroup 1B; Orchard & Goodfellow (1980), cluster 11)]
N 669 ^{d}	*N.farcinica*	S.G. Bradley, MAC 300 [Orchard & Goodfellow (1980), cluster 9 (*N.asteroides*); Hookey (1983), cluster 28 (*N.farcinica*)]
N671	*N.farcinica*	S.G. Bradley, VAC 330; M. Tsukamura, E 7549; K. P. Schaal, N202; ATCC 3318 [Orchard & Goodfellow (1980), cluster 11]
N 687	*N.farcinica*	K.P. Schaal, N; strain Berlin [Orchard & Goodfellow (1980), cluster 11; Hookey (1983), cluster 28]
N 690	*N.farcinica*	K.P. Schaal, N5; strain Karlsruhe [Orchard & Goodfellow (1980), cluster 11]
N 701	*N.farcinica*	M. Ridell, Institute of Medical Microbiology, University of Gothenburg, Sweden, N67; M. Magnusson, 753 (*N.asteroides* ATCC 3399) [Orchard & Goodfellow (1980), cluster 11]
N 702	*N.farcinica*	M. Ridell, N74; M. Magnusson, 751; *N.farcinica* IP 744; bovine farcy, Farcha Laboratory, Fort Lamy, Chad [Orchard & Goodfellow (1980), cluster 11]
N 703	*N.farcinica*	M. Ridell, N76; M. Magnusson, 750; *N.farcinica*,IP 740; bovine farcy, Farcha Laboratory, Fort Lamy, Chad [Orchard & Goodfellow (1980), cluster 11]
N 704	*N.farcinica*	M. Ridell, N118; M. Magnusson, 645; ATCC 6864; NCTC 1935; rabbit in Sumatra [Orchard & Goodfellow (1980), cluster 11]
N 705	*N.farcinica*	M. Ridell, N119; M. Magnusson, 884; isolated from a case of nocardiosis in Sweden [Orchard & Goodfellow (1980), cluster 11]
N 707	*N.farcinica*	M. Ridell, N125; M. Magnusson, 878 [Orchard & Goodfellow (1980), cluster 11]
N 894	*N.farcinica*	M. Tsukamura, M 205 (23087); I Uesaka; N.M. McClung; S.McMillan; K.P. Schaal, N 62, [Hookey (1983), cluster 28]
N 896	*N.farcinica*	M. Tsukamura, R 784, 23098; R.E. Gordon; J. Lacey, A6 [Hookey (1983), cluster 28 (*N.farcinica*)]
N 899	*N.farcinica*	M. Tsukamura, M-300; I. Uesaka; N. M. McClung [Hookey (1983), cluster 28 (*N.farcinica*)]
N 906	*N.farcinica*	K.P. Schaal, N57 [Streptomyces sp.]; ATCC 1474 [Hookey (1983) , cluster 28 (*N.farcinica*)]
N 1136	*N.farcinica*	K.P. Schaal, subgroup B; S.A. Waksman, *N.asteroides*; ATCC 3308
N 1158^T	***N.otitidiscaviarum***	NCTC 19349; ATCC 14629; R.E.Gordon; infected middle ear of guinea pig
N 231	*N.otitidiscaviarum*	R.J. Olds, CN 749; isolated from a dachshund (K.P. Schaal, N 206)
N 232	*N.otitidiscaviarum*	R.J. Olds, CN 751, isolated from a corgi (K.P. Schaal, N 207)
N 313	*N.otitidiscaviarum*	I.P 751; K.P. Schaal, N 208
N 314	*N.otitidiscaviarum*	I.P 771; K.P. Schaal, N 209
N 430	*N.otitidiscaviarum*	R.E. Gordon, IMRU 1370; lung of dog
N 432	*N.otitidiscaviarum*	R.E. Gordon, IMRU 416
N 940	*N.otitidiscaviarum*	M. Ridell, N55; R.E. Bönicke, SN 5602
N 941	*N.otitidiscaviarum*	M. Ridell, N89; R.E. Gordon, 416
N 942	*N.otitidiscaviarum*	M. Ridell, N90; R.E. Gordon, 424
N 943	*N.otitidiscaviarum*	M. Ridell, N91; R.E. Gordon, 737
N 944	*N.otitidiscaviarum*	M. Ridell, N92; R.E. Gordon, 1316
N 945	*N.otitidiscaviarum*	M. Ridell, N93; R.E. Gordon, 1370; SET
N 1142	*N.otitidiscaviarum*	K.P. Schaal, N210; M. Goodfellow, N369; Tsukamura R-1315

^{T}, Type strains.; ^{d}, duplicate strains. Abbreviations: **ATCC**, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA; **IMRU**, Institute of Microbiology, Rutgers State University, New Brunswick, N. J., USA.; **IP**, Institut Pasteur, Rue du Dr. Roux, Paris, France; and **NCTC**, National Collection of Type Cultures, Central Public Health Laboratory, London, UK.

O-rings were placed on the tubes which were then loaded onto the pyrolysis mass spectrometry carousel in batches of 150. The samples were prepared in triplicate.

Pyrolysis was carried out using a Horizon Instruments PyMS 400X mass spectrometer (15). Prior to the analysis, the inlet heater was set at 160 °C and the heated tube loader was set at 120 °C. The assembled tubes were loaded sequentially into the pyrolysis chamber by a robotic arm. Curie-point pyrolysis was carried out at 530 °C for 2.4 seconds under vacuum; the pyrolysis rise time was 0.6 s. The volatile pyrolysis products were ionised by collision with a crossing beam of low-energy (20 eV) electrons and the ions separated in a quadruple mass spectrometer that scanned the pyrolysate at 0.35-second intervals. Integrated ion counts at unit mass intervals from 11 to 400 were recorded on hard disc together with the pyrolysis sequence number and total ion count for each sample.

Data analysis: The raw data was processed and analysed using the PYMENU program (Horizon Instruments Ltd.) and the GENSTAT V statistical package (16), which were run on an IBM PC. Normalised data sets were analysed by principal components analysis. Plots of the first two or three principal components were produced as plots of the spectral scores, the positions of the pyrolysis spectra on the principal component axes. A plot of the mass loadings for the axes provided information about the contribution of masses to the principal component axes. Canonical variate analyses of all of the principal components accounting for more than 0.5 % of the total variance was carried out to give a combined principal component-canonical variate analysis (PC-CVA). The data from PC-CVA were plotted as Mahalonobis distances. The Mahalonobis (17) distance matrix was standardised by dividing the maximum inter-group distance. It was treated as an ordinary Euclidean distance and then converted to a similarity matrix (18). The values in the similarity matrix were examined using unweighted pair grouping method (UPGMA) with an arithmetic averages algorithm (19).

Results and Discussion

Eight *Nocardia brasiliensis*, seventeen *Nocardia farcinica* and fourteen *Nocardia otitidiscaviarum* strains, including duplicated cultures, were analysed using Curie-point pyrolysis mass spectrometry. Excellent agreement was found between the results of the triplicate analyses of all of the test strains. It was also encouraging that most of the duplicated cultures were grouped adjacent to one another, with the exception of *Nocardia farcinica* strain N669 (Figure 1).

Some typical pyrolysis mass spectra are shown in Figure 2. It is evident from these representative data that most of the spectra fall within the mass ranging from 51 to 140 (m/z) with only very low intensity ions found above this mass range. It is also clear from these pyrograms that the spectra are qualitatively similar.

The representative *Nocardia brasiliensis, Nocardia otitidiscaviarum* and *Nocardia farcinica* strains were sharply distinguished from one another with the exception of *Nocardia farcinica*

strain N701, which was observed separate from but adjacent to *Nocardia otitidiscaviarum* (Figure 1). The same relationships were seen when the data were presented in a three-

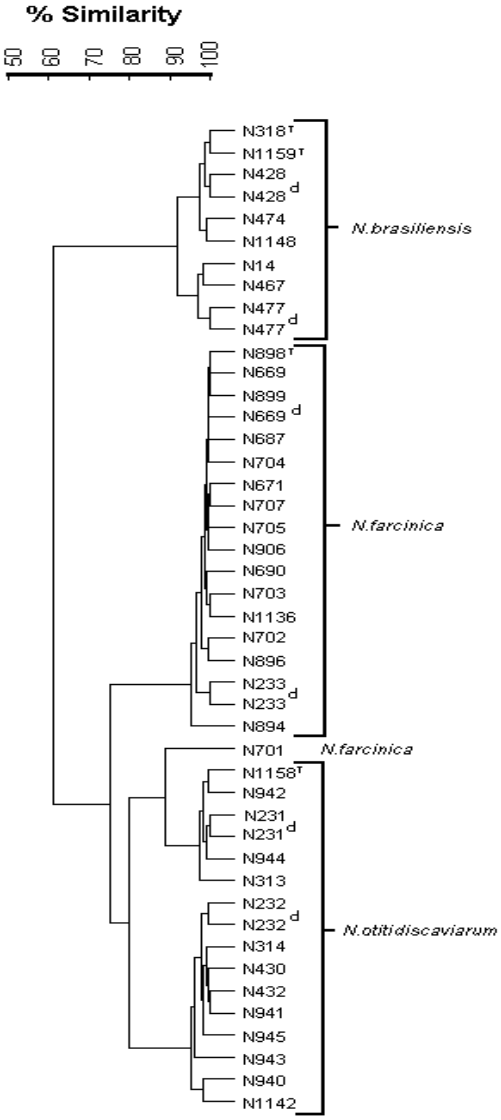

Figure 1. Dendrogram representing relationships found between representatives of some *Nocardia* species are based on pyrolysis mass spectral data. The dendrogram is based on similarity values derived from Mahalanobis distances and the clustering ahcieved using the unweighted pair group method with arithmetic averages algorithm.
[d], duplicated strains; [T], type strain

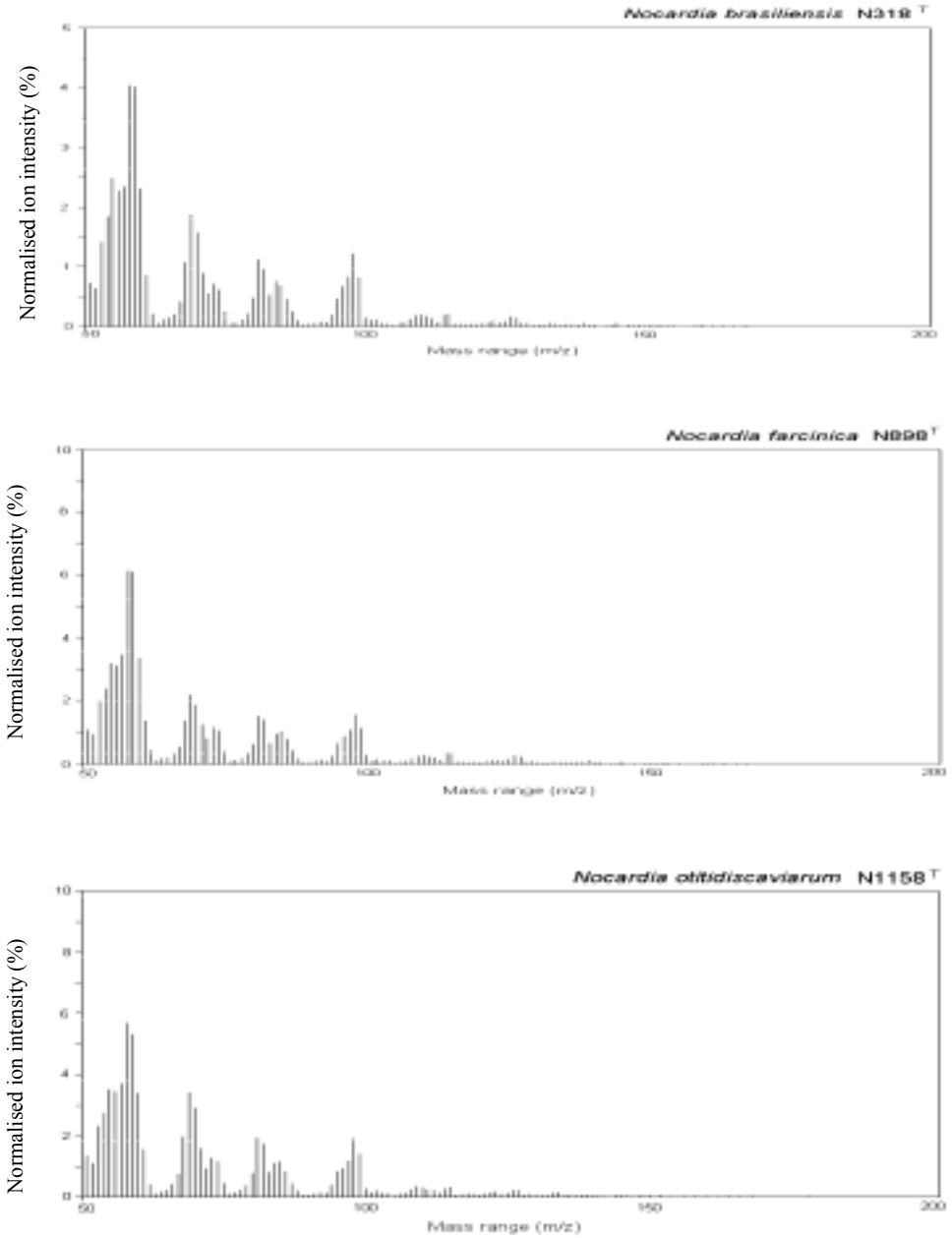

Figure 2. Pyrolysis mass spectra derived from the analysis of *Nocardia brasiliensis* N318^T, *Nocardia farcinica* N898^T and *Nocardia otitidiscaviarum* N1158^T.

dimensional model based on the principal components canonical variates analyses, though, in this case, *Nocardia farcinica* strain N701 was observed together with the other strains bearing the same name (Figure 3). It was very encouraging in the present investigation that the *Nocardia brasiliensis*, *Nocardia farcinica* and *Nocardia otitidiscaviarum* strains formed distinct pyrogroups corresponding to clusters defined in the numerical study.

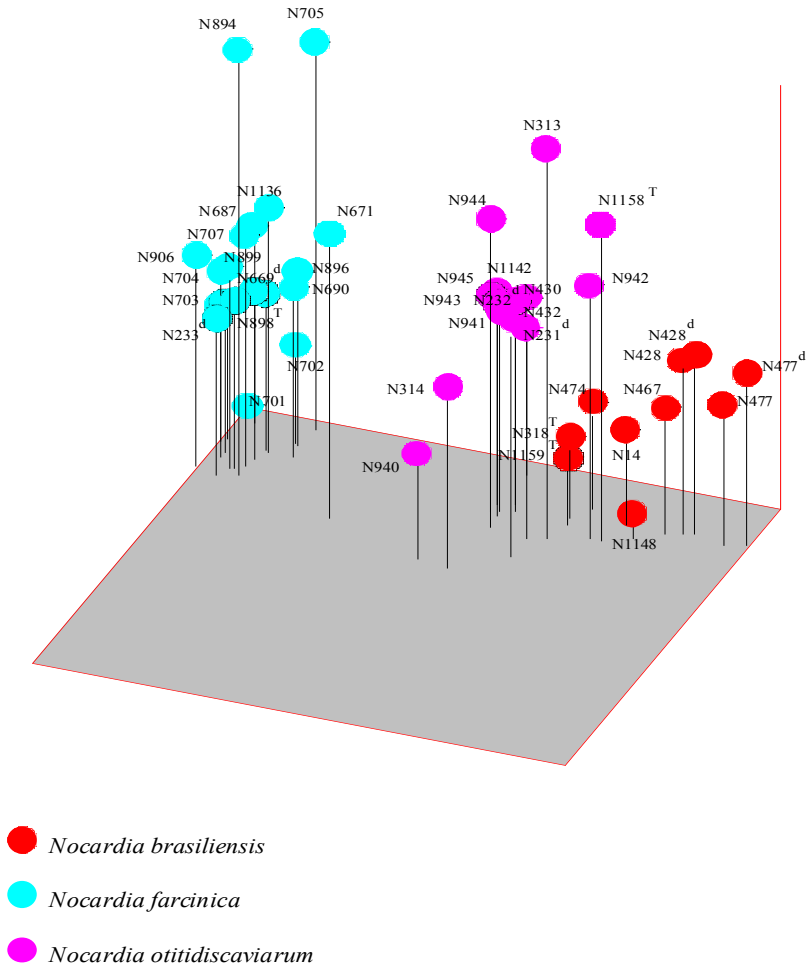

Figure 3. Ordination plot along the first three principal component canonical variates axes showing the mean position of the *Nocardia brasiliensis*, *Nocardia farcinica* and *Nocardia otitidiscaviarum* strains based on pyrolysis mass spectral data. The first three canonical variates account for 72% of the total variation found between the strains.

[d], duplicated strain; [T], type strain.

These results are also in agreement with those of previous taxonomic studies (6, 20, 21, 22, 7, 23). The good agreement found between the pyrolysis mass spectrometric and numerical taxonomic data is in line with the results of earlier studies on actinomadurae (24), rhodococci (25) and streptomycetes (26, 27).

In general, this PyMS may prove to be a valuable method of evaluating *Nocardia species*. It has already been mentioned that rapid, reliable and cost-effective methods are needed to effect the classification, identification and typing of micro-organisms. Curie-point pyrolysis mass specrtometry is being increasingly used for such purposes as it allows rapid automated acquisition of data-derived organism components and requires minimal sample preparation (28, 29, 30). The system can be used to evaluate taxonomic structures derived from the application of other procedures and to select representative strains for taxonomic analyses that require both time-consuming and laborious methods. It can be concluded from the present investigation that the use of PyMS provides an effective procedure for separating representatives of *Nocardia species*.

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