

## Phylogeny and Identification of *Nocardia* Species on the Basis of Multilocus Sequence Analysis<sup>∇</sup>

L. R. McTaggart,<sup>1</sup> S. E. Richardson,<sup>1,2,3</sup> M. Witkowska,<sup>1</sup> and S. X. Zhang<sup>1,2\*</sup>

Medical Mycology, Public Health Laboratories, Ontario Agency for Health Protection and Promotion,<sup>1</sup> Department of Laboratory Medicine and Pathobiology, University of Toronto,<sup>2</sup> and Division of Microbiology, Hospital for Sick Children,<sup>3</sup> Toronto, Ontario, Canada

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***Nocardia* species identification is difficult due to a complex and rapidly changing taxonomy, the failure of 16S rRNA and cellular fatty acid analysis to discriminate many species, and the unreliability of biochemical testing. Here, *Nocardia* species identification was achieved through multilocus sequence analysis (MLSA) of gyrase B of the  $\beta$  subunit of DNA topoisomerase (*gyrB*), 16S rRNA (16S), subunit A of SecA preprotein translocase (*secA1*), the 65-kDa heat shock protein (*hsp65*), and RNA polymerase (*rpoB*) applied to 190 clinical, 36 type, and 11 reference strains. Phylogenetic analysis resolved 30 sequence clusters with high (>85%) bootstrap support. Since most clusters contained a single type strain and the analysis corroborated current knowledge of *Nocardia* taxonomy, the sequence clusters were equated with species clusters and MLSA was deemed appropriate for species identification. By comparison, single-locus analysis was inadequate because it failed to resolve species clusters, partly due to the presence of foreign alleles in 22.1% of isolates. While MLSA identified the species of the majority (71.3%) of strains, it also identified clusters that may correspond to new species. The correlation of the identities by MLSA with those determined on the basis of microscopic examination, biochemical testing, and fatty acid analysis was 95%; however, MLSA was more discriminatory. *Nocardia cyriacigeorgica* (21.58%) and *N. farcinica* (14.74%) were the most frequently encountered species among clinical isolates. In summary, five-locus MLSA is a reliable method of elucidating taxonomic data to inform *Nocardia* species identification; however, three-locus (*gyrB*-16S-*secA1*) or four-locus (*gyrB*-16S-*secA1*-*hsp65*) MLSA was nearly as reliable, correctly identifying 98.5% and 99.5% of isolates, respectively, and would be more feasible for routine use in a clinical reference microbiology laboratory.**

As part of the aerobic actinomycetes, *Nocardia* is a group of filamentous branching bacilli that are characteristically Gram positive and modified acid fast. Although *Nocardia* species normally exist as soil saprophytes, they have increasingly been isolated as infectious agents in immunosuppressed patients and, in some cases, even healthy individuals. Infections range from pulmonary nocardiosis, characterized by necrotizing pneumonia, to cutaneous nocardiosis and even brain abscess (25).

For nearly a century, since its inception in 1888 by Edmund Nocard, the genus *Nocardia* comprised only about a dozen species (26), largely because the somewhat biochemically inert nature of the group inhibited characterization (6). However, in 1988, Wallace et al. (38) uncovered latent diversity when they described six antimicrobial susceptibility pattern types among clinical isolates. DNA (e.g., 16S rRNA [16S] gene) sequencing confirmed and further expanded knowledge of the genetic diversity within the genus (6, 22). To date, the National Center for Biotechnology Information (NCBI) lists 86 recognized species (<http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi>). However, the species differ in their abilities to cause human infection and their responses to antimicrobials (2, 6, 21, 25, 27, 33, 38). For this reason, species identification of *Nocar-*

*dia* isolates from clinical specimens is relevant to patient treatment and provides important epidemiological information.

Beyond Gram and modified-acid-fast staining, species identification of *Nocardia* relies heavily on biochemical tests and cellular fatty acid analysis, which are cumbersome, time-consuming, and not definitive. Various molecular identification schemes investigated to date represent promising alternatives (7, 10, 29, 32, 36). However, 16S rRNA gene sequencing, considered to be the “gold standard” for bacterial identification, fails to discriminate many species (7), and the reliability of identification methods on the basis of the DNA sequence of a single housekeeping gene suffers from stochastic genetic variation and horizontal gene transfer and recombination (12).

Recently, multilocus sequence analysis (MLSA) has been suggested as a method to examine prokaryotic taxonomy. From phylogenetic analysis of a concatenated sequence typically consisting of 5 to 7 housekeeping genes, MLSA assigns a species designation on the basis of the assumption that sequence clusters represent species clusters (12). MLSA has been employed to identify the species of a number of genera with very promising results (1, 4, 5, 11, 14, 15, 16, 18, 20, 24, 28, 40). Furthermore, because of its ease of use, accuracy, and discriminatory power, MLSA may soon surpass DNA-DNA hybridization (DDH) as the gold standard for the investigation of prokaryotic taxonomy, species identification, and determination of genetic diversity (34).

The purpose of this study was to develop an MLSA scheme for the species identification of *Nocardia* clinical isolates. Through phylogenetic analysis of concatenated sequences con-

\* Corresponding author. Present address: Division of Medical Microbiology, The Johns Hopkins University School of Medicine, 600 North Wolfe St., 19 Meyer B1-193, Baltimore, MD 21287. Phone: (410) 955-5077. Fax: (410) 614-8087. E-mail: szhang28@jhmi.edu.

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TABLE 1. *Nocardia* species and species clusters, including the type and reference strains, number of clinical isolates, and average genetic diversity of the *gyrB*-16S-*secA1*-*hsp65*-*rpoB* concatenated sequence within each cluster

Species or species cluster	Culture collection type and reference strains contained in cluster	No. (%) of clinical isolates	Total no. of isolates	% avg genetic diversity <sup>a</sup> (CI <sub>95</sub> <sup>b</sup> )
<i>N. abscessus</i> cluster	DSM <sup>c</sup> 44432 <sup>T</sup>	1 (0.53)	2	0.59
<i>N. africana</i> cluster	DSM 44491 <sup>T</sup> , DSM 44500, NRRL B-1519 ( <i>N. veterana</i> )	0	3	1.07 (0.02, 2.12)
<i>N. amamiensis</i> cluster	DSM 45066 <sup>T</sup>	1 (0.53)	2	1.57
<i>N. anaemiae</i> cluster	DSM 44821 <sup>T</sup>	1 (0.53)	2	3.02
<i>N. aobensis</i> cluster	DSM 44805 <sup>T</sup> , DSM 44543 ( <i>N. paucivorans</i> )	0	2	0.04
<i>N. asiatica</i> cluster	DSM 44668 <sup>T</sup>	6 (3.16)	7	1.10 (0.50, 1.70)
<i>N. asteroides</i> cluster	ATCC <sup>d</sup> 19247 <sup>T</sup>	2 (1.05)	3	1.10 (0.15, 2.05)
<i>N. beijingensis</i> cluster	DSM 44636 <sup>T</sup>	1 (0.53)	2	1.80
<i>N. brasiliensis</i> cluster	DSM 43758 <sup>T</sup> , DSM 43064	8 (4.21)	10	1.39 (0.85, 1.93)
<i>N. brevicatena</i>	ATCC 15333 <sup>T</sup>	0	1	
<i>N. caishijiensis</i>	DSM 44831 <sup>T</sup>	0	1	
<i>N. carnea</i> cluster	NRRL <sup>e</sup> B-1336 <sup>T</sup> , DSM 44558	0	2	1.80
<i>N. cerradoensis</i> cluster	DSM 44546 <sup>T</sup>	2 (1.05)	3	0.58 (0.01, 1.15)
<i>N. cyriacigeorgica</i> cluster	DSM 44484 <sup>T</sup> , DSM 43208	41 (21.58)	43	1.62 (1.34, 1.9)
<i>N. farcinica</i> cluster	NRRL B-2089 <sup>T</sup> , DSM 43289	28 (14.74)	30	0.45 (0.30, 0.60)
<i>N. flavorosea</i>	DSM 44480 <sup>T</sup>	0	1	
<i>N. fluminea</i> cluster	DSM 44489 <sup>T</sup>	1 (0.53)	2	0.91
<i>N. kruczakiae</i> cluster	DSM 44877 <sup>T</sup>	1 (0.53)	2	0.23
<i>N. neocaledoniensis</i> cluster	DSM 44717 <sup>T</sup>	1 (0.53)	2	1.38
<i>N. nova</i> cluster	DSM 44481 <sup>T</sup> , DSM 43207	17 (8.95)	19	0.28 (0.16, 0.40)
<i>N. otitidiscavianum</i> cluster	DSM 43242 <sup>T</sup> , DSM 44565	10 (5.26)	12	0.51 (0.31, 0.71)
<i>N. paucivorans</i>	DSM 44386 <sup>T</sup>	0	1	
<i>N. pneumoniae</i> cluster	DSM 44730 <sup>T</sup>	2 (1.05)	3	1.05 (0.02, 2.08)
<i>N. pseudobrasiliensis</i>	DSM 44290 <sup>T</sup>	0	1	
<i>N. rhammosiphila</i> <sup>f</sup> cluster	NRRL B-24637 <sup>T</sup>	3 (1.58)	4	0.40 (0.10, 0.70)
<i>N. sienata</i> cluster	DSM 44766 <sup>T</sup>	1 (0.53)	2	2.59
<i>N. thailandica</i> cluster	DSM 44808 <sup>T</sup>	1 (0.53)	2	0.27
<i>N. transvalensis</i> cluster	NRRL B-16037 <sup>T</sup> , DSM 46068	0	2	2.69
<i>N. veterana</i> cluster	NRRL B-24136 <sup>T</sup>	1 (0.53)	2	0.0
<i>N. vinacea</i> cluster	DSM 44638 <sup>T</sup>	1 (0.53)	2	0.87
<i>N. wallacei</i> cluster	DSM 45136 <sup>T</sup>	3 (1.58)	4	1.32 (0.69, 1.95)
<i>N. abscessus</i> / <i>N. arthritidis</i> -like	DSM 44557 ( <i>N. abscessus</i> )	18 (9.47)	19	0.36 (0.25, 0.47)
<i>N. nova</i> / <i>N. cerradoensis</i> / <i>N. kruczakiae</i> / <i>N. aobensis</i> -like		20 (10.53)	20	0.29 (0.21, 0.37)
<i>N. arthritidis</i> / <i>N. gamkensis</i> / <i>N. exalbida</i> cluster	DSM 44731 <sup>T</sup> , DSM 44956 <sup>T</sup> , DSM 44883 <sup>T</sup>	7 (3.68)	10	1.12 (0.87, 1.37)
<i>N. ignorata</i> / <i>N. coubleae</i> cluster	DSM 44496 <sup>T</sup> , DSM 44960 <sup>T</sup>	1 (0.53)	3	1.02 (0.78, 1.26)
Nonclustering strains		11 (5.79)	11	
Total	47	190	237	6.53

<sup>a</sup> Calculated using the K2P substitution model.

<sup>b</sup> CI<sub>95</sub>, 95% confidence interval.

<sup>c</sup> DSM, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

<sup>d</sup> ATCC, American Type Culture Collection, Manassas, VA.

<sup>e</sup> NRRL, United States Department of Agriculture, Agricultural Research Service, Peoria, IL.

<sup>f</sup> To date, the taxonomic name *Nocardia rhammosiphila* is not validly published.

sisting of partial fragments of gyrase B, the  $\beta$  subunit of a type II DNA topoisomerase (*gyrB*), 16S, subunit A of the SecA preprotein translocase (*secA1*), the 65-kDa heat shock protein (*hsp65*), and RNA polymerase (*rpoB*) genes, we delineated 30 species clusters. Since most clusters contained a single type strain, the results correlated well with existing knowledge of *Nocardia* taxonomy and provided a means of species assignment for the clinical isolates on the basis of strain placement within the phylogenetic analysis. Furthermore, the MLSA identifications were consistent with, although more discriminatory than, species assignments based on traditional microscopic evaluation, biochemical testing, and cellular fatty acid analysis. We present MLSA as a practical tool for routine *Nocardia* species identification in a clinical reference microbiology laboratory.

## MATERIALS AND METHODS

**Strains.** One hundred ninety clinical isolates of *Nocardia* were used in the study. The isolates were derived from clinical samples submitted to the Mycology Section of the Ontario Public Health Laboratory from December 2005 through January 2010. Microscopic observation of Gram-positive branching, filamentous bacilli, positive modified (Kinyoun)-acid-fast staining, and the production of aerial hyphae on pyruvate agar confirmed that the isolates were *Nocardia* (23). Routine species identification of *Nocardia* isolates was performed by assessing decomposition of adenine, casein, tyrosine, and xanthine; ability to grow at 35°C and 45°C; urease production; and acid production on 19 sugars (adonitol, arabinose, cellobiose, dulcitol, erythritol, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, sorbitol, sucrose, trehalose, and xylose). Results were compared to the reactions reported for six medically important *Nocardia* spp. (6, 23). As well, cellular fatty acid composition was determined by gas chromatography and compared to the compositions of cellular fatty acids in a standard library using the Sherlock Microbial Identification System (MIDI, Inc., Newark, DE). Table 1 lists the 36 type and 11 reference

TABLE 2. PCR primers and DNA amplification conditions for 16S, *gyrB*, *secA1*, *hsp65*, and *rpoB*

Target	Region	Forward primer (5'–3')		Reverse primer (5'–3')		PCR cycling conditions	Reference or source
		Primer name (position)	Sequence	Primer name (position)	Sequence		
16S	bp 31–492	E8F (bp 11–30)	AGAGTTTGATCTGGC TCAG	534r (bp 493–509)	ATTACCGGGCTG CTGG	30 s 98°C; 35 × 5 s 98°C, 5 s 56°C, 20 s 72°C; 1 min 72°C	3, this study
<i>gyrB</i>	bp 1031–1511	Noc- <i>gyrB</i> -F (bp 972–992)	CTTCGCCAACACCATCA ACAC	Noc- <i>gyrB</i> -R (bp 1563–1582)	TGATGATCGACTGGA CCTCG	30 s 98°C; 35 × 5 s 98°C, 5 s 60°C, 20 s 72°C; 1 min 72°C	This study
		Noc- <i>gyrB</i> -F3 (bp 1011–1030)	CGAGGAGGGCTTCCGC GCGG	Noc- <i>gyrB</i> -R3 (bp 1512–1532)	ATCGACTGGACCTCG TTGTTC	30 s 98°C; 35 × 5 s 98°C, 5 s 60°C, 20 s 72°C; 1 min 72°C	This study
<i>secA1</i>	bp 431–875	<i>secA1</i> -F47 (bp 413–430)	GCGACGCCGAGTGG ATGG	<i>secA1</i> -ConR2 (bp 876–896)	TTGGCCTTGATGGCG TTGTTC	30 s 98°C; 35 × 5 s 98°C, 5 s 67°C, 20 s 72°C; 1 min 72°C	This study
		<i>secA1</i> -F47 (bp 413–430)	GCGACGCCGAGTGG ATGG	<i>secA1</i> -ConR (bp 913–933)	GCGACGATGTAGT CCTTGTC	30 s 98°C; 35 × 5 s 98°C, 5 s 63°C, 20 s 72°C; 1 min 72°C	10, this study
<i>hsp65</i>	bp 165–565	Noc- <i>hsp65</i> -F (bp 145–164)	ACCAACGATGGTGTGT CCAT	Noc- <i>hsp65</i> -R (bp 566–585)	CTTGTCGAACCGCAT ACCCT	30 s 98°C; 35 × 5 s 98°C, 5 s 54°C, 20 s 72°C; 1 min 72°C	37
		Noc- <i>hsp65</i> -F2 (bp 109–129)	GTTGTCTGGAGAAGAA GTGG	Noc- <i>hsp65</i> -R (bp 566–585)	CTTGTCGAACCGCAT ACCCT	30 s 98°C; 35 × 5 s 98°C, 5 s 54°C, 20 s 72°C; 1 min 72°C	37, this study
<i>rpoB</i>	bp 1089–1488	Noc- <i>rpoB</i> -F2 (bp 897–919)	CCGTACAAGATCAACAA GAAGC	Noc- <i>rpoB</i> -R4 (bp 1612–1634)	CCC GCGAGGACATC GTCC	30 s 98°C; 35 × 5 s 98°C, 5 s 50°C, 20 s 72°C; 1 min 72°C	This study
		Noc- <i>rpoB</i> -F2 (bp 897–919)	CCGTACAAGATCAACAA GAAGC	Noc- <i>rpoB</i> -R2 (bp 1657–1677)	GCGACGTACTCCAT CTCCTC	30 s 98°C; 35 × 5 s 98°C, 5 s 50°C, 20 s 72°C; 1 min 72°C	This study
		Noc- <i>rpoB</i> -F4 (bp 962–979)	CCC GCGAGGACATCGTCCG	Noc- <i>rpoB</i> -R4 (bp 1612–1634)	CCC GCGAGGACATC GTCC	30 s 98°C; 35 × 5 s 98°C, 5 s 50°C, 20 s 72°C; 1 min 72°C	This study
		Noc- <i>rpoB</i> -F5 (bp 987–1006)	CGAGTACCTGGTGCY CTGC	Noc- <i>rpoB</i> -R5 (bp 1588–1607)	TCGACCGCGAGTTG GCCTG	30 s 98°C; 35 × 5 s 98°C, 5 s 62°C, 20 s 72°C; 1 min 72°C	This study

strains used in this study. (To date, “*Nocardia rhamnosiphila*” is not a validly published taxonomic name.) Type and reference strains were selected on the basis of their known clinical significance and their similarity to 16S sequences of the clinical isolates. All strains were maintained on either brain heart infusion agar (Difco) with 10% sheep’s blood or GYM medium (0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.2% CaCO<sub>3</sub>, 1.2% agar; DSM [German Collection of Microorganisms and Cell Cultures], Braunschweig, Germany) incubated at 37°C.

**DNA extraction, PCR, and sequencing.** DNA was extracted using PrepMan reagent (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. Partial 16S, *secA1*, *hsp65*, *rpoB*, and *gyrB* sequences were amplified using a Phire Hot Start DNA polymerase kit (New England Biolabs, Ipswich, MA) and a Bio-Rad (Hercules, CA) iCycler thermal cycler. The primers and PCR cycling conditions are listed in Table 2. Primers were designed on the basis of sequences found in GenBank. While the majority of *secA1*, *hsp65*, *gyrB*, and *rpoB* sequences were obtained with the first sets of primers listed in Table 2, a few strains required alternate primers, which are listed subsequently for each gene. Forward and reverse cycle sequencing reactions were performed using the PCR primers and a BigDye Terminator (version 1.1) cycle sequencing kit (Applied Biosystems), with the products subsequently being sequenced on a 3130xl genetic analyzer (Applied Biosystems) according to the manufacturer’s instructions.

**Cluster analysis.** In BioNumerics (version 6.0.1) software (Applied Maths, Austin, TX), sequences were aligned and trimmed to defined start and end positions to yield fragments of the following sizes: 462 bp for 16S, 482 bp for *gyrB*, 445 bp for *secA1*, 401 bp for *hsp65*, and 400 bp for *rpoB*. The trimmed sequences were concatenated in the order *gyrB*-16S-*secA1*-*hsp65*-*rpoB* to generate a 2,190-bp sequence. Unrooted trees obtained using individual gene sequences and concatenated sequences were generated by the neighbor-joining (NJ) algorithm following Kimura 2-parameter (K2P) correction and the maximum-parsimony (MP) algorithm using BioNumerics software. Bootstrap analysis (500 replicates) was used to assess the robustness of the clusters.

## RESULTS

**MLSA revealed distinct species clusters.** Among all 237 strains, the diversity of the concatenated *gyrB*-16S-*secA1*-*hsp65*-*rpoB* sequences ranged from 0 to 12.2%, with 0 to 246 nucleotide differences occurring between strains. The NJ tree of the concatenated sequences (Fig. 1) clearly showed 30 sequence clusters with bootstrap values of >85%, many of which contained a single type strain. In keeping with standard MLSA (18, 20, 24, 25, 32, 33, 34), these sequence clusters were considered to represent species clusters, and the isolates within each species cluster were colored coded according to their type strain(s). In total, 93.2% (221/237) of strains were contained within 1 of the 30 species clusters. For 71.3% (169/237) of strains, a species identification was assigned because their MLSA species cluster contained a single type strain. An additional 21.9% (52/237) of strains were contained within 1 of 4 species clusters that lacked a single type strain (Fig. 1; Table 1). On the other hand, 6.8% (16/237) of strains (5 type strains and 11 clinical isolates) failed to cluster with other strains and thus remained uncolored (white).

MLSA revealed two distinct clades that did not cluster with any of the type strains. The first was a set of 20 strains that showed a high level of concatenated sequence similarity to *N. nova* DSM 44481<sup>T</sup> (98.2 to 98.7%), *N. cerradoensis* DSM

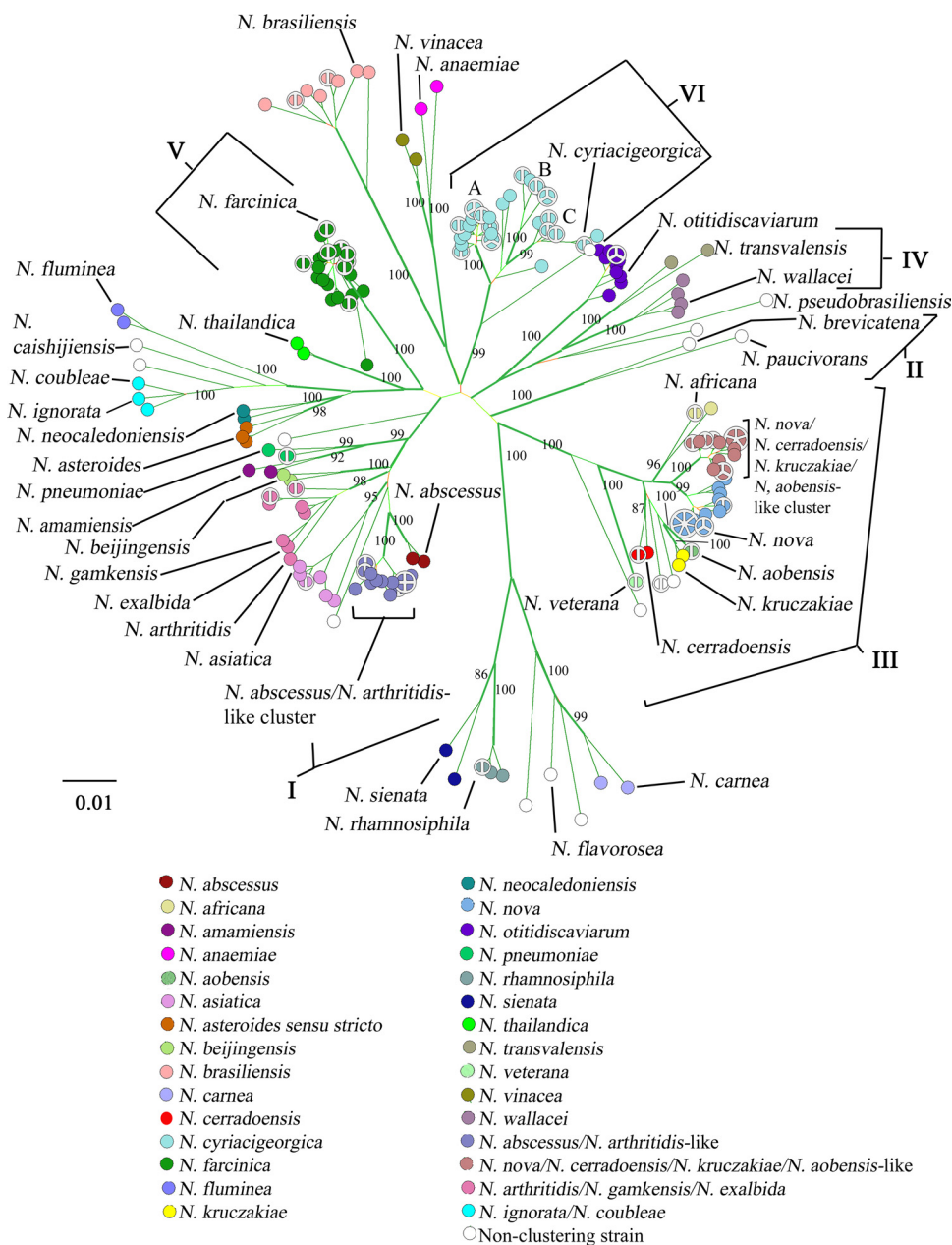


FIG. 1. An NJ tree constructed from the 2,190-bp concatenated *gyrB*-16S-*secA1*-*hsp65*-*rpoB* sequences of 237 strains. Strains were colored to represent species clusters inferred from sequence clusters. Each of the 36 type strains is labeled with its species name. Bootstrap values (500 replicates) for nodes leading to sequence clusters are indicated. Branches indicated in boldface were also recovered in the MP tree. The three subgroups of *N. cyriaciageorgica* are indicated A, B, and C. Also indicated are the six drug-type patterns (I, II, III, IV, V, VI) previously described (3, 4). Bar, 1% nucleotide substitutions.

44546<sup>T</sup> (97.9 to 98.2%), *N. kruzakiae* DSM 44877<sup>T</sup> (97.9 to 98.2%), and *N. aobensis* DSM 44805<sup>T</sup> (97.9 to 98.2%) and yet clustered separately with a high level of bootstrap support (100%) (Fig. 1). The second was a cluster of 19 strains that were similar to *N. abscessus* DSM 44432<sup>T</sup> (97.8 to 98.1%) and *N. arthritis* DSM 44731<sup>T</sup> (97.6 to 97.8%) but again formed a distinct clade with 100% bootstrap support (Fig. 1).

The MLSA scheme also revealed two sets of type strains that fail to form distinct clusters. First, *N. coubleae* DSM 44960<sup>T</sup> and *N. ignorata* DSM 44496<sup>T</sup> along with one clinical isolate

form a distinct clade with 100% bootstrap support. Second, *N. arthritis* DSM 44731<sup>T</sup>, *N. gamkensis* DSM 44956<sup>T</sup>, and *N. exalbida* DSM 44883<sup>T</sup> together with 7 clinical isolates formed a cluster with 98% bootstrap support.

Moreover, although the *N. cyriaciageorgica* group was a well-supported species cluster, it did contain three clearly distinct subgroups supported by bootstrap values of 99%, 100%, and 100%.

Table 1 lists the average genetic diversity present within each species cluster. The average genetic diversity varied depending

on the species cluster. The *N. veterana* cluster had the lowest genetic diversity at 0.0%, while the *N. anaemiae* species cluster had the greatest average genetic diversity at 3.02%.

Three of 11 reference strains failed to cluster according to the species designation reported by their respective culture collections. *N. veterana* NRRL B-1519 clustered with the *N. africana* isolates, *N. paucivorans* DSM 44543 was identical to and therefore clustered with *N. aobensis* DSM 44805<sup>T</sup>, and *N. abscessus* DSM 44557 grouped with the *N. abscessus*/*N. arthritis*-like isolates (Fig. 1; Table 1).

Beyond delineating species clusters, the overall structure of the NJ tree correlated well with the six drug pattern types of the *Nocardia asteroides* complex described by Wallace et al. (38) and clearly differentiated the well-recognized species *N. brasiliensis* and *N. otitidiscaviarum* (6) (Fig. 1).

The concatenated sequences were used to generate a maximum-parsimony tree (data not shown). However, no major differences were observed between the MP tree and the NJ tree. All major branches were supported by both methods, except for a slight rearrangement of *N. fluminea* isolates with respect to *N. coubleae* DSM 44960<sup>T</sup> and *N. ignorata* DSM 44496<sup>T</sup> (Fig. 1).

**Individual gene sequences failed to resolve species clusters.** Phylogenetic analysis using the NJ algorithm applied to each of the five loci individually (data not shown) also demonstrated sequence diversity within the genus. The range of genetic diversity varied for each of the loci: 0.0 to 17.7% for *gyrB*, 0.0 to 9.2% for 16S, 0.0 to 18.2% for *secA1*, 0.0 to 13.0% for *hsp65*, and 0.0 to 22.3% for *rpoB*. Despite the observed sequence diversity, these trees failed to discriminate a number of the species clusters resolved using the concatenated sequence. Species clusters that did form generally received weak bootstrap support. Consequently, attempts to identify individual strains on the basis of cluster analysis of individual loci were unsuccessful.

Of the 179 clinical and 11 reference strains assigned to an MLSA species cluster, 148 (77.9%) had alleles at the *secA1*, *gyrB*, *hsp65*, and *rpoB* loci that were most similar to those of the other strains within its designated MLSA species cluster. However, 20.5% ( $n = 39$ ) of strains contained a foreign allele at one of the loci; that is, the allele was most similar to that found in the type strain representing another species cluster. This caused aberrant clustering when the single-locus tree was compared to trees constructed using the concatenated sequence, 16S, or any of the other three loci. An additional three strains (1.6%) had foreign alleles at 2 loci. Of the loci, *hsp65* was the most promiscuous, with 33 strains (17.4%) exhibiting a foreign allele, compared to *secA1* (3 strains, 1.6%), *rpoB* (6 strains, 3.2%), and *gyrB* (3 strains, 1.6%). Of note, 22 strains from the *N. cyriacigeorgica* species cluster had an *hsp65* allele that exhibited 97.5 to 98.5% similarity to the *hsp65* alleles of *N. asiatica* DSM 44668<sup>T</sup>, *N. abscessus* 44432<sup>T</sup>, and *N. pneumoniae* DSM 44730<sup>T</sup> but only 96.5 to 97.3% similarity to the *N. cyriacigeorgica* DSM 44484<sup>T</sup> *hsp65* allele. The strains exclusively formed subgroup A of the *N. cyriacigeorgica* species cluster (Fig. 1). Apart from the *hsp65* allele of these 22 *N. cyriacigeorgica* strains, the majority of the foreign alleles (78.3%) were most similar to alleles of adjacent species clusters on the NJ tree of concatenated sequences.

As mentioned above, two distinct clades failed to cluster

with a type strain during MLSA. For the first clade, the *rpoB*, *secA1*, and 16S alleles of the *N. nova*/*N. cerradoensis*/*N. kruczakiae*/*N. aobensis*-like cluster were most similar to those of *N. nova* DSM 44481<sup>T</sup> (99.0 to 100%, 98.9 to 99.3%, and 99.6%, respectively), while the *hsp65* alleles were most similar to those of *N. veterana* NRRL B-24136<sup>T</sup> (97.5 to 98.5%) and the *gyrB* alleles were most similar to those of *N. cerradoensis* DSM 44546<sup>T</sup> (98.1 to 98.5%). For the second clade, when examined individually, the *secA1* and *hsp65* sequences of the *N. abscessus*/*N. arthritis*-like cluster were most similar to those of *N. arthritis* DSM 44731<sup>T</sup> (96.0 to 96.3% and 99.2 to 99.5%, respectively), while the *rpoB* and *gyrB* sequences were most similar to those of *N. abscessus* DSM 44432<sup>T</sup> (98.2 to 98.5% and 97.9 to 99.4%, respectively). The 16S sequences were identical to the *N. abscessus* DSM 44432<sup>T</sup> 16S sequence and differed by only 1 bp from that of *N. arthritis* DSM 44731<sup>T</sup>.

**Identification by MLSA cluster analysis is generally concordant with, although more discriminatory, than identification achieved through microscopic examination, biochemical tests, and cellular fatty acid analysis.** Figure 2 shows the NJ tree constructed using the concatenated *gyrB*-16S-*secA1*-*hsp65*-*rpoB* sequences color coded to reflect the species designations achieved through traditional morphological and microscopic examination, biochemical testing, and cellular fatty acid analysis. To correlate these results with the MLSA species designations, we considered 139 clinical isolates. (Of the 190 clinical isolates, 30 were excluded from the comparison because they lacked a microscopic/biochemical/cellular fatty acid identification [ $n = 19$ ], an MLSA species cluster designation [ $n = 7$ ], or both [ $n = 4$ ]. An additional 21 isolates were identified as *N. asteroides* complex by microscopic/biochemical/cellular fatty acid assessment but were assigned to one of the following newly described or rarely encountered species by MLSA: *N. amamiensis*, *N. asiatica*, *N. beijingensis*, *N. exalbida*/*N. gamkensis*/*N. arthritis*, *N. ignorata*/*N. coubleae*, *N. fluminea*, *N. pneumoniae*, *N. rhamnosiphila*, *N. sienata*, *N. thailandica*, and *N. vinacea*. These isolates were also excluded from the comparison because the taxonomic status of these species with respect to the ever-waning *N. asteroides* complex remains undescribed.) In order to accommodate the evolving taxonomy of *Nocardia*, all strains designated *N. asteroides* complex by microscopic/biochemical/cellular fatty acid identification were considered concordant with any MLSA species cluster designation from the following: *N. abscessus*, *N. brevicatena*, *N. paucivorans*, *N. nova*, *N. veterana*, *N. africana*, *N. kruczakiae*, *N. transvalensis*, *N. wallacei*, *N. farcinica*, and *N. cyriacigeorgica* (6, 8). Furthermore, the MLSA identification of isolates of the *N. abscessus*/*N. arthritis*-like species cluster and the *N. nova*/*N. cerradoensis*/*N. kruczakiae*/*N. aobensis*-like species cluster was considered concordant with a microscopic/biochemical/cellular fatty acid identification of *N. asteroides* complex since the species describing these clusters are part of the *N. asteroides* complex. The concordance between the microscopic/biochemical/cellular fatty acid identification and the MLSA species cluster designation was 95% (132/139); however, MLSA was far more discriminatory because it identified the species of 84 isolates defined as *N. asteroides* complex by microscopy, biochemical, and cellular fatty acid testing.

***N. cyriacigeorgica* and *N. farcinica* were the most commonly encountered species among clinical isolates.** Table 1 shows the

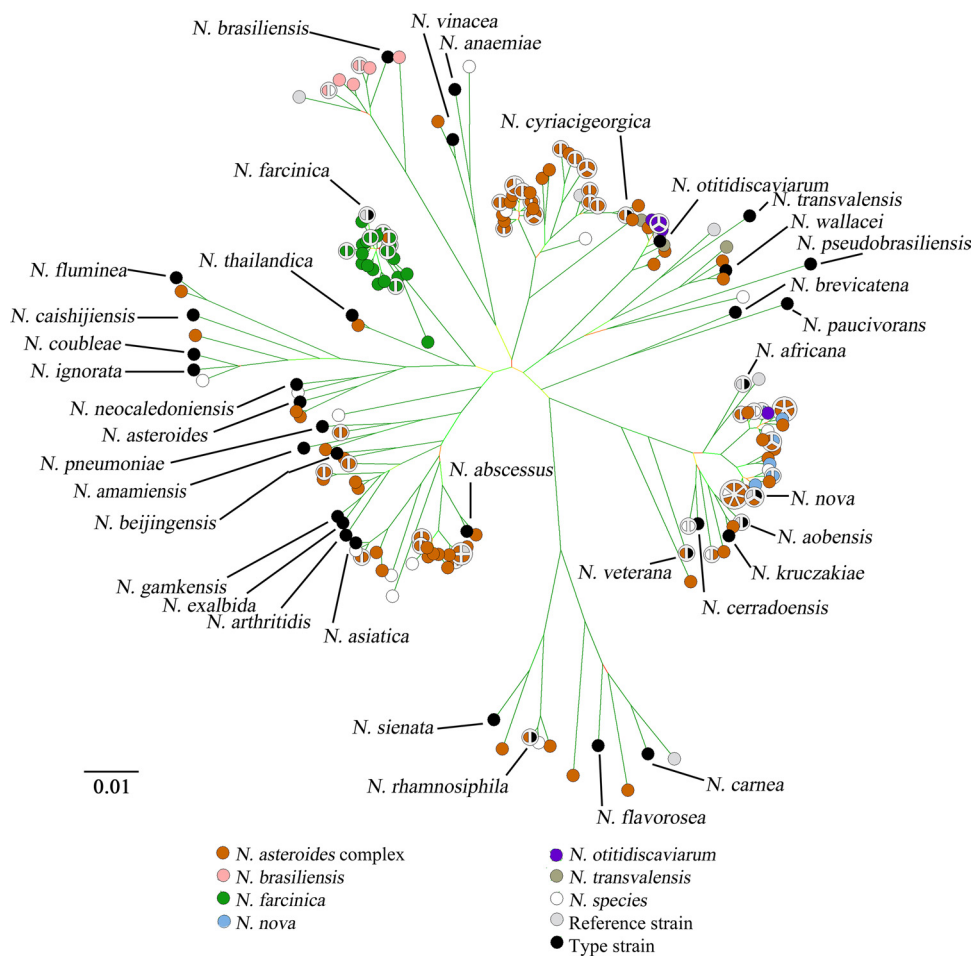


FIG. 2. An NJ tree constructed from the 2,190-bp concatenated *gyrB*-16S-*secA*-*hsp65*-*rpoB* sequences of 237 strains. Strains were colored to represent species identification on the basis of microscopic examination, biochemical testing, and cellular fatty acid analysis. Each of the 36 type strains is labeled with its species name. Bar, 1% nucleotide substitutions.

species distribution on the basis of MLSA for the 190 *Nocardia* clinical isolates received by the Mycology Section at the Ontario Public Health Laboratory from December 2005 through January 2010. *N. cyriaci-georgica* ( $n = 41$ ) and *N. farcinica* ( $n = 28$ ) were the most commonly encountered species, accounting for 36.32% of strains. Also frequently encountered were isolates of the *N. nova*/*N. cerradoensis*/*N. kruczakiae*/*N. aobensis*-like species cluster (10.53%), the *N. abscessus*/*N. arthritis*-like species cluster (9.47%), and *N. nova* (8.95%).

**Toward a species identification scheme for *Nocardia*.** Although five-locus MLSA definitively differentiates many species clusters and facilitates the identification of unknown isolates, the routine amplification of five loci from each and every clinical isolate in a clinical laboratory setting would be too cumbersome. Therefore, we reanalyzed the data in various combinations to produce three- and four-locus NJ trees (data not shown). (Most combinations included the 16S locus because of its predominance in sequence-based identification schemes.) The trees were examined to determine whether they yielded the same species clusters and strain placement as the five-locus tree. As expected, the four-locus trees correctly assigned clinical and reference isolates more frequently than the

three-locus trees. The *gyrB*-16S-*secA*-*hsp65* and *gyrB*-16S-*secA* MLSAs yielded the highest correlations (99.5% and 98.5%, respectively) with the five-locus MLSA, while the 16S-*secA*-*hsp65* MLSA produced the lowest at 88.1%.

## DISCUSSION

The aim of this study was to develop a system for the species identification of *Nocardia* that confidently and powerfully discriminates species, satisfies the most current theories in prokaryotic taxonomy, and yet can be implemented for routine use in a clinical laboratory setting. The MLSA presented here provides such an all-encompassing identification system capable of differentiating currently recognized species and pinpointing anomalous strains potentially representing undescribed species. It is demonstrably superior to identifications based on traditional microscopic examination, biochemical tests, and cellular fatty acid analysis because of its unparalleled ability to comprehensively discriminate a myriad of old and new species recently added to the genus (6, 22).

Of the many approaches to species identification, MLSA represents an ideal method for such a taxonomically diverse

and challenging group as the *Nocardia*. Although DDH has long been held to be the gold standard for prokaryotic species demarcation, MLSA may soon supplant DDH to become the primary measure of bacterial diversity and species identification (34). While the results of MLSA correlate well with DDH taxonomic classifications (5, 28, 30, 31), it is technically easier to perform and allows the rapid concurrent comparison of multiple strains. Compared to single-locus sequence-based identification schemes (e.g., 16S rRNA), it yields a fuller understanding of the genome by sampling multiple alternate regions of the DNA. The use of multiple loci buffers against stochastic genetic variation and horizontal gene transfer with homologous recombination (12, 17, 35). MLSA is less cumbersome than biochemical testing, yet it demonstrates greater discriminatory power (12, 35).

In keeping with the standard MLSA protocol, we chose the following four single-copy housekeeping genes for analysis, in addition to 16S rRNA: *secA1*, which encodes subunit A of a preprotein translocase; *hsp65*, which encodes a 65-kDa heat shock protein; *rpoB*, the gene for RNA polymerase; and *gyrB*, the gene for the  $\beta$  subunit of a type II DNA topoisomerase. Each of these loci has previously been recommended as a conserved core marker suitable for bacterial systematics (19), and most have been used individually for sequence-based identification of *Nocardia* species (7, 10, 29, 32, 36).

MLSA using the NJ phylogenetic tree generated from the concatenated *gyrB*-16S-*secA1*-*hsp65*-*rpoB* sequences of 237 strains demonstrates a tremendous amount of genetic diversity among the *Nocardia*. As recommended for MLSA analysis, species clusters were delineated on the basis of visual inspection of the tree rather than an arbitrary percent similarity threshold largely because intraspecies genetic diversity levels generally vary between species clusters (4, 12). Indeed, our analysis also showed that the degree of genetic diversity within *Nocardia* species clusters fluctuated substantially, discouraging the use of a cutoff value. Remarkably, 30 species clusters were identified within a genus that until recently contained only a few species (26). While individual treatment of the loci produced ambiguous and unreliable species identification, concatenation of all five loci resolved the species clusters with very strong (>85%) bootstrap support, providing a high degree of confidence in the species assignments. As a method of determining the species identity of the strains, MLSA was highly successful: 71.3% received a species assignment, while important taxonomic information was revealed about an additional 21.9% of strains which comprised clusters that did not contain a single type strain.

MLSA ideally utilizes a large number of strains that are representative of the diversity within the genus. Large numbers of isolates produce robust species clusters, which in turn increases confidence in species identification (12, 17). In this study, many of the clusters contained <5 isolates. While this is less than ideal, the MLSA scheme presented here did demarcate and cluster the available strains with a high degree of bootstrap support. The analysis provides a solid foundation to which more strains can be added in the future. As more concatenated sequences are added, the compositions of the clusters and their connections can be expected to change to provide a more accurate picture of the genus (12).

Despite the need for more isolates in some species clusters,

the results of MLSA correlate well with current knowledge of *Nocardia* taxonomy and the results of microscopic/biochemical/cellular fatty acid evaluation. Most (26 of 30) of the species clusters contain a single previously described type strain. The six drug-type patterns of the *N. asteroides* complex plus the well-recognized species *N. otitidiscaviarum* and *N. brasiliensis* (6, 38) are clearly delineated. Furthermore, strain-by-strain comparison of the MLSA species assignment with the identifications provided by microscopic examination, biochemical tests, and cellular fatty acid analysis is generally consistent (95%), although MLSA is more discriminatory. For instance, MLSA confirmed and then further refined the species assignments of a large number ( $n = 84$ ) of *N. asteroides* complex isolates. The 7 inconsistencies in identification among 139 clinical isolates (5%) and 3 of the 11 (27.3%) reference strains reflect the widely acknowledged difficulties with *Nocardia* species identification (6). MLSA is a superior identification method because it identified a number of rarely encountered or newly described species among 21 clinical isolates previously identified as *N. asteroides* complex by traditional microscopic/biochemical/cellular fatty acid analysis. Although DDH has historically been considered the gold standard for prokaryotic species determination, its laborious nature prevented a direct comparison with MLSA in this study. However, the strong concordance with literature reports and the results of microscopic/biochemical/cellular fatty acid species identification methods validates the MLSA system as accurate for *Nocardia* species identification.

As with MLSA studies involving other bacterial genera (4, 11, 16, 18, 28, 40), 22.1% (42/190) of the *Nocardia* isolates had foreign alleles discernible at one or more loci, often from a neighboring cluster, indicating that a history of interspecies recombination has distorted the phylogenetic relationships between species clusters. This phenomenon as well as the discovery of two well-resolved species clusters that lack a type strain discourages the practice of comparing a single housekeeping locus allele sequence to the sequences of a set of type strains as a method for species delineation. However, during MLSA, the phylogenetic signals from multiple loci generally buffered the effect of the foreign allele, such that species clusters were still resolved, albeit fuzzy (16), due to the presence of intermediate genotypes and provided a method for identifying novel species clusters.

Studies with other bacterial genera suggest using MLSA to inform the discovery of new species or taxonomic revisions (5, 13, 14, 20, 24, 30, 31). In our study, MLSA did not distinguish either the *N. arthritidis*, *N. gamkensis*, and *N. exalbida* type strains or the *N. ignorata* and *N. coubleae* type strains. While sequence analysis of additional genes may demarcate these type strains, failure to do so would prompt an extensive evaluation of the legitimacy of their species status. The *N. abscessus*/*N. arthritidis*-like and *N. nova*/*N. cerradoensis*/*N. kruczakiae*/*N. aobensis*-like clusters appear to be a fusion of alleles from two or three type strains. Additional sequences should either reassign these isolates to type strain-defined species clusters or prompt the characterization of new species. The species or subspecies status of the three subclusters of *N. cyriaci-georgica* would benefit from further taxonomic investigation, especially considering its importance as a human pathogen (9, 39). Nonclustering clinical isolates may also represent

new species, provided that they fail to cluster with additional type strains when they are incorporated into the analysis. In all cases, novel species discovery must be supported by MLSA as well as DDH data, extensive biochemical and phenotypic characterizations, and perhaps ecological distinction (12, 13, 20, 30, 31).

The species distribution recovered among Ontario clinical isolates correlates well with the distributions presented in other reports, which also list *N. cyriacigeorgica*, *N. farcinica*, *N. nova*, and *N. abscessus* among the most frequently encountered species from clinical specimens (2, 21, 25, 27, 33). Lack of information regarding clinical symptoms precludes any speculation as to whether the various species infect or simply colonize humans.

While MLSA may involve anywhere from 3 to 8 loci, depending on the genus, at some point additional loci fail to enhance species delineation (4, 17). In order to avoid excess labor and cost in a routine laboratory setting, we sought to identify the best combination of 3 or 4 loci that maintain a good resolution of species clusters. Either the *gyrB*-16S-*secA1*-*hsp65* MLSA or the *gyrB*-16S-*secA1* MLSA is a good option, as each provides the correct species cluster assignment for 99.5% and 98.5% of the isolates, respectively. Frequently encountered species could be confidently assigned on the basis of either of these truncated MLSA methods, with the 5-locus MLSA being reserved for identification of rare species.

In summary, we have developed a MLSA method capable of demarcating various species clusters from among type strains, reference strains, and clinical isolates. This system is superior to biochemical testing for the identification of unknown strains because of its simplicity and ease of use, its discriminatory power, and its ability to concurrently provide taxonomic information on virtually all strains of *Nocardia* encountered in a clinical setting.

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