The 16S ribosomal RNA of *Mycobacterium leprae* contains a unique sequence which can be used for identification by the polymerase chain reaction

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Summary. Nucleotide sequence data for bacterial 16S ribosomal RNA was used to identify oligodeoxyribonucleotide primers suitable for probing the rRNA gene of mycobacteria and related organisms, with the polymerase chain reaction. The method enabled us to distinguish mycobacteria from other closely related genera, and to differentiate between slow- and fast-growing mycobacteria. *Mycobacterium leprae* fell within the slow-growing group of mycobacteria but there are significant differences between the sequence of the *M. leprae* 16S rRNA gene and that of other slow-growing mycobacteria. These differences were used to devise a rapid, non-radioactive method for detecting *M. leprae* in infected tissue.

Introduction

The mycobacteria are gram-positive organisms with guanosine + cytosine-rich DNA. Several members of the genus are important pathogens of man; *Mycobacterium leprae* and *M. tuberculosis* are the causative organisms of leprosy and tuberculosis respectively. The genus can be divided into two broad groups, based on growth rate. The fast-growing species are, for the most part, non-pathogenic, whereas most of the pathogens are slow-growers. *M. leprae* has not been grown *in vitro*, but grows with a generation time of about 12 days in mice.¹

The 16S ribosomal RNA (rRNA) gene is convenient for studying phylogenetic relationships and for developing methods for identifying species. rRNA is present in all free-living organisms, and some stretches of the nucleotide sequence are variable, whereas others are conserved by functional constraints. The conserved sequences permit the alignment of the variable sequences so that only homologous nucleotides are employed in any phylogenetic analysis.² The variable sequences may serve as a means of species identification.

In this study, we have used the polymerase chain reaction (PCR) to probe the variable regions of the 16S rRNA of mycobacteria and related organisms, confirming that the division into slow- and rapid-growers is a phylogenetic division³ and that *M. leprae* falls within the slow-growing group of mycobacteria.^{4,5} The identification of a unique sequence close to the 5' end of the 16S rRNA of *M. leprae* was used to

develop a PCR test for the identification of *M. leprae* in tissue samples.

Materials and methods

Micro-organisms and DNA samples

The bacteria used in this study are listed in table I. DNA was isolated by the method of Katoh and Cox,⁶ except that cells were lysed in 6M guanidinium chloride at –15°C, without using a French pressure cell. The samples were stored in TE buffer (0.01M TrisHCl, 1mM sodium edetate; pH 7.6).

M. leprae-infected tissue

M. leprae-infected mouse footpad tissue was harvested as previously described.⁷ Clinical samples were kindly provided by Dr Niwat Montreewasurat, Raj-Pracha-Samarai Institute, Thailand. They consisted of either skin smears from patients with lepromatous leprosy or paraffin embedded tissues. They were treated as described previously,⁸ before being used in the PCR.

Preparation of cell lysates for use in PCR

Bacteria were harvested by centrifugation, washed with water, resuspended in water (c. 10⁴ cells/μl), boiled for 5 min, then cooled rapidly in ice.⁹ A 10-μl sample was used for each reaction.

Design and synthesis of oligodeoxyribonucleotide primers

An Applied Biosystems 380B DNA synthesiser with the manufacturers chemicals was used to synthe-

Table I. Strains investigated by DNA analysis

Species	Strain	Notes
<i>Mycobacterium asiaticum</i>	Institute Pasteur	a
<i>Mycobacterium avium</i>	NCTC 8559	b
<i>Mycobacterium bovis</i>	NCTC 10268	
<i>Mycobacterium chitae</i>	ATCC 19630	a
<i>Mycobacterium diernhoferi</i>	Environmental isolate	a
<i>Mycobacterium duvalii</i>	NCTC 358	a
<i>Mycobacterium flavescens</i>	NCTC 10270	a
<i>Mycobacterium fortuitum</i>	NCTC 8573	a
<i>Mycobacterium gilvum</i>	Clinical isolate	a
<i>Mycobacterium habana</i>	TMC 5135	c
<i>Mycobacterium intracellulare</i>	NCTC 10682	a
<i>Mycobacterium kansasii</i>	NCTC 10268	
<i>Mycobacterium leprae</i>	Armadillo tissue	
<i>Mycobacterium malmoeense</i>	NCTC 11299	
<i>Mycobacterium marinum</i>	Clinical isolate	
<i>Mycobacterium microti</i>	OV 254	d
<i>Mycobacterium neoaurum</i>	NCTC 10439	a
<i>Mycobacterium paratuberculosis</i>		a
<i>Mycobacterium rhodesiae</i>	ATCC 27024	
<i>Mycobacterium scrofulaceum</i>		c
<i>Mycobacterium smegmatis</i>	NCTC 333	a
<i>Mycobacterium thermoresistibile</i>	ATCC 19528	a
<i>Mycobacterium tuberculosis</i>	H ₃₇ Rv, H ₃₇ Ra Clinical isolates B1453, 79499, 12644, 12646 and 6213	d
<i>Mycobacterium vaccae</i>	R877R	
<i>Corynebacterium minutissimum</i>	NCTC 10284	a
<i>Corynebacterium JK</i>	NCTC 11915	a
<i>Nocardia asteroides</i>	ATCC 3318	a
<i>Nocardia brasiliensis</i>	IMRV 800	a
<i>Propionibacterium acnes</i>	VPI 0162	a
<i>Propionibacterium avidum</i>	VPI 0589	a
<i>Bacillus subtilis</i>	168 trp	e

a, The DNA samples were given by Drs J. Stanford and V. Visuvanathan, Department of Pathology, Middlesex Hospital Medical School, London.

b, DNA was given by T. Hillyer and Dr I. Brown, St Mary's Hospital Medical School, London.

c, The strains were given by Dr N. B. Singh, Central Drug Research Institute, Lucknow, India.

d, Strains were given by Dr J. Stanford.

e, DNA was given by Dr M. Sargent, National Institute for Medical Research. All other strains were grown by standard methods and DNA was isolated as described in the text.

size the primers. The sequences of the primers are shown in table II. 16S rRNA comprises constant regions, in which the nucleotide sequence has been highly conserved during evolution, and eight variable regions (V1–V4, and V6–V9) in which the nucleotide sequence is more variable between species. The g-series primers were designed by identifying conserved sequences from a number of 16S rRNA sequences.¹⁰ The v-series primers were designed to be characteristic of *M. bovis*¹¹ and *M. kansasii*.¹² Primer ML1 was designed to be specific for *M. leprae*, based on the results reported here.

The DNA polymerase chain reaction (PCR)

The 50- μ l reaction mixture contained 0.2–10 ng of DNA, or c. 10^5 cells, 0.8 units of Ampli TaqTM DNA polymerase (Perkin Elmer Cetus), oligodeoxyribonucleotide primers—5 μ l of each 20 μ M stock solution,

10 μ l of 5 $\times$ DNA PCR buffer (0.25 M KCl, 50 mM Tris HCl, pH 8.4), 12.5 mM MgCl₂, 1 mM dATP, 1 mM dTTP, 1 mM dGTP, 1 mM dCTP and gelatine 0.1% w/v—and water (3.3–5 μ l). The reaction mixture was overlaid with mineral oil (35 μ l). The reaction was performed with a Techne PHE2 Dri-block[®] heating block and programmer.

The reaction mixture was heated to 94°C for 1 min, kept at 58°C for 1 min, then heated to 72°C for 2.5 min. There was 35 subsequent cycles of 15 s at 94°C, 1 min at 58°C and 2.5 min at 72°C. Finally, the solution was heated to 94°C for 15 s, then kept at 58°C for 1 min and at 72°C for 5 mins. When the mixtures of primers were used the final concentration of each primer was 1.2 μ M.

Agarose gel electrophoresis

The products of the PCR were separated by

Table II. Oligodeoxyribonucleotide probes used in the PCR

Probe	Position*	Sequence
g series		
g1	4–27	5'TTGGAGAGTTTGATCCTGGCTC3'
g2	46–68	5'GCTTAACACATGCAAGTCGAA3'
cg3	c(514–536)	5'CGTATTACCGCGGCTGCTGGCAC3'
cg4	c(785–810)	5'GGTGGACTACCAGGTATCTAATCC3'
g5	919–942	5'AATTGACGGGGCCCGCACACAA3'
cg5	c(919–942)	5'CGCTTGTGCGGGCCCCCGTCAATT3'
cg6	c(1491–1513)	5'TACGGCTACCTTGTTACGACTTC3'
v-series		
v1	69–88	5'CGGAAAGGTCTCTTCGGAGA3'
cv3	c(441–462)	5'GAACCCGGACCTTCGTGCGATG3'
v6	993–1017	5'CAGGACGCGTCTAGAGATAGCGGTT3'
v7†	1110–1134 (a)	5'CTCATGTTGCCAGCACGTAATGGT3'
	(b)	5'CTCATGTTGCCAGCGGTAATGCC3'
cv9	c(1427–1446)	5'CCCAGGGTTAGGCCAACTGGC3'
ML1		5'CGGAAAGGTCTCTAAAAAATCTT3'

* For the g-series probes, the position in *E. coli* is given; for the v-series probes the position in *M. bovis* is given.

† (a) has the same sequence as *M. bovis*; (b) has the same sequence as *M. kansasii*.

c, as in cg3 etc. indicates a probe complementary to the rRNA sequence.

electrophoresis in agarose gel 1.5–2.0% w/v. A flat-bed submerged configuration of the gel was used. The running buffer was 9 mM Tris-borate—0.25 mM edetic acid, pH 8.2. Ethidium bromide 0.5 µg/ml was included in the gel and the products were made visible with a transilluminator (UVP, Inc.).

Cloning of *M. leprae* DNA

M. leprae was recovered from armadillo tissue, and lysed by the procedure described by Clark-Curtis *et al.*¹³ DNA was deproteinised by treatment with proteinase K, followed by extraction with phenol. This DNA was used to prepare a cosmid library by Ms Jenny Thompson, London School of Hygiene and Tropical Medicine, who kindly supplied us with a copy of the library. The library was prepared briefly as follows: A partial *Sau*3A digest of *M. leprae* DNA was cloned into the *Bam*HI site of the cosmid vector Lorist 4.¹⁴ Recombinant DNA was packaged *in vitro* before transfecting *Escherichia coli* strain 1046 grown in the presence of kanamycin (Sigma) 25 µg/ml. Clones containing rDNA sequences were identified with suitable oligodeoxyribonucleotide probes (e.g., g1 and cg6, table I) made radioactive by labelling the 5' end with ³²P by treatment with λ-[³²P]-dATP (Amersham International) and polynucleotide kinase (Pharmacia, Sweden).

Five clones were identified by such means; two contained the entire *M. leprae* rRNA cistron and, on subsequent analysis, were found to be identical; these were designated KEK4 and KEK5. KEK5 was selected for further analysis.

KEK5 was digested with the restriction endonuclease *Pst*I. The DNA fragments were separated by electrophoresis on agarose 0.8% w/v gels and then transferred to Hybond-NTM nylon membranes by the method of Southern¹⁵ and according to the manufac-

turers instructions. Two *Pst*I fragments were identified by hybridisation with radio-labelled probes. The 1.8-kb fragment containing the 5'-terminus of the 16S rRNA gene was isolated from agarose gels and cloned into plasmid pUC18 cut with *Pst*I by conventional means.¹⁶ The 1.8-kb recombinant plasmid was designated KEK6.

DNA sequencing

Plasmid KEK6 was sequenced by the chain termination method¹⁷ with SequenaseTM (USB) according to the manufacturers instructions. Double stranded plasmid DNA was denatured and prepared for sequencing by the NaOH-precipitation method.¹⁸ Template DNA was then sequenced with oligodeoxyribonucleotides as primers.

Results

An appropriate combination of g-series and v-series primers was used in the PCR to probe the variable regions of the rRNA gene of the various bacteria. The products were analysed by agarose gel electrophoresis. The results of two experiments are shown in fig. 1A and B. A product of 752 bp, resulting from primers g2 and cg3, was seen in all bacterial samples; this serves as a positive control for the presence of 16S rRNA gene sequences. The slow-growing mycobacteria give two additional products of 394 bp (primers v1 and cv3) and 454 bp (primers v6 and cv9). The results obtained with the various primer combinations are summarised in table III.

It is clear that all of the bacteria gave products with the combination of general primers (g2–cg4, and g5–cg6), but only the mycobacteria gave a product with primer cv9. None of the fast-growing mycobacteria

Fig. 1. Comparison of the products of the PCR obtained with DNA from mycobacteria and closely related species. The primers were g2, cg4, v1, cv3, v6 and cv9. (A) a, *B. subtilis*; b, *M. leprae*; c, no DNA; d, *M. tuberculosis* H₃₇Rv; e, *P. avidum*; f, *P. acnes*; g, *N. brasiliensis*; h, *N. asteroides*; i, *Corynebacterium* JK; j, *C. minutissimum*; k, *M. thermoresistibile*; l, *M. smegmatis*; λ, *Hind* III–*Eco*RI double-digest of phage-λ DNA; m, *M. rhodesiae*; n, *M. nonchromogenicum*; o, *M. neoaurum*; p, *M. marinum*; q, *M. malmoense*; r, *M. gilvum*; s, *M. fortuitum*; t, *M. flavescens*; u, *M. duvalii*; v, *M. diernhoferi*; w, *M. chitae*; x, *M. asiaticum*. (B) λ, mol. size markers (*Eco*RI–*Hind* III double digest of phage-λ DNA); a and l, *M. bovis*; b and k, *M. leprae*; c, *M. microti*; d, *Corynebacterium* 91461; e, *Corynebacterium* 10688; f, *M. tuberculosis*; g, *M. paratuberculosis*; h, *M. intracellulare*; i, *M. kansasii*; j, *M. avium*.

gave products with primers designed to probe the other variable regions of the gene, whereas the slow-growers did, suggesting that they are all closely related in the V1, V3, V6, V7 and V9 regions. The V7 region could be further subdivided into *M. bovis*-like or *M. kansasii*-like with primers v7a and v7b.

Based on the analysis described above, *M. leprae* is clearly related to the slow-growing mycobacteria. However, it differed from other species within this group. Firstly, the V1 region is divergent, because, no product was obtained with primers v1 and cg4.

Secondly, the product obtained when *M. leprae* was primed with either g2 and cg4, or g1 and cv3 was slightly larger than the product obtained with other slow-growers (fig. 2), indicating the presence of 10–20 extra nucleotides between nucleotides 68 and 441.

To confirm the insertion of extra nucleotides in the *M. leprae* 16S rRNA gene, the relevant part of the gene was sequenced. The sequences of the relevant region of the *M. leprae* and the *M. bovis* genes are compared in fig. 3. The slightly larger size of the product of the PCR in this region of the *M. leprae* gene is explained by the presence of 12 extra nucleotides (6As and 6Ts) within the V1 region, compared to the other mycobacterial sequences. Because this represents a unique *M. leprae* sequence, we then used a primer (ML1) based on this sequence to develop a PCR test for the detection and identification of *M. leprae*. Fig. 4 shows that we were able to obtain the appropriate size of product with cloned *M. leprae* DNA, with purified genomic DNA and with various *M. leprae*-infected tissues, but not with other mycobacterial species.

Discussion

In this study we have used the PCR to examine the 16S rRNA of mycobacteria. Multiple primers were used to probe the variable regions of the gene. The principal conclusions are: (1) the mycobacteria form a phylogenetically distinct group, readily distinguishable from closely related genera; (2) within the genus, the mycobacteria can be subdivided into two broad groups based on 16S rRNA sequences, which corre-

Fig. 2. Evidence for the presence of additional sequences at the 5'-end of *M. leprae* 16S rRNA compared with *M. bovis* 16S rRNA gene sequences. Products of the PCR were separated by agarose (1.5%) gel electrophoresis, stained with ethidium bromide and visualised with UV light; for A, the primers were g2 and cg4, for B, g1 and cv3. λ, *Hind* III–*Eco*RI double digest of phage-λ DNA; a, *M. leprae*; b, *M. bovis* (752 bp); c, *B. subtilis* (779 bp); d and f, *M. bovis* (459 bp); e, *M. leprae*.

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      •           •           •           •
1  TTTGTTTGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCT
   *****
51 TAACACATGCAAGTCGAACCGGAAAGGTCTCTAAAAAATCTTTTTTAGAGA
   *****-----*-----G****
101 TACTCGAGTGGCGAACGGGTGAGTAACACGTGGGTAATCTGCCCTGCACT
   *****G*****
151 TCAGGGATAAGCTTGGGAAACTGGGTCTAATACCGGATAGGACTTCAAGG
   *-*****C*****CA*GG*A
201 CGCATGTCTTGTGGTGGAAAGCTTTTTT-GCGGTGCAGGATGGGCCCGCGG
   T*****GC***A*****TG*****A*****
252 CCTATCAGCTTGTGGTGGGGTGACGGCCTACCAAGGCGACGACGGGTAG
   *****
302 CCGGCCTGAGAGGGTGTACGGCCACACTGGGACTGAGATACGGCCAGAC
   *****C*****
352 TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA
   *****
402 TGCAGCGACGCCGCGTGGGGGATGACGGCTTTCGGGTGTAAACCTCTTT
   *****C*****
452 CACCATCGACGAAGGTCTGGGTTTTCTCGGATTGACGGTAGGTGGAGAAG
   *****C*****C*****
502 AAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGC GA
   *****
552 GCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTGCGG
   *****
602 TTGTTTCGTGAAATCTCACGGCTTAAGTGTGAGCGTGCGGGCGATACGGGC
   *****
652 AGACTGGAGTACGTGAC
   *****A*****

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Fig. 3. Nucleotide sequence of the 5' end of the *M. leprae* rRNA gene (EMBL Data Library accession number X58888). The top line shows the *M. leprae* sequence, the bottom line the corresponding *M. bovis* sequence (conserved nucleotides are marked *; only where differences occur are the *M. bovis* nucleotides specified). The V1 region is boxed.

Fig. 4. Detection of *M. leprae* with primers based on the *M. leprae*-specific 16S rRNA sequence. Products of the PCR were separated by agarose (2%) gel electrophoresis, stained with ethidium bromide and visualised with UV light; the primers were g5 and cg6, and cv3 and ML1. a, H₂O; b, cloned *M. leprae* DNA; c, genomic *M. leprae* DNA; d, *M. leprae*-infected mouse footpad tissue; e, lepromatous leprosy patient skin smear; f, leprosy patient tissue section; g, leprosy patient tissue section (negative); h, human placental DNA; i, *M. avium* DNA; j, *M. bovis* DNA; k, *M. kansasii* DNA; l, *M. habana* DNA; m, *M. tuberculosis* DNA; n, H₂O; o, *Hind* III-*Eco*RI double digest of phage- λ DNA.

spond to fast- and slow-growing species; (3) *M. leprae* falls within the slow-growing group of mycobacteria; (4) there are significant differences between the *M. leprae* 16S rRNA sequences and other mycobacterial sequences; (5) these differences can be exploited to detect and identify *M. leprae* in infected tissue.

The mycobacteria could be differentiated from the other bacteria tested on the basis of the V9 region, which was probed with primers g5 and cv9. All of the mycobacterial species gave a product of c. 670 bp which was not given by the non-mycobacterial species. This is in agreement with the sequence data obtained for V9 with both fast growing (e.g., *M. smegmatis*) and slow-growing (*M. leprae*) species.^{5,19} The V9 region of 16S rRNA is believed to be involved in the binding of the smaller and larger ribosomal subparticles.²⁰ Functional constraints could account for the more highly conserved nature of the V9 region compared with the V1, V3 and V6 regions whose functions have yet to be identified.

The results show that the V1, V3, V6, V7 and V9 regions of the slow-growing mycobacteria are all closely related. However, the fast-growing species

Table III. Summary of the results of the PCR obtained with various primer combinations

Species	Product obtained with							
	cg4 v1	g1- cv3	cg6- v6	cg6- v7a	cg6- v7b	g5- cv9	cg4- g2	cg6- g5
Slow growing mycobacteria								
<i>M. tuberculosis</i>	+	+	+	+	-	+	+	+
<i>M. bovis</i>	+	+	+	+	-	+	+	+
<i>M. microti</i>	+	+	+	+	-	+	+	+
<i>M. avium</i>	+	+	+	-	+	+	+	+
<i>M. intracellulare</i>	+	+	+	-	+	+	+	+
<i>M. scrofulaceum</i>	+	-	+	-	+	+	+	+
<i>M. simiae</i>	+	+	+	-	+	+	+	+
<i>M. kansasii</i>	+	+	+	-	+	+	+	+
<i>M. asiaticum</i>	+	+	+	-	+	+	+	+
<i>M. malmoense</i>	+	+	+	-	+	+	+	+
<i>M. paratuberculosis</i>	+	+	+	-	+	+	+	+
<i>M. leprae</i>	-	+	+	+	-	+	+	+
<i>M. marinum</i>	+	+	+	+	-	+	+	+
Fast growing mycobacteria								
<i>M. fortuitum</i>	-	-	-			+	+	+
<i>M. chitae</i>	-	-	-			+	+	+
<i>M. smegmatis</i>	-	-	-			+	+	+
<i>M. diernhoferi</i>	-	-	-			+	+	+
<i>M. neoaurum</i>	-	-	-			+	+	+
<i>M. vaccae</i>	-	-	-			+	+	+
<i>M. flavescens</i>	-	-	-			+	+	+
<i>M. phlei</i>	-	-	-			+	+	+
<i>M. thermoresistibile</i>	-	-	-			+	+	+
<i>M. duvalii</i>	-	-	-			+	+	+
<i>M. gilvum</i>	-	-	-			+	+	+
Non-mycobacterial species								
<i>P. acnes</i>	-	-	-			-	+	+
<i>P. avidum</i>	-	-	-			-	+	+
<i>N. asteroides</i>	-	-	-			-	+	+
<i>N. brasiliensis</i>	-	-	-			-	+	+
<i>C. minutissimum</i>	-	-	-			-	+	+
<i>Corynebacterium JK</i>	-	-	-			-	+	+
<i>Corynebacterium 91461</i>	-	-	-			-	+	+
<i>Corynebacterium 10668</i>	-	-	-			-	+	+

+ Product of correct size; - no product of correct size.

were distinguished by not producing products when probed in the V1 (primers cg4-v1), V3 (primers g1-cv3) and V6 (primers cg6-v6) regions. Partial sequence data for a number of mycobacteria, obtained by directly sequencing 16S rRNA, has been reported.³ If the sequence data for the V3 region are compared with the cv3 primer sequence, the comparison reveals a perfect match for the slow growers. In contrast, fast-growing mycobacteria have a deletion of 12 nucleotides in the helix 18(b) region of V3,³ leading to extensive mismatches with primer cv3 which provides the basis for the differentiation between fast- and slow-growing mycobacteria.

Although our results are compatible with others which have assigned *M. leprae* to the slow-growing group of mycobacteria,⁴ we have identified significant differences within the V1 region. Sequencing of this

region has revealed an extra 12 nucleotides which has enabled us to design PCR primers that provide a rapid, unequivocal and non-radioactive test for the presence of *M. leprae*. PCR tests for the presence of *M. leprae* have been devised based on other *M. leprae* sequences;^{8,9,21,22} the availability of an additional test based on the 16S rRNA gene sequence will greatly enhance our ability to identify the presence of the organism in infected tissue or in putative in-vitro cultures, and could provide the basis for tests aimed at detecting RNA rather than DNA.

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Note added in proof.

The PCR method for the identification of *M. leprae*, including the partial 16S rRNA sequence was presented at the Sixth Santa Margherita Ligure Meeting; Symposium on Leprosy Research (Genoa, Italy), 3–7 October 1990; the report will appear in the Proceedings of the meeting (in press).

After the manuscript was submitted for publication, the complete sequence of *M. leprae* 16S rRNA was published (Liesack W, Pitulle C, Sela S, Stackebrandt E. Nucleotide sequence of the 16S rRNA of *Mycobacterium leprae*, *Nucleic Acids Research* 1990; **18**: 5558). A partial sequence was also reported (Teske A, Walters J, Böttger EC. The 16S rRNA nucleotide sequence of *Mycobacterium leprae*: phylogenetic position and development of DNA probes. *FEMS Microbiology Letters* 1991; **80**: 231–238). The sequence data of fig. 3 and the corresponding data reported above are in complete accord.

We now know that no product is obtained with *M. scrofulaceum* and primers g1 and cv3 (see table III) because primer cv3 and its potential binding site are mis-matched in eight positions (Rogall T, Flohr T, Böttger EC. Differentiation of mycobacterial species by direct sequencing of amplified DNA. *J Gen Microbiol* 1990; **136**: 1915–1920).