Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA

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Nucleotide sequences specific for a range of *Mycobacterium* species were defined by computer-assisted sequence comparisons of small subunit ribosomal RNA. A polymerase chain reaction-based sequencing strategy was used to demonstrate that the 16S rRNA sequence can be used for the rapid identification of mycobacterial isolates. Identification at the species level can be obtained within 2 d, requiring <10 000 bacteria. This procedure reliably differentiates *Mycobacterium* spp. which are difficult to identify by classical methods, such as *M. malmoeense*, *M. szulgai* and *M. flavescens*.

Introduction

Mycobacteria are aerobic, acid-fast, slow-growing bacteria, which are widespread in nature and include some important human pathogens, e.g. *Mycobacterium tuberculosis* and *M. avium* (Kubica, 1984). A major descriptive division of mycobacteria is related to growth rate and pigmentation, and four groups are recognized. Group I consists of the photochromogenic species of slow growers, members of group II are scotochromogenic slow growers, group III contains the nonphotochromogenic slow growers and group IV consists of rapid growers, defined as maturing in less than 7 d (Wayne & Kubica, 1986).

Current methods for identification of mycobacteria rely primarily on phenotypic, chemotaxonomic and serotaxonomic tests performed on cultures. Identification of mycobacterial isolates based on phenotypic characters is a time-consuming procedure, requiring an additional 2–4 week period after the initial isolation. Many different features requiring complex and specialized tests are necessary to identify mycobacteria at the species level (Wayne & Kubica, 1986; Goodfellow & Cross, 1983). Numerous investigations have been undertaken to define reliable features and test assays for differentiating between species (Wayne *et al.*, 1974, 1976, 1983, 1989). Attempts to subdivide mycobacterial species on the basis of immunological methods, DNA homology and related analyses (Baess, 1979, 1983; McFadden *et al.*, 1987; Gross & Wayne, 1970; Imaeda *et al.*, 1988; Wayne & Kubica, 1986) have proved useful in

determining taxonomic relationships, but the reliable identification of mycobacteria remains problematical.

An alternative approach to the identification of mycobacterial isolates is the definition of species-specific nucleotide sequences, combined with a convenient and rapid procedure for obtaining these sequences. Small subunit ribosomal RNAs (rRNA) are an attractive target for this purpose. rRNA is an essential constituent of bacterial and eukaryotic ribosomes (Fox *et al.*, 1980; Woese *et al.*, 1983), and the 16S rRNA molecule is functionally constrained such that it is highly conserved, with rare sequence changes in certain positions. However, the location of these changes is specific to the group or species in which they occur (Gray *et al.*, 1984; Stackebrandt & Woese, 1981; Woese, 1987; Dams *et al.*, 1988). This specificity of the 16S rRNA sequence, and the information content of the molecule, is sufficient to allow both statistically valid phylogenetic analysis and species identification, once the sequence has been determined (Woese, 1987).

We have recently described a method for the rapid and reliable determination of bacterial rRNA sequences by direct sequencing of DNA (Böttger, 1989; Edwards *et al.*, 1989) amplified by the polymerase chain reaction (PCR) (Saiki *et al.*, 1985). In this paper, we define stretches of nucleotides in the structure of small subunit rRNA, based upon comparative 16S rRNA sequencing within a wide range of *Mycobacterium* species, which are highly specific for the various *Mycobacterium* species investigated. This information offers a new approach for the rapid and definitive identification of mycobacterial isolates at the species level.

Abbreviation: PCR, polymerase chain reaction.

Methods

Bacterial strains. The strains whose 16S rRNA sequences were determined in this study are listed in Table 1. Clinical isolates of the *M. tuberculosis* complex including *M. bovis* and *M. africanum* were kindly provided by K. Schröder, Forschungsinstitut Borstel.

Determination of sequences. Cultures grown on Löwenstein-Jensen agar slants (*M. paratuberculosis* required mycobactin) were harvested and washed in 10 mM-Tris, 1 mM-EDTA (TE). The pellet was redissolved in 10 mM-Tris, 10 mM-EDTA, 0.1% Tween 80, 2 mg lysozyme ml⁻¹, incubated for 2 h at 37 °C with shaking and centrifuged. The pellet was lysed by redissolving in TE containing 100 µg Proteinase K ml⁻¹ and 1% (w/v) SDS and incubated for 1 h at 37 °C. The DNA was extracted by adding an equal volume of TE-saturated phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). The aqueous phase was transferred to another tube and 0.05 vols of cold 3 M-sodium acetate pH 5.2 were added. After mixing by inversion, samples were placed on ice for 10 min, before centrifugation in a microcentrifuge for 10 min. The supernatant fluid was transferred to another tube, and the DNA precipitated by adding 0.1 vol. 3 M-sodium acetate and 2.5 vols of ethanol (Maniatis *et al.*, 1982). The precipitated nucleic acids were washed, dried, redissolved in TE and stored at -20 °C.

The 16S rRNA sequences from the species given in Table 1 were obtained as described previously (Edwards *et al.*, 1989) and the complete sequences will be reported elsewhere. The nucleotide sequences of the relevant regions of the small subunit rRNA investigated here were determined by PCR-mediated amplification of the appropriate DNA fragment followed by direct sequence determination. Briefly, 1–100 ng of bacterial DNA was subjected to PCR in

a total volume of 100 µl, with 2.5 units of *Taq* polymerase (Perkin-Elmer Cetus or Gibco/BRL), 50 mM-KCl, 10 mM-Tris/HCl pH 8.3, 1.5 mM-MgCl₂, 0.01% gelatin, 100 pmol of each of the two primers, and 200 µM of each dNTP (dATP, dCTP, dGTP, TTP). The 100 µl mixture was covered by 100 µl of light mineral oil (Sigma). The relevant gene fragment coding for 16S rRNA was synthesized using either primer combination 1 (pA, AGA GTT TGA TCC TGG CTC AG; and pE*, CCG TCA ATT CCT TTG AGT TT) or primer combination 2 (pA, AGA GTT TGA TCC TGG CTC AG; and pI*, TGC ACA CAG GCC ACA AGG GA). The thermal profile involved 36 cycles of denaturation at 93 °C for 1 min, primer annealing for 2 min, and extension at 72 °C for 6 min. The annealing temperature chosen was dependent on the primers used: 37 °C for primer combination 1 and 60 °C for primer combination 2. Following amplification, the DNA was extracted with phenol and precipitated with ethanol. Alternatively, the volume of the amplification reaction (100 µl) was simply reduced under vacuum. The relevant fragment was isolated by 0.8% (w/v) agarose gel electrophoresis and electroeluted (Maniatis *et al.*, 1982), then purified on Elutip-D columns (Schleicher & Schuell) and ethanol-precipitated. For sequencing, template DNA (0.05–0.2 pmol; the PCR routinely yielded 2–4 pmol of the desired fragment) was annealed to 2.0 pmol of the respective sequencing primer in 10 µl (40 mM-Tris/HCl pH 7.5, 20 mM-MgCl₂, 50 mM-NaCl; 5 min at 94 °C, 2 min at 65 °C, 15 min at 37 °C). Sequencing primers used were pB, TAA CAC ATG CAA GTC GAA CG; pC*, CCC ACT GCT GCC TCC CGT AG; pC, CTA CGG GAG GCA GCA GTG GG; and pD*, GTA TTA CCG CGG CTG CTG. The labelling step was carried out for 5 min at 25 °C by adding 1.0 µl 0.1 M-dithiothreitol, 5.0 µCi (185 kBq) [α -³²P]dCTP, 2.0 µl labelling nucleotide mix (dGTP, dATP, TTP at 200 nM) and 3 units sequenase (United States Biochemical Corporation) (total volume 15.0 µl). Of this reaction mix, 3.5 µl was

Table 1. *Mycobacteria used in this study*

Species	Source*
<i>M. tuberculosis</i>	Inst. Med. Microbiology, Hannover
<i>M. bovis</i>	
<i>M. bovis</i> BCG	
<i>M. tuberculosis</i> H37	
<i>M. avium</i>	DSM 43216
<i>M. chelonae</i>	ATCC 14472
' <i>M. cookii</i> '	Dr Kazda, Forschungsinst. Borstel
<i>M. flavescens</i>	ATCC 14474 ^T
<i>M. fortuitum</i>	ATCC 6841 ^T
<i>M. gastri</i>	ATCC 15754 ^T
<i>M. goodii</i>	ATCC 14470 ^T
<i>M. intracellulare</i>	ATCC 15985
<i>M. kansasii</i>	DSM 43224
<i>M. komossense</i>	ATCC 33013; Dr Kazda
<i>M. marinum</i>	Dr Schröder, Forschungsinst. Borstel
<i>M. malmoense</i>	ATCC 29571 ^T
<i>M. nonchromogenicum</i>	ATCC 19530 ^T
<i>M. paratuberculosis</i>	ATCC 19698; Dr Jorgensen, National Veterinary Lab., Copenhagen
<i>M. phlei</i>	ATCC 35784; Dr Kazda
<i>M. scrofulaceum</i>	ATCC 19981 ^T
<i>M. simiae</i>	ATCC 25275 ^T
<i>M. sphagni</i>	ATCC 33027 ^T ; Dr Kazda
<i>M. smegmatis</i>	ATCC 14468
<i>M. szulgai</i>	ATCC 25799 ^T
<i>M. terrae</i>	ATCC 15755 ^T
<i>M. xenopi</i>	ATCC 19250 ^T

* ATCC, American Type Culture Collection, Rockville, USA; DSM, Deutsche Stammsammlung für Mikroorganismen, Braunschweig, FRG; ^T, type strain.

added to 2.5 µl of the appropriate termination mix (80 µM-dNTPs, 8 µM-ddNTP, 50 mM-NaCl) and incubated for 5 min at 37 °C. Formamide/dye stop mix (4 µl) was added, and samples were heated for 5 min at 94 °C before loading 2.5 µl onto a 6% sequencing gel (Maniatis *et al.*, 1982). After electrophoresis, gels were fixed in 10% (v/v) acetic acid/12% (v/v) methanol, dried, and exposed to X-ray film for 12 h.

Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia) and purified by shadow-casting polyacrylamide gel electrophoresis (Maniatis *et al.*, 1982).

The sequences were aligned using a multisequence alignment algorithm (Krüger & Osterburg, 1983) provided in the program package SAGE (Technoma) designed for the IBM XT/AT/PS2 and compatible systems. The alignment was adjusted manually to account for common secondary structure.

Results and Discussion

The PCR primers directed the synthesis of an approximately 1 kb gene fragment containing the 5' part of the gene coding for 16S rRNA. Fig. 1 shows the physical location of the oligonucleotides used in this study. As can be seen in Fig. 2, as little as 1 ng of purified DNA, is sufficient to yield a fragment by the PCR which can be sequenced.

An alignment of the sequences from the organisms investigated is given in Fig. 3. The depicted part of the small subunit rRNA gene (positions 123–273) contains a region known to be highly variable in eubacteria (Woese *et al.*, 1983), i.e. positions 200–240 according to the numbering system for *Escherichia coli* (Brosius *et al.*, 1978). By comparing the aligned sequences obtained, regions specific for each species can be defined.

In detail, the *M. tuberculosis* complex can be identified at positions 123–157, 175–213 and 215–273. *M. tuberculosis* is highly related to *M. bovis* and *M. africanum* (Baess, 1979), such that a proposal has been put forward to reduce *M. bovis* and *M. africanum* to synonyms of *M. tuberculosis*, with a possible subdivision at the sub-specific or infrasubspecific level (Wayne & Kubica, 1986). Accordingly, *M. tuberculosis* cannot be differentiated from *M. bovis* and *M. africanum* at the 16S rRNA sequence level.

Mycobacterium avium and *M. paratuberculosis* share an identical sequence for positions 136–273, but can be

Fig. 1. Physical map of the primers used with respect to their target sites in the 16S rRNA. The direction of sequence determination is indicated by an arrow. The nucleotide positions of the synthetic DNA oligomers in prokaryotic 16S rRNA as represented by *E. coli* (Brosius *et al.*, 1978) are: pA, 8–28; pB, 50–70; pC, 361–341; pC*, 341–361; pD, 536–518; pE, 928–908; and pI, 1047–1027.

differentiated at position 135, where an A residue occurs for *M. avium* and a G residue for *M. paratuberculosis*.

Mycobacterium gastri, *M. kansasii*, *M. scrofulaceum* and *M. simiae* show an identical rRNA sequence from positions 147 to 257. However, these species have different residues at positions 129/130, 144/145, 261 and 275, thus allowing them to be distinguished:

129/130	144/145	261	275	
...CA.....	AC....(..)	T....(..)	...C	<i>M. gastri/kansasii</i>
...CA.....	TT....(..)	A....(..)	...T	<i>M. scrofulaceum</i>
...TA.....	TT....(..)	T....(..)	...C	<i>M. simiae</i>

Mycobacterium gastri cannot be differentiated from *M. kansasii* at the small subunit ribosomal RNA level, because the 16S rRNA sequence from both species is identical, indicating a previously unreported close phylogenetic relationship between these two species (unpublished observation).

M. nonchromogenicum, *M. terrae*, *M. xenopi*, *M. gordonae*, *M. szulgai*, *M. malmoeense*, *M. intracellulare*, *M. marinum*, *M. flavesens*, *M. cookii*, *M. chelonae*, *M. smegmatis*, *M. fortuitum*, *M. sphagni*, *M. komossense* and *M. phlei* each show a distinct stretch of nucleotides at positions 178–273.

Fig. 2. PCR-mediated synthesis of a 1000 bp fragment of the gene coding for 16S rRNA, using varying amounts of DNA and primer combination pA/pI*. Lane 1, 100 ng; lane 2, 10 ng; lane 3, 1 ng; lane 4, no DNA. Molecular mass ladder is the 1 kb ladder (BRL).

130	140	150	160	170	180	190	
CGT GGG TGA TCT GCC CTG CAC TTC GGG ATA AGC CTG GGA AAC TGG GTC TAA TAC CGG ATA GG-ACCA CGG GAT GCA							<i>M. tuberculosis</i>
... .. CA. ... A. ... CTG CACT .AA .C ...							<i>M. avium</i>
... .. CA.T .AA .C ...							<i>M. paratuberculosis</i>
... .. CA. AC.TT .GC ...							<i>M. gastri</i>
... .. CA. AC.TT .GC ...							<i>M. kansasii</i>
... .. CA.TT .GC ...							<i>M. scrofulaceum</i>
... .. A.TT .GC ...							<i>M. simiae</i>
... .. .TAG .AT .C ...							<i>M. nonchromogenicum</i>
... .. .CTT							<i>M. terrae</i>
... .. C. TTC TGC ...							<i>M. xenopi</i>
... .. A. A. A.A .C A. ...							<i>M. gordonae</i>
... .. A.C .A .GC ...							<i>M. szulgai</i>
... .. A.C .A .GC ...							<i>M. malmoense</i>
... .. CA. TTA .GC ...							<i>M. intracellulare</i>
... .. C. T.							<i>M. marinum</i>
... .. .T AC. ...							<i>'M. cookii'</i>
... .. .TA ... TT-C. T NT. TC ...							<i>M. flavescens</i>
... .. .TA ... T-...G .C AC. TC							<i>M. chelonae</i>
... .. .TA ... CACC.TG .T. TC ...							<i>M. smegmatis</i>
... .. .TA ... T-... .C .C. T.							<i>M. fortuitum</i>
... .. .TA ... T-... T. TC TC. T.							<i>M. sphagni</i>
... .. .T T. T. AC. T.							<i>M. komossense</i>
... .. .CT CACC.TT .T. .T. ...							<i>M. phlei</i>

200	210	220	230	240	250	260	270	
TG--TCT-TGT GGT GGA AAG C GC TT T--AG CGG TGT GGG ATG AGC CCG CGG CCT ATC AGC								<i>M. tuberculosis</i>
..-...-C.T.								<i>M. avium</i>
..-...-C.T.								<i>M. paratuberculosis</i>
..-C-... .. .T.								<i>M. gastri</i>
..-C-... .. .T.								<i>M. kansasii</i>
..-C-... .. .T.								<i>M. scrofulaceum</i>
..-C-... .. .T.								<i>M. simiae</i>
..-GTG-... .. .T. A. T.								<i>M. nonchromogenicum</i>
..-T-... .. .T. T.								<i>M. terrae</i>
..-GG-G-... .. .GGT. T.								<i>M. xenopi</i>
..-C-... .. .T. T.								<i>M. gordonae</i>
..-C-...GT.								<i>M. szulgai</i>
..-C-...GT.								<i>M. malmoense</i>
..-...TAT. T.								<i>M. intracellulare</i>
..-C-... .. .T. A. T.								<i>M. marinum</i>
..G-... .. .T. T.								<i>'M. cookii'</i>
..GCCTGG. A-T. T.								<i>M. flavescens</i>
..G-... .. .T. A. T.								<i>M. chelonae</i>
..GCCTGG. A-T. T.								<i>M. smegmatis</i>
..G-... .. .T. A. T.								<i>M. fortuitum</i>
..-GGG-... .. .T. A. T.								<i>M. sphagni</i>
..G-... .. .T. A. T.								<i>M. komossense</i>
..G-...-G.G A.G.T.								<i>M. phlei</i>

Fig. 3. Alignment of 16S rRNA sequences. The first nucleotide shown corresponds to *E. coli* 16S rRNA position 123 (Brosius *et al.*, 1978). The nucleotide sequence of *M. bovis*, *M. bovis* BCG, *M. tuberculosis* H37 and *M. africanum* is identical to that of *M. tuberculosis* and therefore not shown. *M. tuberculosis* was used as the reference sequence; nucleotides different from those of *M. tuberculosis* are indicated; bars indicate deletions. The nucleotide sequence was determined using oligonucleotides pB or pC*. Both primers result in identical sequence information, but obtain it from opposite strands.

As expected from the location of this thoroughly investigated region in an rRNA segment characterized by its interspecies variability (Woese *et al.*, 1983; Woese, 1987), an intensive search of published 16S rRNA sequences (Dams *et al.*, 1988) gave no hint of convergent sequence homology in unrelated organisms within this part of the gene. Another less conserved region, which may be useful for confirmation of mycobacterial species

already characterized by sequencing region 123–273, is shown in Fig. 4. The sequence determination in this part of the 16S rRNA is especially useful for differentiating *M. gastri*/*M. kansasii* from *M. simiae* and *M. scrofulaceum*. Sequencing with the appropriate primer, pC or pD*, can be performed using the same amplified DNA fragment as that for determining the sequence of region 123–273.

430	440				450				460				470				480				490				500				
o	o				o				o				o				o				o				o				
AAA	CC-T	CTT	TCA	CCA	TCG	ACG	AAG	G--	TC	CGG	GTT	CTC	TCG	GAT	TGA	CGG	TAG	GTG	GAG	AAG	AAG	CAC	CGG	CC	<i>M. tuberculosis</i>				
...	...	...	...	...	...	...	...	...	...	...	...	T...	...	...	...	...	...	...	...	...	...	...	...	...	<i>M. avium</i>				
...	...	...	...	...	...	...	...	...	...	...	...	T...	...	...	...	...	...	...	...	...	...	...	...	...	<i>M. paratuberculosis</i>				
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	<i>M. gastri</i>				
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	<i>M. kansasii</i>				
...	...	...	...	...	...	...	...	...	CT	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	<i>M. scrofulaceum</i>				
...	...	...	...	G	GG	...	...	C--	G	A--	---	---	...	...	...	...	...	C	...	C	...	...	...	...	<i>M. simiae</i>				
...	...	...	...	GT	...	...	...	CTC	CA	A	...	TCT	...	...	...	...	...	...	A	C	...	...	...	...	<i>M. nonchromogenicum</i>				
...	...	...	...	GT	...	...	...	CTC	CG	T	...	TCT	G	...	...	...	...	...	A	C	...	...	...	...	<i>M. terrae</i>				
...	...	C	...	...	G	C	...	...	C--	G	...	...	...	...	TGG	...	...	...	G	C	...	...	...	...	<i>M. xenopi</i>				
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	<i>M. goodii</i>				
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	<i>M. szulgai</i>				
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	<i>M. malmoense</i>				
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	<i>M. intracellulare</i>				
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	<i>M. marinum</i>				
...	...	...	...	G	GT	GG	...	C--	G	A--	---	---	...	...	...	...	...	C	C	A	C	...	...	G	...	<i>M. flavescens</i>			
...	...	...	...	AT	GG	...	...	C--	G	A--	---	---	---	...	...	...	...	C	C	A	T	...	...	G	...	<i>M. chelonae</i>			
...	...	...	...	G	CA	...	...	C--	G	A--	---	---	---	...	...	...	...	...	T	...	C	...	...	G	...	<i>M. smegmatis</i>			
...	...	...	...	AT	GG	...	...	C--	G	A--	---	---	---	...	...	...	...	C	C	A	T	...	...	G	...	<i>M. fortuitum</i>			

Fig. 4. Alignment of 16S rRNA sequences. The first nucleotide shown corresponds to *E. coli* 16S rRNA position 429 (Brosius *et al.*, 1978). *M. tuberculosis* was used as the reference sequence; nucleotides different from those of *M. tuberculosis* are indicated; bars indicate deletions. The nucleotide sequence was determined using oligonucleotides pC or pD*. Both primers result in identical sequence information, but obtain it from opposite strands.

It is well established that the variability of 16S rRNA sequences is conserved at the species level (Woese, 1987). In order to prove that different strains belonging to the same species exhibit identical variable regions within the 16S rRNA molecule, clinical isolates from the *M. tuberculosis* complex were obtained from K. Schröder, Forschungsinstitut Borstel, FRG. Thirteen isolates of *M. tuberculosis*, two isolates of *M. bovis* and two isolates of *M. africanum* were investigated, all of which were found to possess a 16S rRNA sequence identical to that of the *M. tuberculosis* strain shown in Fig. 3 (data not shown). These results demonstrate conservation of the variable nucleotides within the 16S rRNA molecule at the species level.

Nucleic acid technology has allowed bacterial classification to include elucidation of evolutionary relationships by comparative analysis of functionally equivalent macromolecules (Zuckerandl & Pauling, 1965). Indeed, the determination of rRNA sequences has become a powerful tool for the systematic classification of bacteria (Fox *et al.*, 1980; Woese *et al.*, 1983; Gray *et al.*, 1984; Woese, 1987). It has only recently emerged that parts of the 16S rRNA may be used to identify micro-organisms (Göbel & Stanbridge, 1987; Musial *et al.*, 1988; Rossau *et al.*, 1989; Stackebrandt *et al.*, 1989), thus providing an alternative to the classical determinative approach, in which phenotypic criteria are used to identify species. Because of the overall conserved nature of rRNA, it is difficult to define stretches of nucleotides specific for very closely related taxa which may only be divided at a subspecific level. Thus, phenotypic distinctions can be made for *M. tuberculosis*, *M. bovis* and *M. africanum*, but

100% homology in the 16S rRNA structure rules out differentiation at this level. Similarly, *M. gastri* differs from *M. kansasii* in phenotypic features, although both species share an identical small subunit rRNA sequence.

Until now, the identification of cultural isolates of mycobacteria based upon phenotypic and chemotaxonomic features has traditionally been laborious and time-consuming. In addition, identification of mycobacterial isolates is complicated by disparities in the growth and metabolic rates of members of different species, and by the need for a variety of specialized and complex tests. In contrast, our approach is directed at the rapid determination of 16S rRNA sequences shown to be species-specific. Barely grown primary cultures suffice, circumventing the need for complex biochemical tests, and for subcultivating strains after their initial isolation. Currently 5000 mycobacterial cells are sufficient to yield a sequenceable amplified gene fragment. This level of sensitivity can be further improved (to 100 cells) by first reverse-transcribing the rRNA into cDNA before using it in the PCR (data not shown). The rapidity (from cell pellet to information on the nucleotide sequence within 2 d) and technical simplicity of the approach presented in this paper compare favourably with the traditional determination of phenotypic characters.

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