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Comparative evaluation of sequence analysis of 16S rRNA and *rpoB* genes for identification of aquatic mycobacteria

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Abstract: The nucleotide sequences of partial 16S rRNA and bacterial RNA polymerase β-subunit (*rpoB*) genes for 57 mycobacterial strains were determined. Compared to the 16S rRNA gene sequences, variable regions were scattered along the whole fragment sequence, indicating that the *rpoB* gene is more polymorphic. Unlike 16S rRNA sequences, species variation was observed within *M. fortuitum* strains. The topology of the *rpoB*-based phylogenetic tree was almost the same as that of the 16S rRNA sequence analysis. These results suggest that the *rpoB* gene is a highly conserved gene, and taxonomical studies based on this gene may be comparable with similar studies based on the 16S rRNA gene. The overall mean distance for *rpoB*-gene–based sequences was 2.5 times greater than that of the 16S rRNA gene for all 57 mycobacterial strains examined. However, some slowly growing mycobacteria could not be differentiated based on *rpoB* gene sequences. Moreover, a bootstrap value above 70% was observed for 13 nodes, while this value was 14 nodes in the case of 16S rRNA sequences. To the best of our knowledge, this is the first investigation evaluating the use of 16S rRNA and *rpoB* sequence analyses for identification of aquatic mycobacteria obtained from diverse geographical locations.

Key words: Sequence analyses, 16S rRNA, *rpoB*, aquatic mycobacteria

1. Introduction

Mycobacterium spp. are common fish pathogens frequently detected in marine and estuarine environments (1,2). Molecular identification of such microorganisms is emerging as an alternative and complementary method to established phenotypic techniques. Genotypic identification of bacteria typically involves the use of conserved sequences within phylogenetically informative genetic targets, such as the 16S rRNA gene. Sequence analysis of the 16S rRNA gene has been widely used for identification and phylogenetic studies of bacteria including mycobacteria (3,4).

However, despite the extensive contribution of 16S-rRNA–based methods in identification of known and novel species of mycobacteria, the use of this genotypic target is not without limitations. Unlike other bacteria, the 16S rRNA sequences of mycobacteria are very similar, and in some species are even identical, with similarities between 94.3% and 100% (5).

Hence, during the last few years alternative molecular targets have been explored. Sequence analyses of the *hsp65* gene (6), *recA* (7), *SecA1* (8), DNA gyrase (9), internal transcribed spacer of the 16S-23S rRNA (10), and *rpoB* (11) have been attempted. Of these, analysis of the *rpoB* gene has been strongly recommended as a supplementary or alternative method to 16S rRNA gene analysis of mycobacteria (11–13). In all cases, the results of *rpoB* were superior to those of 16S rRNA. Thus, the aims of this study were to evaluate the use of partial sequence analysis of these two genes for identification and phylogenetical studies of aquatic mycobacteria.

2. Materials and methods

2.1. Bacterial isolates

Fifty-seven aquatic mycobacterial strains, including 10 reference strains and 47 field isolates obtained from different geographical locations, were used in this study (Table 1). All strains were grown on Middlebrook 7H10 medium supplemented with oleic acid–albumin–dextrose–catalase (OADC) (both from Becton-Dickinson, Franklin Heights, NJ, USA) and 0.5% glycerol and incubated at 22 °C or 30 °C for 1 to 4 weeks depending on their growth rate.

2.2. DNA preparation

DNA templates were prepared following methods described by Pourahmad et al. (14) with no modification.

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Table 1. Species of mycobacteria used in this study with source and origin.

Species as received	Strain	Source	Origin
M. marinum	NCIMB 1297[a]	Unspeciated marine fish	USA
M. fortuitum	NCIMB 1294	Chinook salmon (*Oncorhynchus tshawytscha*)	Unknown
M. fortuitum	NCIMB 1295	Unknown fish	Unknown
M. chelonae	NCIMB 13533	Atlantic salmon (*Salmo salar*)	UK
M. peregrinum	NCTC 10264[b]	Human	Mexico
M. senegalense	NCTC 10956	Bovine lymph node	France
M. shottsii	NCTC 13215[T *]	Striped bass (*Morone saxatilis*)	USA
M. pseudoshottsii	NCTC 13318[T]	Striped bass (*Morone saxatilis*)	USA
M. conceptionense	CIP 108544[T c]	Human	France
'*M. angelicum*'[d]	DSM 45057 [T e]	Freshwater angelfish (*Pterophyllum scalare*)	Slovenia
'*M. aemonae*'	DSM 45058[T]	Goldfish (*Carassius auratus*)	Slovenia
M. stomatepiae	DSM 45059[T]	Striped barombi nsess (*Stomatepia mariae*)	UK
M. fortuitum	TB1[f]	Siamese fighting fish (*Betta splendens*)	Thailand
M. salmoniphilum	MT1900	Atlantic salmon (*Salmo salar*)	UK
M. marinum	S7	Snakehead fish (*Channa striata*)	Thailand
M. fortuitum	S11	Snakehead fish (*Channa striata*)	Thailand
M. fortuitum	S12	Snakehead fish (*Channa striata*)	Thailand
M. fortuitum	S13	Snakehead fish (*Channa striata*)	Thailand
M. fortuitum	S14	Snakehead fish (*Channa striata*)	Thailand
M. fortuitum	S18	Snakehead fish (*Channa striata*)	Thailand
M. fortuitum	S269	Snakehead fish (*Channa striata*)	Thailand
M. fortuitum	IoA4	Unknown fish	Unknown
M. fortuitum	IoA5	Unknown fish	Unknown
M. fortuitum	IoA6	Unknown fish	Unknown
M. gordonae	79/02[g]	Goldfish (*Carassius auratus*)	Slovenia
M. fortuitum	277/2/01	Three-spot gourami (*Trichogaster trichopterus*)	Slovenia
M. fortuitum	55/02	Sterlet (*Acipenser ruthenus*)	Slovenia
M. fortuitum	276/7/01	Guppy (*Poecilia reticulata*)	Slovenia
M. gordonae	126/1/03	Freshwater angelfish (*Pterophyllum scalare*)	Slovenia
M. fortuitum	42/04	Goldfish (*Carassius auratus*)	Slovenia
M. fortuitum	276/3/01	Goldfish (*Carassius auratus*)	Slovenia
M. fortuitum	276/5/01	Three-spot gourami (*Trichogaster trichopterus*)	Slovenia
M. marinum	131/03	Three-spot gourami (*Trichogaster trichopterus*)	Slovenia
M. gordonae	126/1/03	Freshwater angelfish (*Pterophyllum scalare*)	Slovenia
M. fortuitum	42/04	Goldfish (*Carassius auratus*)	Slovenia

Table 1. (Continued).

Species as received	Strain	Source	Origin
M. fortuitum	276/3/01	Goldfish (*Carassius auratus*)	Slovenia
M. fortuitum	276/5/01	Three-spot gourami (*Trichogaster trichopterus*)	Slovenia
M. marinum	131/03	Three-spot gourami (*Trichogaster trichopterus*)	Slovenia
M. gordonae	277/3/01	Guppy (*Poecilia reticulata*)	Slovenia
M. marinum	5/9/03	Human	Slovenia
M. chelonae	29/02	Goldfish (*Carassius auratus*)	Slovenia
M. fortuitum	11/02	Dwarf gourami (*Colisa lalia*)	Slovenia
M. gordonae	49/21/03	Tap water	Slovenia
M. stomatepiae	T3	Striped barombi nsess (*Stomatepia mariae*)	UK
M. stomatepiae	T4	Striped barombi nsess (*Stomatepia mariae*)	UK
M. chelonae	T5-2	Rosy barb (*Puntius conchonius*)	UK
M. chelonae	T9	Lumpsucker (*Cyclopterus lumpus*)	UK
M. chelonae	T14	Yellow seahorse (*Hippocampus kuda*)	UK
M. marinum	T16	Otjikoto tilapia *(Tilapia guinasana*)	UK
M. marinum	SO020195[i]	Red drum (*Scianops ocellatus*)	Israel
M. marinum	SR180194	Rabbit fish (*Siganus rivulatus*)	Israel
M. marinum	DL180892	Sea bass (*Dicentrarchus labrax*)	Israel
M. marinum	DL150191	Sea bass (*Dicentrarchus labrax*)	Israel
M. marinum	DL240490	Sea bass (*Dicentrarchus labrax*)	Israel
M. marinum	CF030494	Butterfly fish (*Chaetodon fasciatus*)	Israel
M. marinum	DL045	Thalassa (*Dicentrarchus labrax*)	Greece
M. marinum	DP241194	Sharp snout sea bream (*Diplodus puntazzo*)	Israel
M. marinum	1521/68	Unknown	Germany
M. marinum	L.L. ATCC	Unknown	USA
M. marinum	DL921110	Sea bass (*Dicentrarchus labrax*)	Denmark
Mycobacterium sp.	DL049	Thalassa (*Dicentrarchus labrax*)	Greece
M. marinum	“Hernandez”	Unknown fish	Germany

[a]: National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK.
[b]: National Collection of Type Cultures, UK.
[c]: Collection de l’Institut Pasteur.
[d]: Not yet standing in nomenclature.
[e]: Deutsche Sammlung von Mikroorganismen und Zellkulturen.
[f]: Strains obtained from different countries and held at –70 in the Institute of Aquaculture (IoA), University of Stirling.
[g]: Mycobacterial isolates from Slovenia were kindly provided by Dr Mateja Pate of the Veterinary Faculty Ljubljana, University of Ljubljana, Gerbiceva 60, 1115 Ljubljana, Slovenia.
[h]: Mycobacterial strains isolated from London Zoo Aquarium during this study.
[i]: Isolates from SO020195 to DP241194 were kindly provided by Dr Angelo Colorni of the Israel Oceanographic and Limnological Research Ltd., National Centre for Mariculture, Eilat, Israel.
[*]: Type strain.

2.3. PCR

Primers used to amplify a part (c. 550 bp) of the 16S rRNA gene were pA (5′-AGAGTTTGATCCTGGCTCAG-3′) (15) in combination with primer 266 (5′-CACGCTCACAGTTAAGCCGT-3′) (16). Ten to 50 ng of DNA was added to 50-µL PCR reactions containing a mixture of 25 µL of PCR MasterMix (Thermo, Epsom, UK), ultrapure water (Millipore, Watford, UK), and 25 pmol of each primer with the following thermal profile: 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s, and a final extension at 72 °C for 7 min. In the case of the *rpoB* gene, primer RPO5′ (5′-TCAAGGAGAAGCGCTACGA-3′) in combination with RPO3′ (5′-GGATGTTGATCAGGGTCTGC-3′) (10 pmol of each) was used to amplify a 360-bp fragment of *this target* gene following the thermal profile described previously (17). In both cases, PCR reactions were performed in an automated T-gradient thermocycler (Biometra, Göttingen, Germany). To confirm successful PCR amplification, the presence of an amplicon of the expected size was visualized by electrophoresis (using 10 µL of completed PCR reaction on a 2.0% agarose gel stained with ethidium bromide and visualized under UV light).

2.4. Sequencing and analysis

Using all the PCR primers mentioned, sequence analysis was carried out following the method described by Pourahmad et al. (18). Briefly, PCR amplicons were purified using a QIAquick purification kit (Qiagen, Crawley, UK) and sequenced in both directions using the original amplicon primers. Fluorescently labelled templates were generated using a Genome Lab Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter Ltd., High Wycombe, UK) and read on a CEQ8800 automated capillary sequencer (Beckman, Coulter). Both forward and reverse sequences were assembled and edited using the SeqMan II module of Lasergene, version 6 (DNASTAR). The resulting consensus sequences were compared with published nucleotide sequences by BLASTn analysis.

2.5. Nucleotide sequence accession numbers

The nucleotide sequences for 16S rRNA gene were deposited in GenBank under accession numbers AM885899–AM884333 and EF535601-2. In addition, the *rpoB* gene sequences were deposited in GenBank under accession numbers AM885871–AM885927.

3. Results

3.1. Sequence and phylogenetic analysis the 16S rRNA gene

Amplification products of the 16S rRNA were obtained from all mycobacteria tested. A representative selection of the amplicons obtained from PCR amplification is shown in Figure 1. Using PCR primers pA and 266 for the sequence analysis resulted in a band approximately 550 bp in length that completely covered both variable regions A (helix 10) and B (helix 18) in all the mycobacteria isolates studied.

The nucleotide sequences of partial 16S rRNA gene for 57 mycobacterial isolates were determined, and the results are summarised in Table 2. Species identification by sequence analysis, percentage similarity with other mycobacteria (the first two best matches), and the number of the nucleotides (bp) obtained from the two primers used (pA and 266) for the partial sequencing of 16S rRNA are presented.

The type strain *M. shottsii* with 2 bp and 4 bp differences from *M. marinum* and *M. pseudoshottsii* was 99.6% and 99.3% similar to these two species, respectively.

Isolates of *M. peregrinum*, including the reference strain (NCTC 10264) were identical but gave a 100%

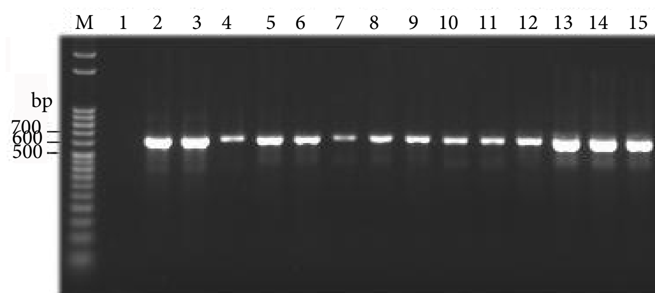

Figure 1. The PCR products obtained from amplification of 16S rRNA for representative selection of aquatic mycobacteria.
M = molecular size marker (50 bp ladder).
Lanes: 1) –ve control; 2) *M. marinum* (NCIMB 1297); 3) *M. shottsii* (NCTC 13215^T); 4) *M. pseudoshottsii* (NCTC 13318^T); 5) *M. peregrinum* (NCTC 10264); 6) *M. conceptionense* (CIP 108544^T); 7) *M. fortuitum* (NCIMB 1295); 8) *M. senegalense* (NCTC 10956); 9) *M. marinum* L.L. ATCC; 10) *M. marinum* DP241194; 11) *M. marinum* DL240490; 12) *M. conceptionense* TB1; 13) *M. conceptionense* 42/04; 14) *M. fortuitum* S7; 15) *M. fortuitum* S269.

Table 2. Identification of aquatic mycobacteria by partial and almost complete sequence analysis of the 16S rRNA gene.

Species	No. of isolates	Size (bp)	First match (%)	Second match (%)
M. marinum	12	565	*M. marinum* (100)	*M. ulcerans* (100)
M. marinum	4	565	*M. pseudoshottsii* (100)	*M. marinum* (99.6)
M. shottsii	1	565	*M. shottsii* (100)	*M. marinum* (99.6)
M. pseudoshottsii	1	565	*M. pseudoshottsii* (100)	*M. marinum* (99.7)
M. fortuitum	6	551	*M. fortuitum* (100)	*M. farcinogenes* (99.6)
M. peregrinum	3	551	*M. peregrinum* (100)	*M. septicum* (100)
M. conceptionense	8	551	*M. farcinogenes* (99.6)	*M. conceptionense*
M. conceptionense	3	551	*M. conceptionense* (100)	*M. farcinogenes* (99.6)
M. senegalense	1	553	*M. farcinogenes* (100)	*M. senegalense* (100)
M. mucogenicum	2	551	*M. ratisbonense* (100)	*M. mucogenicum* (100)
M. gordonae	3	568	*M. gordonae* (100)	*M. gordonae* (99–100)
M. arupense	1	567	*M. arupense*	*M. nonchromogenicum* (99.2)
M. chelonae	4	551	*M. chelonae* (100)	*M. chelonae* (99)
M. salmoniphilum	2	551	*M. salmoniphilum* (100)	*M. abscessus* (99)
M. stomatepiae	3	553	*M. stomatepiae* (100)	*M. florentinum* (99.7)
'M. angelicum'	1	565	*M. malmoense* (99.1)	*M. bohemicum* (98.9)
'M. aemonae'	1	563	*M. bohemicum* (98.9)	*M. malmoense* (98.9)
Mycobacterium sp. DL049	1	551	*Mycobacterium* sp.	*M. chubuense* (98.7)

match with both *M. peregrinum* and *M. septicum*. Likewise, all strains of *M. fortuitum* were assigned as this species (100% matched), and they were also very similar to *M. farcinogenes*, *M. senegalense*, and *M. conceptionense* (99.6% similarity).

Mycobacterium conceptionense and *M. senegalense* type strains, as well as isolates IoA4, IoA6, TB1, S14, S18, 55/02, 276/5/01, 276/7/01, 11/02, and 42/04 were 99.6% similar to *M. farcinogenes*, *M. senegalense*, *M. conceptionense*, and *M. fortuitum* over the first 550 bp of the 16S rRNA gene.

Isolates of *M. stomatepiae* had 2 nucleotide mismatches with the most closely related species, *M. florentinum*, in variable region A.

A phylogenetic tree based on ~550 bp sequences of the 16S rRNA clearly placed slowly and rapidly growing mycobacteria in two major branches. An isolate of *M. arupense*, which is an intermediate species, was clustered with the rapidly growing group in the middle part of the tree, very close to slowly growing mycobacteria (Figure 2). A bootstrap value above 70% was observed for 14 nodes.

3.2. Sequence and phylogenetic analysis of the rpoB gene

The amplified product of a 360-bp fragment of the *rpoB* gene for a representative selection of aquatic mycobacteria is shown in Figure 2.

Using both of the primers used in PCR, nucleotide sequences were obtained for 57 of the mycobacterial strains examined. The results of species identification by sequence analysis and percentage similarity with other mycobacteria (the first two best matches) are presented in Table 3. With some exceptions, sequences were 321 bp in size (after removing primer fragments). The isolate of *M. arupense* had 24-bp insertions. In addition, the *M. chelonae* and *M. salmoniphilum* isolates showed 6-bp insertion nucleotides in positions 61–67 of *M. tuberculosis* strain H37Rv (GenBank accession no.: BX842574).

Compared to the 16S rRNA gene sequences, variable regions were scattered along the whole fragment sequence, indicating that this fragment of the *rpoB* gene is more polymorphic (data not shown).

Unlike the 16S rRNA sequences, species variation was observed within *M. fortuitum* strains. Accordingly,

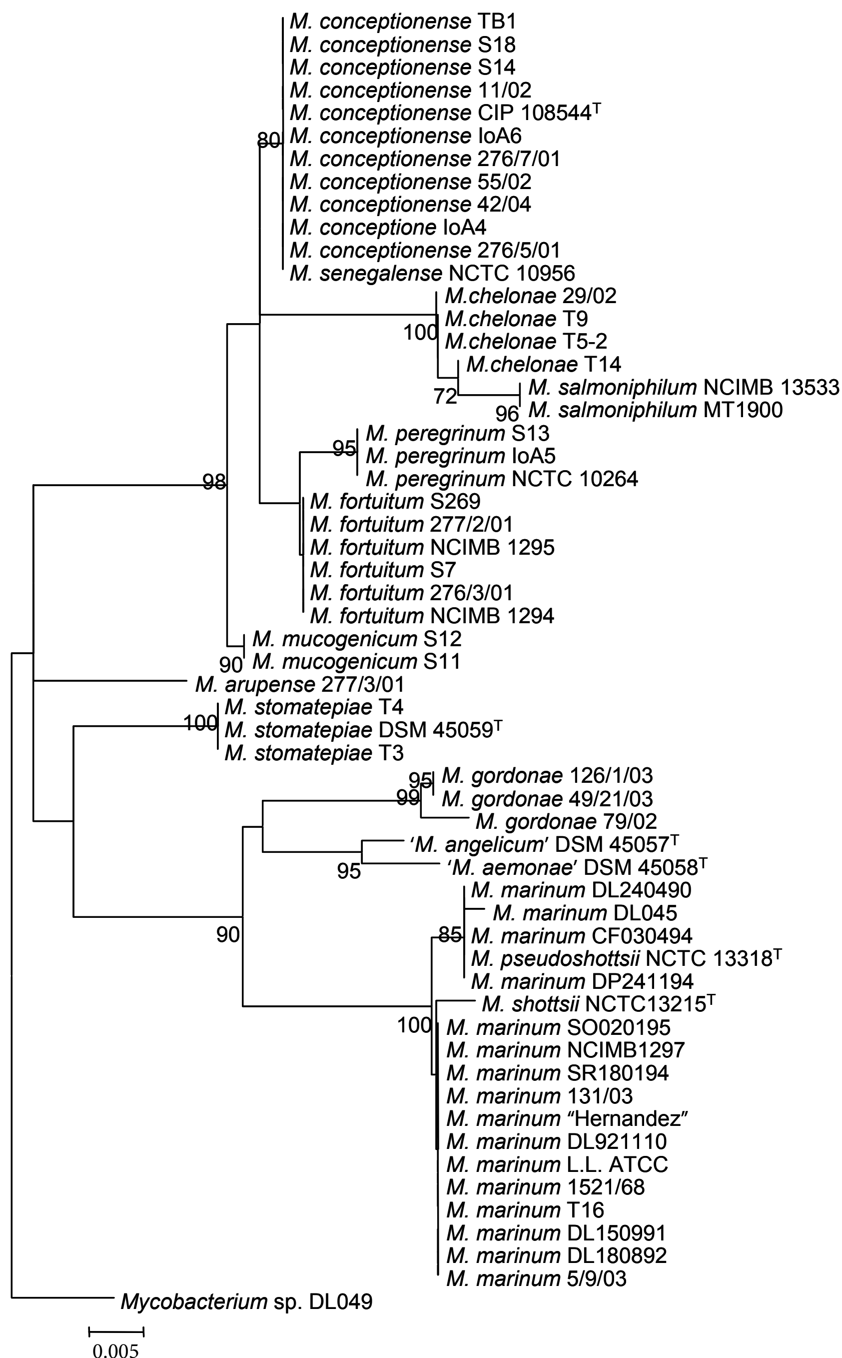

Figure 2. Phylogenetic relationships based on approximately 550 bases of the 16S rRNA genes of aquatic mycobacteria calculated by neighbour-joining method with 1000 replicates. Only bootstrap values above 70% are shown. The scale bar indicates 0.05% nucleotide difference.

isolates S7, S269, and 277/2/01 had a 1-bp difference from the reference strains of *M. fortuitum* and isolate 276/3/01, showing 99.7% similarity to this particular species. Likewise, strains of *M. conceptionense* were divided into two groups. Isolates IoA4, IoA6, S18, S14, and TB1 clustered with *M. conceptionense* CIP 108544^{T} (100% matched); all *M. conceptionense* isolates from Slovenia grouped with *M. senegalense* (NCTC 10956) and differed from the first group by 1 nucleotide.

Table 3. Identification of aquatic mycobacteria by sequence analysis of the *rpoB* gene (360 bp).

Species	No. of isolates	First match (%)	Second match (%)
M. marinum	12	'*M. liflandii*' (98.8)	*M. ulcerans* (98.4)
M. marinum	4	'*M. liflandii*' (99.7)	*M. ulcerans* (99.4)
M. shottsii	1	'*M. liflandii*' (98.4)	*M. ulcerans* (98.1)
M. pseudoshottsii	1	'*M. liflandii*' (99.7)	*M. ulcerans* (99.4)
M. fortuitum	3	*M. fortuitum* (100)	*M. fortuitum* (98.8)
M. fortuitum	3	*M. fortuitum* (99.7)	*M. fortuitum* (98.4)
M. peregrinum	1	*M. peregrinum* (100)	*M. fortuitum* (96.5)
M. peregrinum	2	*M. peregrinum* (99.7)	*M. fortutitum* (96.9)
M. conceptionense	7	*M. conceptionense* (100)	*M. farcinogenes* (98.8)
M. conceptionense	4	*M. conceptionense* (99.7)	*M. farcinogenes* (99.1)
M. senegalense	1	*M. conceptionense* (99.7)	*M. farcinogenes* (99.1)
M. mucogenicum	2	*M. peregrinum* (99.7)	*M. fortuitum* (96.9)
M. gordonae	3	*M. avium* (93.8)	*M. gordonae* (100)
M. arupense	1	*M. avium* (93.7)	'*M. liflandii*' (93.5)
M. chelonae	4	*M. chelonae* (100)	*M. phocaicum* (99.1)
M. salmoniphilum	2	*M. chelonae* (95.4)	*M. chelonae* (94.8)
M. stomatepiae	3	*M. avium* (92.2)	*M. mageritense* (91.9)
'*M. angelicum*'	1	*M. avium* (93.1)	*M. goodii* (90.4)
'*M. aemonae*'	1	*M. avium* (92.2)	*M. mageritense* (91.9)
Mycobacterium sp. DL049	1	*M. goodii* (90.7)	*M. avium* (90.0)

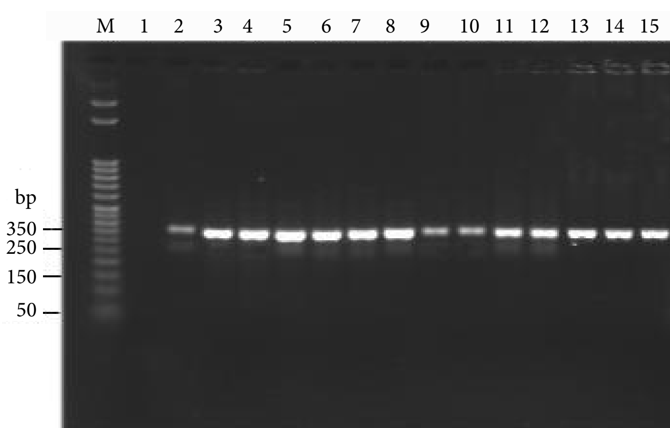

Figure 3. The PCR products obtained from amplification of *rpoB* gene for a representative selection of aquatic mycobacteria.
M = molecular size marker (50 bp ladder).
Lanes: 1) –ve control; 2) *M. marinum* (NCIMB 1297); 3) *M. shottsii* (NCTC 13215^T); 4) *M. pseudoshottsii* (NCTC 13318^T); 5) *M. peregrinum* (NCTC 10264); 6) *M. conceptionense* (CIP 108544^T); 7) *M. fortuitum* (NCIMB 1295); 8) *M. senegalense* (NCTC 10956); 9) *M. marinum* L.L. ATCC; 10) *M. marinum* DP241194; 11) *M. marinum* DL240490; 12) *M. conceptionense* TB1; 13) *M. conceptionense* 42/04; 14) *M. fortuitum* S7; 15) *M. fortuitum* S269.

However, in using the sequence analysis of the *rpoB* gene, some species could not be differentiated. For instance, isolates of *M. mucogenicum* could not be distinguished from *M. peregrinum* field isolates. These isolates diverged from *M. peregrinum* (NCTC 10264) by 1 bp. Similarly, '*M. aemonae*' DSM 45058^T shared the same sequence with *M. stomatepiae* DSM 45059^T (Table 3). Isolates of *M. gordonae*, *M. stomatepiae*, '*M. aemonae*', and '*M. angelicum*' resembled *M. avium*, but with different percentages of similarity. Furthermore, the first best match for all field isolates and reference strains of *M. marinum*, *M. shottsii*, and *M. pseudoshottsii* was '*M. liflandii*' (98.4%–99.7% similarity). The second best match to these species was *M. ulcerans* (98.1%–99.4%).

Using MEGA software version 4.0, the overall mean distance for *rpoB*-gene–based sequences was 2.5 times greater than that of the 16S rRNA gene for all 57 mycobacterial strains examined (data not shown).

The topology of the *rpoB*-based phylogenetic tree was almost the same as that of the 16S rRNA sequence analysis. Again, excluding *M. arupense* isolate 277/3/01, which was placed with slowly growing mycobacteria, all slowly and rapidly growing mycobacteria were clearly separated (Figure 4). However, a bootstrap value above 70% was observed for 13 nodes.

4. Discussion

Sequence analysis of the 16S rRNA gene has been widely used to identify veterinary and human clinical mycobacteria species and has been used for phylogenetic studies (19,20). However, few studies have employed this method for identification or for taxonomic relationships of aquatic mycobacteria. Ucko *et al.* (16) used sequences of almost the entire 16S rRNA gene to characterize and seek heterogeneity of *M. marinum* strains isolated in Israel and other geographical regions. Furthermore, sequence analysis of this gene has been used to analyse several novel aquatic mycobacteria species (21–23). However, in these studies only a few species were included. Indeed, no evaluation study comprising different species of aquatic mycobacteria has been carried out. This is the first investigation evaluating the use of 16S rRNA sequence analysis for identification of known and novel species of aquatic mycobacteria obtained from diverse geographical locations. To validate this research, a reference strain of each species, when available, was also included.

The partial sequencing performed in this study could not be used to discriminate between *M. peregrinum* and *M. septicum*, although the second species has not apparently been isolated from aquatic organisms.

The gene encoding the β-subunit of the bacterial RNA polymerase (*rpoB*) was proposed as an alternative molecular marker for identification of many bacteria including mycobacteria (11,17). Sequence analysis and the PRA method using a 360-bp fragment of *rpoB* was previously employed for identification of mycobacteria in clinical human specimens (13,17) and aquatic sources (24,25). Using PRA analysis, Seok *et al.* (26) was able to differentiate between the isolates of *M. abscessus* and *M. chelonae* responsible for mycobacterial infection in zebrafish.

In this study, sequences of this region of the *rpoB* gene were analysed. The sequence divergence among 57 mycobacteria was 2.5 times greater than that of the 16S rRNA. In a comparison between sequence analyses of 16S rRNA and *rpoB* genes for identification of *Legionella* spp., the latter was 3.5 times more variable (27). Furthermore, the inferred phylogenetic tree resulting from the analysis of the *rpoB* gene placed all slowly and rapidly growing mycobacteria in the same group as in the 16S rRNA. In addition, the isolate of *M. arupense* was clustered with slowly growing mycobacteria in the *rpoB* gene analysis, while the 16S-based tree failed to produce this outcome. *Mycobacterium arupense* was recently described as an intermediate species, between slowly and rapidly growing mycobacteria (28). However, as it belongs to the *M. terrae*-like group (29), it should be considered a slow-growing *Mycobacterium*. These results suggest that the *rpoB* gene is highly conserved, and taxonomical studies based on this gene may be comparable with similar studies based on the 16S rRNA gene. The latter gene has been proposed as a molecular clock based on its slow and constant rate of mutation over time (30).

When sequences from the present study were compared with sequences available in databanks, well-known species such as *M. marinum*, *M. gordonae*, and more recently described species, *M. shottsii* and *M. pseudoshottsii*, could not be correctly identified with their corresponding species. For instance, isolates of *M. marinum* isolated from frog were assigned to '*M. liflandii*', a *M. ulcerans*-like species that has not yet been validated (31). This is most likely due to lack of sufficient *rpoB*-gene–based sequences of slowly growing mycobacteria in the databases. Hence, this 360-bp fragment of the *rpoB* gene has very poor coverage in the databases, allowing for correct identification of only a limited number of mycobacteria.

Sequencing of the 16S rRNA gene has great advantages both for identifying and revealing taxonomic relationships among aquatic mycobacteria. Nevertheless, due to high similarities in 16S rRNA gene-based sequences for many mycobacteria, a supplementary gene is required. The *rpoB* gene seems to be an appropriate candidate, provided a more polymorphic fragment than the 360-bp sequence examined in this study is selected. In other words, the

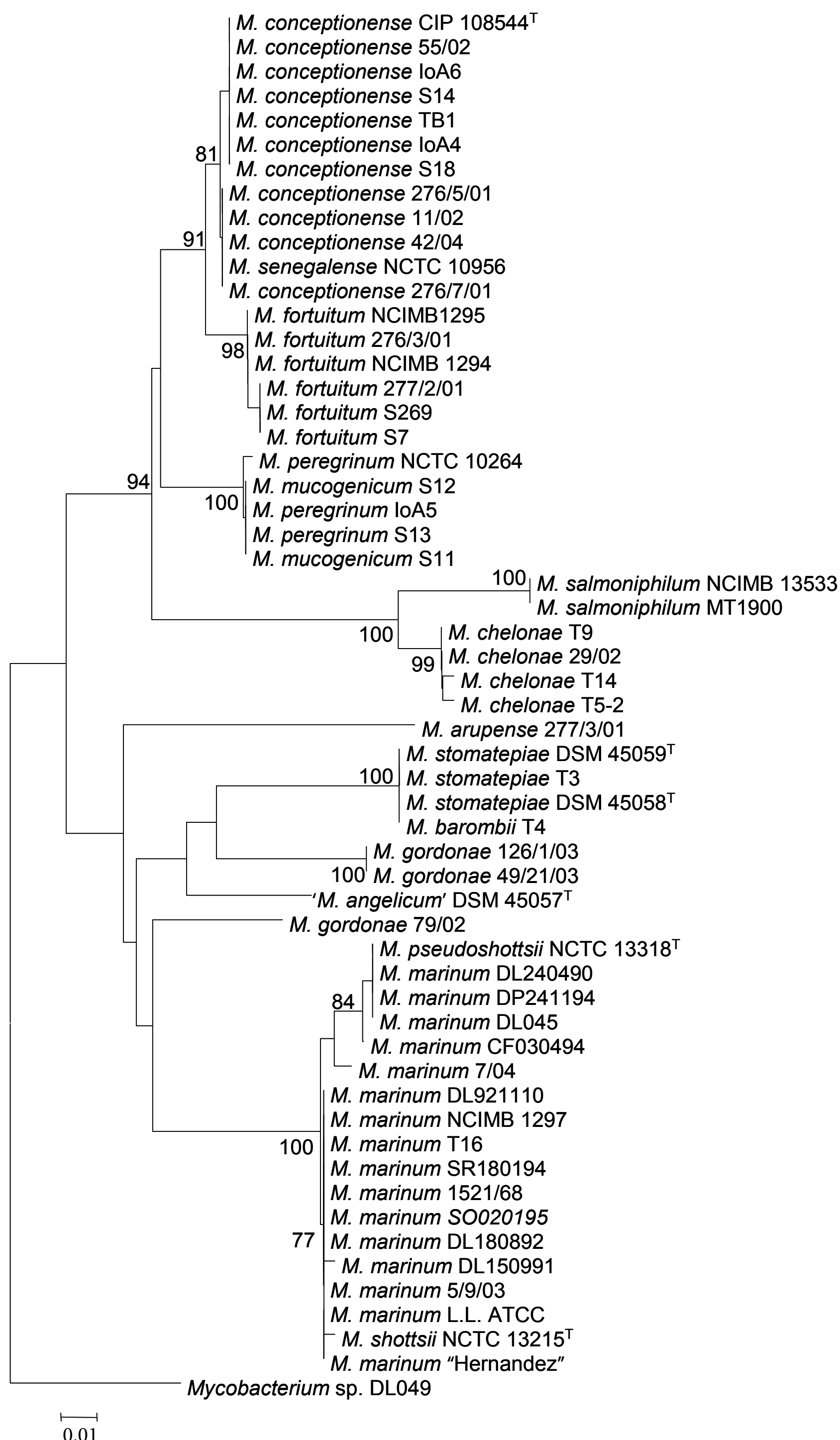

Figure 4. Phylogenetic relationships based on *rpoB* gene sequences of aquatic mycobacteria calculated by neighbour-joining method with 1000 replicates. Only bootstrap values above 70% are shown. The scale bar indicates 0.1% nucleotide difference.

360 bp of the *rpoB* gene analysed in this study was less reliable than the 16S rRNA gene for identification of aquatic mycobacteria, particularly in cases involving novel species. More reliable results are likely to be obtained if other fragments of the *rpoB* genes, i.e. 764 bp (12), are investigated.

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