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Abstract:	Mycobacteria are common causative agents of bacterial infections in many species of freshwater and marine fish. Identification of mycobacteria to the species level based on phenotypic tests is inappropriate and time consuming. Molecular methods such as partial or entire gene sequence determination in mycobacteria have been employed to resolve these problems. The objective of this study was to assess the use of sequence analysis of the mycobacterial 16S-23S internal transcribed spacer (ITS) region for the identification of different aquatic mycobacteria species. Using published primers, ITS sequences of 64 field and reference strains were determined. The identity of all isolates previously identified as M. marinum by restriction fragment length polymorphism (RFLP) DNA profiling was confirmed as M. marinum by sequence analysis. With the exception of five rapidly growing mycobacteria isolates, all other mycobacteria were easily identified by sequencing of the ITS region. Using this spacer region, it was possible to differentiate between slowly growing and rapidly growing mycobacteria, even before sequence analysis, by the size of the PCR product, although species identification could not be made by size alone. Overall, direct sequencing of this genetic element following PCR has been shown to be useful in the identification of aquatic mycobacteria species.

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Identification of Aquatic Mycobacteria based on Sequence analysis of the 16S-23S rRNA Internal Transcribed Spacer (ITS) region

Running title: ITS sequences for Identification of Aquatic Mycobacteria

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Abstract

Mycobacteria are common causative agents of bacterial infections in many species of freshwater and marine fish. Identification of mycobacteria to the species level based on phenotypic tests is inappropriate and time consuming. Molecular methods such as partial or entire gene sequence determination in mycobacteria have been employed to resolve these problems. The objective of this study was to assess the use of sequence analysis of the mycobacterial 16S-23S internal transcribed spacer (ITS) region for the identification of different aquatic mycobacteria species. Using published primers, ITS sequences of 64 field and reference strains were determined. The identity of all isolates previously identified as *M. marinum* by restriction fragment length polymorphism (RFLP) DNA profiling was confirmed as *M. marinum* by sequence analysis. With the exception of five rapidly growing mycobacteria isolates, all other mycobacteria were easily identified by sequencing of the ITS region. Using this spacer region, it was possible to differentiate between slowly growing and rapidly growing mycobacteria, even before sequence analysis, by the size of the PCR product, although species identification could not be made by size alone. Overall, direct sequencing of this genetic element following PCR has been shown to be useful in the identification of aquatic mycobacteria species.

Key words: ITS sequence analysis, Identification, Aquatic mycobacteria

Introduction

The genus *Mycobacterium* are aerobic, non-motile bacteria with surprisingly diverse phenotypes related to growth rate, colony appearance, environmental distribution, and pathogenic potential for eukaryotic hosts (Smole *et al.*, 2002). Commonly, mycobacteria are further characterised into slowly and rapidly growing mycobacteria. Rapidly growing isolates are classified as species, which under optimal nutrient and temperature regimes, produce grossly visible colonies from dilute inocula in solid media in less than 7 days. Slowly growing isolates are classified as those species taking more than 7 days to exhibit visible colonies, again under optimal temperature and nutrient regimes (Lévy-Frébault and Portaels, 1992).In recent years, the number of species of mycobacteria reported in the aquatic environment has greatly increased (Herbst, Costa, Weiss, Johnson, Bartell, Davis, Walsh, & Levi 2001; Pourahmad, Cervellione, Thompson, Taggart, Adams & Richards 2008; Rhodes, Kator, Kotob, van Berkum, Kaattari, Vogelbein, Quinn, Floyd, Butler & Ottinger 2003; Rhodes, Kator, McNabb, Deshayes, Reyrat, Brown-Elliott, Wallace, Trott, Parker, Lifland, Osterhout, Kaattari I., Reece, Vogelbein & Ottinger 2005; Whipps, Butler, Pourahmad, Watral & Kent 2007). Phenotypic methods for identifying mycobacteria, such as acid fast staining, are not useful for identifying mycobacteria to the species level and biochemical tests are time consuming, and even then may not be able to differentiate between the different species of mycobacteria being examined. Molecular methods attempt to resolve these problems. For many years, PCR has been used clinically for the rapid identification of *Mycobacterium* species. However, due to the number of closely related species within the genus *Mycobacterium*, cross reactivity of different species in the PCR frequently occurs, and thus additional methods are required to identify mycobacteria to the species level.

Nucleic acid sequence determination of mycobacteria is another method frequently used to identify different mycobacteria to the species level. With this approach, a fragment or the entire gene from mycobacteria is amplified by PCR. The amplified segment is sequenced and compared to known sequences within available databases i.e. GenBank. Although sequence analysis requires more specialized equipment than other molecular methods, this technology is becoming less expensive. Sequencing also provides the highest level of resolution when looking for differences in a molecular target (Patel, Leonard, Pan, Musser, Berman & Nachamkin 2000).

Several different regions in the *Mycobacterium* genome have been investigated and compared between species and these include the 16S rRNA gene (Kirschner, Springer, Vogel, Meier, Wrede, Kiekenbeck, Bange & Bottger 1993), the heat shock protein 65 gene (*hsp65*) (Ringuet, Koua-Koffi, Honore, Varnerot, Vincent, Berche, Gaillard & Pierre-Audigier 1999), the internal transcribed spacer (ITS) region sequence located between the 16S rRNA and the 23S rRNA genes (Roth, Fischer, Hamid, Michalke, Ludwig & Mauch 1998) and the ß subunit of RNA polymerase gene (*rpoB*) (Kim, Lee, Lyu, Kim, Bai, Kim, Chae, Kim, Cha & Kook 1999). Sequence analysis of the 16S rRNA has been widely used; however, the presence of identical or highly similar 16S rRNA sequences between species limits the use of this target for differentiation (Clarridge 2004; Dobner, Feldmann, Rifai, Loscher & Rinder 1996). Due to sequence variability of the 16S-23S spacer region, several ITS sequence based assays have been developed as an alternative approach for the identification of mycobacteria (Gürtler, Harford, Bywater & Mayall 2006; Mohamed, Kuyper, Iwen, Ali, Bastola & Hinrichs 2005).

The aim of this study was to assess the use of sequence analysis of the mycobacterial 16S-23S internal transcribed spacer region for the identification of different aquatic mycobacteria species.

Materials and Methods

Bacterial strains

Sixty-nine aquatic mycobacterial strains including 12 reference strains and 57 field isolates obtained from different geographical locations were used in this study (Table 1). All strains had been previously identified by RFLP (Pourahmad & Richards, 2013). Isolates were grown on Middlebrook 7H10 medium supplemented with oleic acid–albumin–dextrose–catalase (OADC) (both from Becton-Dickinson, USA) and 0.5% glycerol, and incubated at 22ºC or 30ºC for 1 to 4 weeks depending on their growth rate.

DNA preparation

DNA templates were prepared following methods described by Pourahmad, Thompson, Taggart, Adams & Richards (2008).

PCR

The ITS region in mycobacteria was amplified using forward primer Sp1 (5´-ACCTCCTTTCTA AGGAGCACC-3´) and reverse primer Sp2 (5´-GATGCTCGCAACCACTATCCA-3´) designed by Roth et al. (2000). The PCR was carried out using the following thermal profile: 2 min initial denaturation at 94 ºC followed by 35 cycles of denaturation at 94 ºC, annealing at 60 ºC, and extension at 72 ºC each for 1 min and final incubation at 72 ºC for 5 min. To confirm successful PCR amplification, the presence of an amplicon of 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).

Sequence and analysis

Using both PCR primers (Sp1& Sp2), sequence analysis was carried out following the method described by Pourahmad, Thompson, Adams &Richards (2009).

Nucleotide sequence accession numbers

The nucleotide sequences described herein have been deposited in the GenBank database under accession numbers AM396443-AM396482, AM902922-AM902940 and AM902944-AM902951.

Results

Sequence analysis

The nucleotide sequences of the 16S-23S rRNA spacer region for 64 out of 69 mycobacterial isolates were determined following PCR, the results of which are summarised in Table 2. Species identification by sequence analysis, percentage similarity with other mycobacteria, as well as the actual number of the nucleotides (bp) obtained in the PCR product with the two primers used (Sp1 and Sp2) are presented.

The identity of all isolates, previously identified as *M. marinum*, were confirmed as *M. marinum* by sequence analysis of the ITS region following PCR and all isolates tested gave PCR products of the same size i.e. amplicons of 220 bp.

The results from analysis of the *M. fortuitum* isolates were more varied and for some isolates the sequence analysis could not be completed. Although the majority of the *M. fortuitum* isolates gave variable amplicon sizes in the PCR (from 243 bp to 361 bp), and could easily be distinguished from the slowly growing *M. marinum* isolates. Of the 20 isolates identified as *M. fortuitum* subtype V by RFLP (Pourahmad & Richards, 2013), three isolates (including two reference strains) could not be identified following PCR, as it was not possible to

147 sequence them, despite repeating the extractions and DNA purification. With regard to
148 sequence analysis, many of the *M. fortuitum* isolates were identified as other species and not
149 *M. fortuitum*. Isolate 276/3/01 was clearly assigned as *M. fortuitum* (100% matched), and
150 although isolates S7 and 277/2/01 had identical sequences (Fig. 1), they could not be
151 identified as definite species using ITS sequencing. Overall, they had 93.9% identity with *M.*
152 *senegalense*, but, over a shorter (148 bp) fragment, and showed 98.6% identity with *M.*
153 *fortuitum* (Fig. 2). The remaining 15 isolates of *M. fortuitum* (subtype V), had DNA
154 sequences resembling *M. conceptionense* (99.5-100% matched). Of these, the strains 55/02,
155 276/7/01, 276/5/01, 42/04 and 11/02, isolated in Slovenia, had a transition of A to G at the
156 position of 118 as illustrated in Fig. 2, while isolates IoA5 and S13 were similar to *M.*
157 *peregrinum* (95%) based on 121 bp out of the 309 and 312 bp sequenced, respectively.
158 However, despite several attempts of sequencing the reference strain of *M. peregrinum*, its
159 sequence could not be determined. In addition, isolates S11 and S12, also previously assumed
160 to be *M. fortuitum*, resembled *M. mucogenicum* (98.3%) based on 121bp of the 303 and 319
161 sequenced, respectively (Table 2). *Mycobacterium* sp. DL049 showed a close match (98.1%)
162 with *M. fortuitum*, but this was only for 54 bp in a 253 bp fragment.

163 The alignment of partial sequences of isolates belonging to *M. chelonae* is displayed in Fig. 3
164 and mismatches are shown in rectangles. The ITS sequences of *M. chelonae* (NCIMB 13533)
165 and *M. chelonae* MT1900 are shown to be more polymorphic than the other *M. chelonae*
166 isolates sequenced. When compared against databank sequences, these two strains were
167 similar to *M. salmoniphilum* (99.5-100%), a *M. chelonae*-like species, isolated mostly from
168 salmonid fish (Whipps *et al.* 2007).

169 A variety of other species were also analysed. The nucleotide sequences of *M. stomatepiae*
170 isolates T3, T4 and T11^{T} (DSM 45059^{T}) were identical and unique, with the closest similarity

to *M. lentiflavum* (98.4%). '*Mycobacterium angelicum'* and '*Mycobacterium aemonae'* type strains were assigned as *M. szulgai* (98.3% matched) and *M. kansasii* (92.7% matched), respectively. Of the four isolates of *M. gordonae* examined, isolates 49/21/03 and 126/1/03 had a 100% match and isolate 79/02 had a 96.4% match to this species. Sequence analysis of isolate 277/3/01 was not successful however, despite extracting DNA and sequencing PCR amplicons several times.

The phylogenetic relationships between the aquatic mycobacteria included in this study were analysed by using the neighbour-joining method with Kimura's two parameter distance correction model with 1,000 bootstrap replications in the MEGA, version 4.0, software package (Tamura, Dudley, Nei & Kumar 2007) as illustrated in Fig. 4.

Discussion

Compared to phenotypic methods, genotyping methods and in particular PCR-based methods, have provided more rapid, reliable and cost-effective alternatives for detection and identification of mycobacteria to the species level. Several PCR-based methods such as sequence analysis of various genes have been investigated for clinically important mycobacteria. Amongst the gene regions examined, the spacer region between 16S and 23S gene (ITS) has more sequence variability and as a result can effectively differentiate between closely related mycobacteria. Most reports of using ITS region amplification by PCR followed by sequencing or restriction fragment length analysis have focused on slow growing mycobacteria (Gürtler *et al.*, 2006; Novi, Rindi, Lari & Garzelli 2000; Roth *et al.*, 1998) and to a lesser extent, rapidly growing mycobacteria (Hamid, Rot, Landt, Kroppenstedt, Goodfellow & Mauch 2002; Khan, Selvaraju & Yadav 2005). To date, the use of this variable spacer region as a target to differentiate aquatic mycobacteria has not been reported.

In this study, genus-specific primers designed by Roth *et al.* (2000) were used in PCR to amplify the ITS spacer region of a large number of mycobacteria isolates, and the potential of using sequencing of this region to differentiate and identify aquatic mycobacteria was investigated. Variable sizes of amplicons obtained in the PCR (Table 2) allowed the differentiation of rapidly growing mycobacteria from slowly growing ones even before performing the sequencing.

As mentioned earlier, the number of reports targeting the ITS identifying slowly growing mycobacteria exceed those for rapidly growing mycobacteria. The latter group is known to have more than one copy of rRNA (rrn) per genome (Lappayawichit, Rienthong, Rienthong, Chuchottaworn, Chaiprasert, Panbangred, Saringcarinkul & Palittapongarnpim 1996; Roth *et al.*, 1998) which makes sequencing complicated due to interoperon heterogeneity (Hamid *et al.*, 2002). This was shown to be the case in this study for type strains of *M. fortuitum*, and *M. peregrinum* as well as the presumed *M. gordonae* isolate 277/3/01 as their PCR products could not be directly sequenced. However, all other rapidly growing mycobacteria, including isolates of *M. mucogenicum*, *M. chelonae*, *M. peregrinum* and *M. conceptionense* were easily identified by sequencing. *Mycobacterium fortuitum* isolates 277/2/01 and S7 which resemble *M. senegalense*, had a longer sequence compared to this species and a shorter sequence compared to *M. fortuitum* isolate 276/3/01 (256 bp against 243 bp and 327 bp, respectively) and were 98.6% identical to existing *M. fortuitum* isolates in the databanks for only 148 bp. Whether factors other than interoperon heterogeneity are responsible for the unsuccessful sequencing of those aforementioned strains or not, remains to be established. The co-existence of two or more different species in one sample is another factor which can affect the results of sequence analysis (Xiong, Kong, Yang, Cheng & Gilbert 2006). The cultures used here, however, were morphologically pure and DNA was extracted from a single colony. Cloning of a single copy of the ITS spacer region followed by sequencing, may

tackle the ambiguous results obtained (Hamid *et al.*, 2002; Cloud, Meyer, Pounder, Jost, Sweeney, Carroll & Woods 2006). Further investigations including sequence analysis of other genetic markers such as *hsp65*, 16S rRNA and *rpoB* genes are also warranted.

Using this spacer region, it is possible to differentiate between slowly growing and rapidly growing mycobacteria even before sequence analysis, although it is not possible to identify to species level without sequencing. Therefore, the ITS-based amplification method could be used as a primary screening test to detect mixed infections as well as to differentiate slowly growing from rapidly growing mycobacteria.

Strain differentiation within a particular species has many potential uses (Ucko, Colorni, Kvitt, Diamant, Zlotkin & Knibb 2002). In this study, isolates of *M. conceptionense* from Slovenia showed a nucleotide transition compared to other isolates of this species from different geographical regions. This observation may indicate the value of this target in epidemiological studies relating to mycobacteria.

The constructed phylogenetic tree clustered sequences of new *Mycobacterium* species together. The isolates assigned as *M. mucogenicum* and *M. peregrinum* were placed in the same clade as *M. fortuitum*, whereas the slowly growing *Mycobacterium.* sp. 126/5/01 clustered with *M. gordonae*, also a slowly growing *Mycobacterium*. However, unlike the phylogenetic tree constructed for *hsp65* gene sequences, which clearly separated the clade for slowly growing from rapidly growing mycobacteria (Devulder, de Montclos & Flandrois 2005), this separation was not as clear for ITS sequences.

In the study carried out by Leclerc, Haddad, Moreau & Thorel (2000), ITS sequences were more informative than 16S rRNA sequences since the total number of variable sites was similar for the two markers (103 for ITS versus 142 for 16S rRNA), whereas the length of 16S rRNA sequences (934 nucleotides) was four times longer than the length of ITS

sequences (242 nucleotides). Strains with the same 16S rRNA sequence had different ITS sequences. In the present study, due to variability and short sequence of the ITS spacer (which only needs two primers for getting a clear sequence, compared to 16S rRNA gene which is conserved and has a longer sequence), the direct sequencing of this genetic element has been shown to be useful in the identification of new aquatic mycobacteria species.

In conclusion, this study has shown that sequencing of the 16S-23S ITS region is useful for the detection and identification of different aquatic mycobacteria. With regard to the variability observed with the results of the sequence analysis of ITS region for different mycobacteria isolates, this may be a useful tool in epidemiological studies. With the genus-specific primer set used, it is possible to rapidly and reliably detect mixed cultures of *Mycobacterium* and differentiate slowly from rapidly growing mycobacteria in microbiological laboratories.

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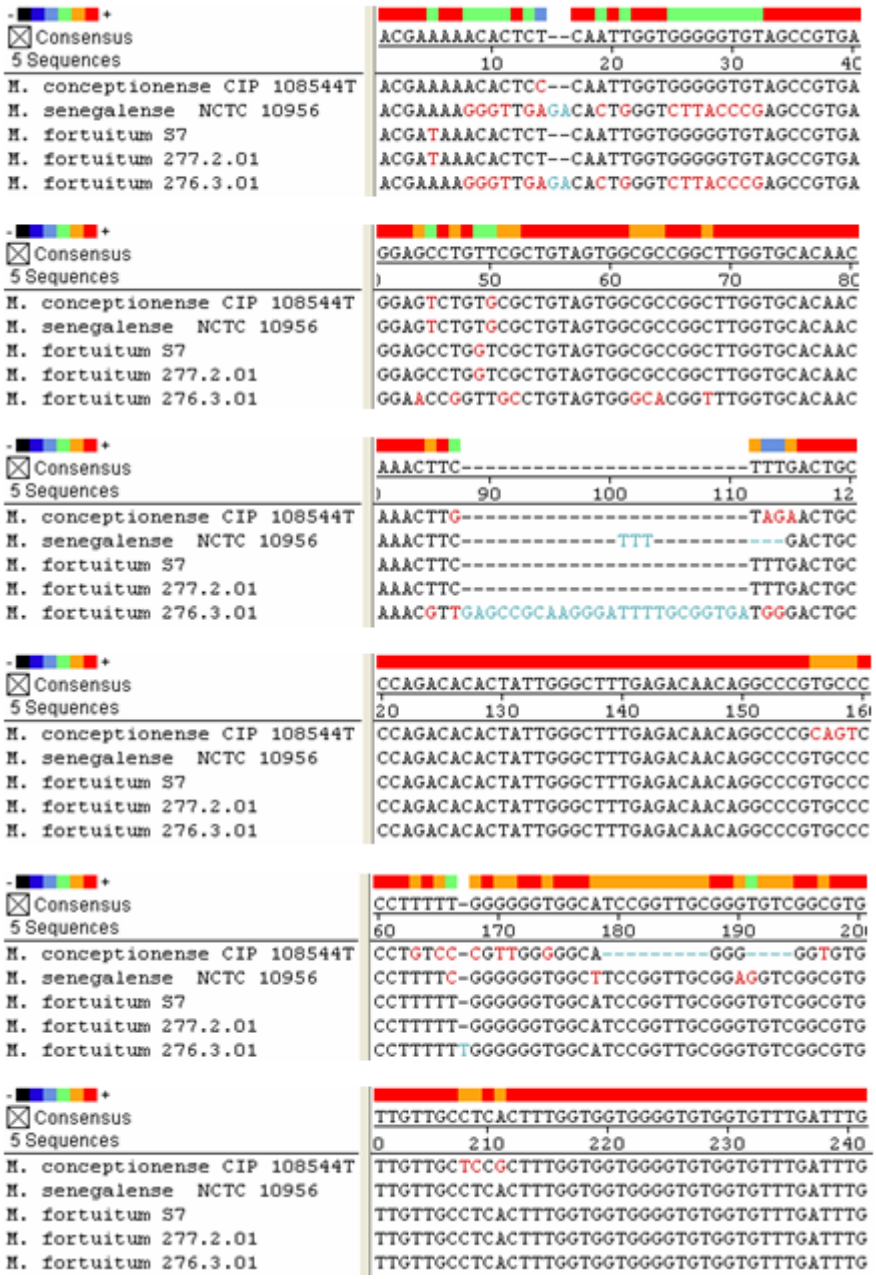

Fig. 1: Alignment of a representative selection of sequences of 16S-23S rRNA ITS for aquatic mycobacteria using SeqMan II programme of Lasergene, Version 6.0 (DNASTAR). The bar indicates the degree of conserved areas of the sequences.

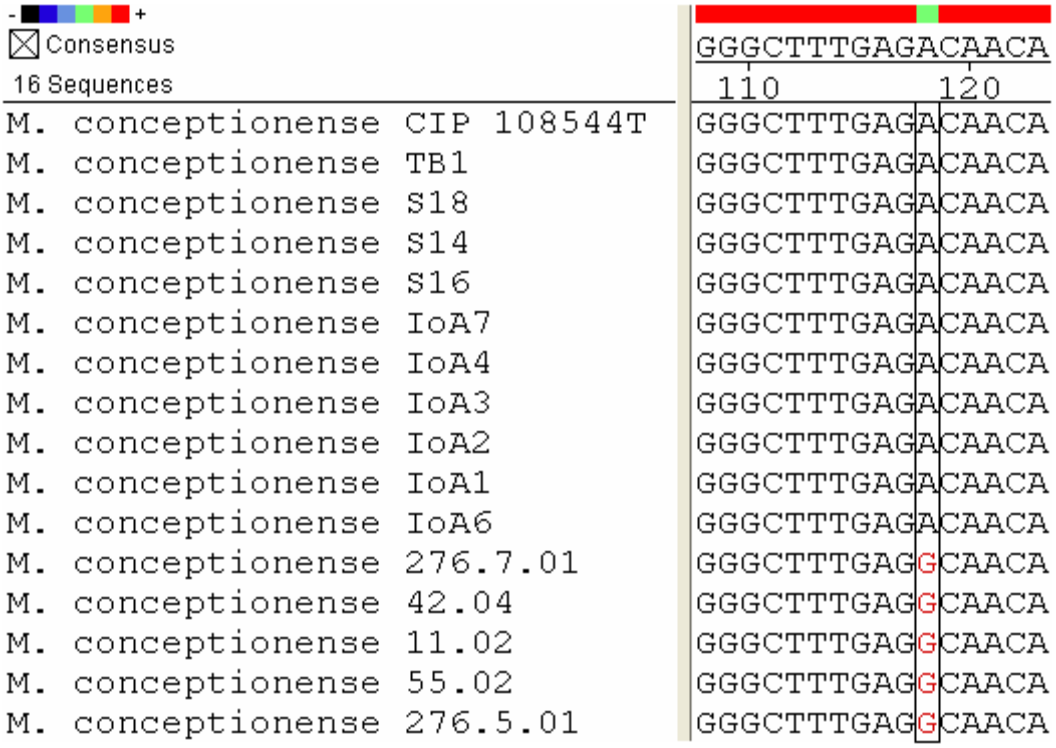

Fig. 2: Alignment of the 16S-23S rRNA ITS sequences for *M. conceptionense* isolated from different geographical regions by using Clustal W of MegAlign programme of Lasergene, Version 6.0 (DNASTAR). The bar indicates the degree of conserved areas of the sequences

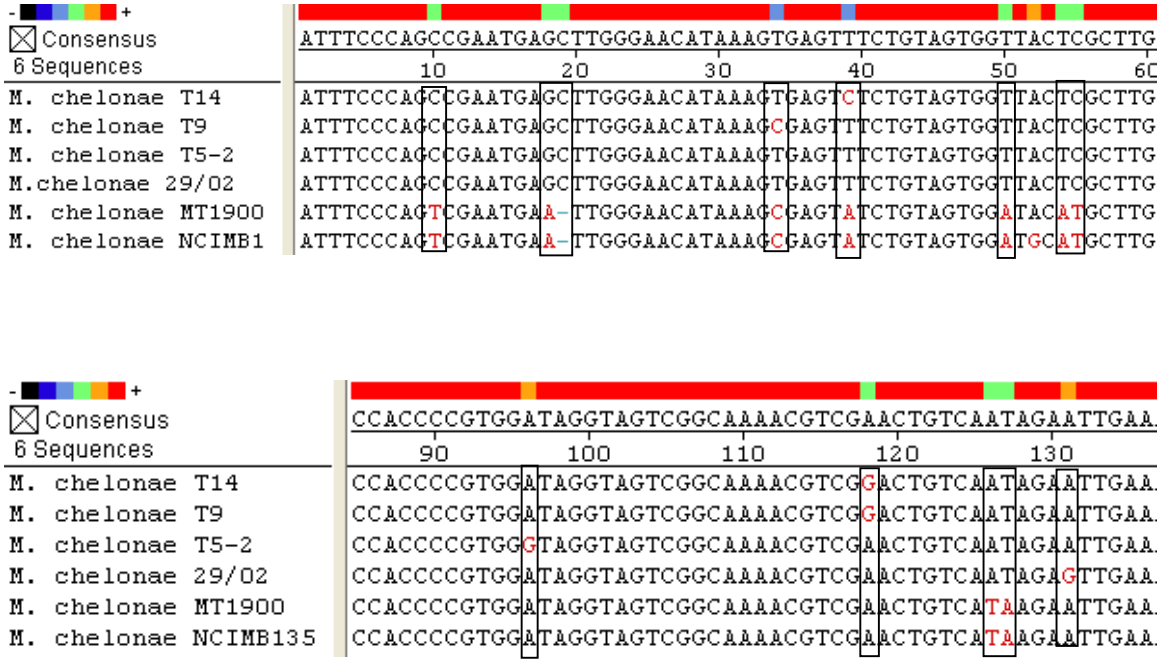

Fig. 3: Alignment of the 16S-23S ITS sequences of *M. chelonae* isolates using Clustal W programme of Lasergene Version 6.0 (DNASTAR) (position numbers are considered after the removal of the primer sequence). The bar indicates the degree of conserved areas of the sequences

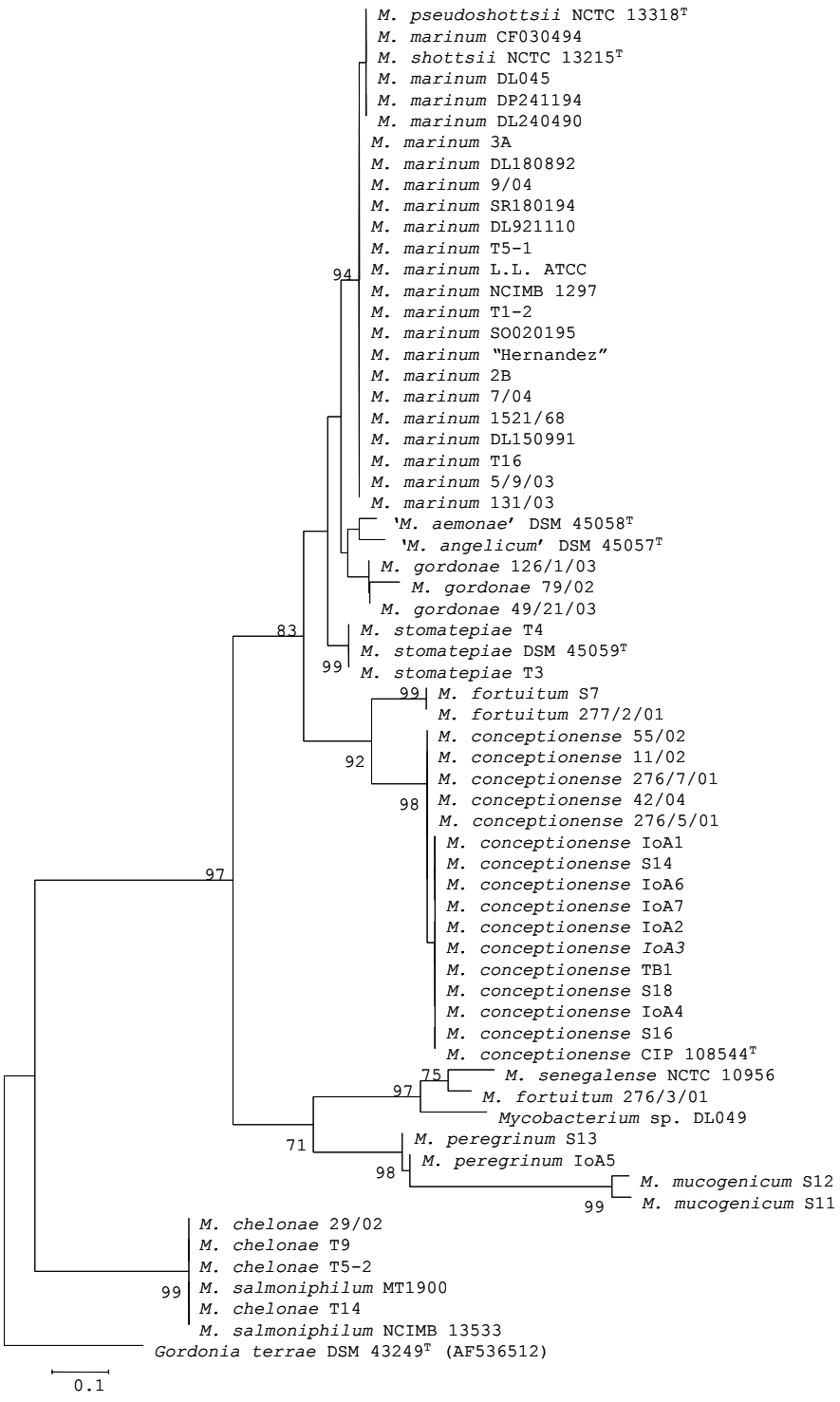

Fig. 4: Phylogenetic tree of the ITS1 sequences of aquatic mycobacteria. Prepared by using the neighbour-joining method and Kimura's two-parameter distance correction model. The support of each branch, as determined from 1,000 bootstrap samples, is indicated by the value at each node (as a percentage). Bootstrap values less than 70% are not shown. *Gordonia terrae* was used as the out-group. The scale bar represents a 1% difference in nucleotide sequences.

Table 1: Species of mycobacteria, source and origin used in this study

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. chelonae	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	S16	Snakehead fish (*Channa striata*)	Thailand
M. fortuitum	S18	Snakehead fish (*Channa striata*)	Thailand
M. fortuitum	IoA1	Unknown fish	Unknown
M. fortuitum	IoA2	Unknown fish	Unknown
M. fortuitum	IoA3	Unknown fish	Unknown
M. fortuitum	IoA4	Unknown fish	Unknown
M. fortuitum	IoA5	Unknown fish	Unknown
M. fortuitum	IoA6	Unknown fish	Unknown
M. fortuitum	IoA7	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. fortuitum	32/02	Goldfish (*Carassius auratus*)	Slovenia

Table 1 (cont.): Species of mycobacteria, source and origin used in this study

Species as received	Strain	Source	Origin
M. gordonae	126/1/03	Freshwater angelfish (*Pterophyllum scalare*)	Slovenia
M. fortuitum	32/02	Goldfish (*Carassius auratus*)	Slovenia
M. fortuitum	42/04	Goldfish (*Carassius auratus*)	Slovenia
M. marinum	7/04	Catfish (*Corydoras* sp.)	Slovenia
M. fortuitum	276/3/01	Goldfish (*Carassius auratus*)	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. fortuitum	50/04	Aquarium water	Slovenia
M. chelonae	29/02	Goldfish (*Carassius auratus*)	Slovenia
M. fortuitum	11/02	Dwarf gourami (*Colisa lalia*)	Slovenia
M. marinum	09/04	Platyfish (*Xiphophorus maculatus*)	Slovenia
M. gordonae	49/21/03	Tap water	Slovenia
M. marinum	T1-1[h]	Lumpsucker (*Cyclopterus lumpus*)	UK
M. marinum	T1-2	Lumpsucker (*Cyclopterus lumpus*)	UK
M. stomatepiae	T3	Striped barombi nsess (*Stomatepia mariae*)	UK
M. stomatepiae	T4	Striped barombi nsess (*Stomatepia mariae*)	UK
M. marinum	T5-1	Rosy barb (*Puntius conchonius*)	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	L.L. ATCC	Unknown	USA
M. marinum	SO020195[i]	Red Drum (*Scianops ocellatus*)	Israel
M. marinum	SR180194	Rabbit fish (*Siganus rivulatus*)	Israel
Mycobacterium sp.	DL049	Thalassa (*Dicentrarchus labrax*)	Greece
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	DL921110	Sea bass (*Dicentrarchus labrax*)	Denmark
M. marinum	"Hernandez"	Unknown fish	Germany

Table 1 (cont.): Species of mycobacteria, source and origin used in this study

Species as received	Strain	Source	Origin
M. marinum	1521/68	Unknown	Germany
M. marinum	DP241194	Sharp snout sea bream (*Diplodus puntazzo*)	Israel
M. marinum	2B	Unknown fish	UAE[j]
M. marinum	3A	Unknown fish	UAE

[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], Yet, not 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 from 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 from Israel Oceanographic and Limnological Research Ltd., National Centre for Mariculture, Eilat, Israel

[j], , United Arab Emirates

[*], Type strain.

Table 2: Results of the 16S-23S ITS sequence analysis for aquatic mycobacteria

Species name as received	No. of isolates	Amplicon size	Results of sequencing (% of match)
M. marinum	19	220	*M. marinum* (100)
M. marinum	4	220	*M. marinum* (98)
M. shottsii (NCTC 13318^T)	1	220	*M. marinum* (98)
M. pseudoshottsii (NCTC 13318^T)	1	220	*M. marinum* (98)
M. fortuitum 276/3/01	1	283	*M. fortuitum* (100)
M. fortuitum	2	345-361	*M. mucogenicum* (98.3)[a]
M. fortuitum	15	243	*M. conceptionense* (99.5-100)
M. fortuitum	2	256	*M. senegalense* (93.9)[b]
M. fortuitum	2	351-354	*M. peregrinum* (95) [c]
M. senegalense (NCTC 10956)	1	258	*M. senegalense* (100)
M. conceptionense (CIP 108544^T)	1	243	*M. conceptionense* (100)
M. chelonae	4	257	*M. chelonae* (99.5-100)
M. chelonae	2	256	*M. salmoniphilum* (99.5-100)
M. gordonae 79/02	1	211	*M. gordonae* (96.4)
M. gordonae	2	211	*M. gordonae* (100)
M. stomatepiae (T11^T, T3, T4)	3	225	*M. lentiflavum* (98.4)
'M. angelicum' (DSM 45057^T)	1	220	*M. szulgai* (98.3)
'M. aemonae' (DSM 45058 ^{T})	1	226	*M. kansasii* (92.7)
Mycobacterium sp. DL049	1	295	*M. fortuitum* (98.1)[d]

[a], similarity only over 121 bp

[b], similarity of 98.6% match with *M. fortuitum* for only 148 bp

[c], similarity only over 121 bp

[d], similarity for only 54 bp