Comparative evaluation of Polymerase Chain Reaction.-.Restriction Enzyme Analysis (PRA) and Sequencing of Heat shock protein 65 (*hsp65*) gene for Identification of Aquatic Mycobacteria

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Running title: PRA and sequencing of *hsp65* gene for identification of aquatic mycobacteria

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Abstract

Traditional identification of mycobacteria based on cultural and biochemical tests can take several weeks and may fail to provide a precise identification. Polymerase Chain Reaction-restriction analysis (PRA) of the gene encoding heat shock protein 65 kDa (*hsp65*) gene has been proposed as a rapid and inexpensive alternative approach**.** Despite being widely used for differentiation of mammalian mycobacteria, this method has only been applied in the identification of a small number of aquatic mycobacteria. The present study aimed to evaluate the potential use of PRA of *hsp65* for the identification of aquatic mycobacteria compared with sequence analysis. Seventy one mycobacterial isolates including, 10 type/ reference strains and the remainder field isolates, were subjected to PRA of a 441 bp fragment of this gene. For 68 representative isolates, sequence analysis was performed. All rapidly and slowly growing mycobacteria had best matches with 99.3% to 100% similarity with their corresponding species in the databanks.

PRA proved to be a simple and rapid method for identifying aquatic mycobacteria. However, the incidence of similar or identical restriction patterns for some species of mycobacteria, and in particular, identification of new species of mycobacteria is a major problem using such a method. In contrast, the nucleic acid sequencing of the *hsp65* gene yielded unambiguous results.

1. Introduction

Mycobacteriosis (fish tuberculosis) is a progressive disease of a wide range of wild and captive marine and freshwater fish species (Chinabut, 1999). The zoonotic nature of the organism, the massive economical losses which have occurred to the aquaculture industry due to this disease and the lack of effective treatment highlight the need for mycobacteria to be rapidly detected and identified to species level.

Traditional identification of mycobacteria based on cultural and biochemical tests may take several weeks, and sometimes even then fail to provide a precise identification. In addition, the procedures of these tests are time consuming and complicated. Therefore, various molecular based methods have been developed for the rapid detection and identification of mycobacteria species. There are currently several commercially available methods, including the AccuProbe system (Genprobe, San Diego, Calif), INNO-LiPA (Innogenetics, Ghent, Belgium), or GenoType *Mycobacterium* assay (Hain, Lifescience, Germany). Although such tests provide desirable sensitivity and specificity, they are limited to the identification of a small number of mycobacteria.

Among other molecular methods, Polymerase Chain Reaction (PCR) -Restriction Enzyme Analysis (PRA), and sequence analysis have been applied to several mycobacteria genes. In this regard, PCR of the 441 bp of heat shock protein 65 kD gene (*hsp65*), followed by restriction fragment pattern analysis by *BstE*II and *Hae*III enzymes proposed by Telenti et al. (1993), has provided a rapid method for identifying mycobacteria and closely related species (Brunello et al., 2001; Devallois et al., 1997). In addition, an internet database (PRAsite) (http://app.chuv.ch/prasite/index.html) has been established that contains an algorithm

of *hsp65* PRA patterns to assist with identification. Three hundreds and forty eight patterns of 113 species have so far been included in the PRAsite database.

Due to the hypervariability of the *hsp65* gene, sequence analysis of Telenti fragment (441 bp) has become a routine method in taxonomical studies (Murcia et al., 2006; Wilson et al., 2001) and in identification of clinical isolates of mycobacteria (Li et al., 2003; McNabb et al., 2004; Ringuet et al., 1999; Turenne et al., 2006). The PRA technique is relatively inexpensive and does not need specialised equipment; whereas, sequencing is more discriminative but requires highly sophisticated equipment.

Unlike the extensive use of these methods in clinical medicine, there are limited reports of the application of these techniques for detection and identification of fish mycobacteria.

The aims of this study were (i) to evaluate the possible use of PRA and sequence analysis based on the 441 bp fragment of *hsp65* gene and (ii) to compare the reliability of these methods in identification and differentiation of aquatic mycobacteria.

2. Materials and Methods

2.1 Bacterial strains

The 10 type/reference strains and 61 field isolates used in this study are listed in Table 1. Identification at the species level had previously been carried out by a variety of molecular methods (Pate et al., 2005; Pourahmad, 2008; Puttinaowarat et al., 2002). All strains were grown on Middlebrook 7H10 medium supplemented with oleic acid-albumin-dextrose-catalase (OADC) and 0.5% glycerol.

2.2 DNA preparation

DNA was extracted from cultures using NucleoSpin® columns (Macherey–Nagel, Germany) following manufacturer's instructions.

2.3 PCR amplification of *hsp65*

PCR mixtures were prepared with 12. 5 µl of a PCR master mix (AB gene®, UK) containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 10% glycerol, 200 µM (each) deoxynucleoside triphosphates, 10 pmol of each primer Tb11 (5′-ACCAACGATGGTGTGTCCAT- 3′) and Tb12 (5′-CTTGTCGAACCGCATACCCT- 3′), as described by Telenti et al., (1993), 1.25 U of *Taq* polymerase, 2 µl of each template containing 10-50 ng DNA and ultra-pure water (Millipore) to final volume of 25µl . PCR reactions of a 441 bp fragment of *hsp65* gene were performed in an automated T-gradient thermocycler (Biometra®, Göttingen, Germany) with the following amplification profile: initial step of 5 min incubation at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and a final extension of 5 min at 72°C. The PCR product specificity was checked by electrophoresis on a 1.2% agarose gel for 45 min at 120 V.

2.4 Restriction enzyme analysis of the *hsp65* PCR product

Two separate restriction digests (*Bst*EII and *Hae*III, both purchased from Sigma) were enzyme, 1.5 µl of appropriate restriction buffer, and 15 µl of ultra-pure water. The mixtures were incubated overnight at 60°C for *Bst*EII digestion and for 60 min at 37°C for *Hae*III digestion. Restriction fragments were electrophoresed on a 2.5% Low Melting Point agarose gel (Promega, UK) at 70 V for 2.5 h and visualized by ethidium bromide staining. A 50 bp ladder (Sigma) served as an external molecular size marker, and was added at the both ends of each gel to reduce migration-related

errors. The restriction pattern was compared to the published algorithm (Telenti et al., 1993) and with available PRA profiles from PRAsite.

2.5 Sequencing of the *hsp65* PCR product

The remaining PCR amplicons from the PRA were purified using the Qiaquick purification kit as described by the supplier (Qiagen, UK) and quantified by Nanodrop spectrophotometry, before being sequenced in both directions, using the PCR primers (Tb11 and Tb12) as well as an in house design forward primer, F927 (5´-GGACCCGTACGAGAAGAT- 3´). Sequencing was carried out using GenomeLab™ Dye Terminator Cycle Sequencing Quick Start Kit (as per manufacturer's instructions) and detected on an automated capillary sequencer, CEQ™ 8800 (Beckman Coulter, UK).

2.6 Sequence and phylogenetic analysis

Both forward and reverse sequences were assembled and edited using the SeqMan II module of Lasergene, Version 6 (DNASTAR, Inc., Madison, USA). The resulting consensus sequences were compared with published nucleotide sequences by BLASTn analysis. Phylogenetic trees were constructed, using the neighbour-joining method in MEGA version 3.1 software (Kumar et al., 2004) applying the Kimura two-parameter distance correction model. The tree was rooted using *Mycobacterium* sp. isolate DL049 (accession no AM902972) as the outgroup. Statistical confidence in tree branches were generated by performing 1000 bootstrap replicates

2.7 Nucleotide sequence accession numbers

The nucleotide sequences described herein have been deposited in the GenBank database under accession no. AM398442- AM398480; AM902952- AM902978 and EF535603-EF535604

3. Results

3.1 Sequence analysis of *hsp65*

Primer Tb11 gave inconsistent sequencing results and was later replaced by primer F927 (see Section 2.5). Use of this primer, which is 39 bp internal to primer Tb11, resulted in greatly improved sequence data. Sixty-eight of the 71 isolates mentioned above were sequenced. With the exception of isolates of *Mycobacterium* sp. isolate 126/5/03, *M. szulgai* /*M. intremedium* isolate 54/02, *M. gordonae* isolate 277/3/01 and *Mycobacterium* sp. isolate DL049, all rapidly and slowly growing mycobacteria had best matches with 99.3% to 100% similarity with their corresponding species in the databanks. The phylogenetic relationships among these isolates is presented in Fig. 3.

The resulting tree demonstrated two major clusters where slowly growing and rapidly growing mycobacteria could be distinguished. Sequence data resolved some ambiguous results with the previous methods, i.e. conventional PCR and PRA. All species screened were readily discriminated from each other. *M. fortuitum*, *M. senegalense* and *M. conceptionense*, the three species with the highest degree of similarity for the16S rRNA gene (Kirschner et al., 1993**)**, had sequences differing by 7 and 9 bp, respectively (Figs.4). Similarly, the isolates of *M. peregrinum* and *M. fortuitum* could be differentiated by 12 nucleotides. Furthermore, using a programme such as RestrictionMapper (version 3) http://www.restrictionmapper.org, enabled precise definition of the sizes of the PRA restriction fragments from sequences (Table 3).

Despite repeating the DNA extraction and purification from *M. chelonae* isolates, 2 of 5 *M. chelonae* isolates, *M. chelonae* (NCIMB 13533) and *M. chelonae* MT1900, could only be amplified by primers Tb11 and Tb12 when higher cycles (38 times

compare to 35 cycles) of PCR were applied. More interestingly, when their sequences were compared against databanks, they were more closely related (99.3% similarity) to *M. salmoniphilum*, a new *Mycobacterium chelonae*-like organism (Whipps et al., 2007). However, using overlapping primers to Tb11 and Tb12, F927 and R927, those two isolates were easily amplified and sequenced.

3.2 PRA of *hsp65*

The results obtained from the PRA analysis of 71 mycobacterial isolates are summarized in Table 2. For easy interpretation of the PRA profiles generated by each field isolate, a 50 bp ladder size marker was used as a reference and the PRA profile of a type strain was used as an internal control.

*Hae*III and *Bst*EII digestion of the 441 bp *hsp65* PCR amplicon generated fragments ranging from 50 to 441 bp. Restriction fragments smaller than 50 bp were omitted in order to reduce confusion with primer-dimer bands, and restriction fragment band sizes were rounded to the nearest 5 bp, as recommended by Telenti et al. (1993).

For some species, such as *M. gordonae*, that are known to contain several subtypes, their subtypes generated distinctive restriction profiles (Fig. 1, lanes 3, 6, 18). Therefore, the results clearly indicated that the PRA method could differentiate mycobacteria at the species and even the subspecies level.

All *M. marinum*, *M. shottsii*, *M. pseudoshottsii*, *M. peregrinum*, *Mycobacterium* sp. DL049 and *M. gordonae* type V (Fig. 1 lane 3) displayed similar patterns on *Bst*EII digestion (240 and 210 bp); however, with the exception of the first 3 species, the remaining species could be discriminated from the *Hae*III digestion

Although 6 strains of *M. fortuitum* including two type strains (NCIMB 1294 and 1295) and 4 field isolates had an identical PRA pattern, they were indistinguishable from *M. conceptionense* and *M. senegalense* strains due to the same digestion pattern for *Bst*EII and very similar *Hae*III pattern (Fig. 2 lanes 1-8 and 12)

There were 6 isolates with patterns not represented in the literature or in the PRA website (Fig. 1 lanes 5, 12 and 14). The type strain and field isolates of *M. peregrinum* had the pattern of *M. peregrinum* II as described by Devallois et al. (1997) and was present on the PRA website (240 and 210 bp upon digestion with *Bst*EII and the pattern of 140/120/100/60 for *Hae*III digestion) as illustrated in Fig 3 lane 11.

The advantage of sequence analysis over PRA was most obvious for the *M. fortuitum* group, presumed *M. gordonae* 277/3/01 and species with new PRA patterns which could only be specified via sequencing (Table 3). Of the *M. fortuitum* group, 15 isolates were matched with *M. conceptionense* (99.7% - 100% similarity); isolates S11 and S12 resembled *M. mucogenicum* (99.3% matched) and *M. gordonae* 277/3/01 was most closely related to *M. arupene* (99.5%). In addition, *M. stomatepiae* isolates T3, T4 and T11^{T}, '*M. angelicum*' 126/5/03^{T}, '*M. aemonae*' 54/02^{T} were closely related to *M. triplex*/*M. lentiflavum* (98% similar), *M. avium* (96.8% matched) and *M. gordonae* (95.5% similarity), respectively.

4. Discussion

The molecular identification of mycobacteria is of growing interest for routine speciation of these organisms. Several genetic based methods have been carried out

including sequence analysis of the 16S rRNA (Turenne et al., 2001), the spacer between 16S-23S rRNA (Roth et al., 1998), the gene coding for the beta subunit of RNA polymerase (*rpoB*) gene (Kim et al., 1999) and partial sequencing (Ringuet et al., 1999; McNabb et al., 2004) and PRA of *hsp65* gene (Brunello et al., 2001; Devallois et al., 1997). Of these, a relatively simple method providing promising results is PRA of *hsp65* gene. Despite being widely used for differentiation of mammalian mycobacteria, this method has only been used in the identification of a small number of aquatic mycobacteria (Rhodes et al., 2005; Ucko et al., 2002).

Seventy-one mycobacterial isolates were subjected to PRA of the *hsp65* gene. All isolates of the well-characterised species, *M. marinum*, *M. fortuitum* and *M. gordonae*, could be correctly identified using the PRA method. However, because of the number of fragment patterns not yet reported, this technique could not be used solely as a tool for identification of rare or novel species of mycobacteria. Both isolates of *M. mucogenicum* included in the study had patterns most similar to *M. chitae* type I when compared to patterns in the PRA web site.

The use of PRA for subtyping mycobacteria may improve our epidemiological understanding of these organisms in the aquatic environment. In this study, *M. gordonae* type I, one of the most frequently reported types of this species in clinical laboratories constituted 1/3 of the fish isolates of *M. gordonae*, probably indicting the importance of this type among aquatic isolates of this species. Using PRA of *hsp65*, other researchers showed that *M. kansasii* type I is the most common human isolate of this species (Alcaide et al., 1997; Cheunoy et al., 2005).

A comparison was performed between restriction fragment size from running agarose gels and the real size of fragments deduced from sequence analysis (Table 3). The

agarose gel-based PRA fragments normally differed in size between the two methods by up to 9 bp, apart from patterns for *Hae*III digestion of *M. gordonae* IV which were very different (200, 60 bp for the estimated compared to 161, 110, 60 bp obtained from gel) (Table 3). Brunello et al. (2001) showed that PRA patterns with a more accurate size were obtained using a 10% polyacrylamide gel. However, in practice polyacrylamide gels are more difficult to use than agarose gels as discussed elsewhere (Devallois et al., 1997).

The sequence variation present within the mycobacterial *hsp65* gene has made it an attractive locus for species identification and molecular phylogeny (Smole et al., 2002). In spite of the difficulty in amplifying the two isolates of *M. chelonae*, their patterns were identical and very similar with *Bst*EII and *Hae*III restriction, respectively. Nevertheless, intraspecies polymorphism in the 441 bp target region observed in individual *M. chelonae* complex species, such as *M. chelonae*, was shown to cause different restriction patterns for individual isolates of the same species, thereby increasing the ambiguity of the method (Wilson et al., 2001). As reported by other workers, differences between calculated sizes of restriction fragments and published patterns because of running conditions, type of agarose used and the computer programme used, as well as confusion in interpretation of patterns due to lack of standardisation are major drawbacks of PRA (Devallois et al., 1997; Hafner et al., 2004; Rocha et al., 1999). Another difficulty with PRA is that a single base change may lead to the appearance or disappearance of a restriction site (Ringuet et al., 1999). For instance, a point mutation in one of the restriction sites had shifted typical PRA pattern of *M. malmoense* to the *M. marinum* specific PRA pattern (Nolte et al., 2005). The existence of the PRA database web site for PRA of *hsp65* provides an easy way of interpreting the patterns (Cheunoy et al., 2005), however, there was no

identical match when the actual size of fragments in Table 3 were compared against those in the database. This observation suggests that the PRA web site has been designed based on published patterns rather than the real size of restricted fragments obtained by sequence analysis.

Application of at least two restriction endonucleoase enzymes is required in PRA methods used for the identification of mycobacteria to species level. In this regard, *Hae* III in combination with *Bst*E II have been traditionally employed in the PRA of 441 bp *hsp*65 gene. However, in this study the use of the *Bst*E II enzyme resulted in the production of identical fragment patterns for different species of mycobacteria, e.g. *M. peregrinum* type II, *M. marnium* and *M gordonae* type V. Thus, replacement of this restriction enzyme by a more discriminative restriction enzyme seems necessary.

In contrast, the nucleic acid sequencing of *hsp65* provided unambiguous results. Two isolates which were previously described as *M. fortuitum* by conventional PCR and identified as *M. chitae* type I by means of PRA, were clearly assigned as *M. mucogenicum* (99.3% similarity). The lack of overlap between *hsp65* sequences from *M. fortuitum* and *M. conceptionense* was particularly valuable for the accurate identification of these species. The novel patterns in Table 2 obtained by PRA were confirmed by sequence analysis. Fish strain 277/3/01 was a 99.5% match with *M. arupense*, a novel species of mycobacteria that has recently been described (Cloud et al., 2006). Masaki et al. (2006) have also recently reported two human isolates of this species, in Japan. However, strain 277/3/01, isolated from fish in Slovenia and using the GenoType *Mycobacterium* assay (Hain, Lifescience) had been identified as *M. gordonae* (Pate et al., 2005). This finding may highlight the zoonotic significance of

this newly emerging *Mycobacterium* species. Alternatively, with the assumption of environmental contamination for the isolate 277/3/01, this may provide evidence for an environmental source of *M. arupense*.

All field and reference strains of *M. fortuitum* had an identical *hsp65* sequence; whilst, the ITS sequences of isolates S7 and 277/2/01 were only partially matched with this species and the sequence of the type strains could not be determined by direct sequencing of PCR amplicons (Pourahmad, 2008). The snakehead fish isolate, S7, has been previously identified as *M. poriferae* by means of HPLC and biochemical tests (Tortoli et al., 1996). However, using PCR-RCBH, Puttinaowarat et al. (Puttinaowarat et al., 2002) re-characterised this isolate as *M. fortuitum*. The results of sequencing of the *hsp65* gene in this study were in agreement with this group. This observation may emphasise the intraspecies variability of ITS sequences that have been reported, elsewhere (Gürtler et al., 2006; Roth et al., 1998; Yoon et al., 1997). Nevertheless, sequence analysis of other phylogenetically important genes, i.e., the 16S rRNA and the *rpoB* genes warranted precise identification of the aforementioned species.

The current *hsp65*-based protocol (441 bp fragment) yields inefficient amplification in *M. chelonae* complex due to sequence variability in the primer-binding region (Khan et al, 2005). Likewise, in this study the difficulty in amplification of presumed *M. chelonae* (NCIMB 13533) and *M. chelonae* MT1900 which had a better match with *M. salmoniphilum* indicated the drawback of the Telenti et al. fragment. This problem as well as the uncertainty in differentiation of field and type strains of *M. marinum* and *M. pseudoshottsii* may highlight the need to develop methods based on alternative genetic targets with better resolution for differentiation of members of the *M.*

chelonae complex and *M. marinum* which are frequently isolated from fish (Bruno et al., 1998; dos Santos et al., 2002; Kent et al., 2004).

The construction of a phylogenetic tree (Fig. 3) was useful for indicating when isolates were either rapid or slow-growing mycobacteria. *Mycobacterium arupense*, for instance, which has been characterised as an intermediate species between fast and slowly growing mycobacteria (Cloud et al., 2006), though grouped with rapidly growing mycobacteria, was located between these two major groups. Furthermore, when an isolate could not be matched as a certain species, the phylogenetic tree showed the relatedness of that isolate to a known species or group of mycobacteria.

In conclusion, PRA is simple, rapid and inexpensive method for the identification of mycobacteria. However, sequence analysis of the *hsp65* gene has proven a useful method to integrate into species identification to indicate both slowly- and rapidly-growing species of aquatic mycobacteria. As sequencing facilities are becoming more accessible, sequencing of this 441 bp fragment may increasingly be used instead of PRA as a means of molecular identification of *Mycobacterium* species.

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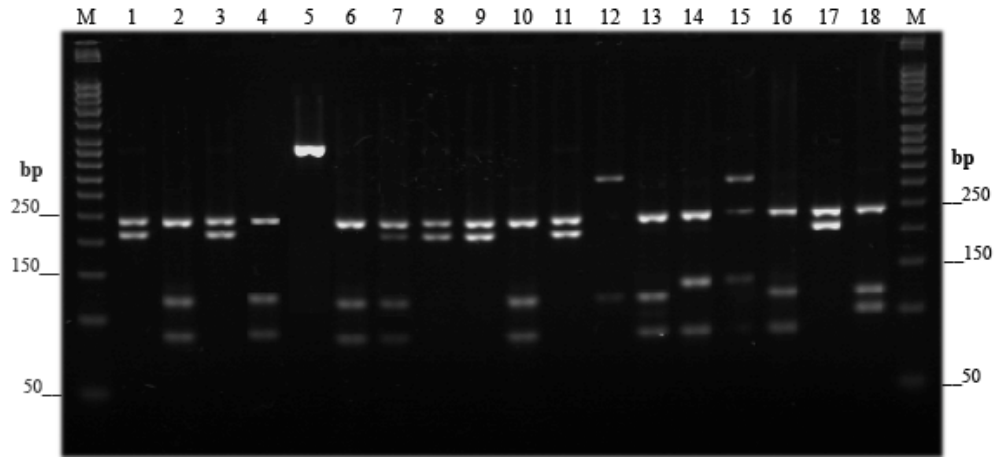

Fig.1: A representative selection of PRA profiles of *hsp65* gene PCR products using *Bst*EII enzyme

Lanes: 1) *M. marinum* (NCIMB1297); 2) *M. fortuitum* (NCIMB 1294); 3) *M. gordonae* 79/02; 4) *M. fortuitum* 277/2/01; 5) '*M. angelicum*' (DSM 45057^{T}); 6) *M. gordonae* 126/1/03; 7) *Mycobacterium* sp. 32/02; 8) *M. marinum* 7/04; 9) *M. marinum* 131/03; 10) *M. fortuitum* 276/3/01; 11) *M. marinum* 5/9/03; 12) *M. arupense* 277/3/01; 13) *M. conceptionense* 42/04; 14) '*M. aemonae*' (DSM 45058^{T}); 15) *Mycobacterium* sp. 50/04, 16) *M. conceptionense* 11/02; 17) *M. marinum* 9/04; 18) *M. gordonae* 49/21/03

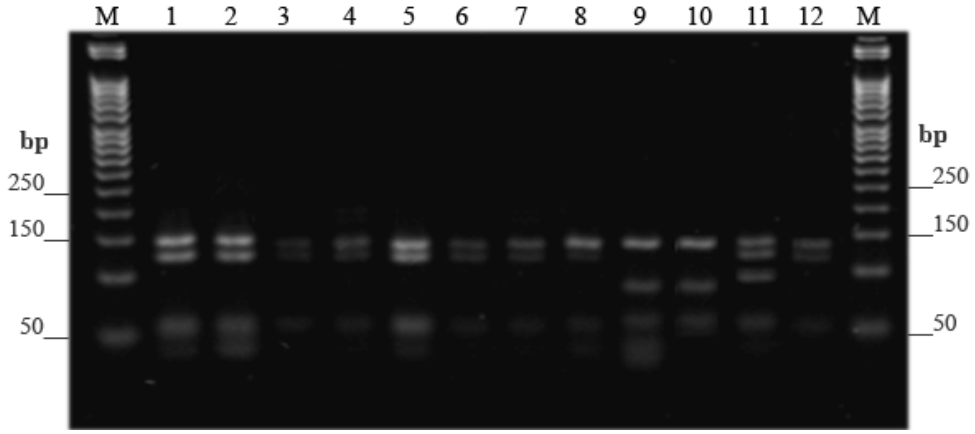

Fig.2: A representative selection of PRA profiles of *hsp65* gene PCR products using *Hae*III enzyme

Lanes 1) *M. fortuitum* (NCIMB 1295); 2) *M. fortuitum* (NCIMB 1294); 3) *M. conceptionense* IoA1; 4) *M. conceptionense* IoA2; 5) *M. conceptionense* IoA3; 6) *M. conceptionense* IoA4; 7) *M. conceptionense* IoA6; 8) *M. conceptionense* IoA7; 9) *M. mucogenicum* S11; 10) *M. mucogenicum* S12; 11) *M. peregrinum* S13; 12) *M. conceptionense* S14

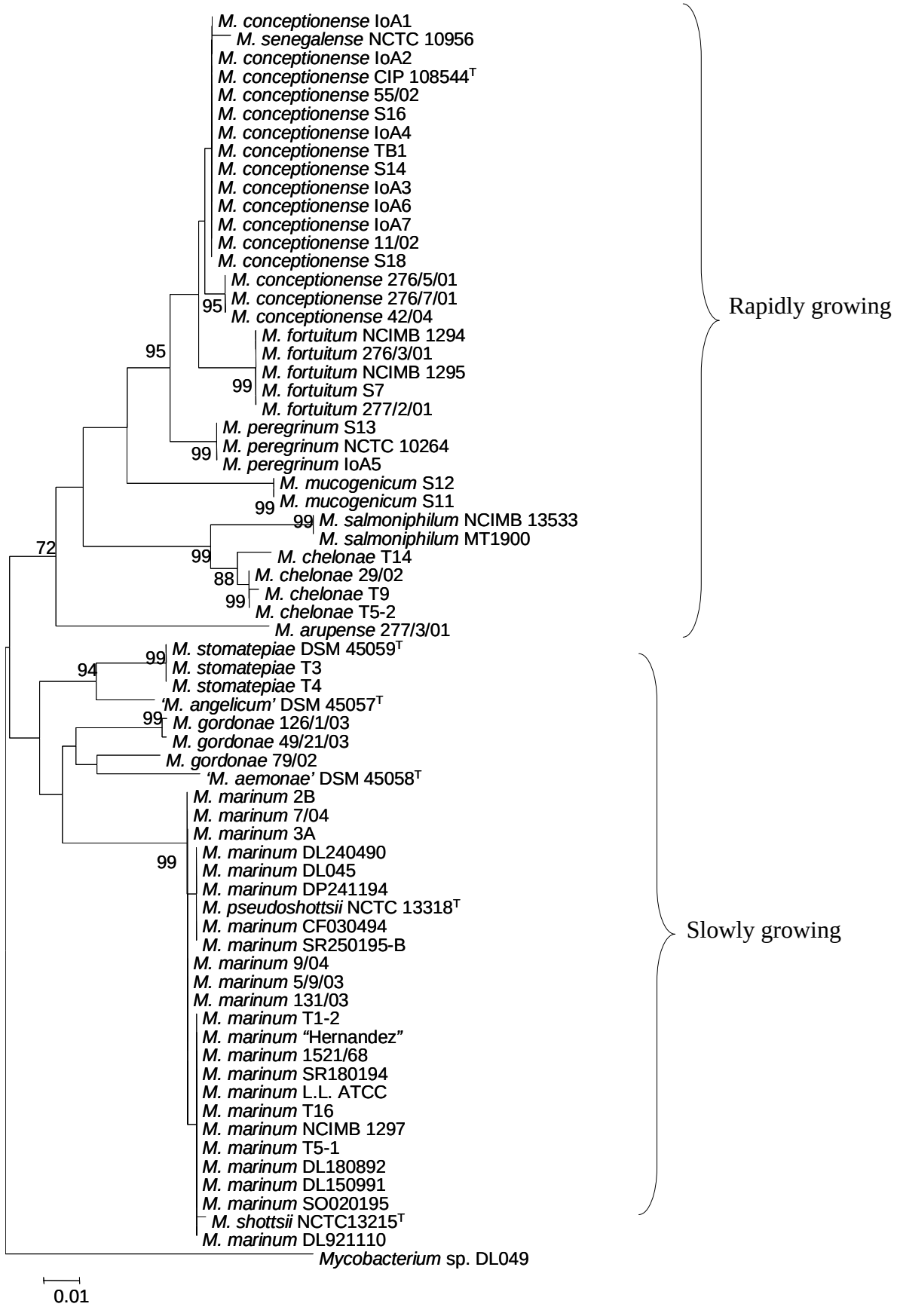
M. conceptionense IoA1
M. senegalense NCTC 10956
M. conceptionense IoA2
M. conceptionense CIP 108544T
M. conceptionense 55/02
M. conceptionense S16
M. conceptionense IoA4
M. conceptionense TB1
M. conceptionense S14
M. conceptionense IoA3
M. conceptionense IoA6
M. conceptionense IoA7
M. conceptionense 11/02
M. conceptionense S18
M. conceptionense 276/5/01
M. conceptionense 276/7/01
M. conceptionense 42/04
M. fortuitum NCIMB 1294
M. fortuitum 276/3/01
M. fortuitum NCIMB 1295
M. fortuitum S7
M. fortuitum 277/2/01
M. peregrinum S13
M. peregrinum NCTC 10264
M. peregrinum IoA5
M. mucogenicum S12
M. mucogenicum S11
M. salmoniphilum NCIMB 13533
M. salmoniphilum MT1900
M. chelonae T14
M. chelonae 29/02
M. chelonae T9
M. chelonae T5-2
M. arupense 277/3/01
M. stomatepiae DSM 45059T
M. stomatepiae T3
M. stomatepiae T4
'M. angelicum' DSM 45057T
M. gordonae 126/1/03
M. gordonae 49/21/03
M. gordonae 79/02
'M. aemonae' DSM 45058T
M. marinum 2B
M. marinum 7/04
M. marinum 3A
M. marinum DL240490
M. marinum DL045
M. marinum DP241194
M. pseudoshottsii NCTC 13318T
M. marinum CF030494
M. marinum SR250195-B
M. marinum 9/04
M. marinum 5/9/03
M. marinum 131/03
M. marinum T1-2
M. marinum "Hernandez"
M. marinum 1521/68
M. marinum SR180194
M. marinum L.L. ATCC
M. marinum T16
M. marinum NCIMB 1297
M. marinum T5-1
M. marinum DL180892
M. marinum DL150991
M. marinum SO020195
M. shottsii NCTC13215T
M. marinum DL921110
Mycobacterium sp. DL049
95
95
99
99
99
72
99
88
99
99
94
99
99
0.01
Rapidly growing
Slowly growing

Fig.3: Phylogenetic tree of the *hsp65* gene 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. *Mycobacterium* sp. DL049 was used as the out-group. The scale bar indicates a 1% difference in nucleotide sequences.

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

Species*	Strain	Source	Origin
M. conceptionense	CIP 108544[Tc]	Human	France
M. pseudoshottsii	NCTC 13318[T]	Striped bass (*Morone saxatilis*)	USA
M. shottsii	NCTC 13215[T]	Striped bass (*Morone saxatilis*)	USA
M. stomatepiae	DSM 45059[T]	Barombi Mbo Cichlid (*Stomatepia mariae*)	UK
M. chelonae	NCIMB 13533	Atlantic salmon (*Salmo salar*)	UK
M. fortuitum	NCIMB 1294	Chinook salmon (*Oncorhynchus tshawytscha*)	Unknown
M. fortuitum	NCIMB 1295	Unknown fish	Unknown
M. marinum	NCIMB 1297[a]	Unspeciated marine fish	USA
M. peregrinum	NCTC 10264[b]	Human	Mexico
M. senegalense	NCTC 10956	Bovine lymph node	France
M. marinum	DL921110[d]	Sea bass (*Dicentrarchus labrax*)	Denmark
M. marinum	DL150191	Sea bass (*Dicentrarchus labrax*)	Israel
M. marinum	SO020195	Red Drum (*Scianops ocellatus*)	Israel
M. marinum	SR180194	Rabbit fish (*Siganus rivulatus*)	Israel
M. marinum	SR250195-B	Rabbitfish (*Siganus rivulatus*)	Israel
M. marinum	DL180892	Sea bass (*Dicentrarchus labrax*)	Israel
M. marinum	DL240490	Sea bass (*Dicentrarchus labrax*)	Israel
M. marinum	“Hernandez”	Unknown fish	Germany
M. marinum	1521/68	Unknown	Germany
M. marinum	DL045	Thalassa (*Dicentrarchus labrax*)	Greece
Mycobacterium sp.	DL049	Thalassa (*Dicentrarchus labrax*)	Greece
M. marinum	L.L. ATCC	Unknown	USA
M. chelonae	29/02[e]	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	32/02	Goldfish (*Carassius auratus*)	Slovenia
M. fortuitum	276/3/01	Goldfish (*Carassius auratus*)	Slovenia
M. fortuitum	50/04	Aquarium water	Slovenia
M. fortuitum	11/02	Dwarf gourami (*Colisa lalia*)	Slovenia
M. fortuitum	276/5/01	Three-spot gourami (*Trichogaster trichopterus*)	Slovenia
M. gordonae	277/3/01	Guppy (*Poecilia reticulata*)	Slovenia
M. gordonae	79/02	Goldfish (*Carassius auratus*)	Slovenia
M. gordonae	126/1/03	Freshwater angelfish (*Pterophyllum scalare*)	Slovenia
M. gordonae	49/21/03	Tap water	Slovenia
M. marinum	5/9/03	Human	Slovenia
M. marinum	7/04	Catfish (*Corydoras* sp.)	Slovenia

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

Species*	Strain	Source	Origin
M. marinum	131/03	Three-spot gourami (*Trichogaster trichopterus*)	Slovenia
M. marinum	09/04	Platyfish (*Xiphophorus maculatus*)	Slovenia
Mycobacterium sp.	42/04	Goldfish (*Carassius auratus*)	Slovenia
Mycobacterium sp.	126/5/01	Freshwater angelfish (*Pterophyllum scalare*)	Slovenia
Mycobacterium sp.	276/7/01	Guppy (*Poecilia reticulata*)	Slovenia
M. szulgai/ *M. intermedium*	54/02	Goldfish (*Carassius auratus*)	Slovenia
M. fortuitum	TB1[f]	Siamese fighting fish (*Betta splendens*)	Thailand
M. fortuitum	TB40	Siamese fighting fish (*Betta splendens*)	Thailand
M. fortuitum	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. marinum	S4	Snakehead fish (*Channa striata*)	Thailand
M. marinum	2B	Unknown fish	UAE[g]
M. marinum	3A	Unknown fish	UAE
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. chelonae	MT1900	Atlantic salmon (*Salmo salar*)	UK
M. chelonae	T5-2[h]	Rosy barb (*Puntius conchonius*)	UK
M. chelonae	T9	Lumpsucker (*Cyclopterus lumpus*)	UK
M. chelonae	T14	Yellow seahorse (*Hippocampus kuda*)	UK
M. marinum	T1-1	Lumpsucker (*Cyclopterus lumpus*)	UK
M. marinum	T1-2	Lumpsucker (*Cyclopterus lumpus*)	UK
M. marinum	T5-1	Rosy barb (*Puntius conchonius*)	UK
M. marinum	T16	Otjikoto tilapia *(Tilapia guinasana*)	UK
M. stomatepiae	T3	Barombi Mbo Cichlid (*Stomatepia mariae*)	UK
M. stomatepiae[i]	T4	Barombi Mbo Cichlid (*Stomatepia mariae*)	UK

*, Names as received

[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], Isolates from DL921110 to L.L. ATCC were kindly provided by Dr. Angelo Colorni from Israel Oceanographic and Limnological Research Ltd., National Centre for Mariculture, Eilat, Israel.

[e], Mycobacterial isolates from Slovenia were kindly provided by Dr. Mateja Pate from Veterinary Faculty Ljubljana, University of Ljubljana, Gerbiceva 60, 1115 Ljubljana, Slovenia

[f], Strains obtained from different countries and held at –70 °C in the Institute of Aquaculture (IoA), University of Stirling

[g], United Arab Emirates

[h], Mycobacterial strains isolated from London Zoo Aquarium during this study

[i], Pourahmad et al.(2008), article in press

The superscript letter "T" denotes Type strain.

Table 2: Summary of patterns obtained by PRA of *hsp65* for aquatic *Mycobacterium* spp.

Species* and/or isolate name	No. of isolates	PRA pattern (bp)		Results
		***Hae*III**	***Bst*EII**	
M. conceptionense CIP 108544^T	1	145, 120, 50	240, 120, 85	*M. fortuitum*
M. pseudoshottsii. NCTC 13318^T	1	145, 105, 80	240, 210	*M. marinum*
M. shottsii NCTC 13215^T	1	145, 105, 80	240, 210	*M. marinum*
M. stomatepiae DSM 45059^T	1	145, 130	330, 115	New pattern
M. chelonae NCIMB 13533	1	200, 60	310, 130	*M. chelonae*
M. fortuitum NCIMB 1294	1	145, 120, 50	240, 120, 85	*M. fortuitum*
M. fortuitum NCIMB 1294	1	145, 120, 50	240, 120, 85	*M. fortuitum*
M. marinum NCIMB 1297	1	145, 105, 80	240, 210	*M. marinum*
M. peregrinum NCTC 10264	1	140, 120, 100, 60	240, 210	*M. peregrinum* II [b]
M. senegalense NCTC 10956	1	145, 120, 50	240, 120, 85	*M. fortuitum*
M. chelonae	5	200, 60	310, 130	*M. chelonae*
M. fortuitum	18	145, 120, 50	240, 120, 85	*M. fortuitum*
M. fortuitum IoA5, S13	2	140, 120, 100, 60	240, 210	*M. peregrinum* II [b]
M. fortuitum	3	multiple	multiple	Mixed culture
M. fortuitum S11, S12	2	140, 85, 60	320, 115	*M. chitae* I
M. gordonae 79/02	1	130, 110	240, 210	*M. gordonae* V [a]
M. gordonae 126/1/03	1	155, 110, 60	240, 120, 105	*M. gordonae* I [b]
M. gordonae 49/21/03	1	200, 60	325, 130	*M. gordonae* IV [c]
M. gordonae 277/3/01	1	145, 60	330, 115	New pattern
M. marinum	22	145, 105, 80	240, 210	*M. marinum*
M. stomatepiae	2	145, 130	330, 115	New pattern
M. szulgai /*M. intermedium*	1	145, 130, 60	240, 130, 80	New pattern
M.ycobacterium sp. 126/5/01	1	130, 10	Not digested	New pattern
Mycobacterium sp. DL049	1	150, 80, 65, 55	240, 210	New pattern

*, Names as received

[a], Published pattern of Telenti et al. (1993)

[b], Published pattern of Brunello et al. (2001)

[c], Published pattern of Devallois et al. (1997)

Table 3: Mycobacteria identified by sequencing of the *hsp65* PCR products and comparison of fragment length after digestion by *Hae*III and *Bst*EII with fragments deduced from sequence analysis

Sequencing (% of best match)	No. of isolates	PRA results	PRA pattern (bp)*		Sequence (bp)**	
			***Hae*III**	***Bst*EII**	***Hae*III**	***Bst*EII**
M. marinum	18	*M. marinum*	145, 105, 80	240, 210	145, 106, 78	231, 210
M. shottsii (100)	1	*M. marinum*	145, 105, 80	240, 210	145, 106, 78	231, 210
M. pseudoshottsii (100)	6	*M. marinum*	145, 105, 80	240, 210	145, 106, 78	231, 210
M. fortuitum (100)	5	*M. fortuitum*	145, 120, 50	240, 120, 85	145, 123, 58	231, 115, 80
M. peregrinum (100)	3	*M. peregrinum* II	140, 120, 100, 60	240, 210	145, 139, 98, 53	231, 210
ND	3	Multiple fragments	Multiple fragments	Multiple fragments	NS	NS
M. conceptionense (100)	16	*M. fortuitum******	145, 120, 50	240, 120, 85	139, 123, 58	231, 115, 80
M. senegalense (100)	1	*M. fortuitum******	145, 120, 50	240, 120, 85	139, 123, 58	231, 115, 80
M. mucogenicum (99.3)	2	*M. chitae* I	140, 85, 60	320, 115	139, 87, 58	325, 116
M. gordonae (99.7)	1	*M. gordonae* V	130, 110	240, 210	127, 110	231, 210
M. gordonae (99.8)	1	*M. gordonae* I	155, 110, 60	240, 120, 105	161, 110, 60	23, 115, 80
M. gordonae (99.5)	1	*M. gordonae* IV	200, 60	325, 130	161, 110, 60	325, 116
M. chelonae (100)	4	*M. chelonae*	200, 60	310, 130	197, 60, 58, 54	310, 131
M. salmoniphilum (100)	2	*M. chelonae*	200, 60	310, 130	197, 60, 58,54	310, 131
		New patterns for:				
M. stomatepiae (100)	3	DSM 45059^{T}, T3, T4	145, 130	330, 115	145, 127, 50	325, 116
M. avium (96.8)	1	*Mycobacterium* sp.126/5/03	130, 110	Not digested	127, 112	Not digested
M. arupense (99.5)	1	*M. gordonae* 277/3/01	145, 60	330, 115	145, 60	325, 116
M. gordonae (95.5)	1	*M. szulgai*//*M. intermedium*	145, 130, 60	240, 130, 80	145, 127, 60	231, 130, 80
M. poriferae (94)	1	*Mycobacterium* sp. DL049	145, 80, 65, 55	240, 210	140, 80, 58, 50	231, 210

ND, Not done;

NS, Not stated;

*, Estimation of fragment size obtained by agarose gel;

**, Estimation of fragment size deduced from sequences;

***, The type strains of *M. senegalense* and *M. conceptionense* have also had PRA patterns very similar to *M. fortuitum*.