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

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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 <i>M. marinum</i> by restriction fragment length polymorphism (RFLP) DNA profiling was confirmed as <i>M. marinum</i> 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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1 **Identification of Aquatic Mycobacteria based on Sequence**
2 **analysis of the 16S-23S rRNA Internal Transcribed Spacer (ITS)**
3 **region**

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6 **Running title: ITS sequences for Identification of Aquatic Mycobacteria**

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31 **Abstract**

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

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49 Key words: ITS sequence analysis, Identification, Aquatic mycobacteria

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52 **Introduction**

53 The genus *Mycobacterium* are aerobic, non-motile bacteria with surprisingly diverse
54 phenotypes related to growth rate, colony appearance, environmental distribution, and
55 pathogenic potential for eukaryotic hosts (Smole *et al.*, 2002). Commonly, mycobacteria are
56 further characterised into slowly and rapidly growing mycobacteria. Rapidly growing isolates
57 are classified as species, which under optimal nutrient and temperature regimes, produce
58 grossly visible colonies from dilute inocula in solid media in less than 7 days. Slowly
59 growing isolates are classified as those species taking more than 7 days to exhibit visible
60 colonies, again under optimal temperature and nutrient regimes (Lévy-Frébault and Portaels,
61 1992). In recent years, the number of species of mycobacteria reported in the aquatic
62 environment has greatly increased (Herbst, Costa, Weiss, Johnson, Bartell, Davis, Walsh, &
63 Levi 2001; Pourahmad, Cervellione, Thompson, Taggart, Adams & Richards 2008; Rhodes,
64 Kator, Kotob, van Berkum, Kaattari, Vogelbein, Quinn, Floyd, Butler & Ottinger 2003;
65 Rhodes, Kator, McNabb, Deshayes, Reyrat, Brown-Elliott, Wallace, Trott, Parker, Lifland,
66 Osterhout, Kaattari I., Reece, Vogelbein & Ottinger 2005; Whipps, Butler, Pourahmad,
67 Watral & Kent 2007). Phenotypic methods for identifying mycobacteria, such as acid fast
68 staining, are not useful for identifying mycobacteria to the species level and biochemical tests
69 are time consuming, and even then may not be able to differentiate between the different
70 species of mycobacteria being examined. Molecular methods attempt to resolve these
71 problems. For many years, PCR has been used clinically for the rapid identification of
72 *Mycobacterium* species. However, due to the number of closely related species within the
73 genus *Mycobacterium*, cross reactivity of different species in the PCR frequently occurs, and
74 thus additional methods are required to identify mycobacteria to the species level.

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

83 Several different regions in the *Mycobacterium* genome have been investigated and compared
84 between species and these include the 16S rRNA gene (Kirschner, Springer, Vogel, Meier,
85 Wrede, Kiekenbeck, Bange & Bottger 1993), the heat shock protein 65 gene (*hsp65*)
86 (Ringuet, Koua-Koffi, Honore, Varnerot, Vincent, Berche, Gaillard & Pierre-Audigier 1999),
87 the internal transcribed spacer (ITS) region sequence located between the 16S rRNA and the
88 23S rRNA genes (Roth, Fischer, Hamid, Michalke, Ludwig & Mauch 1998) and the β subunit
89 of RNA polymerase gene (*rpoB*) (Kim, Lee, Lyu, Kim, Bai, Kim, Chae, Kim, Cha & Kook
90 1999). Sequence analysis of the 16S rRNA has been widely used; however, the presence of
91 identical or highly similar 16S rRNA sequences between species limits the use of this target
92 for differentiation (Clarridge 2004; Dobner, Feldmann, Rifai, Loscher & Rinder 1996). Due
93 to sequence variability of the 16S-23S spacer region, several ITS sequence based assays have
94 been developed as an alternative approach for the identification of mycobacteria (Gürtler,
95 Harford, Bywater & Mayall 2006; Mohamed, Kuyper, Iwen, Ali, Bastola & Hinrichs 2005).

96 The aim of this study was to assess the use of sequence analysis of the mycobacterial 16S-
97 23S internal transcribed spacer region for the identification of different aquatic mycobacteria
98 species.

99 **Materials and Methods**

100 **Bacterial strains**

101 Sixty-nine aquatic mycobacterial strains including 12 reference strains and 57 field isolates
102 obtained from different geographical locations were used in this study (Table 1). All strains
103 had been previously identified by RFLP (Pourahmad & Richards, 2013). Isolates were grown
104 on Middlebrook 7H10 medium supplemented with oleic acid–albumin–dextrose–catalase
105 (OADC) (both from Becton-Dickinson, USA) and 0.5% glycerol, and incubated at 22°C or
106 30°C for 1 to 4 weeks depending on their growth rate.

107

108 **DNA preparation**

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

111 **PCR**

112 The ITS region in mycobacteria was amplified using forward primer Sp1 (5'-
113 ACCTCCTTTCTA AGGAGCACCC-3') and reverse primer Sp2 (5'-
114 GATGCTCGCAACCACTATCCA-3') designed by Roth et al. (2000). The PCR was carried
115 out using the following thermal profile: 2 min initial denaturation at 94 °C followed by 35
116 cycles of denaturation at 94 °C, annealing at 60 °C, and extension at 72 °C each for 1 min and
117 final incubation at 72 °C for 5 min. To confirm successful PCR amplification, the presence of
118 an amplicon of expected size was visualized by electrophoresis (using 10 µL of completed
119 PCR reaction on a 2.0% agarose gel stained with ethidium bromide and visualized under UV
120 light).

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122

123 **Sequence and analysis**

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

126 **Nucleotide sequence accession numbers**

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

130

131 **Results**

132 *Sequence analysis*

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

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

141 The results from analysis of the *M. fortuitum* isolates were more varied and for some isolates
142 the sequence analysis could not be completed. Although the majority of the *M. fortuitum*
143 isolates gave variable amplicon sizes in the PCR (from 243 bp to 361 bp), and could easily be
144 distinguished from the slowly growing *M. marinum* isolates. Of the 20 isolates identified as
145 *M. fortuitum* subtype V by RFLP (Pourahmad & Richards, 2013), three isolates (including
146 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

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

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

181 **Discussion**

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

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

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

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

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

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

232 The constructed phylogenetic tree clustered sequences of new *Mycobacterium* species
233 together. The isolates assigned as *M. mucogenicum* and *M. peregrinum* were placed in the
234 same clade as *M. fortuitum*, whereas the slowly growing *Mycobacterium*. sp. 126/5/01
235 clustered with *M. gordonae*, also a slowly growing *Mycobacterium*. However, unlike the
236 phylogenetic tree constructed for *hsp65* gene sequences, which clearly separated the clade for
237 slowly growing from rapidly growing mycobacteria (Devulder, de Montclos & Flandrois
238 2005), this separation was not as clear for ITS sequences.

239 In the study carried out by Leclerc, Haddad, Moreau & Thorel (2000), ITS sequences were
240 more informative than 16S rRNA sequences since the total number of variable sites was
241 similar for the two markers (103 for ITS versus 142 for 16S rRNA), whereas the length of
242 16S rRNA sequences (934 nucleotides) was four times longer than the length of ITS

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

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

255 **References**

256 Clarridge J.E., III (2004) Impact of 16S rRNA Gene Sequence Analysis for Identification of
257 Bacteria on Clinical Microbiology and Infectious Diseases. *Clinical Microbiology Reviews*
258 **17**, 840-862.

259 Cloud J.L., Meyer J.J., Pounder J.I., Jost K.C., Jr., Sweeney A., Carroll K.C. & Woods G.L.
260 (2006) *Mycobacterium arupense* sp. nov., a non-chromogenic bacterium isolated from
261 clinical specimens. *International Journal of Systematic and Evolutionary Microbiology* **56**,
262 1413-1418.

263 Devulder G., de Montclos M.P. & Flandrois J.P. (2005) A multigene approach to
264 phylogenetic analysis using the genus *Mycobacterium* as a model. *International Journal of*
265 *Systematic and Evolutionary Microbiology* **55**, 293-302.

266 Dobner P., Feldmann K., Rifai M., Loscher T. & Rinder H. (1996) Rapid identification of
267 mycobacterial species by PCR amplification of hypervariable 16S rRNA gene promoter
268 region. *Journal of Clinical Microbiology* **34**, 866-869.

269 Gürtler V., Harford C., Bywater J. & Mayall B.C. (2006) Direct identification of slowly
270 growing *Mycobacterium* species by analysis of the intergenic 16S-23S rDNA spacer region
271 (ISR) using a GelCompar II database containing sequence based optimization for restriction
272 fragment site polymorphisms (RFLPs) for 12 enzymes. *Journal of Microbiological Methods*
273 **64**, 185-199.

274 Hamid M.E., Roth A., Landt O., Kroppenstedt R.M., Goodfellow M. & Mauch H. (2002)
275 Differentiation between *Mycobacterium farcinogenes* and *Mycobacterium senegalense*
276 strains based on 16S-23S ribosomal DNA internal transcribed spacer sequences. *Journal of*
277 *Clinical Microbiology* **40**, 707-711.

278 Herbst L.H., Costa S.F., Weiss L.M., Johnson L.K., Bartell J., Davis R., Walsh M. & Levi M.
279 (2001) Granulomatous Skin Lesions in Moray Eels Caused by a Novel *Mycobacterium*
280 Species Related to *Mycobacterium triplex*. *Infection and Immunity* **69**, 4639-4646.

281 Khan I.U.H., Selvaraju S.B. & Yadav J.S. (2005a) Method for Rapid Identification and
282 Differentiation of the Species of the *Mycobacterium chelonae* Complex Based on 16S-23S
283 rRNA Gene Internal Transcribed Spacer PCR-Restriction Analysis. *Journal of Clinical*
284 *Microbiology* **43**, 4466-4472.

285 Kim B.J., Lee S.H., Lyu M.A., Kim S.J., Bai G.H., Kim S.J., Chae G.T., Kim E.C., Cha C.Y.
286 & Kook Y.H. (1999) Identification of Mycobacterial Species by Comparative Sequence
287 Analysis of the RNA Polymerase Gene (*rpoB*). *Journal of Clinical Microbiology* **37**, 1714-
288 1720.

289 Kirschner P., Springer B., Vogel U., Meier A., Wrede A., Kiekenbeck M., Bange F. &
290 Bottger E. (1993) Genotypic identification of mycobacteria by nucleic acid sequence
291 determination: report of a 2-year experience in a clinical laboratory. *Journal of Clinical*
292 *Microbiology* **31**, 2882-2889.

293 Lappayawichit P., Rienthong S., Rienthong D., Chuchottaworn C., Chaiprasert A.,
294 Panbangred W., Saringcarinkul H. & Palittapongarnpim P. (1996) Differentiation of
295 *Mycobacterium* species by restriction enzyme analysis of amplified 16S-23S ribosomal DNA
296 spacer sequences. *Tubercle and Lung Disease* **77**, 257-263.

297 Leclerc M.C., Haddad N., Moreau R. & Thorel M.F. (2000) Molecular characterization of
298 environmental *Mycobacterium* strains by PCR-restriction fragment length polymorphism of
299 *hsp65* and by sequencing of *hsp65*, and of 16S and ITS1 rDNA. *Research in Microbiology*
300 **151**, 629-638.

301 Lévy-Frébault V.V. & Portaels F. (1992) Proposed minimal standards for the genus
302 *Mycobacterium* and for description of new slowly growing *Mycobacterium* species.
303 *International Journal of Systematic and Evolutionary Microbiology* **42**, 315-323.

304 Mohamed A.M., Kuyper D.J., Iwen P.C., Ali H.H., Bastola D.R. & Hinrichs S.H. (2005)
305 Computational Approach Involving Use of the Internal Transcribed Spacer 1 Region for
306 Identification of *Mycobacterium* Species. *Journal of Clinical Microbiology* **43**, 3811-3817.

307 Novi C.L.A.U., Rindi L.A.U.R., Lari N.I.C.O. & Garzelli C.A.R.L. (2000) Molecular typing
308 of *Mycobacterium avium* isolates by sequencing of the 16S-23S rDNA internal transcribed
309 spacer and comparison with IS1245-based fingerprinting. *The Journal of Medical*
310 *Microbiology* **49**, 1091-1095.

311 Patel J.B., Leonard D.G.B., Pan X., Musser J.M., Berman R.E. & Nachamkin I. (2000)
312 Sequence-Based Identification of *Mycobacterium* Species Using the MicroSeq 500 16S
313 rDNA Bacterial Identification System. *Journal of Clinical Microbiology* **38**, 246-251.

314 Pourahmad, F., Cervellione, F., Thompson, K.D., Taggart, J.B., Adams, A., Richards R.H.,
315 2008. *Mycobacterium stomatepiae* sp. nov., a slowly growing, non-chromogenic species
316 isolated from fish. *International Journal of Systematic and Evolutionary Microbiology* 58,
317 2821-2827.

318 Pourahmad, F., Thompson, K.D., Taggart, J.B., Adams, A., Richards R.H., 2008. Evaluation
319 of the INNO-LiPA mycobacteria v2 assay for identification of aquatic mycobacteria. *Journal*
320 *of Fish Diseases* 31, 931–940.

321 Pourahmad, F., Thompson, K.D., Adams, A. & Richards R.H. (2009) Detection and
322 identification of aquatic mycobacteria in formalin-fixed, paraffin-embedded fish tissues.
323 *Journal of Fish Diseases* 32, 409–419.

324 Rhodes M.W., Kator H., Kotob S., van Berkum P., Kaattari I., Vogelbein W., Quinn F.,
325 Floyd M.M., Butler W.R. & Ottinger C.A. (2003) *Mycobacterium shottsii* sp. nov., a slowly
326 growing species isolated from Chesapeake Bay striped bass (*Morone saxatilis*). *International*
327 *Journal of Systematic and Evolutionary Microbiology* **53**, 421-424.

328 Rhodes M.W., Kator H., McNabb A., Deshayes C., Reyrat J.M., Brown-Elliott B.A., Wallace
329 R., Jr., Trott K.A., Parker J.M., Lifland B., Osterhout G., Kaattari I., Reece K., Vogelbein W.
330 & Ottinger C.A. (2005) *Mycobacterium pseudoshottsii* sp. nov., a slowly growing
331 chromogenic species isolated from Chesapeake Bay striped bass (*Morone saxatilis*).
332 *International Journal of Systematic and Evolutionary Microbiology* **55**, 1139-1147.

333 Ringuet H., Koua-Koffi C., Honore S., Varnerot A., Vincent V., Berche P., Gaillard J.L. &
334 Pierre-Audigier C. (1999) *hsp65* Sequencing for Identification of Rapidly Growing
335 Mycobacteria. *Journal of Clinical Microbiology* **37**, 852-857.

336 Roth A., Fischer M., Hamid M.E., Michalke S., Ludwig W. & Mauch H. (1998)
337 Differentiation of Phylogenetically Related Slowly Growing Mycobacteria Based on 16S-23S

338 rRNA Gene Internal Transcribed Spacer Sequences. *Journal of Clinical Microbiology* **36**,
339 139-147.

340 Roth A., Reischl U., Streubel A., Naumann L., Kroppenstedt R.M., Habicht M., Fischer M. &
341 Mauch H. (2000) Novel Diagnostic Algorithm for Identification of Mycobacteria Using
342 Genus-Specific Amplification of the 16S-23S rRNA Gene Spacer and Restriction
343 Endonucleases. *Journal of Clinical Microbiology* **38**, 1094-1104.

344 Smole S.C., McAleese F., Ngampasutadol J., von Reyn C.F. & Arbeit R.D. (2002) Clinical
345 and Epidemiological Correlates of Genotypes within the *Mycobacterium avium* Complex
346 Defined by Restriction and Sequence Analysis of *hsp65*. *Journal of Clinical Microbiology*
347 **40**, 3374-3380.

348 Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4: Molecular Evolutionary Genetics
349 Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596-1599.

350 Ucko M., Colorni A., Kvitt H., Diamant A., Zlotkin A. & Knibb W.R. (2002) Strain
351 Variation in *Mycobacteriu marinum* Fish Isolates. *Applied and Environmental Microbiology*
352 **68**, 5281-5287.

353 Whipps C.M., Butler W.R., Pourahmad F., Watral V.G. & Kent M.L. (2007) Molecular
354 systematics support the revival of *Mycobacterium salmoniphilum* (ex Ross 1960) sp. nov.,
355 nom. rev., a species closely related to *Mycobacterium chelonae*. *International Journal of*
356 *Systematic and Evolutionary Microbiology* **57**, 2525-2531.

357 Xiong L., Kong F., Yang Y., Cheng J. & Gilbert G.L. (2006) Use of PCR and Reverse Line
358 Blot Hybridization Macroarray Based on 16S-23S rRNA Gene Internal Transcribed Spacer
359 Sequences for Rapid Identification of 34 *Mycobacterium* Species. *Journal of Clinical*
360 *Microbiology* **44**, 3544-3550.

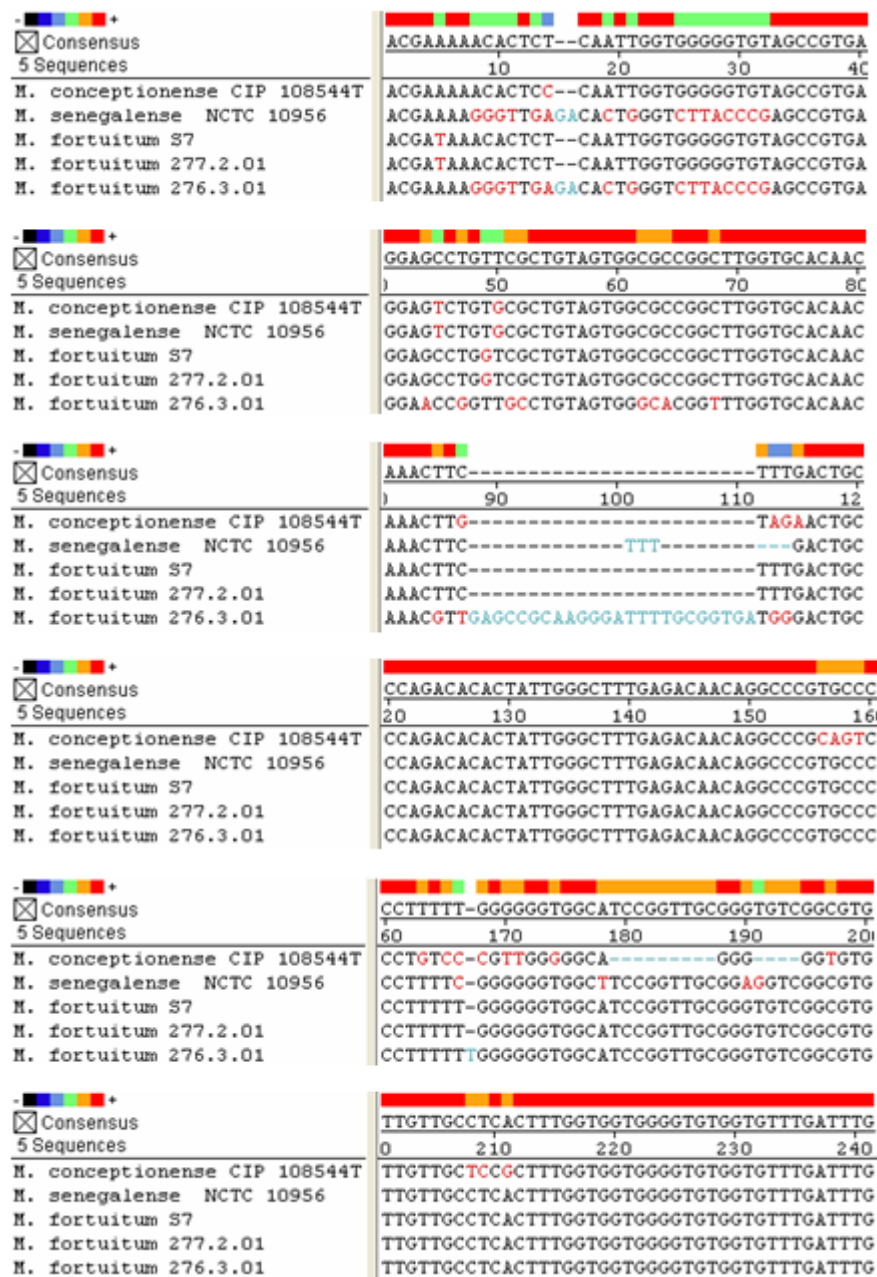


Fig. 1: Alignment of a representative selection of sequences of 16S-23S rRNA ITS for aquatic mycobacteria using SeqMan II programme of Lasergene, Version 6.0 (DNASTAR). The bar indicates the degree of conserved areas of the sequences.

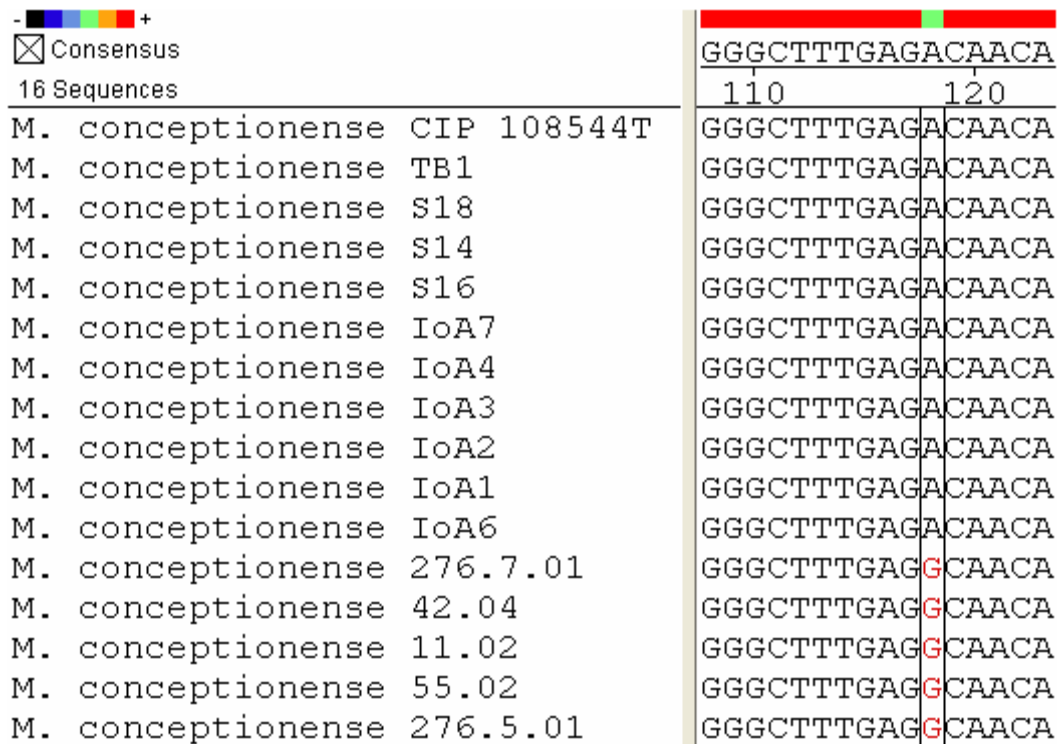


Fig. 2: Alignment of the 16S-23S rRNA ITS sequences for *M. conceptionense* isolated from different geographical regions by using Clustal W of MegAlign programme of Lasergene, Version 6.0 (DNASTAR). The bar indicates the degree of conserved areas of the sequences

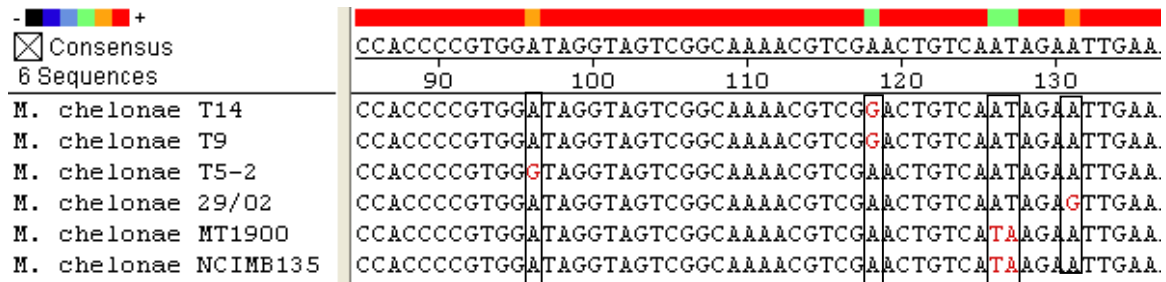
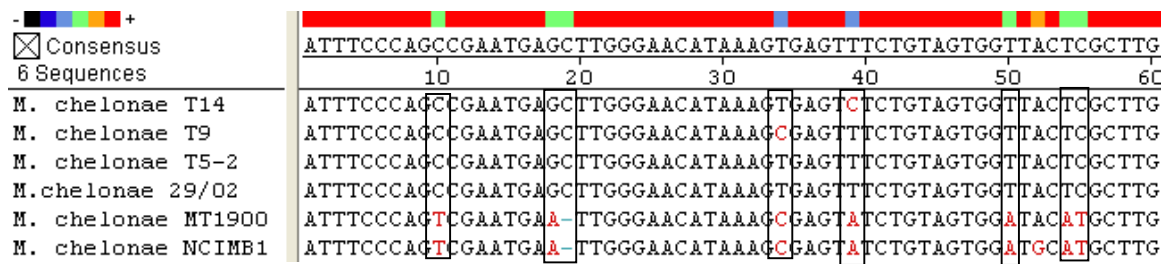


Fig. 3: Alignment of the 16S-23S ITS sequences of *M. chelonae* isolates using Clustal W programme of Lasergene Version 6.0 (DNASTAR) (position numbers are considered after the removal of the primer sequence). The bar indicates the degree of conserved areas of the sequences

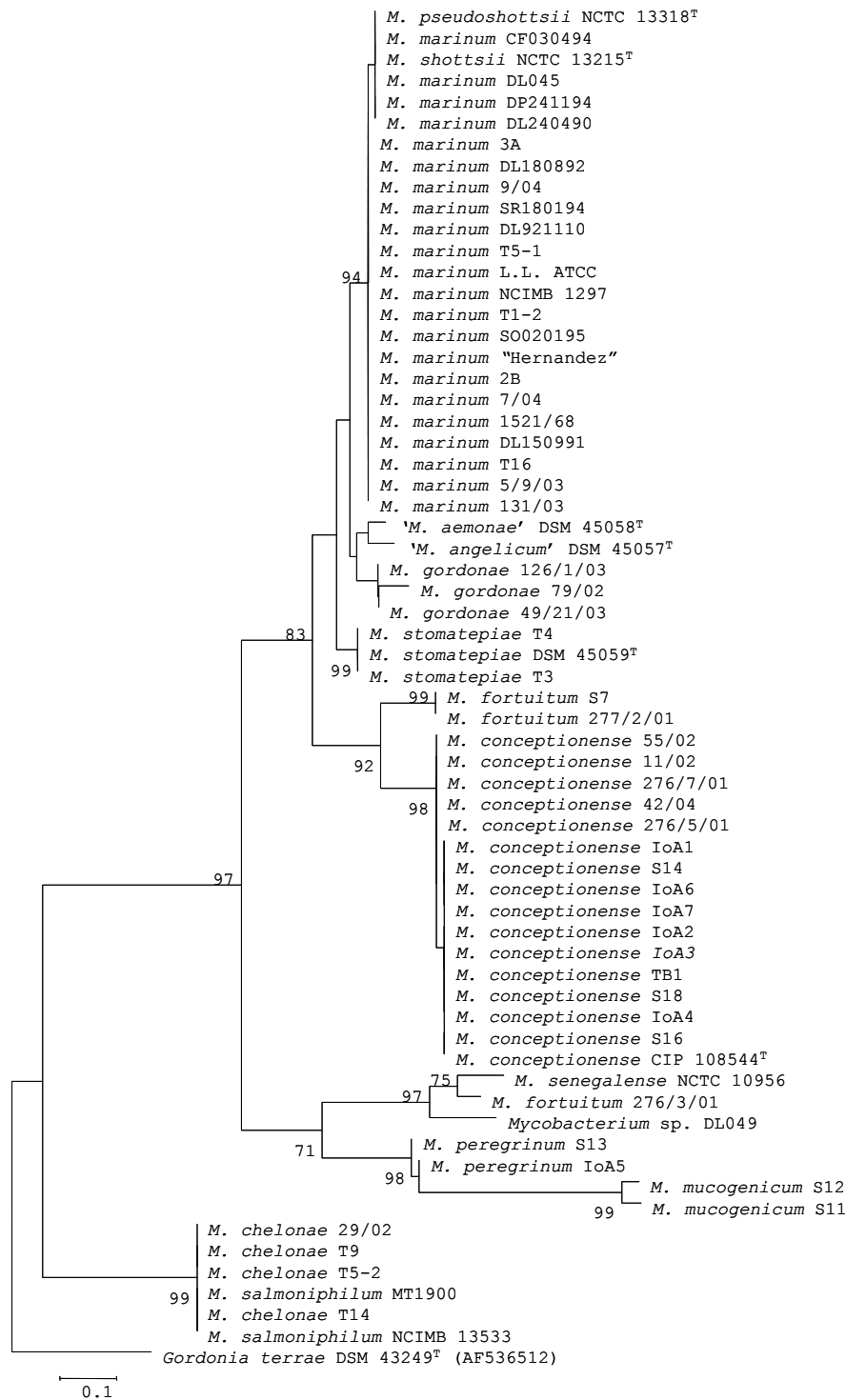


Fig. 4: Phylogenetic tree of the ITS1 sequences of aquatic mycobacteria. Prepared by using the neighbour-joining method and Kimura's two-parameter distance correction model. The support of each branch, as determined from 1,000 bootstrap samples, is indicated by the value at each node (as a percentage). 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
<i>M. marinum</i>	NCIMB 1297 ^a	Unspeciated marine fish	USA
<i>M. fortuitum</i>	NCIMB 1294	Chinook salmon (<i>Oncorhynchus tshawytscha</i>)	Unknown
<i>M. fortuitum</i>	NCIMB 1295	Unknown fish	Unknown
<i>M. chelonae</i>	NCIMB 13533	Atlantic salmon (<i>Salmo salar</i>)	UK
<i>M. peregrinum</i>	NCTC 10264 ^b	Human	Mexico
<i>M. senegalense</i>	NCTC 10956	Bovine lymph node	France
<i>M. shottsii</i>	NCTC 13215 ^{T*}	Striped bass (<i>Morone saxatilis</i>)	USA
<i>M. pseudoshottsii</i>	NCTC 13318 ^T	Striped bass (<i>Morone saxatilis</i>)	USA
<i>M. conceptionense</i>	CIP 108544 ^{T c}	Human	France
' <i>M.angelicum</i> ' ^d	DSM 45057 ^{T e}	Freshwater angelfish (<i>Pterophyllum scalare</i>)	Slovenia
' <i>M.aemonae</i> '	DSM 45058 ^T	Goldfish (<i>Carassius auratus</i>)	Slovenia
<i>M. stomatepia</i>	DSM 45059 ^T	Striped barombi nness (<i>Stomatepia mariae</i>)	UK
<i>M. fortuitum</i>	TB1 ^f	Siamese fighting fish (<i>Betta splendens</i>)	Thailand
<i>M. chelonae</i>	MT1900	Atlantic salmon (<i>Salmo salar</i>)	UK
<i>M. marinum</i>	S7	Snakehead fish (<i>Channa striata</i>)	Thailand
<i>M. fortuitum</i>	S11	Snakehead fish (<i>Channa striata</i>)	Thailand
<i>M. fortuitum</i>	S12	Snakehead fish (<i>Channa striata</i>)	Thailand
<i>M. fortuitum</i>	S13	Snakehead fish (<i>Channa striata</i>)	Thailand
<i>M. fortuitum</i>	S14	Snakehead fish (<i>Channa striata</i>)	Thailand
<i>M. fortuitum</i>	S16	Snakehead fish (<i>Channa striata</i>)	Thailand
<i>M. fortuitum</i>	S18	Snakehead fish (<i>Channa striata</i>)	Thailand
<i>M. fortuitum</i>	IoA1	Unknown fish	Unknown
<i>M. fortuitum</i>	IoA2	Unknown fish	Unknown
<i>M. fortuitum</i>	IoA3	Unknown fish	Unknown
<i>M. fortuitum</i>	IoA4	Unknown fish	Unknown
<i>M. fortuitum</i>	IoA5	Unknown fish	Unknown
<i>M. fortuitum</i>	IoA6	Unknown fish	Unknown
<i>M. fortuitum</i>	IoA7	Unknown fish	Unknown
<i>M. gordonae</i>	79/02 ^g	Goldfish (<i>Carassius auratus</i>)	Slovenia
<i>M. fortuitum</i>	277/2/01	Three-spot gourami (<i>Trichogaster trichopterus</i>)	Slovenia
<i>M. fortuitum</i>	55/02	Sterlet (<i>Acipenser ruthenus</i>)	Slovenia
<i>M. fortuitum</i>	276/7/01	Guppy (<i>Poecilia reticulata</i>)	Slovenia
<i>M. fortuitum</i>	32/02	Goldfish (<i>Carassius auratus</i>)	Slovenia

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

Species as received	Strain	Source	Origin
<i>M. gordonae</i>	126/1/03	Freshwater angelfish (<i>Pterophyllum scalare</i>)	Slovenia
<i>M. fortuitum</i>	32/02	Goldfish (<i>Carassius auratus</i>)	Slovenia
<i>M. fortuitum</i>	42/04	Goldfish (<i>Carassius auratus</i>)	Slovenia
<i>M. marinum</i>	7/04	Catfish (<i>Corydoras</i> sp.)	Slovenia
<i>M. fortuitum</i>	276/3/01	Goldfish (<i>Carassius auratus</i>)	Slovenia
<i>M. marinum</i>	131/03	Three-spot gourami (<i>Trichogaster trichopterus</i>)	Slovenia
<i>M. gordonae</i>	277/3/01	Guppy (<i>Poecilia reticulata</i>)	Slovenia
<i>M. marinum</i>	5/9/03	Human	Slovenia
<i>M. fortuitum</i>	50/04	Aquarium water	Slovenia
<i>M. chelonae</i>	29/02	Goldfish (<i>Carassius auratus</i>)	Slovenia
<i>M. fortuitum</i>	11/02	Dwarf gourami (<i>Colisa lalia</i>)	Slovenia
<i>M. marinum</i>	09/04	Platyfish (<i>Xiphophorus maculatus</i>)	Slovenia
<i>M. gordonae</i>	49/21/03	Tap water	Slovenia
<i>M. marinum</i>	T1-1 ^h	Lumpsucker (<i>Cyclopterus lumpus</i>)	UK
<i>M. marinum</i>	T1-2	Lumpsucker (<i>Cyclopterus lumpus</i>)	UK
<i>M. stomatepiae</i>	T3	Striped barombi nness (<i>Stomatepia mariae</i>)	UK
<i>M. stomatepiae</i>	T4	Striped barombi nness (<i>Stomatepia mariae</i>)	UK
<i>M. marinum</i>	T5-1	Rosy barb (<i>Puntius conchonius</i>)	UK
<i>M. chelonae</i>	T5-2	Rosy barb (<i>Puntius conchonius</i>)	UK
<i>M. chelonae</i>	T9	Lumpsucker (<i>Cyclopterus lumpus</i>)	UK
<i>M. chelonae</i>	T14	Yellow seahorse (<i>Hippocampus kuda</i>)	UK
<i>M. marinum</i>	T16	Otjikoto tilapia (<i>Tilapia guinasana</i>)	UK
<i>M. marinum</i>	L.L. ATCC	Unknown	USA
<i>M. marinum</i>	SO020195 ⁱ	Red Drum (<i>Scianops ocellatus</i>)	Israel
<i>M. marinum</i>	SR180194	Rabbit fish (<i>Siganus rivulatus</i>)	Israel
<i>Mycobacterium</i> sp.	DL049	Thalassa (<i>Dicentrarchus labrax</i>)	Greece
<i>M. marinum</i>	DL180892	Sea bass (<i>Dicentrarchus labrax</i>)	Israel
<i>M. marinum</i>	DL150191	Sea bass (<i>Dicentrarchus labrax</i>)	Israel
<i>M. marinum</i>	DL240490	Sea bass (<i>Dicentrarchus labrax</i>)	Israel
<i>M. marinum</i>	CF030494	Butterfly fish (<i>Chaetodon fasciatus</i>)	Israel
<i>M. marinum</i>	DL921110	Sea bass (<i>Dicentrarchus labrax</i>)	Denmark
<i>M. marinum</i>	“Hernandez”	Unknown fish	Germany

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

Species as received	Strain	Source	Origin
<i>M. marinum</i>	1521/68	Unknown	Germany
<i>M. marinum</i>	DP241194	Sharp snout sea bream (<i>Diplodus puntazzo</i>)	Israel
<i>M. marinum</i>	2B	Unknown fish	UAE ^j
<i>M. marinum</i>	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)
<i>M. marinum</i>	19	220	<i>M. marinum</i> (100)
<i>M. marinum</i>	4	220	<i>M. marinum</i> (98)
<i>M. shottsii</i> (NCTC 13318 ^T)	1	220	<i>M. marinum</i> (98)
<i>M. pseudoshottsii</i> (NCTC 13318 ^T)	1	220	<i>M. marinum</i> (98)
<i>M. fortuitum</i> 276/3/01	1	283	<i>M. fortuitum</i> (100)
<i>M. fortuitum</i>	2	345-361	<i>M. mucogenicum</i> (98.3) ^a
<i>M. fortuitum</i>	15	243	<i>M. conceptionense</i> (99.5-100)
<i>M. fortuitum</i>	2	256	<i>M. senegalense</i> (93.9) ^b
<i>M. fortuitum</i>	2	351-354	<i>M. peregrinum</i> (95) ^c
<i>M. senegalense</i> (NCTC 10956)	1	258	<i>M. senegalense</i> (100)
<i>M. conceptionense</i> (CIP 108544 ^T)	1	243	<i>M. conceptionense</i> (100)
<i>M. chelonae</i>	4	257	<i>M. chelonae</i> (99.5-100)
<i>M. chelonae</i>	2	256	<i>M. salmoniphilum</i> (99.5-100)
<i>M. gordonae</i> 79/02	1	211	<i>M. gordonae</i> (96.4)
<i>M. gordonae</i>	2	211	<i>M. gordonae</i> (100)
<i>M. stomatepiae</i> (T11 ^T , T3, T4)	3	225	<i>M. lentiflavum</i> (98.4)
' <i>M. angelicum</i> ' (DSM 45057 ^T)	1	220	<i>M. szulgai</i> (98.3)
' <i>M. aemonae</i> ' (DSM 45058 ^T)	1	226	<i>M. kansasii</i> (92.7)
<i>Mycobacterium</i> sp. DL049	1	295	<i>M. fortuitum</i> (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