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1 **Rapid detection and identification of non-tuberculous mycobacterial pathogens in fish**  
2 **using high resolution melting analysis (HRMA)**

3

4

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19

20 **Running title:** identification of fish mycobacteria by HRMA

**21 Abstract**

**22** Mycobacterial infections in fish are commonly referred to as piscine mycobacteriosis,  
**23** irrespectively of the specific identity of the causal organism. They usually cause a chronic  
**24** disease and sometimes may result in high mortalities and severe economic losses. Nearly 20  
**25** species of *Mycobacterium* have been reported to infect fish. Among them, *M. marinum*, *M.*  
**26** *fortuitum* and *M. chelonae* are generally considered as the major agents responsible for fish  
**27** mycobacteriosis. As no quick and inexpensive diagnostic test exists, we tested the potential of  
**28** high resolution melting analysis (HRMA) to rapidly identify and differentiate several  
**29** *Mycobacterium* species involved in fish infections. By analyzing both the melting temperature  
**30** and melting profile of the 16S-23S rRNA internal transcribed spacer (ITS), we were able to  
**31** discriminate 12 different species simultaneously. Sensitivity tests conducted on *M. marinum*  
**32** and *M. fortuitum* purified DNA revealed a limit of detection of 10 genome equivalents per  
**33** reaction. The primers used in this procedure did not lead to any amplification signal with 16  
**34** control non-*Mycobacterium* species, thereby demonstrating their specificity for the genus  
**35** *Mycobacterium*.

**36**

## 37 Introduction

38 The non-tuberculous mycobacteria (NTM) are Gram positive, acid-fast and aerobic bacteria  
39 that belong to the order *Actinomycetales*. They are widespread in the aquatic environment,  
40 both in fresh and marine waters, where they can survive in hostile conditions by forming  
41 biofilms (1). Some of them are the causative agents of fish mycobacteriosis, which  
42 predominantly occurs as a chronic disease and occasionally in an acute form (2, 3). The  
43 primary pathological lesions associated with the disease are grayish white nodules  
44 (granulomas) in the internal organs such as liver, spleen or kidney, which may further lead to  
45 high fish mortality and severe losses in aquaculture industry (4-6). Fish mycobacteriosis has  
46 been reported to affect nearly 200 freshwater and saltwater species (7). *Mycobacterium*  
47 *marinum*, *M. fortuitum* and *M. chelonae* are considered the main causative agents of fish  
48 mycobacteriosis (2). However, many other *Mycobacterium* species have been found to be  
49 associated with granulomas in aquarium, cultured and wild fish, among which *M. abscessus*,  
50 *M. gastri*, *M. smegmatis*, *M. bohemicum*, *M. gordonae*, etc. (3, 6). NTM causing infection in  
51 fish are divided between rapid growers (which develop visible colonies on solid media within  
52 7 days) and slow growers (which require longer incubation times) (3, 8). Although  
53 comprehensive surveys are rare, the frequency of NTM infection in cultured fish seems to  
54 increase (9). For illustration, 135 out of 312 ornamental fish collected during an 18-month  
55 survey were found positive for NTM, with 55% of them being positive by Ziehl-Neelsen  
56 staining (10). Another study reported the isolation of *Mycobacterium sp.* from 29.9% of 127  
57 ornamental fish batches imported into Italy (11). It was also shown that a low dose infection  
58 of *M. marinum* results in the development of a latent disease (12). Yet, there are no validated  
59 treatments for mycobacteriosis in fish: complete depopulation of asymptomatic carriers and  
60 disinfection are the primary methods for controlling the disease (13). Some NTM species are  
61 also responsible for human infections (9, 14), with an increasing incidence.

62 The rapid development of fish farming and of the ornamental fish trade has led to a  
63 worldwide increase in the number of reports of mycobacterial infections in fish, with two  
64 major consequences: (i) a substantial financial loss in the two above-mentioned industries and  
65 (ii) an increased risk of contamination for people who handle fish (15-18). Therefore, early  
66 surveillance systems based on a rapid identification of fish pathogens are critical for effective  
67 disease control in aquaculture, and improved epidemiological surveys. Furthermore, some  
68 authors have discussed specific recommendations for the policy on the importation of  
69 ornamental fish (18), which should include the evaluation of bio-security procedures and  
70 disease monitoring.

71 Over the last years, a great number of molecular methods, mostly based on nucleic acid  
72 amplification, have been developed for the diagnosis of fish mycobacteriosis (reviewed in (6)).  
73 Recently, a commercial kit, the GenoType Mycobacterium CM (common Mycobacteria) kit  
74 was introduced for identification of mycobacterial cultures (mainly from clinical origin). This  
75 kit is able to identify 25 different species based on 16S rDNA gene hybridization. Combined  
76 with another version, the AS (additional species) kit, the test can discriminate among 44  
77 species in total, with success rate of ~96% over 219 tested isolates (19). However, the use of  
78 this kit remains both time-consuming and costly, since it requires in most cases the prior  
79 growth of mycobacterial isolates and the cost for one reaction remains far more expensive than  
80 sequencing. Another commercial test exclusively targeting fish pathogens, the INNO-LipA  
81 Mycobacteria v2 assay, was developed (20). This kit is also based on the hybridization  
82 between Mycobacterial 16S-23S rRNA internal transcribed spacer and the corresponding  
83 oligonucleotide probes immobilized on membrane strips. Although this kit is able to  
84 distinguish 16 different *Mycobacterium* species with a rather good success rate (21), it still  
85 relies on the isolate cultivation, which does not fulfill the speed requirements for large-scale  
86 prevalence studies and epidemiological surveys.

87 Therefore, there is an urgent need for a fast, accurate, sensitive and cost-effective method  
88 adapted to veterinary needs. Over the last few years, real-time PCR methods have been  
89 developed and widely evaluated in studies for detection of *Mycobacterium* (22, 23). High  
90 resolution melting analysis (HRMA), further developed from real-time PCR, is an emerging  
91 technique in medical microbiology that may allow simultaneous detection and diagnosis of  
92 pathogens at species and sub-species levels (24-26). This technique, first reported in 2002, is  
93 based on the difference in melting behaviors of DNA molecules, according to their sequence,  
94 length and GC content (25, 27). HRMA requires only ng amounts of DNA and has the  
95 potential to discriminate closely related microorganisms with high accuracy, speed and  
96 sensitivity.

97 The present study specifically aimed at developing an HRMA-based identification test of the  
98 major *Mycobacterium* species affecting fishes. Targeting the 16S-23S rRNA internal  
99 transcribed spacer (ITS), this assay relies on the measurement of differences in both the  
100 melting temperature and melting profile.

101

## 102 **Material and methods**

103

### 104 *Bacterial strains*

105 Twelve *NTM* isolates were used as "reference" species in the present study. Among them, 5  
106 were purchased from Pasteur Institute (Paris, France) as pure isolates, 2 were obtained from  
107 the Laboratoire Départemental Vétérinaire (LDV, Montpellier, France) and consisted of  
108 strains isolated from fish tissues, and 5 were isolated from human patients in Arnaud de  
109 Villeneuve Hospital (Montpellier, France). A list of these strains is presented in table 1. All of  
110 these strains, which had previously been identified by biochemical tests and/or sequencing,  
111 were cultured on Lowenstein-Jensen (LJ) slants, and grown at 37°C for several days to

112 several weeks. In addition, 16 non-mycobacteria field isolates, including both fish  
113 opportunistic and pathogenic bacteria, were also used for evaluating the specificity of the  
114 assay (Table 1). These bacteria, which comprised 6 Gram positive and 10 Gram negative  
115 species, were grown in their specific culture medium. All isolates were manipulated in a  
116 biosafety level 2 containment laboratory.

117

#### 118 *Fish tissue samples*

119 As one of the French approved laboratories, the Laboratoire Départemental Vétérinaire  
120 (LDV34) of Montpellier (France) is requested by local fish farmers or owners to diagnose  
121 their fish when these latter are suspected to carry infections. In this context, LDV34 holds a  
122 small collection of fish samples that were either diagnosed for mycobacterial infections or  
123 collected from infected farms. From this collection, 30 fish samples were used for validating  
124 the PCR-HRM test described in this study, together with 3 DNA samples extracted from  
125 mycobacterial cultures isolated from some of these fish. The list and origin of these samples  
126 are presented in table 2.

127

#### 128 *Genomic DNA isolation*

129 DNA from all samples (bacterial strains and fish tissues) was purified with the Wizard®  
130 Genomic DNA Purification kit (Promega), following the appropriate protocol provided in the  
131 kit. For Gram positive cultures, a slightly modified protocol was used. Bacterial colonies were  
132 resuspended in 480 µl of EDTA solution (50 mM, pH8). After addition of 120 µl of lysozyme  
133 (10 mg/ml), bacterial cells were incubated for 1 hr at 37°C, centrifuged for 5 min at 13,000 g,  
134 resuspended in 600 µl of nuclei lysis solution, incubated again for 10 min at 100°C and  
135 cooled to room temperature. This lysate was then supplemented with 20 µl of proteinase K  
136 (20 mg/ml) and incubated for another 3 hrs at 55°C, under gentle shaking. The rest of the

137 procedure was performed according to the manufacturer's instructions. DNA from Gram  
138 negative isolates and from fish tissues was extracted following the Gram negative and animal  
139 tissue protocols provided in the kit, respectively. In all cases, DNA was eluted in 70  $\mu\text{l}$  of the  
140 provided Tris-EDTA solution and its concentration measured by UV spectrometry  
141 (NanoDrop® ND-1000 spectrophotometer, NanoDrop Technologies Inc). Bacterial genomic  
142 DNA was adjusted to approximately  $0.5 \times 10^4$  or  $0.5 \times 10^6$  genome equivalents  $\mu\text{l}^{-1}$ , based on an  
143 average genome size of 6.6 Mb, whereas fish DNA was diluted to  $10 \text{ ng } \mu\text{l}^{-1}$ .

144

#### 145 *Assay design*

146 Since the assay aimed at being as simple as possible, it relied on the use of a double-strand  
147 intercalating - and thus non-sequence specific - fluorophore for measuring differences in  
148 melting profiles of amplification products. Under such conditions, the targeted genomic  
149 region had (i) to be unique for each of the investigated species and (ii) to harbor conserved  
150 sequences at its extremities enabling genus-specific amplification. Multiple alignments of  
151 different genomic regions (including the 16S-23S ribosomal operon, the  $\beta$ -subunit of RNA  
152 polymerase (*rpoB*), the 65-kD heat shock protein (*hsp65*) and the B-subunit of DNA gyrase  
153 (*gyrB*) genes) were realized with ClustalX v2 (28) on sequences imported from the NCBI  
154 collection ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). They revealed that the internal transcribed spacer (ITS)  
155 region could fulfill these requirements. From these alignments, a single primer pair was  
156 subsequently designed to amplify a fragment of  $\sim 220$  to  $\sim 320$  bp in all the targeted  
157 mycobacterial species (forward GCTGGATCACCTCCTTTCTA and reverse  
158 AGATGCTCGCAACCACTAT). The primers were verified for the absence of secondary  
159 structures with GeneRunner v3.01 (Hasting Software, Inc. 1994) and purchased from  
160 Eurofins-MWG-Operon.

161 The amplification and melting steps were achieved using the LightCycler®480 high  
162 resolution melting master kit (Roche). The reaction mixture was composed of 2X Master mix,  
163 MgCl<sub>2</sub>, forward and reverse primers, genomic DNA and PCR-grade water, in a final volume  
164 of 10 µl. The amplification procedure consisted of an initial denaturation followed by 45  
165 cycles of denaturation, annealing and elongation. After amplification, the melting program  
166 was set up by heating to 95°C for 1 min, cooling to 40°C for 1 min and applying a temperature  
167 ramp from 65 to 95°C with a transition rate of 0.2°C s<sup>-1</sup> and a continuous fluorescence  
168 monitoring. Each reaction was run in triplicate in 96-well plates, with the LightCycler® 480  
169 System (Roche). Each PCR-HRM run included one negative control where the DNA template  
170 was replaced by water.

171

#### 172 *Sensitivity and specificity*

173 For determination of the assay sensitivity, serial 10-fold dilutions of known genomic DNA  
174 concentrations of *M. fortuitum* and *M. marinum* were prepared in (i) sterilized distilled water  
175 and (ii) 100 ng of genomic DNA extracted from *Pangasianodon hypophtalmus* fish liver. The  
176 number of genome equivalents was estimated from the measured DNA concentrations and the  
177 size of the fully sequenced *M. marinum* genome (6.66 Mb). Serial dilutions of *M. fortuitum*  
178 and *M. marinum* DNA covered the range of 10<sup>6</sup> to 1 genome equivalents, and standard curves  
179 were drawn from these measurements performed under the same conditions as described  
180 above. Because mycobacterial genomes may carry 1 or 2 ribosomal RNA (rRNA) operons  
181 (29, 30), results were always expressed as genome copies or genome equivalents, and not as  
182 16S-23S (ITS) copies.

183 Specificity of the assay was evaluated on 16 non-mycobacterial isolates, including 7 Gram  
184 positive and 9 Gram negative species (Table 1). The amount of non-mycobacterial DNA in  
185 each reaction was adjusted to approximately 10<sup>6</sup> genome equivalents (based on an average

186 genome size of 6 Mb). Positive controls consisted of 2 mycobacterial species (*M. marinum*  
187 and *M. fortuitum*) and their DNA amount was set to  $\sim 10^4$  genome equivalents only. To ensure  
188 the integrity of these 18 genomic DNAs, they were subsequently amplified with a pair of 16S  
189 universal primers (31) in a 2720 thermal cycler (Applied Biosystems). PCR mixtures  
190 contained 5  $\mu$ l of 2X master mix (Fast-Start PCR kit, Roche), 0.8  $\mu$ M of forward (5'-  
191 GCACAAGCGGTGGAGCATGTGG-3') and reverse (5'- GCCCGGGAACGTATTCACCG-  
192 3') primers and 2  $\mu$ l of template DNA, in a final volume of 10  $\mu$ l. Amplification consisted of  
193 30 cycles of denaturation (95°C, 30 sec), annealing (60°C, 30 sec) and elongation (72°C, 30  
194 sec) and PCR products were observed on 1% agarose gel electrophoresis containing SYBR  
195 Safe DNA gel stain (Invitrogen).

196

#### 197 *High resolution melting analysis*

198 The LightCycler®480 software package (version 1.5.0.39) was utilized for both PCR and  
199 HRM analyses. After each run, cycles of quantification (Cq) were calculated in order to  
200 ensure that each DNA template had been successfully amplified. All amplifications that  
201 resulted in Cq values  $> 30$  were arbitrarily considered negative and were therefore excluded  
202 from subsequent analyses. Melting profiles were analyzed with the gene scanning function, in  
203 a standardized way. First, melting curves were normalized in the pre-melting and post-melting  
204 regions; the normalization temperature range was 81 to 81.5°C and 92 to 92.5°C, respectively.  
205 Then, a temperature shift was applied on normalized curves with a threshold set at 5% of  
206 normalized fluorescence. Finally, melting curves were classified into groups with a default  
207 sensitivity of 0.3 and using the auto-group function. This way, curves showing nearly  
208 identical shapes were grouped together and were depicted in the same color for easy  
209 visualization. Yet, since the software enables a classification into a maximum of 6 groups  
210 only, a  $T_m$  calling was also applied in order to record the melting temperature of each

211 product. Species were then discriminated according to both their melting profile and melting  
212 temperature (T<sub>m</sub>).

213

#### 214 *Validation of the assay with blind samples*

215 To evaluate the ability of this PCR-HRM assay to identify unknown specimens, 30 tissues  
216 originating from either infected fish or fish collected from infected ponds, as well as 3 DNA  
217 samples extracted from mycobacterial cultures isolated from some of these fish, were  
218 investigated (Table 2). DNAs from these samples were blindly subjected to PCR-HRM,  
219 together with the 12 *Mycobacterium* "reference" species (*M. marinum*, *M. fortuitum*, *M.*  
220 *chelonae*, *M. gordonae*, *M. smegmatis*, *M. phlei*, *M. bohemicum*, *M. pseudoshottsii*, *M.*  
221 *abscessus*, *M. haemophilum*, *M. avium*, *M. gastri*) and one negative control (no DNA).  
222 Amplification yield was first verified and samples that displayed a C<sub>q</sub> value < 30 were  
223 tentatively assigned a species name through the successive analysis of the melting profile and  
224 T<sub>m</sub>. For confirmation, samples that displayed one single amplification product were sent for  
225 sequencing to Genoscreen company (Lille, France).

226

## 227 **Results**

228

#### 229 *Specificity of the primers*

230 Tested on genomic DNA extracted from pure bacterial cultures, the primers specifically  
231 designed for this assay successfully amplified all of the 12 assessed *Mycobacterium* species,  
232 including *M. marinum*, *M. fortuitum* and *M. chelonae* (3). The amplification resulted in  
233 products of the expected size, comprised between approximately 220 and 320 bp. For 7 of the  
234 analyzed strains, a single product was amplified, as revealed by melting peaks obtained from  
235 the first derivative of fluorescence over temperature. Yet, for *M. abscessus*, *M. gastri* and *M.*

236 *haemophilum* strains, a small secondary melting peak was always observed, whereas the peak  
237 shape of *M. chelonae* and *M. fortuitum* contained a minor "shoulder" (not shown). Probably  
238 because the secondary melting peaks were small, the LightCycler<sup>®</sup> 480 software detected two  
239 T<sub>m</sub> values solely for *M. gastri*. In this case, only the T<sub>m</sub> corresponding to the main peak was  
240 taken into consideration. The occurrence of secondary melting peaks, mostly in fast-growing  
241 species, may be due to the existence of 2 rRNA operons (29, 32). However, the melting  
242 curves for these species were highly reproducible over experiments, as they were identical in  
243 all subsequent HRM runs.

244 When tested on a range of non-mycobacterial species (see table 1), the primers always yielded  
245 C<sub>q</sub> values above 30, *i.e.* below the detection threshold, with no detectable band on agarose gel  
246 electrophoresis (Figure 1). It is noteworthy that the amount of tested genomic DNA for these  
247 16 non-mycobacterial strains was elevated, as it approximated  $\sim 10^6$  genome equivalents. In  
248 comparison, the two major pathogens *M. marinum* and *M. fortuitum*, tested at a 100-fold lower  
249 DNA concentration (about  $10^4$  genome copies), resulted in C<sub>q</sub> values around 19-20. When  
250 universal primers for 16S rDNA were tested on the same DNAs, all the strains yielded an  
251 amplification product of nearly the same intensity, demonstrating that neither PCR inhibition  
252 nor DNA degradation occurred (Figure 1c).

253

#### 254 *Sensitivity of the PCR-HRM assay*

255 Sensitivity tests were conducted on *M. marinum* and *M. fortuitum* DNAs, assuming that  
256 results would be comparable for the other strains, since the use of equivalent DNA  
257 concentrations yielded nearly similar C<sub>q</sub> values for all the tested strains. As described  
258 previously, the detection threshold was set to 30 cycles, because C<sub>q</sub> values of negative  
259 controls were always comprised between 30 and 35, probably because of a slight primer-  
260 dimer formation, undetectable neither on agarose gel electrophoresis nor by melting curve

261 analysis. Using this threshold, the assay was able to accurately and reproducibly detect as low  
262 as 10 copies of *M. marinum* and *M. fortuitum* genomes in two experimental backgrounds, *i.e.*  
263 water and fish DNA. Figure 2 presents the amplification results obtained for *M. marinum* and  
264 *M. fortuitum* diluted in 100 ng of fish DNA. The faint band observed on gel electrophoresis  
265 for 1 genome equivalent of *M. marinum* (Figure 2b) was not considered significant.  
266 Furthermore, the relation between fluorescence intensity and DNA quantity was linear over 6  
267 logs for the dilutions in fish DNA ( $R^2= 0.999$  and  $0.998$  for *M. marinum* and *M. fortuitum*,  
268 respectively) as well as for the dilutions in water ( $R^2= 0.997$  and  $0.998$  for *M. marinum* and  
269 *M. fortuitum*, respectively) (Figure 3). Therefore, the presence of fish DNA, which reflects the  
270 actual nature of biological samples, did not alter the sensitivity of *Mycobacterium* detection.

271

#### 272 *Analysis of melting profiles*

273 Twelve strains were tested for their ability to be discriminated according to their melting  
274 profile and melting temperature. Analysis of the melting curves showed the presence of 9  
275 distinct melting profiles, as illustrated by the difference plots in figure 4. Yet, the strains that  
276 shared identical melting profiles could be differentiated by a distinct  $T_m$  (Table 3), thereby  
277 enabling a total discrimination of the 12 species. Reliability of such strain classification was  
278 evaluated through different means. First, the intra-run repeatability of difference plots was  
279 verified by (i) running 6 replicates of each strain and (ii) running 6 serial dilutions of *M.*  
280 *marinum* and *M. fortuitum*, each in triplicate. As attested by figure 5, the replicate curves  
281 could be superimposed. More importantly, the difference plots, and hence the grouping  
282 ability, was not affected by the template DNA concentration, at least between  $10$  and  $10^6$   
283 genome copies in the reaction. Secondly, measurement of the melting temperature of all of the  
284 12 mycobacterial strains from three separate runs revealed a high reproducibility, as shown in  
285 table 3. All but 3 standard deviations (SD) were  $\leq 0.04^\circ\text{C}$ , and the maximum SD was  $0.12^\circ\text{C}$

286 (for *M. fortuitum*). Finally, when repeated 3 times from 3 independent runs, the HRM analysis  
287 showed consistent grouping patterns. It is noteworthy that the 3 most frequent *Mycobacterium*  
288 fish pathogens *M. marinum*, *M. fortuitum* and *M. chelonae* display somewhat different Tms.  
289 Therefore, their identification could be simply done according to their Tm (Table 3).

290

#### 291 *Mycobacterium detection and species assignment in blind samples*

292 Among the 30 fish tissue samples that were tested, only 10 could be successfully amplified  
293 with the HRM primers, since the 20 others yielded Cq values higher than 30. Concentration of  
294 these 10 samples varied between 20 and 1200 genome equivalents per reaction, or between ~1  
295 and ~60 genome equivalents ng<sup>-1</sup> of total genomic DNA since 20 ng of total DNA were used  
296 in each reaction. Considering the mass of tissue used (20 mg) and the DNA yield (ng of total  
297 DNA g<sup>-1</sup> of tissue) for each extraction, such concentrations corresponded to approximately  
298 7.5x10<sup>5</sup> to 2.5x10<sup>7</sup> genome copies g<sup>-1</sup> tissue. The HRM analysis allowed assigning 3 of these  
299 samples as *M. marinum* and 6 as *M. phlei* (Figure 6). Sequencing of the 9 corresponding  
300 amplification products confirmed the species identification for 8 of them, while the remaining  
301 one turned out to belong to *M. malmoeense*, which is not included in the present assay. The last  
302 positive sample presented a melting curve with double inflection, with Tm values comparable  
303 to those of *M. marinum* (86.7°C) and *M. phlei* (90.9°C), suggesting the presence of a mixed  
304 infection with those 2 species (Figure 6C). Regarding the 3 unknown DNAs, 2 could be  
305 unambiguously assigned to *M. marinum*, while the third one was associated with *M.*  
306 *abscessus* (not shown). Sequencing of the corresponding amplification products confirmed  
307 this species identification for the 3 samples.

308

309

310

## 311 Discussion

312 To our knowledge, this is the first report of an HRM-based assay that enables rapid detection  
313 and identification of several non-tuberculous mycobacterial species without relying on costly  
314 probes such as molecular beacons or Taqman probes. When genomic DNA extracted from  
315 pure cultures was used, the assay was able to confidently detect approximately 10  
316 mycobacterial genomes per reaction. This low detection limit was comparable to that obtained  
317 by Zerihum et al. (33), who used a Taqman probe, and slightly better than that reported by  
318 Salati et al. (34) with a nested-PCR test. It is also comparable to the detection levels described  
319 by Pakarinen et al. (35), whose results showed that the use of a hybridization probe did not  
320 significantly improve the assay sensitivity. Moreover, the ability of species identification was  
321 not affected by the initial DNA template amount, as melting curves obtained from 10-fold  
322 dilutions of genomic DNA (corresponding to  $10^7$ -10 genome equivalents) could be  
323 superimposed (Figure 5B).

324 The mycobacterial concentrations of the unknown fish samples were estimated to range  
325 between 20 and 1200 genome equivalents per reaction, which corresponded to approximately  
326  $7.5 \times 10^5$  and  $2.5 \times 10^7$  genome equivalents  $g^{-1}$  of tissue. The lowest concentration was close to  
327 the detection limit of the present assay, indicating that lower bacterial loads would be hardly  
328 detected under the conditions used here. It is difficult to compare these values with those  
329 obtained by other groups (33, 34), as the procedures used to estimate the minimal detectable  
330 bacterial load from fish tissues were different. Technical culture may be considered as the  
331 gold standard with a limit of detection close to 10 colony forming units (cfu)  $g^{-1}$  tissue (36).  
332 However, in some situations, culture-based methods may underestimate the amount of  
333 mycobacterial cells because of: i) the presence of viable but non-cultivable mycobacteria(37),  
334 and ii) non-optimal culture conditions. The detection limit obtained in our study (~10  
335 bacterial genomes per reaction) is comparable to that reported by others (33) on DNA

336 extracted from pure cultures (~6.5 cfu per reaction). Based on their calculations, our lowest  
337 estimated concentration would thus correspond to ~150 cfu g<sup>-1</sup> tissue. Such a detection limit  
338 would therefore allow to reveal the presence of mycobacterium in most infected fish, as the  
339 scarce information reporting mycobacterial loads in tissues of infected fish indicate values  
340 comprised between 10<sup>2</sup> to 10<sup>9</sup> cfu g<sup>-1</sup> tissue (38, 39). Optimization of the DNA extraction  
341 protocol from fish tissue would possibly improve this sensitivity level. Moreover, our present  
342 results confirm previous findings obtained by LDV34 (not published), i.e.: the 4 samples  
343 found positive for *M. marinum* correspond to organs showing granulomas and sampled from  
344 sick fishes that had been diagnosed as *M. marinum*; the 26 remaining ones corresponded to  
345 fish that did not show any lesion, but were in contact with other infected fishes. However, the  
346 fact that *M. phlei* was identified in 6 of them is rather surprising and requires further  
347 investigation, as *M. phlei* is not known as a common NTM species in fish.

348 High resolution melting analysis has already been successfully used for discriminating related  
349 species of bacteria (26, 40). Besides, by measuring the T<sub>m</sub> of a small amplicon of the *hsp65*  
350 gene, it was possible to differentiate *Mycobacterium abscessus* from *M. chelonae*, 2 very  
351 close rapidly growing species that cannot be discriminated biochemically (41). The authors  
352 hypothesized that with a T<sub>m</sub> SD of less than 0.1°C, it should be possible to differentiate many  
353 different species. In our case, *M. abscessus* and *M. chelonae* had pretty different melting  
354 profiles, and could be discriminated by both their melting profile and melting temperature,  
355 which differed by approximately 1.2°C. However, even though most T<sub>m</sub> SDs were lower than  
356 0.1°C, some species could not be identified solely according to their T<sub>m</sub> (e.g. *M. chelonae*  
357 and *M. haemophilum* or *M. pseudoshottsii* and *M. bohemicum*,). Thus, the combination of  
358 both T<sub>m</sub> and melting profile analysis increased the discriminative power of the assay.

359 Selection of the target sequence was made bearing in mind that some mycobacterial species  
360 carry 2 rRNA operons, and that strains belonging to the same species may display sequence

361 differences. Therefore, the relative high length of the target amplicon (~220-320 bp,  
362 depending on the species) was expected to confer a greater tolerance of the melting profile to  
363 sequence changes. Among the unknown samples that were identified as *M. marinum* (Table  
364 2), the sequence of two of them differed from the strain used as reference by 2 mismatches  
365 located near the 5' extremity of the amplification product. Yet, the presence of 2 mismatches  
366 did not significantly modify the T<sub>m</sub> and did not prevent to classify these 2 strains as *M.*  
367 *marinum*. The extent to which sequence variations affect melting profiles and temperatures  
368 has not been quantified precisely. However, it is known that melting differences decrease as  
369 the amplicon size increases (42), and the use of such a long amplicon in the present study is  
370 likely to represent a limitation for the number of species that can be simultaneously  
371 discriminated.

372 Anyhow, the results presented here show that this assay confidently discriminates 12  
373 mycobacterial species, *i.e.* *M. phlei*, *M. smegmatis*, *M. gastri*, *M. bohemicum*, *M. marinum*, *M.*  
374 *fortuitum*, *M. chelonae*, *M. gordonae*, *M. pseudoshottsii*, *M. abscessus*, *M. haemophilum* and  
375 *M. avium*. Though it includes the most frequent reported fish pathogens, we cannot exclude  
376 the possibility of a wrong species determination in the case of fish carrying uncommon  
377 mycobacterial species not accounted for here. An alternative solution to increase the number  
378 of species that can be simultaneously differentiated and reduce the probability of wrong  
379 species assignment has recently been described (43). Although it looks very attractive, the use  
380 of a combination of labeled probes dramatically increases the cost of the experiment. Besides,  
381 it also seems to decrease the assay sensitivity, since the lowest bacterial concentration that  
382 was tested (100 genome copies) yielded relatively high C<sub>q</sub> values (around 35). This drop in  
383 sensitivity is probably the result of a higher level of constraints induced by the simultaneous  
384 use of 4 different probes that require consensus experimental conditions for optimal behavior.

385 Finally, compared to the 2 existing commercial kits (19, 21), the assay presented here presents  
386 many advantages. First, the real-time PCR format makes possible to analyze many samples at  
387 the same time. Indeed, using the 384-well plate format and running both reference and target  
388 samples in triplicate, it is possible to simultaneously analyze more than 100 samples.  
389 Secondly, since this PCR-HRM assay does not require prior mycobacterial culture, it takes  
390 only a couple of hours to obtain results from fish tissue samples. Thirdly, the cost of such a  
391 test will be much lower than that of the existing kits, since it is a one-step assay and it does  
392 not rely on any costly labeled probe. A first estimation would place the cost of one reaction,  
393 from sample to result, below 5 €. To conclude, the present PCR-HRM assay is accessible,  
394 quick and inexpensive. It enables the detection of the presence of any mycobacterial genome,  
395 since it uses genus-specific primers, as well as the identification of 12 mycobacterial species  
396 directly from fish samples, without prior bacterial cultivation. Its format allows the analysis of  
397 more than 100 unknown samples simultaneously, thus making possible to investigate the  
398 prevalence of these pathogens at large scales and at a reasonable cost.

399

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407 2013-104.

408

409

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530

531

532 Table 1. List of *Mycobacterium* and non-*Mycobacterium* species used in this study

Strain	Gram	Source
Mycobacteria		
<i>M. phlei</i> (CIP 105389T)	+	Pasteur Institute
<i>M. bohemicum</i> (CIP 105811T)	+	Pasteur Institute
<i>M. gastri</i> (CIP 104530T)	+	Pasteur Institute
<i>M. pseudoshottsii</i> (CIP109775 T)	+	Pasteur Institute
<i>M. smegmatis</i> (CIP 104444 T)	+	Pasteur Institute
<i>M. fortuitum ssp fortuitum</i>	+	Arnaud de Villeneuve Hospital
<i>M. marinum</i>	+	Arnaud de Villeneuve Hospital
<i>M. chelonae</i>	+	Arnaud de Villeneuve Hospital
<i>M. abscessus</i>	+	Arnaud de Villeneuve Hospital
<i>M. goodii</i>	+	Arnaud de Villeneuve Hospital
<i>M. avium</i>	+	LDV Montpellier
<i>M. haemophilum</i>	+	LDV Montpellier
Non-mycobacteria		
<i>Flavobacterium psychrophilum</i>	-	LDV Montpellier
<i>Pseudomonas fluorescens</i>	-	LDV Montpellier
<i>Aeromonas sobria</i>	-	LDV Montpellier
<i>Aeromonas hydrophila</i>	-	LDV Montpellier
<i>Vibrio vulnificus</i>	-	LDV Montpellier
<i>Citrobacter braaki</i>	-	LDV Montpellier
<i>Shewanella putrefaciens</i>	-	LDV Montpellier
<i>Photobacterium damsela</i>	-	LDV Montpellier
<i>Chryseobacterium indologenes</i>	-	LDV Montpellier
<i>Citrobacter freundii</i>	-	LDV Montpellier
<i>Lactococcus garvieae</i>	+	LDV Montpellier
<i>Carnobacterium piscicola</i>	+	LDV Montpellier
<i>Streptococcus parauberis</i>	+	LDV Montpellier
<i>Carnobacterium maltaromaticum</i>	+	LDV Montpellier
<i>Enterococcus faecalis</i>	+	LDV Montpellier
<i>Nocardia sp.</i>	+	CVI Wageningen <sup>a</sup>

533 <sup>a</sup> Central Veterinary Institute

**535** Table 2. List of the analyzed fish tissue samples

Original sample name (fish species)	Concentration <sup>a</sup>	NTM Species identification
080109000133-01 ( <i>Anableps anableps</i> )	7,41E+05	<i>M. phlei</i>
070213001086-01 ( <i>Dicentrarchus labrax</i> )	6,17E+06	<i>M. marinum</i>
061107005301-01 ( <i>Hemigrammus bleheri</i> )	1,61E+07	Mix of <i>M. phlei</i> / <i>M. marinum</i> ?
070821004737-01 (undetermined cichlid)	1,67E+06	<i>M. phlei</i>
070821004737-01 (undetermined cichlid)	1,04E+07	<i>M. phlei</i>
090805003940-01( <i>Sparus aurata</i> )	1,45E+06	<i>M. malmoense</i>
090805003940-01 ( <i>Sparus aurata</i> )	3,89E+06	<i>M. phlei</i>
090805003940-01 ( <i>Sparus aurata</i> )	2,07E+06	<i>M. phlei</i>
120124000342-01 ( <i>Scophthalmus maximus</i> )	5,39E+06	<i>M. marinum</i>
071108006385-02 ( <i>Sciaenops ocellatus</i> )	2,49E+07	<i>M. marinum</i>
DNA samples		
120417001884-01 ( <i>Danio rerio</i> )		<i>M. abscessus</i>
120124000342-01 ( <i>Scophthalmus maximus</i> )		<i>M. marinum</i>
120127000443-01 ( <i>Danio rerio</i> )		<i>M. marinum</i>

**536** <sup>a</sup> Concentration is expressed as genome equivalents g<sup>-1</sup> tissue

**537**

**538** Table 3. Reproducibility of the melting temperature measurement

Strain	T <sub>m1</sub>	T <sub>m2</sub>	T <sub>m3</sub>	Mean T <sub>m</sub> ± SD
<i>M. abscessus</i>	86.12	86.07	86.09	86.10 ± 0.03
<i>M. marinum</i>	86.71	86.68	86.67	86.68 ± 0.02
<i>M. chelonae</i>	87.31	87.23	87.26	87.27 ± 0.04
<i>M. haemophilum</i>	87.20	87.14	87.16	87.16 ± 0.03
<i>M. gordonae</i>	88.21	88.20	88.17	88.19 ± 0.02
<i>M. fortuitum ssp fortuitum</i>	89.13	89.34	89.33	89.27 ± 0.12
<i>M. gastri</i>	89.46	89.39	89.46	89.44 ± 0.04
<i>M. avium</i>	89.05	88.87	88.88	88.94 ± 0.10
<i>M. phlei</i>	90.92	90.92	90.82	90.89 ± 0.06
<i>M. smegmatis</i>	90.12	90.11	90.10	90.11 ± 0.01
<i>M. pseudoshottsii</i>	89.97	89.97	89.97	89.97 ± 0.01
<i>M. bohemicum</i>	89.98	89.96	89.93	89.96 ± 0.03

**539** Each measurement is the average T<sub>m</sub> of 3 replicates

**540**

**541**

542 **Legend to figures**

543

544 Figure 1: Specificity of the PCR-HRM assay. Amplification curves of the 16 non-  
545 mycobacterial species ( $\sim 10^6$  genome equivalents) are presented together with those of *M.*  
546 *marinum* and *M. fortuitum* at a 100-fold lower concentration ( $\sim 10^4$  genome equivalents) (A)  
547 and corresponding gel electrophoresis with the *Mycobacterium*-specific primers (B) and 16S  
548 universal primers (C). Lane 1: molecular weight marker; lanes 2-19: *Mycobacterium*  
549 *marinum*, *Mycobacterium fortuitum*, *Carnobacterium piscicola*, *Streptococcus parauberis*,  
550 *Carnobacterium maltaromaticum*, *Enterococcus faecalis*, *Citrobacter freundii*, *Lactococcus*  
551 *garvieae*, *Nocardia sp.*, *Flavobacterium psychrophilum*, *Pseudomonas fluorescens*,  
552 *Aeromonas sobria*, *Aeromonas hydrophila*, *Vibrio vulnificus*, *Citrobacter braaki*, *Shewanella*  
553 *putrefaciens*, *Photobacterium damsela* and *Chryseobacterium indologenes*.

554

555 Figure 2: Sensitivity of the PCR-HRM assay. Serial dilutions of bacterial DNA were prepared  
556 in 100 ng of fish genomic DNA and subsequently used as template for PCR amplification. (A)  
557 amplification curves; (B) corresponding gel electrophoresis.

558

559 Figure 3: Standard curves obtained from serial dilutions of bacterial DNA in H<sub>2</sub>O (A) and in  
560 100 ng of fish DNA resuspended in Tris-EDTA (B).

561

562 Figure 4: Strain grouping by HRMA. Difference plots were obtained after a temperature shift  
563 on the normalized melting curves, using *M. pseudoshottsii* as base curve. For clarity, the  
564 strain differentiation is displayed into 2 plots showing 5 groups (A) and 4 groups (B). This  
565 experiment was repeated 3 times independently and yielded to exactly the same grouping  
566 results. When 2 species grouped together, they were differentiated by their  $T_m$ . This was the

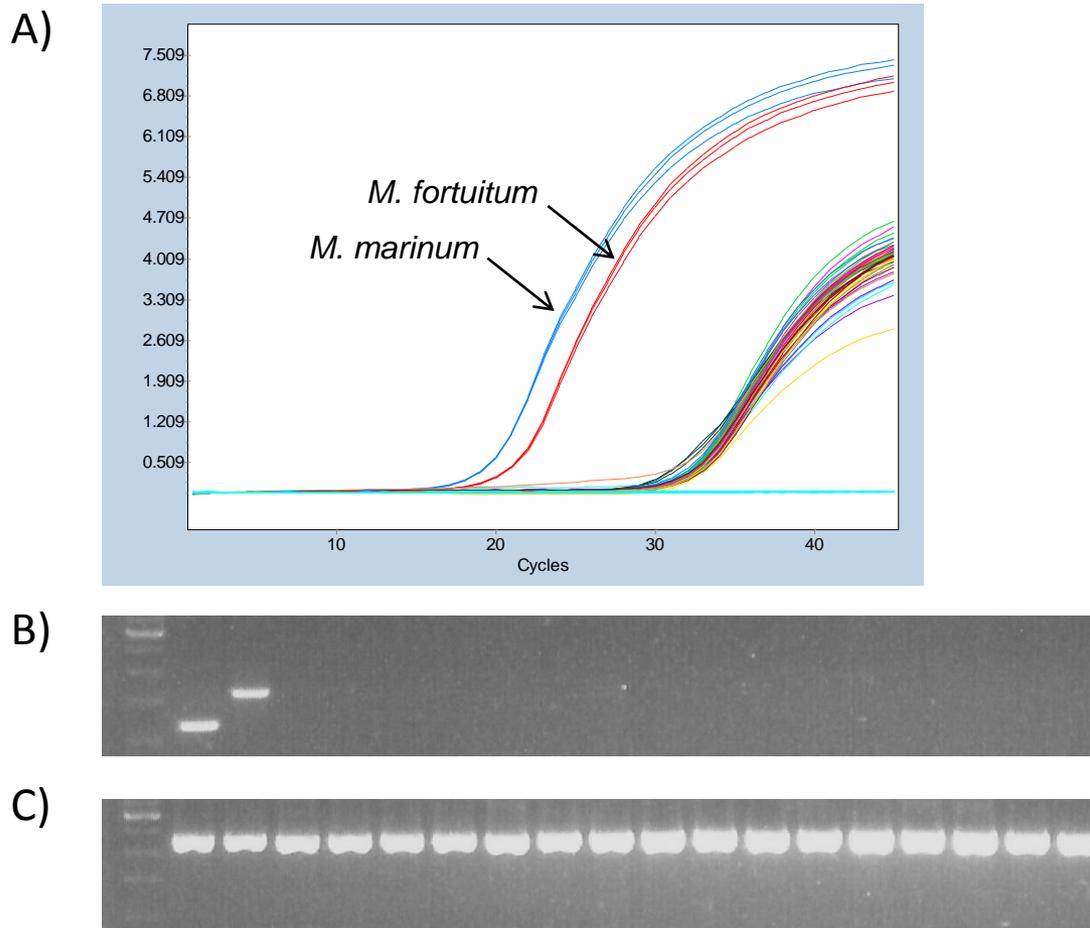
567 case for group 1 (*M. marinum* and *M. gordonae*), group 2 (*M. phlei* and *M. pseudoshottsii*)  
568 and group 3 (*M. fortuitum* and *M. haemophilum*). Refer to table 3 for T<sub>m</sub> values.

569

570 Figure 5: Reproducibility of difference plots. (A) Each mycobacterial DNA was replicated 6  
571 times and difference plots were drawn for the 6 replicates. For clarity, only 6 species are  
572 presented on the graph. (B) Difference plots were drawn from serial dilutions of *M. marinum*  
573 and *M. fortuitum* (10<sup>6</sup> to 10 genomes/reaction), using 3 replicates per dilution.

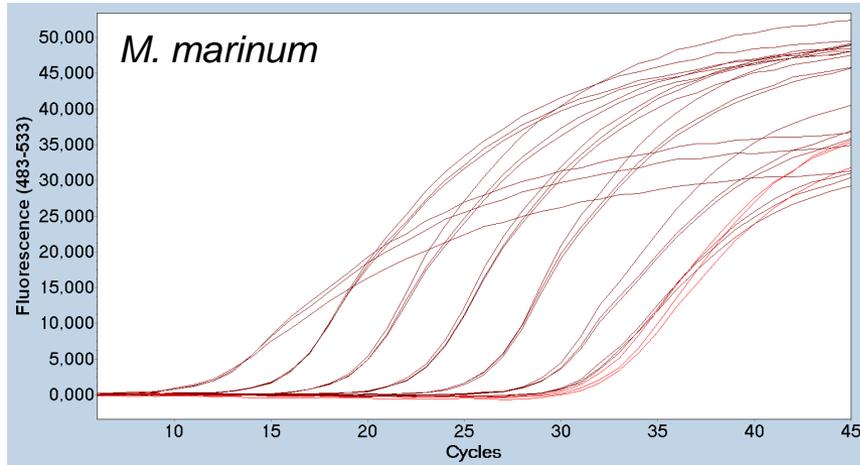
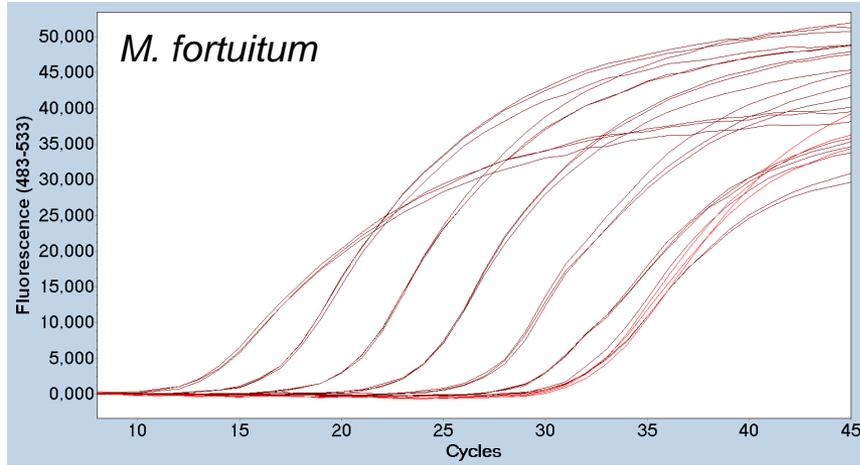
574

575 Figure 6: Identification of unknown samples. Difference plots of 4 reference strains (*M.*  
576 *marinum*, *M. fortuitum*, *M. chelonae* and *M. phlei*) are displayed alone (A) and together with  
577 9 unknown samples identified as *M. marinum* or *M. phlei* (B), using *M. fortuitum* as base  
578 curve. (C) Melting curve of an unknown sample showing two inflexions with T<sub>m</sub> comparable  
579 to those of *M. marinum* and *M. phlei*.

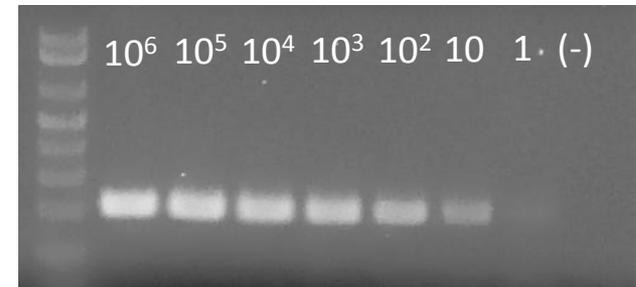
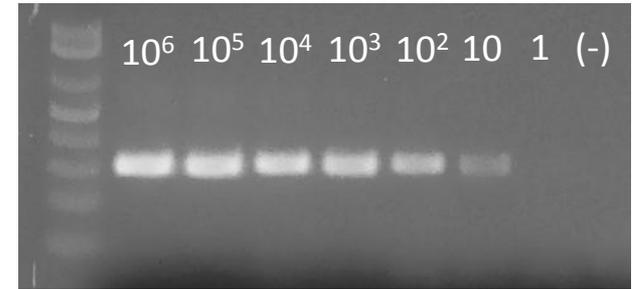


**Figure 1:** Figure 1: Specificity of the PCR-HRM assay. Amplification curves of the 16 non-mycobacterial species ( $\sim 10^6$  genome equivalents) are presented together with those of *M. marinum* and *M. fortuitum* at a 100-fold lower concentration ( $\sim 10^4$  genome equivalents) (A) and corresponding gel electrophoresis with the *Mycobacterium*-specific primers (B) and 16S universal primers (C). Lane 1: molecular weight marker; lanes 2-19: *Mycobacterium marinum*, *Mycobacterium fortuitum*, *Carnobacterium piscicola*, *Streptococcus parauberis*, *Carnobacterium maltaromaticum*, *Enterococcus faecalis*, *Citrobacter freundii*, *Lactococcus garvieae*, *Nocardia* sp., *Flavobacterium psychrophilum*, *Pseudomonas fluorescens*, *Aeromonas sobria*, *Aeromonas hydrophila*, *Vibrio vulnificus*, *Citrobacter braaki*, *Shewanella putrefaciens*, *Photobacterium damsela* and *Chryseobacterium indologenes*.

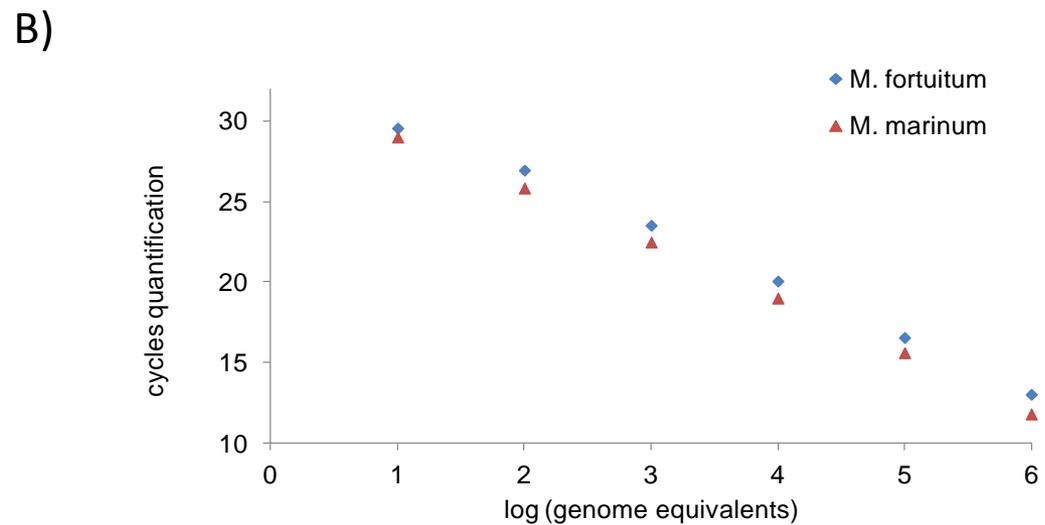
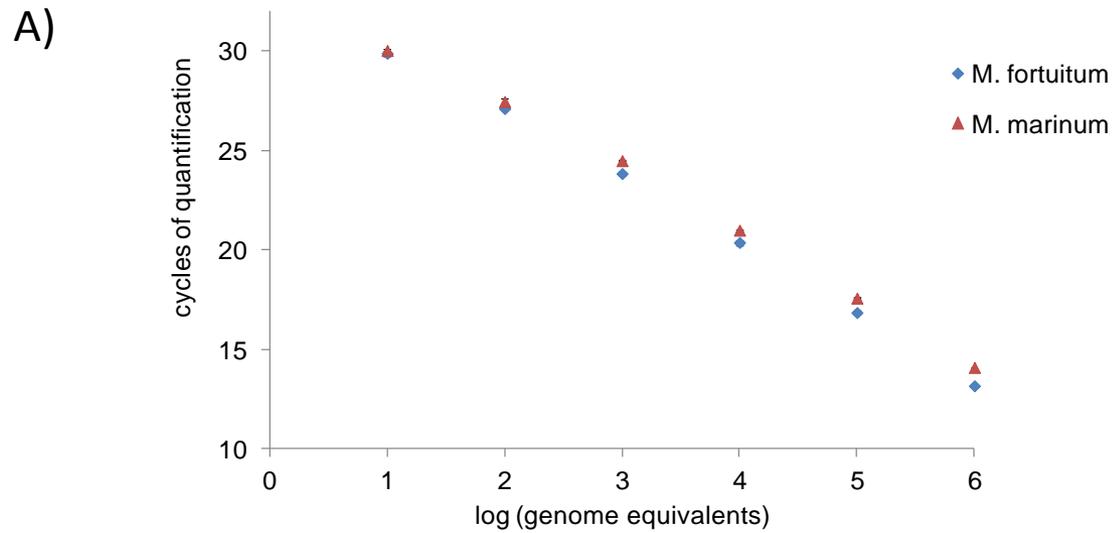
A)



B)

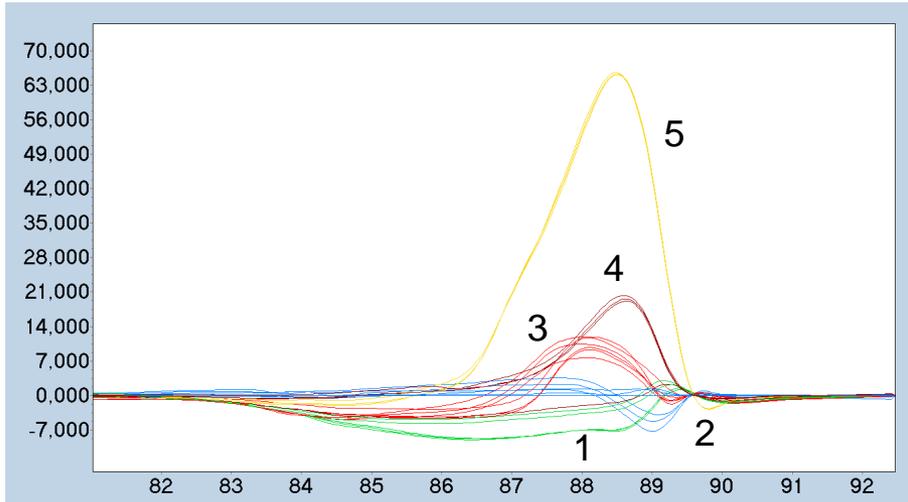


**Figure 2:** Sensitivity of the PCR-HRM assay. Serial dilutions of bacterial DNA were prepared in 100 ng of fish genomic DNA and subsequently used as template for PCR amplification. (A) amplification curves; (B) corresponding gel electrophoresis.

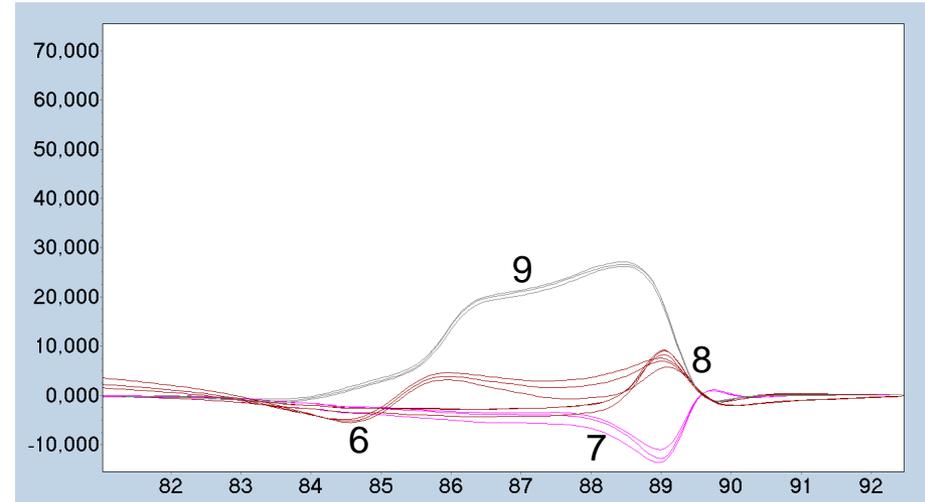


**Figure 3:** Standard curves obtained from serial dilutions of bacterial DNA in H<sub>2</sub>O (A) and in 100 ng of fish DNA resuspended in Tris-EDTA (B).

A)

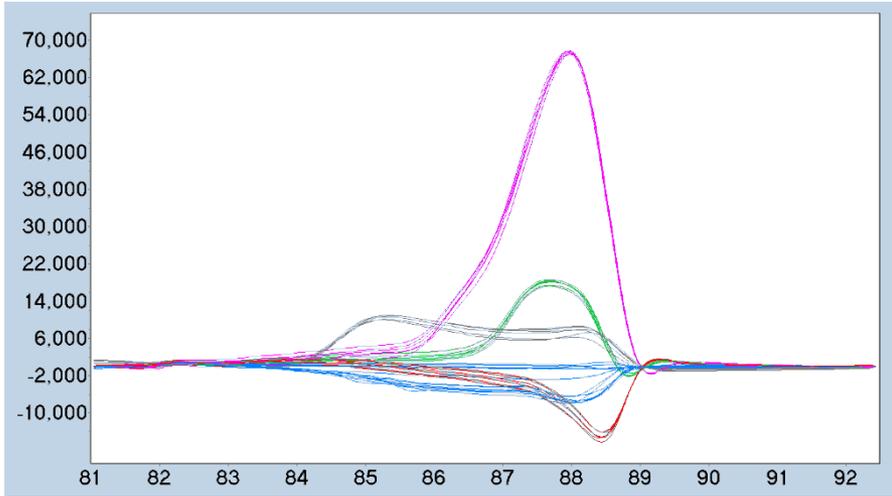


B)

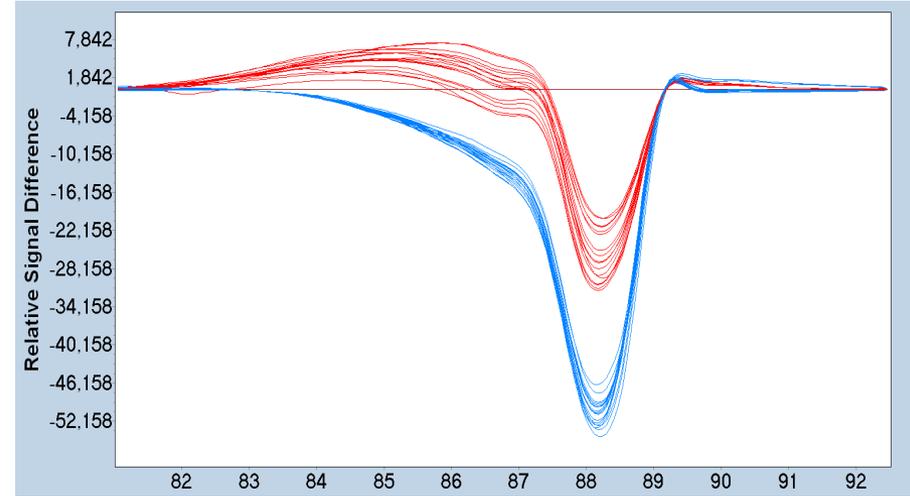


**Figure 4:** Strain grouping by HRMA. Difference plots were obtained after a temperature shift on the normalized melting curves, using *M. pseudoshottsii* as base curve. For clarity, the strain differentiation is displayed into 2 plots showing 5 groups (A) and 4 groups (B). This experiment was repeated 3 times independently and yielded to exactly the same grouping results. When 2 species grouped together, they were differentiated by their  $T_m$ . This was the case for group 1 (*M. marinum* and *M. gordonae*), group 2 (*M. phlei* and *M. pseudoshottsii*) and group 3 (*M. fortuitum* and *M. haemophilum*). Refer to table 3 for  $T_m$  values.

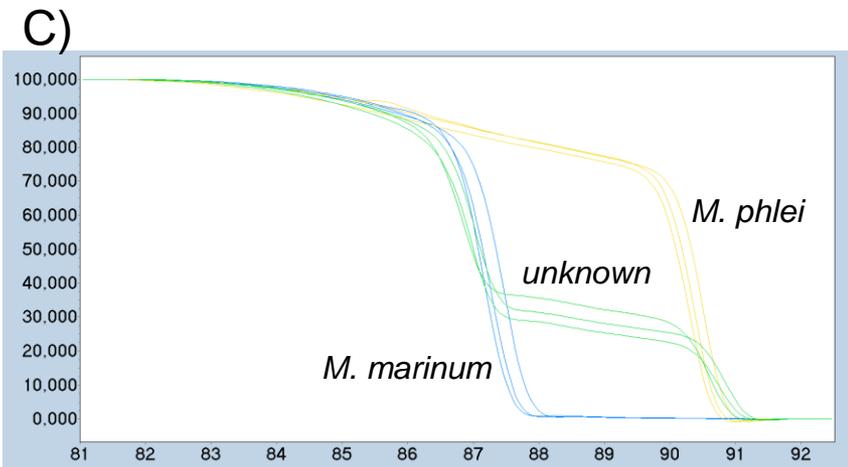
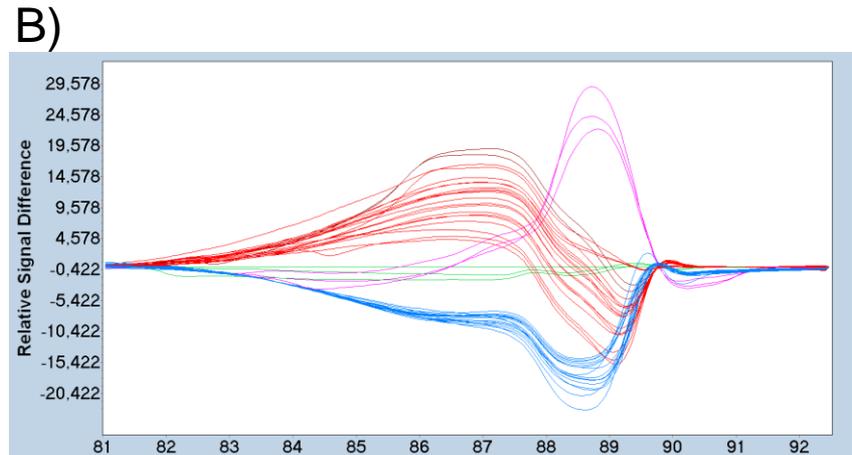
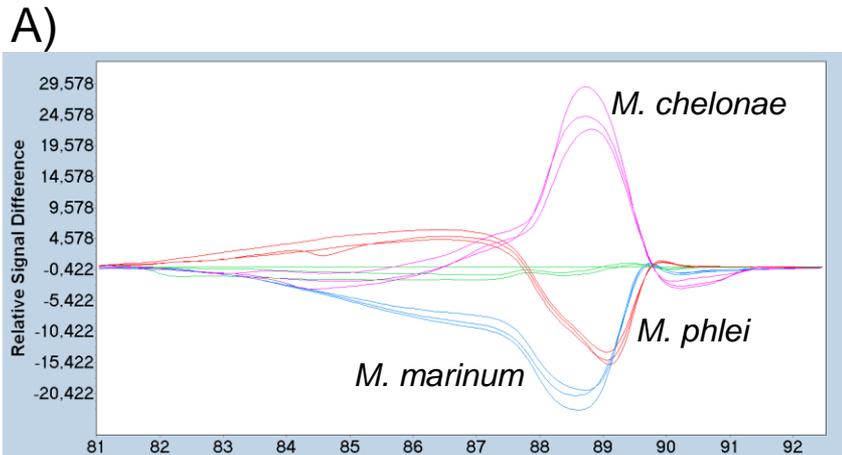
A)



B)



**Figure 5:** Reproducibility of difference plots. (A) Each mycobacterial DNA was replicated 6 times and difference plots were drawn for the 6 replicates. For clarity, only 6 species are presented on the graph. (B) Difference plots were drawn from serial dilutions of *M. marinum* and *M. fortuitum* ( $10^6$  to  $10$  genomes/reaction), using 3 replicates per dilution.



**Figure 6:** Identification of unknown samples. Difference plots of 4 reference strains (*M. marinum*, *M. fortuitum*, *M. chelonae* and *M. phlei*) are displayed alone (A) and together with 9 unknown samples identified as *M. marinum* or *M. phlei* (B), using *M. fortuitum* as base curve. (C) Melting curve of an unknown sample showing two inflexions with  $T_m$  comparable to those of *M. marinum* and *M. phlei*.