

Rapid detection and identification of nontuberculous mycobacterial pathogens in fish by using high-resolution melting analysis

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Jean-Christophe Avarre

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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

5 Thu Nguyet Phung ^{1,2}, Domenico Caruso ², Sylvain Godreuil ³, Nicolas Keck ⁴, Tatiana

6 Vallaeys ⁵, Jean-Christophe Avarre ^{2#}

7

8 ¹ Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology (VAST), 18 Hoang
9 Quoc Viet Road, Cau Giay, Hanoi, Vietnam

10 ² Institut Recherche pour le Développement (IRD), UMR226 ISEM, 361 rue Jean Francois Breton,
11 34196 Montpellier cedex 05, France

12 ³ INSERM U1058, CHU Arnaud de Villeneuve, 371 avenue du doyen Gaston Giraud, 34295
13 Montpellier Cedex 05, France

14 ⁴ Laboratoire Départemental Vétérinaire de l'Hérault, 306 rue Croix de Las Cazes, CS 69013, 34967
15 Montpellier Cedex 2, France

16 ⁵ Université Montpellier 2, UMR5119 ECOSYM, Place Eugène Bataillon, 34095 Montpellier cedex
17 05, France

18 #jean-christophe.avarre@ird.fr

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 µl 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 0.5×10^4 or 0.5×10^6 genome equivalents μl^{-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 β -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). 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 ~220 to ~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 MgCl₂, 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⁻¹ 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 10⁶ 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 10⁶ 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 μ l of 2X master mix (Fast-Start PCR kit, Roche), 0.8 μ M of forward (5'-
191 GCACAAGCGGTGGAGCATGTGG-3') and reverse (5'- GCCCGGGAACGTATTCACCG-
192 3') primers and 2 μ l of template DNA, in a final volume of 10 μ 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_m).

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_q value < 30 were
223 tentatively assigned a species name through the successive analysis of the melting profile and
224 T_m. 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[®] 480 software detected two
239 T_m values solely for *M. gastri*. In this case, only the T_m 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_q 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_q 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_q values for all the tested strains. As described
258 previously, the detection threshold was set to 30 cycles, because C_q 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°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⁻¹ 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⁻¹ of tissue) for each extraction, such concentrations corresponded to approximately
298 7.5x10⁵ to 2.5x10⁷ genome copies g⁻¹ 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 7.5×10^5 and 2.5×10^7 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⁻¹ 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 10² to 10⁹ cfu g⁻¹ 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_m 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_m 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_m SDs were lower than
356 0.1°C, some species could not be identified solely according to their T_m (e.g. *M. chelonae*
357 and *M. haemophilum* or *M. pseudoshottsii* and *M. bohemicum*,). Thus, the combination of
358 both T_m 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_m 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_q 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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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. goodnae</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 ^a

533 ^a Central Veterinary Institute

535 Table 2. List of the analyzed fish tissue samples

Original sample name (fish species)	Concentration ^a	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 ^a Concentration is expressed as genome equivalents g⁻¹ tissue

537

538 Table 3. Reproducibility of the melting temperature measurement

Strain	T _{m1}	T _{m2}	T _{m3}	Mean T _m ± 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_m 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 H₂O (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_m 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* (10⁶ 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_m comparable
579 to those of *M. marinum* and *M. phlei*.

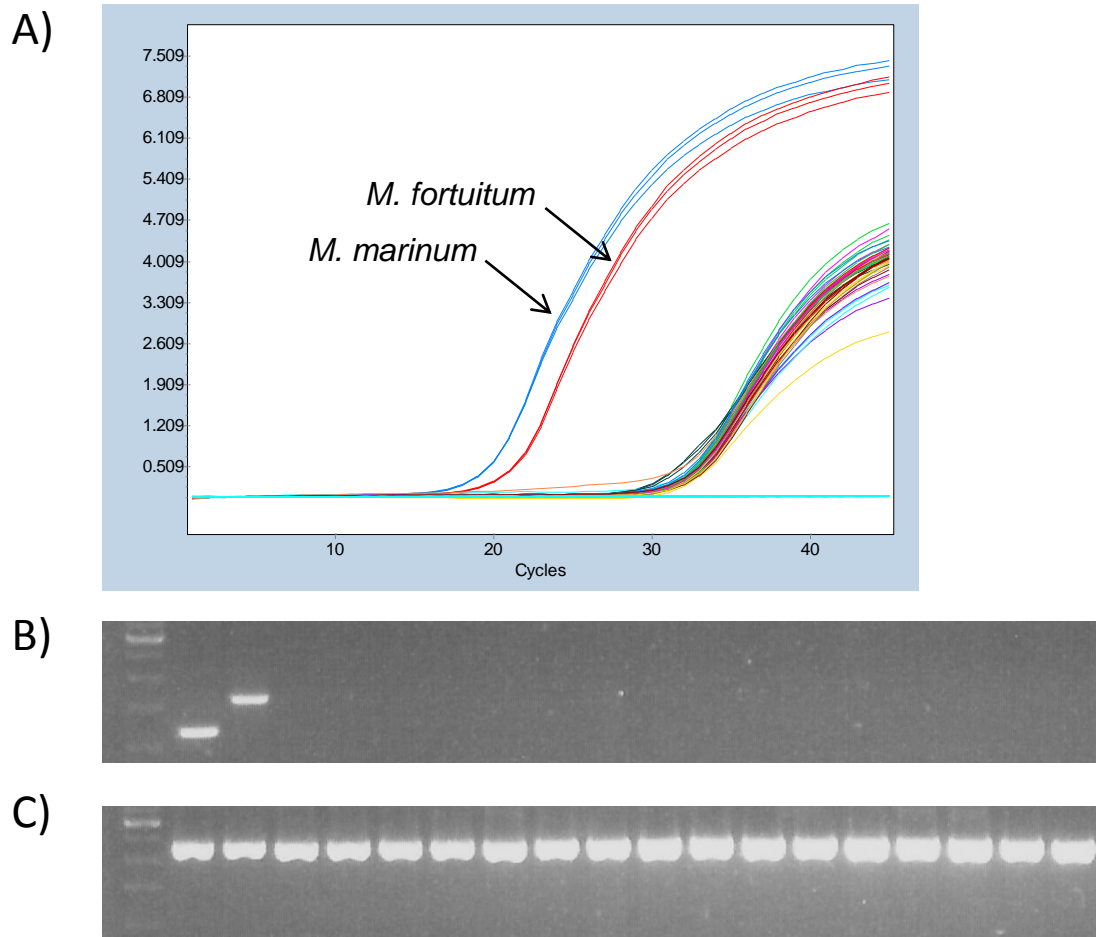
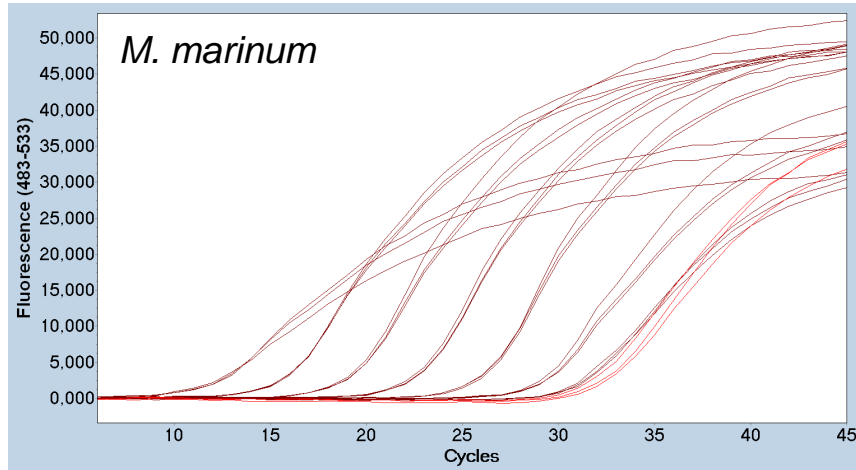
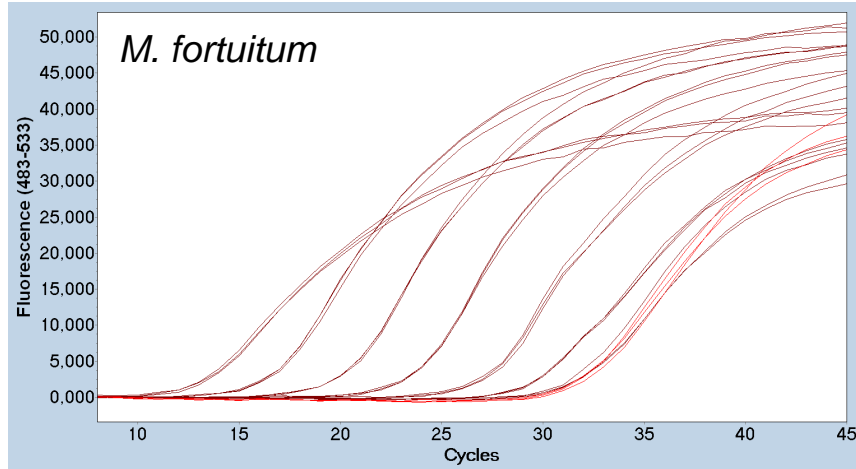


Figure 1: Figure 1: Specificity of the PCR-HRM assay. Amplification curves of the 16 non-mycobacterial species ($\sim 10^6$ genome equivalents) are presented together with those of *M. marinum* and *M. fortuitum* at a 100-fold lower concentration ($\sim 10^4$ genome equivalents) (A) and corresponding 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)

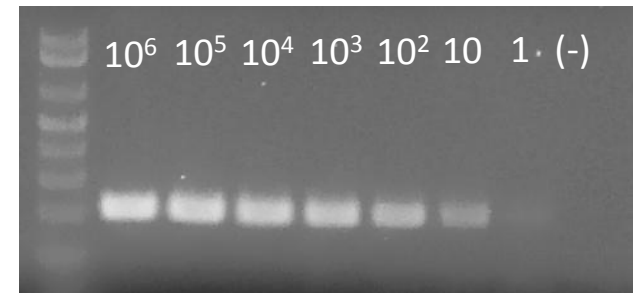
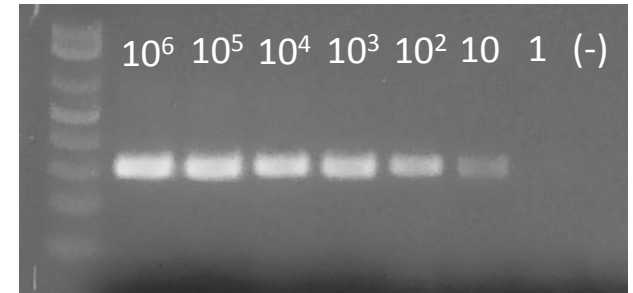


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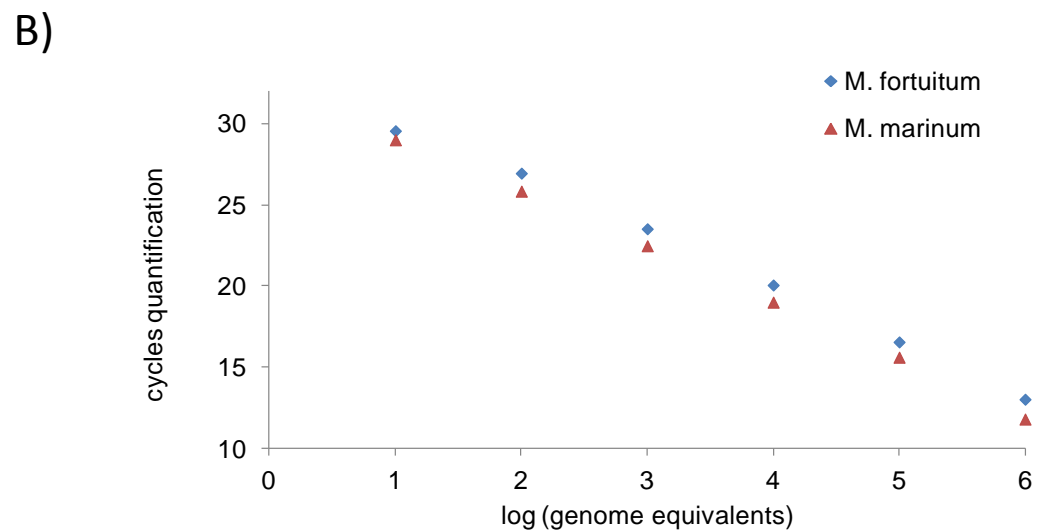
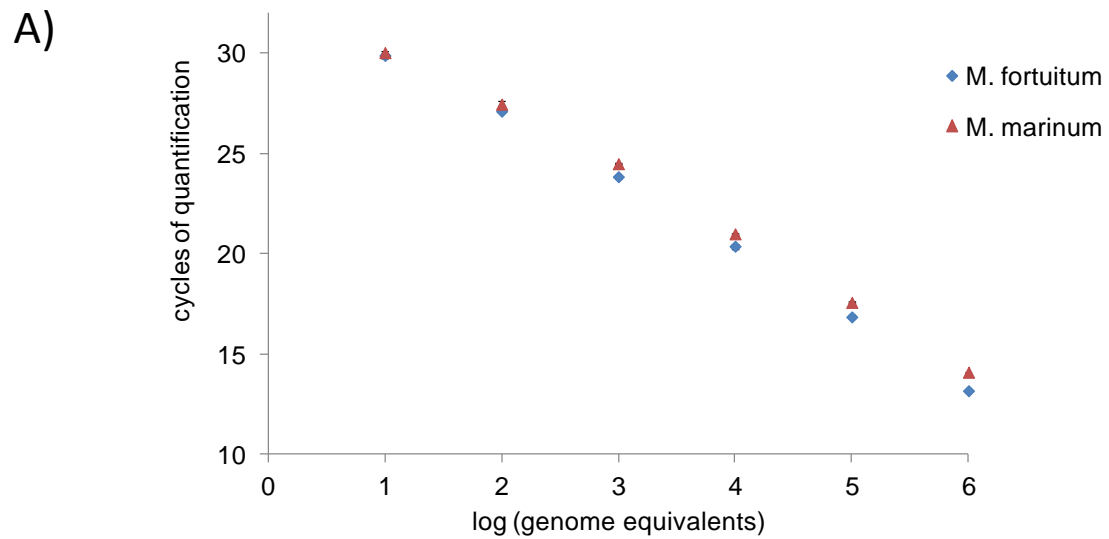
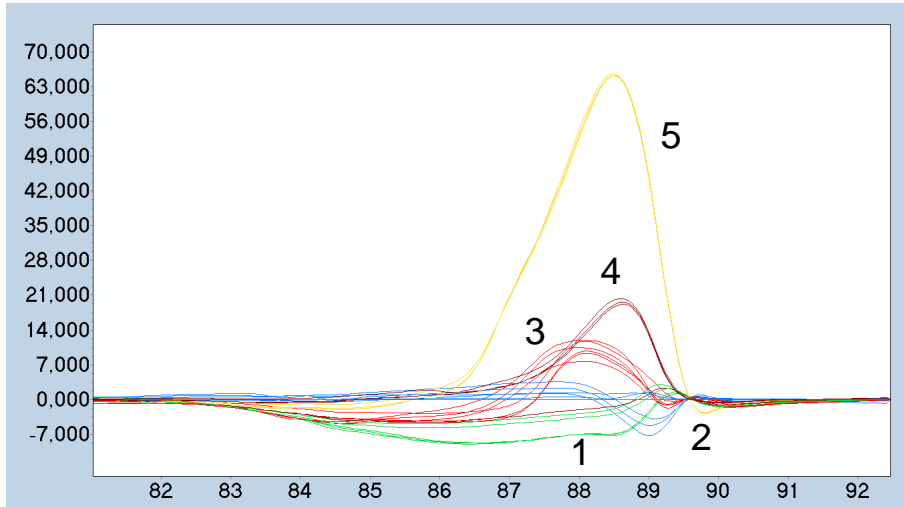


Figure 3: Standard curves obtained from serial dilutions of bacterial DNA in H₂O (A) and in 100 ng of fish DNA resuspended in Tris-EDTA (B).

A)



B)

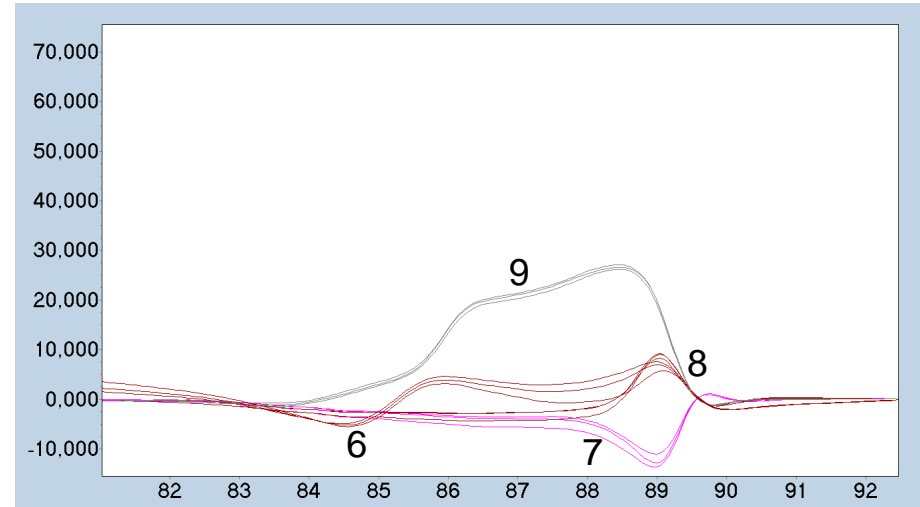
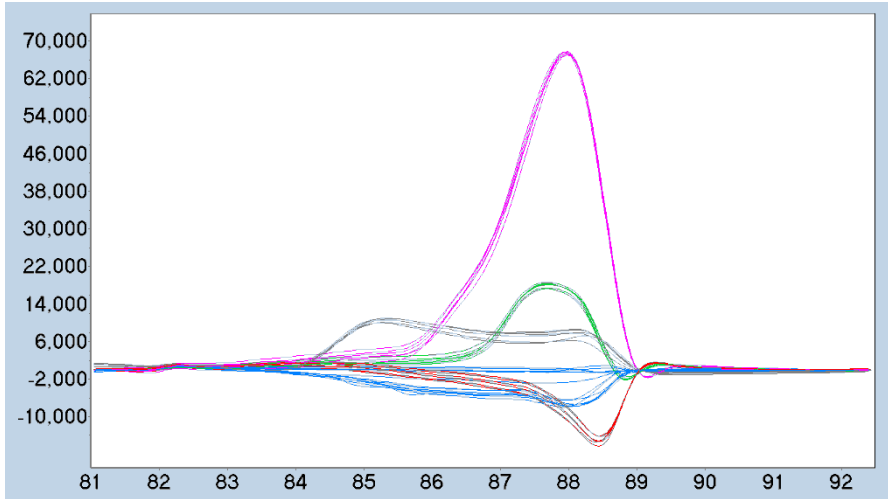


Figure 4: Strain grouping by HRMA. Difference plots were obtained after a temperature shift on the normalized melting curves, using *M. pseudoshottsii* as base curve. For clarity, the strain differentiation is displayed into 2 plots showing 5 groups (A) and 4 groups (B). This experiment was repeated 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)

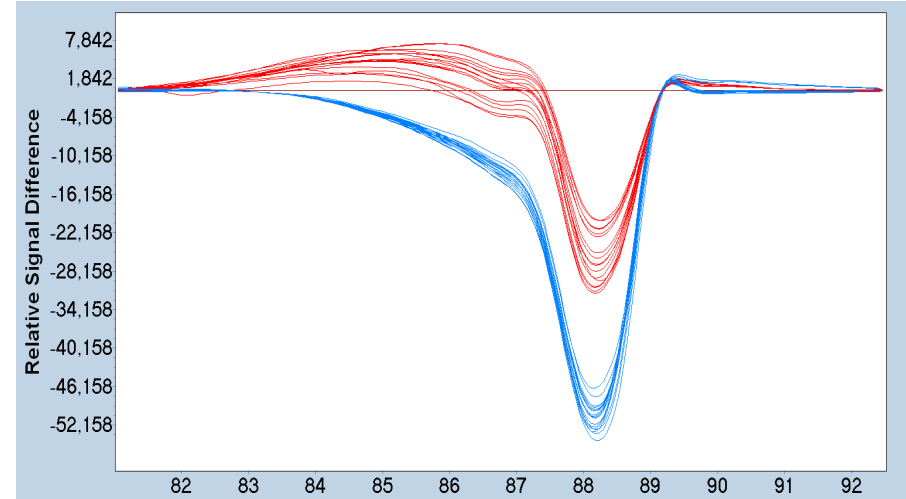


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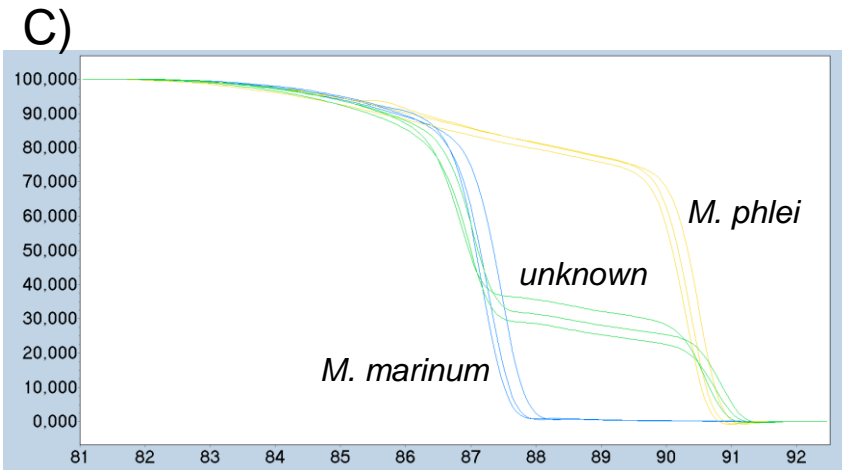
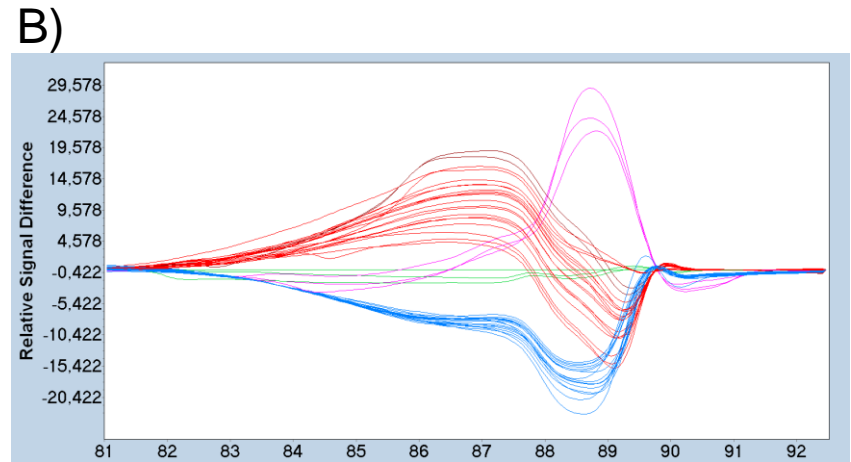
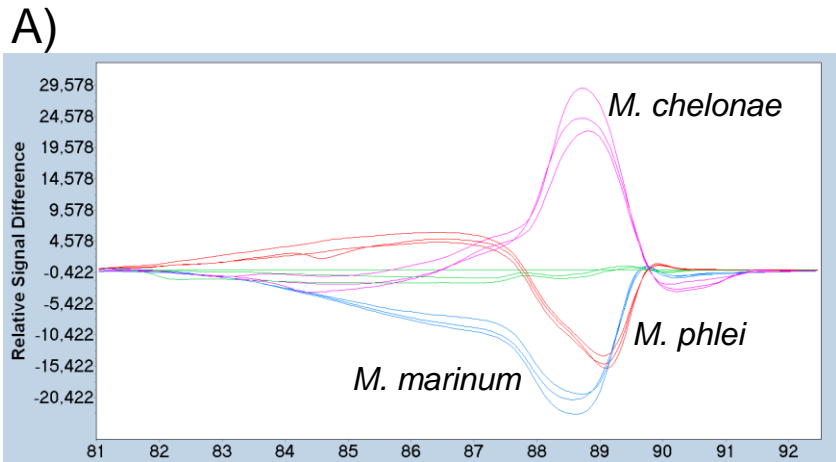


Figure 6: Identification of unknown samples. Difference plots of 4 reference strains (*M. marinum*, *M. fortuitum*, *M. chelonae* and *M. phlei*) are displayed alone (A) and together with 9 unknown samples identified as *M. marinum* or *M. phlei* (B), using *M. fortuitum* as base curve. (C) Melting curve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