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

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Running title: identification of fish mycobateria by HRMA

Abstract

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

Introduction

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

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

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

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

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

Material and methods

Bacterial strains

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

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

Fish tissue samples

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

Genomic DNA isolation

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

procedure was performed according to the manufacturer's instructions. DNA from Gram negative isolates and from fish tissues was extracted following the Gram negative and animal tissue protocols provided in the kit, respectively. In all cases, DNA was eluted in 70 µl of the provided Tris-EDTA solution and its concentration measured by UV spectrometry (NanoDrop® ND-1000 spectrophotometer, NanoDrop Technologies Inc). Bacterial genomic DNA was adjusted to approximately $0.5x10^4$ or $0.5x10^6$ genome equivalents μl^{-1}, based on an average genome size of 6.6 Mb, whereas fish DNA was diluted to 10 ng μl^{-1}.

Assay design

Since the assay aimed at being as simple as possible, it relied on the use of a double-strand intercalating - and thus non-sequence specific - fluorophore for measuring differences in melting profiles of amplification products. Under such conditions, the targeted genomic region had (i) to be unique for each of the investigated species and (ii) to harbor conserved sequences at its extremities enabling genus-specific amplification. Multiple alignments of different genomic regions (including the 16S-23S ribosomal operon, the β-subunit of RNA polymerase (*rpoB*), the 65-kD heat shock protein (*hsp65*) and the B-subunit of DNA gyrase (*gyrB*) genes) were realized with ClustalX v2 (28) on sequences imported from the NCBI collection (www.ncbi.nlm.nih.gov). They revealed that the internal transcribed spacer (ITS) region could fulfill these requirements. From these alignments, a single primer pair was subsequently designed to amplify a fragment of ~220 to ~320 bp in all the targeted mycobacterial species (forward GCTGGATCACCTCCTTTCTA and reverse AGATGCTCGCAACCACTAT). The primers were verified for the absence of secondary structures with GeneRunner v3.01 (Hasting Software, Inc. 1994) and purchased from 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 10^6 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 10^6 genome equivalents (based on an average

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

High resolution melting analysis

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

product. Species were then discriminated according to both their melting profile and melting temperature (Tm).

Validation of the assay with blind samples

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

Results

Specificity of the primers

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

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

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

Sensitivity of the PCR-HRM assay

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

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

Analysis of melting profiles

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

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

Mycobacterium detection and species assignment in blind samples

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

Discussion

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

The mycobacterial concentrations of the unknown fish samples were estimated to range between 20 and 1200 genome equivalents per reaction, which corresponded to approximately $7.5x10^5$ and $2.5x10^7$ genome equivalents g^{-1} of tissue. The lowest concentration was close to the detection limit of the present assay, indicating that lower bacterial loads would be hardly detected under the conditions used here. It is difficult to compare these values with those obtained by other groups (33, 34), as the procedures used to estimate the minimal detectable bacterial load from fish tissues were different. Technical culture may be considered as the gold standard with a limit of detection close to 10 colony forming units (cfu) g^{-1} tissue (36). However, in some situations, culture-based methods may underestimate the amount of mycobacterial cells because of: i) the presence of viable but non-cultivable mycobacteria(37), and ii) non-optimal culture conditions. The detection limit obtained in our study (~10 bacterial genomes per reaction) is comparable to that reported by others (33) on DNA

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

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

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

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

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

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

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410 **References**

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412 1. Hall-Stoodley L, Brun OS, Polshyna G, Barker LP. 2006. *Mycobacterium marinum*
413 biofilm formation reveals cording morphology. FEMS Microbiol. Lett. 257:43-49.

414 2. Decostere A, Hermans K, Haesebrouck F. 2004. Piscine mycobacteriosis: a literature
415 review covering the agent and the disease it causes in fish and humans. Vet.
416 Microbiol. 99:159-166.

417 3. Gauthier DT, Rhodes MW. 2009. Mycobacteriosis in fishes: A review. Vet. J. 180:33-
418 47.

419 4. Beran V, Matlova L, Dvorska L, Svastova P, Pavlik I. 2006. Distribution of
420 mycobacteria in clinically healthy ornamental fish and their aquarium environment. J.
421 Fish Dis. 29:383-393.

422 5. Bruno DW, Griffiths J, Mitchell CG, Wood BP, Fletcher ZJ, Drobniewski FA,
423 Hastings TS. 1998. Pathology attributed to *Mycobacterium chelonae* infection among
424 farmed and laboratory-infected Atlantic salmon *Salmo salar*. Dis. Aquat. Org. 33:101-
425 109.

426 6. Jacobs JM, Stine CB, Baya AM, Kent ML. 2009. A review of mycobacteriosis in
427 marine fish. J. Fish Dis. 32:119-130.

428 7. Toranzo AE, Magarinos B, Romalde JL. 2005. A review of the main bacterial fish
429 diseases in mariculture systems. Aquaculture 246:37-61.

430 8. Stahl DA, Urbance JW. 1990. The division between fast- and slow-growing species
431 corresponds to natural relationships among the mycobacteria. J. Bacteriol. 172:116-
432 124.

433 9. Falkinham JO. 1996. Epidemiology of infection by nontuberculous mycobacteria.
434 Clin. Microbiol. Rev. 9:177-215.

435 10. Prearo M, Zanoni RG, Dall'Orto BC, Pavoletti E, Florio D, Penati V, Ghittino C.
436 2004. Mycobacterioses: Emerging pathologies in aquarium fish. Vet. Res. Commun.
437 28:315-317.
438 11. Zanoni RG, Florio D, Fioravanti ML, Rossi M, Prearo M. 2008. Occurrence of
439 *Mycobacterium spp*. in ornamental fish in Italy. J. Fish Dis. 31:433-441.
440 12. Parikka M, Hammaren MM, Harjula S-KE, Halfpenny NJA, Oksanen KE, Lahtinen
441 MJ, Pajula ET, Iivanainen A, Pesu M, Ramet M. 2012. *Mycobacterium marinum*
442 causes a latent infection that can be reactivated by gamma irradiation in adult
443 zebrafish. PLoS pathogens 8:e1002944.
444 13. Noga EJ. 1996. Fish disease: diagnosis and treatment. Iowa State University Press.
445 14. Wagner D, Young LS. 2004. Nontuberculous mycobacterial infections: A clinical
446 review. Infection 32:257-270.
447 15. Durborow RM. 1999. Health and safety concerns in fisheries and aquaculture. Occup.
448 Med.-State Art Rev. 14:373-406.
449 16. Finkelstein R, Oren I. 2011. Soft tissue infections caused by marine bacterial
450 pathogens: epidemiology, diagnosis, and management. Current infectious disease
451 reports 13:470-477.
452 17. Lehane L, Rawlin GT. 2000. Topically acquired bacterial zoonoses from fish: a
453 review. Med. J. Aust. 173:256-259.
454 18. Passantino A, Macri D, Coluccio P, Foti F, Marino F. 2008. Importation of
455 mycobacteriosis with ornamental fish: Medico-legal implications. Travel medicine and
456 infectious disease 6:240-244.
457 19. Makinen J, Marjamaki M, Marttila H, Soini H. 2006. Evaluation of a novel strip test,
458 GenoType Mycobacterium CM/AS, for species identification of mycobacterial
459 cultures. Clin. Microbiol. Infect. 12:481-483.

460 20. Tortoli E, Mariottini A, Mazzarelli G. 2003. Evaluation of INNO-LiPA
461 MYCOBACTERIA v2: Improved reverse hybridization multiple DNA probe assay for
462 mycobacterial identification. J. Clin. Microbiol. 41:4418-4420.
463 21. Pourahmad F, Thompson KD, Taggart JB, Adams A, Richards RH. 2008. Evaluation
464 of the INNO-LiPA mycobacteria v2 assay for identification of aquatic mycobacteria.
465 J. Fish Dis. 31:931-940.
466 22. O'Mahony J, Hill C. 2002. A real time PCR assay for the detection and quantitation of
467 *Mycobacterium avium subsp paratuberculosis* using SYBR Green and the Light
468 Cycler. J. Microbiol. Methods 51:283-293.
469 23. Parashar D, Chauhan DS, Sharma VD, Katoch VM. 2006. Applications of real-time
470 PCR technology to mycobacterial research. Indian J. Med. Res. 124:385-398.
471 24. Castellanos E, Aranaz A, De Buck J. 2010. PCR amplification and high-resolution
472 melting curve analysis as a rapid diagnostic method for genotyping members of the
473 *Mycobacterium avium-intracellulare* complex. Clin. Microbiol. Infect. 16:1659-1662.
474 25. Ruskova L, Raclavsky V. 2011. The potential of high resolution melting analysis
475 (HRMA) to streamline, facilitate and enrich routine diagnostics in medical
476 microbiology. Biomed. Pap-Olomouc 155:239-252.
477 26. Šimenc J, Potočnik U. 2011. Rapid differentiation of bacterial species by high
478 resolution melting curve analysis. Prikladnaia biokhimiia i mikrobiologiia 47:283-290.
479 27. Reed GH, Kent JO, Wittwer CT. 2007. High-resolution DNA melting analysis for
480 simple and efficient molecular diagnostics. Pharmacogenomics 8:597-608.
481 28. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H,
482 Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG.
483 2007. Clustal W and clustal X version 2.0. Bioinformatics 23:2947-2948.

484 29. Bercovier H, Kafri O, Sela S. 1986. Mycobacteria possess a surprisingly small number
485 of ribosomal-RNA genes in relation to the size of their genome. Biochem. Biophys.
486 Res. Commun. 136:1136-1141.
487 30. Klappenbach JA, Saxman PR, Cole JR, Schmidt TM. 2001. rrndb: the Ribosomal
488 RNA Operon Copy Number Database. Nucleic Acids Res. 29:181-184.
489 31. Iwamoto T, Tani K, Nakamura K, Suzuki Y, Kitagawa M, Eguchi M, Nasu M. 2000.
490 Monitoring impact of in situ biostimulation treatment on groundwater bacterial
491 community by DGGE. FEMS Microbiol. Ecol. 32:129-141.
492 32. Menendez MC, Garcia MJ, Navarro MC, Gonzalez-y-Merchand JA, Rivera-Gutierrez
493 S, Garcia-Sanchez L, Cox RA. 2002. Characterization of an rRNA operon (rrnB) of
494 Mycobacterium fortuitum and other mycobacterial species: Implications for the
495 classification of mycobacteria. J. Bacteriol. 184:1078-1088.
496 33. Zerihun MA, Hjortaas MJ, Falk K, Colquhoun DJ. 2011. Immunohistochemical and
497 Taqman real-time PCR detection of mycobacterial infections in fish. J. Fish Dis.
498 34:235-246.
499 34. Salati F, Meloni M, Fenza A, Angelucci G, Colorni A, Orru G. 2010. A sensitive
500 FRET probe assay for the selective detection of *Mycobacterium marinum* in fish. J.
501 Fish Dis. 33:47-56.
502 35. Pakarinen J, Nieminen T, Tirkkonen T, Tsitko I, Ali-Vehmas T, Neubauer P,
503 Salkinoja-Salonen MS. 2007. Proliferation of mycobacteria in a piggery environment
504 revealed by mycobacterium-specific real-time quantitative PCR and 16S rRNA
505 sandwich hybridization. Vet. Microbiol. 120:105-112.
506 36. Kaattari IM, Rhodes MW, Kator H, Kaattari SL. 2005. Comparative analysis of
507 mycobacterial infections in wild striped bass *Morone saxatilis* from Chesapeake Bay.
508 Dis. Aquat. Org. 67:125-132.

509 37. Lescenko P, Matlova L, Dvorska L, Bartos M, Vavra O, Navratil S, Novotny L, Pavlik
510 I. 2003. Mycobacterial infection in aquarium fish. Vet Med-Czech 48:71-78.
511 38. Gauthier DT, Rhodes MW, Vogelbein WK, Kator H, Ottinger CA. 2003.
512 Experimental mycobacteriosis in striped bass *Morone saxatilis*. Dis. Aquat. Org.
513 54:105-117.
514 39. Yanong RPE, Pouder DB, Falkinham JO. 2010. Association of Mycobacteria in
515 Recirculating Aquaculture Systems and Mycobacterial Disease in Fish. J. Aquat.
516 Anim. Health 22:219-223.
517 40. Cheng JC, Huang CL, Lin CC, Chen CC, Chang YC, Chang SS, Tseng CP. 2006.
518 Rapid detection and identification of clinically important bacteria by high-resolution
519 melting analysis after broad-range ribosomal RNA real-time PCR. Clin. Chem.
520 52:1997-2004.
521 41. Odell ID, Cloud JL, Seipp M, Wittwer CT. 2005. Rapid species identification within
522 the *Mycobacterium chelonae-abscessus* group by high-resolution melting analysis of
523 hsp65 PCR products. Am. J. Clin. Pathol. 123:96-101.
524 42. Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J, Wittwer CT. 2003.
525 Amplicon melting analysis with labeled primers: A closed-tube method for
526 differentiating homozygotes and heterozygotes. Clin. Chem. 49:396-406.
527 43. El-Hajj HH, Marras SAE, Tyagi S, Shashkina E, Kamboj M, Kiehn TE, Glickman
528 MS, Kramer FR, Alland D. 2009. Use of Sloppy Molecular Beacon Probes for
529 Identification of Mycobacterial Species. J. Clin. Microbiol. 47:1190-1198.

530
531

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

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

533 [a] Central Veterinary Institute

534

535 Table 2. List of the analyzed fish tissue samples

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

536 [a] Concentration is expressed as genome equivalents g^{-1} tissue

537

538 Table 3. Reproducibility of the melting temperature measurement

Strain	Tm_1	Tm_2	Tm_3	Mean Tm ± SD
M. abscessus	86.12	86.07	86.09	86.10 ± 0.03
M. marinum	86.71	86.68	86.67	86.68 ± 0.02
M. chelonae	87.31	87.23	87.26	87.27 ± 0.04
M. haemophilum	87.20	87.14	87.16	87.16 ± 0.03
M. gordonae	88.21	88.20	88.17	88.19 ± 0.02
M. fortuitum ssp fortuitum	89.13	89.34	89.33	89.27 ± 0.12
M. gastri	89.46	89.39	89.46	89.44 ± 0.04
M. avium	89.05	88.87	88.88	88.94 ± 0.10
M. phlei	90.92	90.92	90.82	90.89 ± 0.06
M. smegmatis	90.12	90.11	90.10	90.11 ± 0.01
M. pseudoshottsii	89.97	89.97	89.97	89.97 ± 0.01
M. bohemicum	89.98	89.96	89.93	89.96± 0.03

539 Each measurement is the average Tm of 3 replicates

540
541

Legend to figures

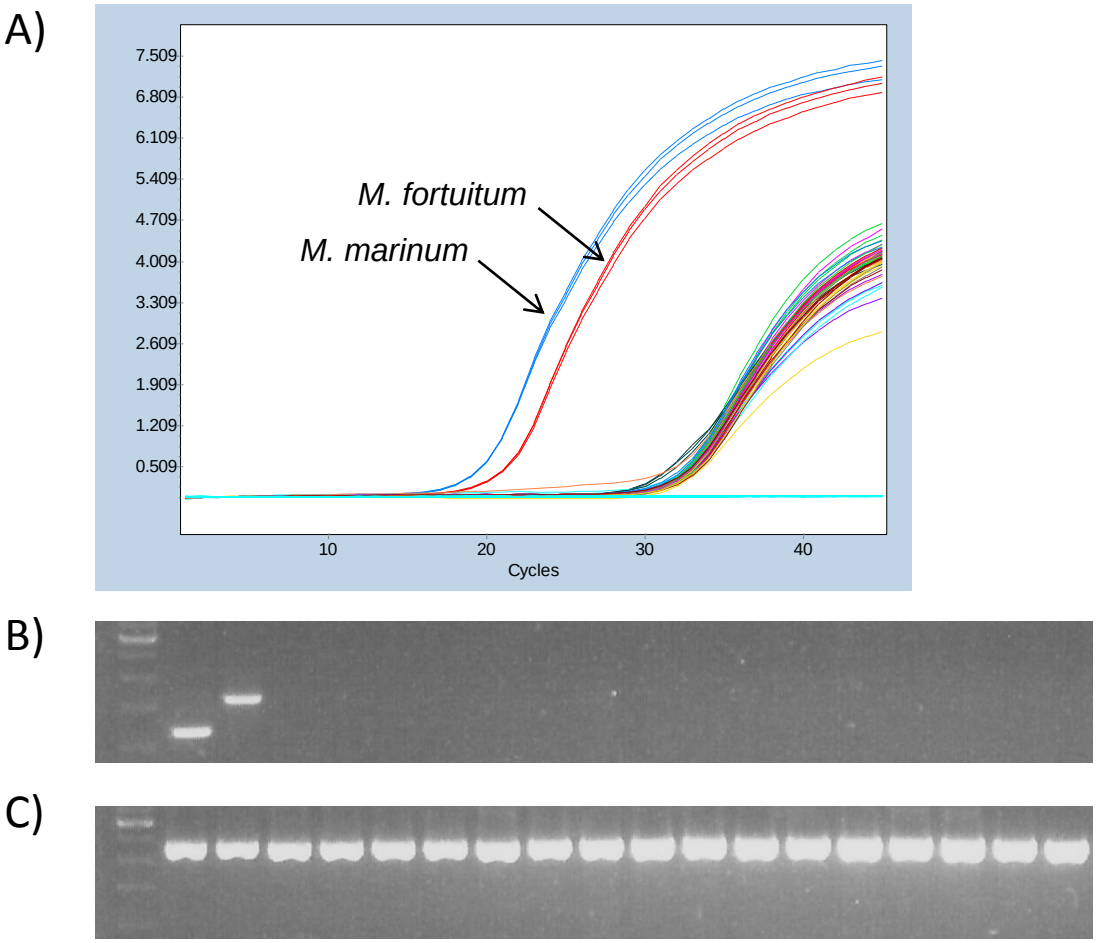
Figure 1: Specificity of the PCR-HRM assay. Amplification curves of the 16 non-mycobacterial species (~10^6 genome equivalents) are presented together with those of *M. marinum* and *M. fortuitum* at a 100-fold lower concentration (~10^4 genome equivalents) (A) and corresponding gel electrophoresis with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

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 H2O (A) and in 100 ng of fish DNA resuspended in Tris-EDTA (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 Tm. 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 Tm 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* (10^6 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 *marin*um, *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 Tm comparable
579 to those of *M. marinum* and *M. phlei*.



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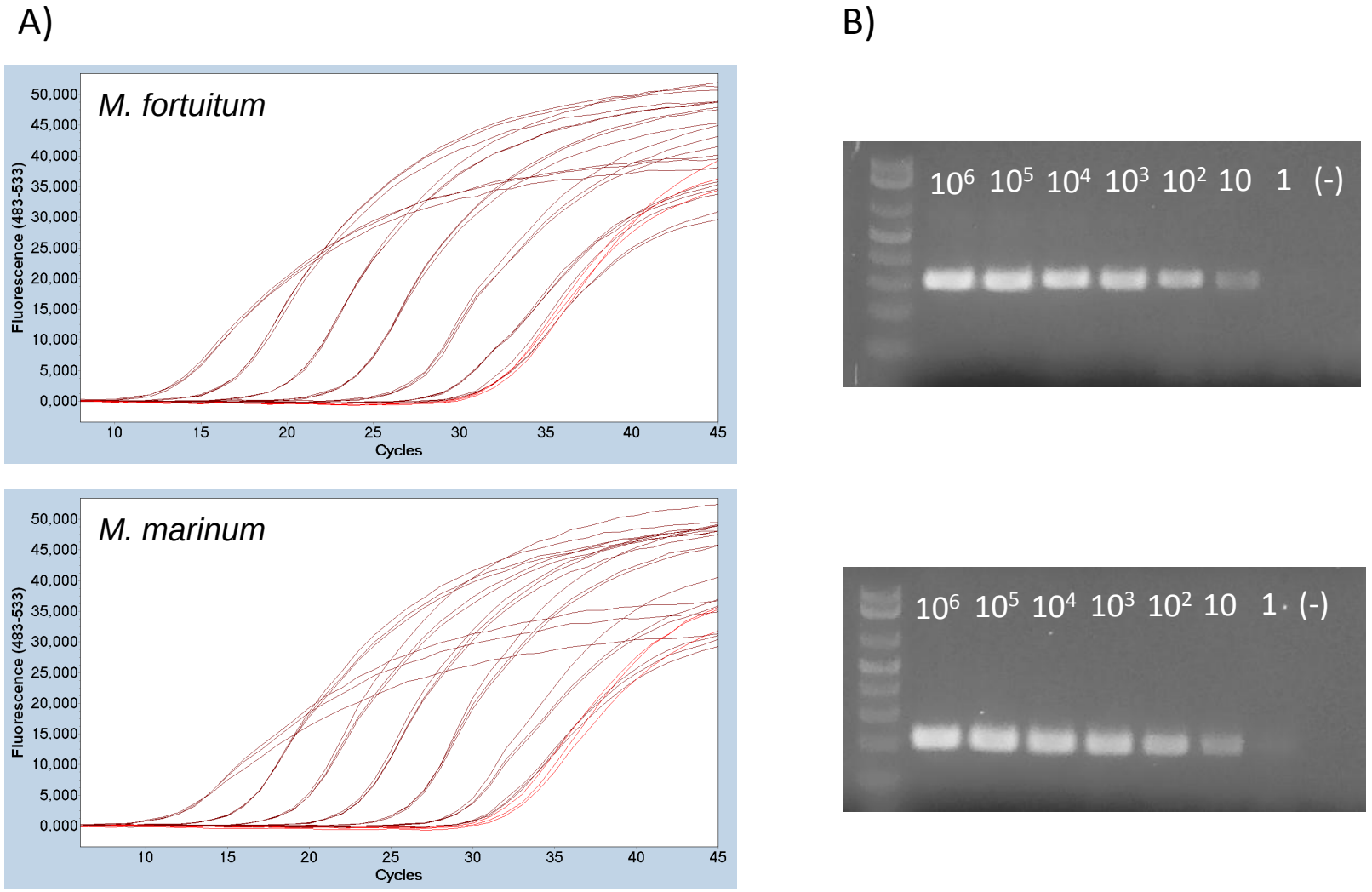

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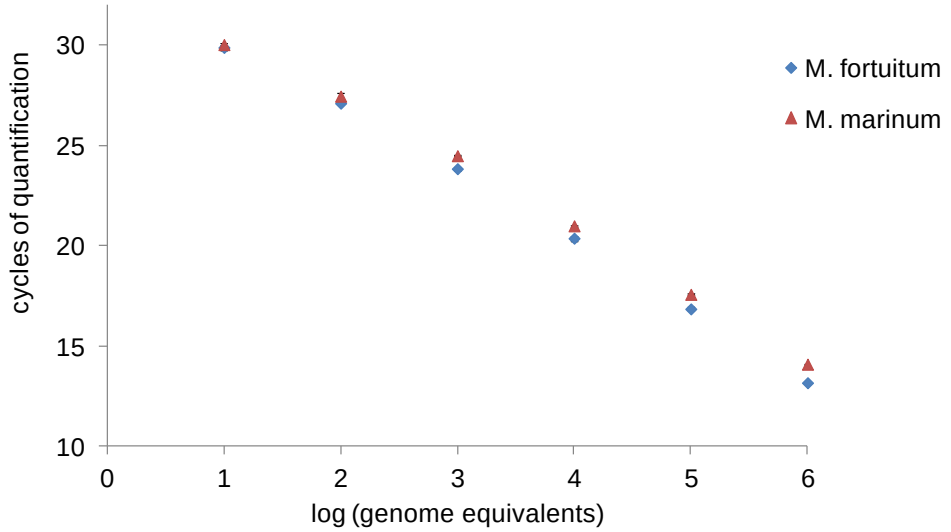
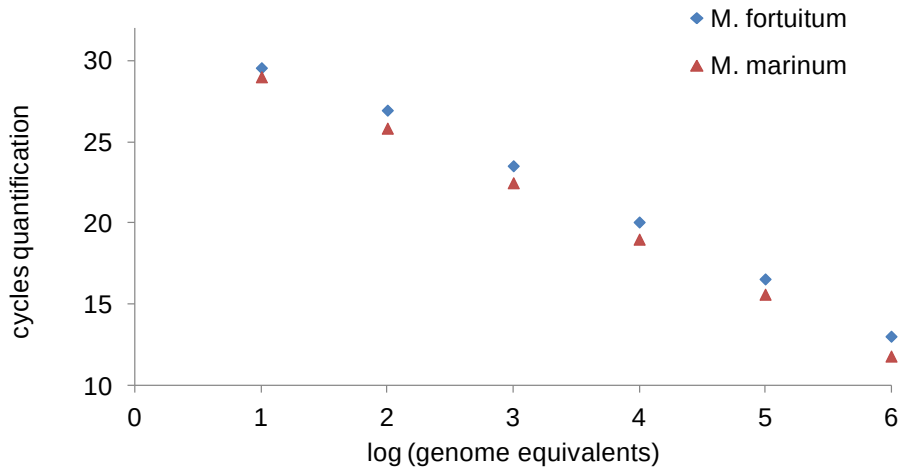
A)



B)



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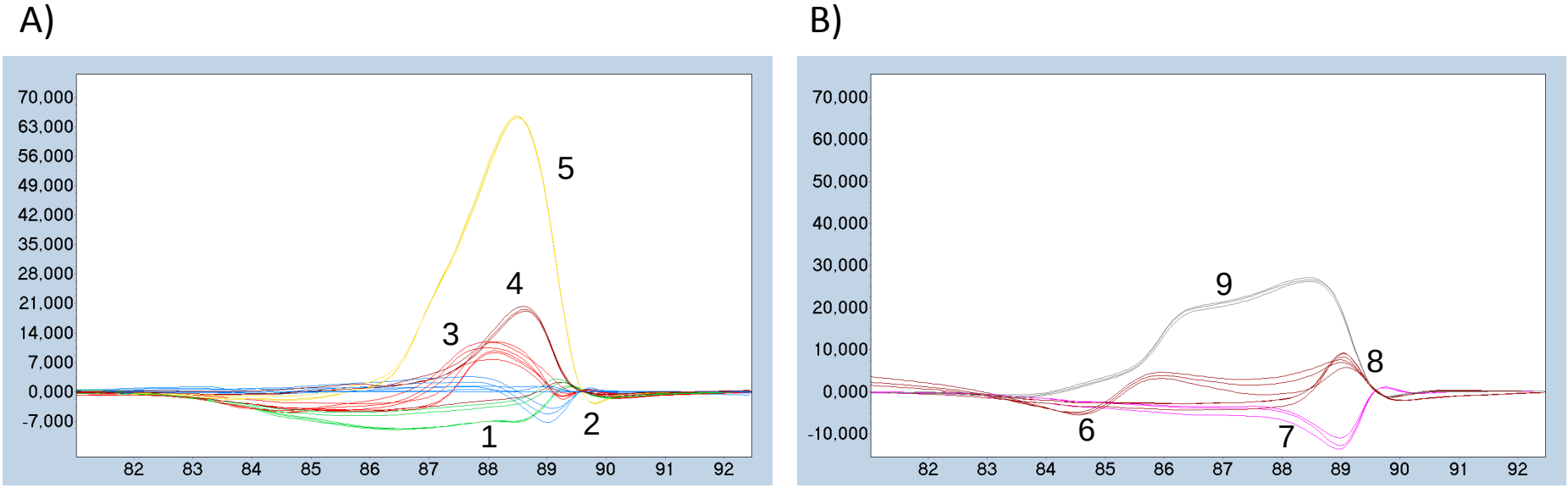

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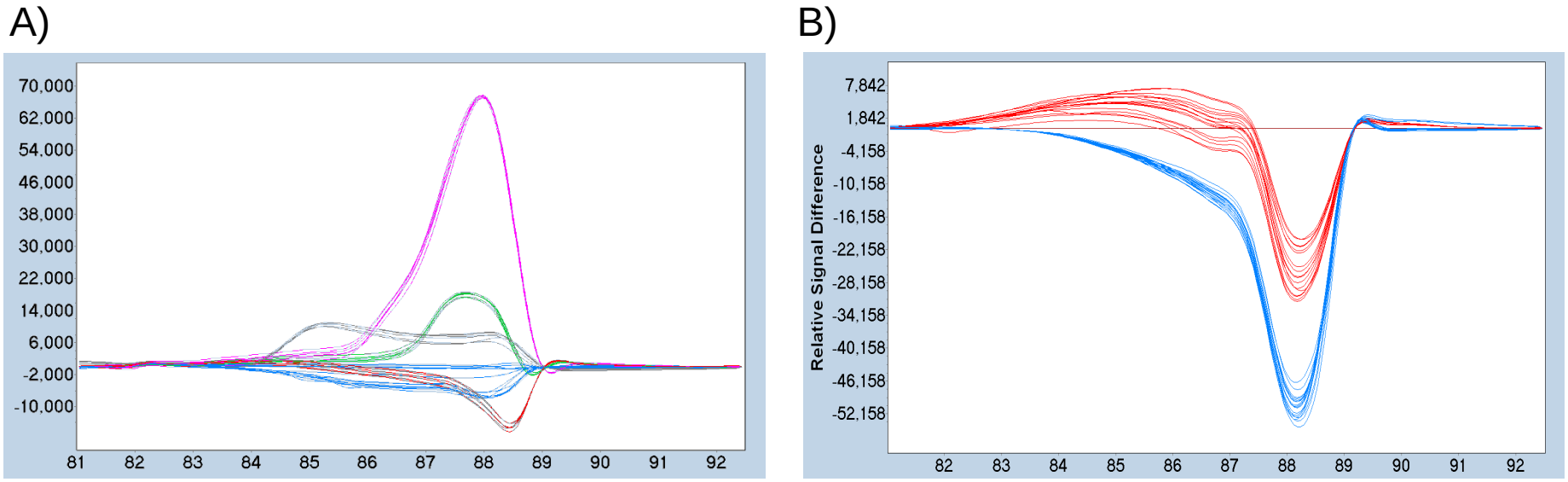

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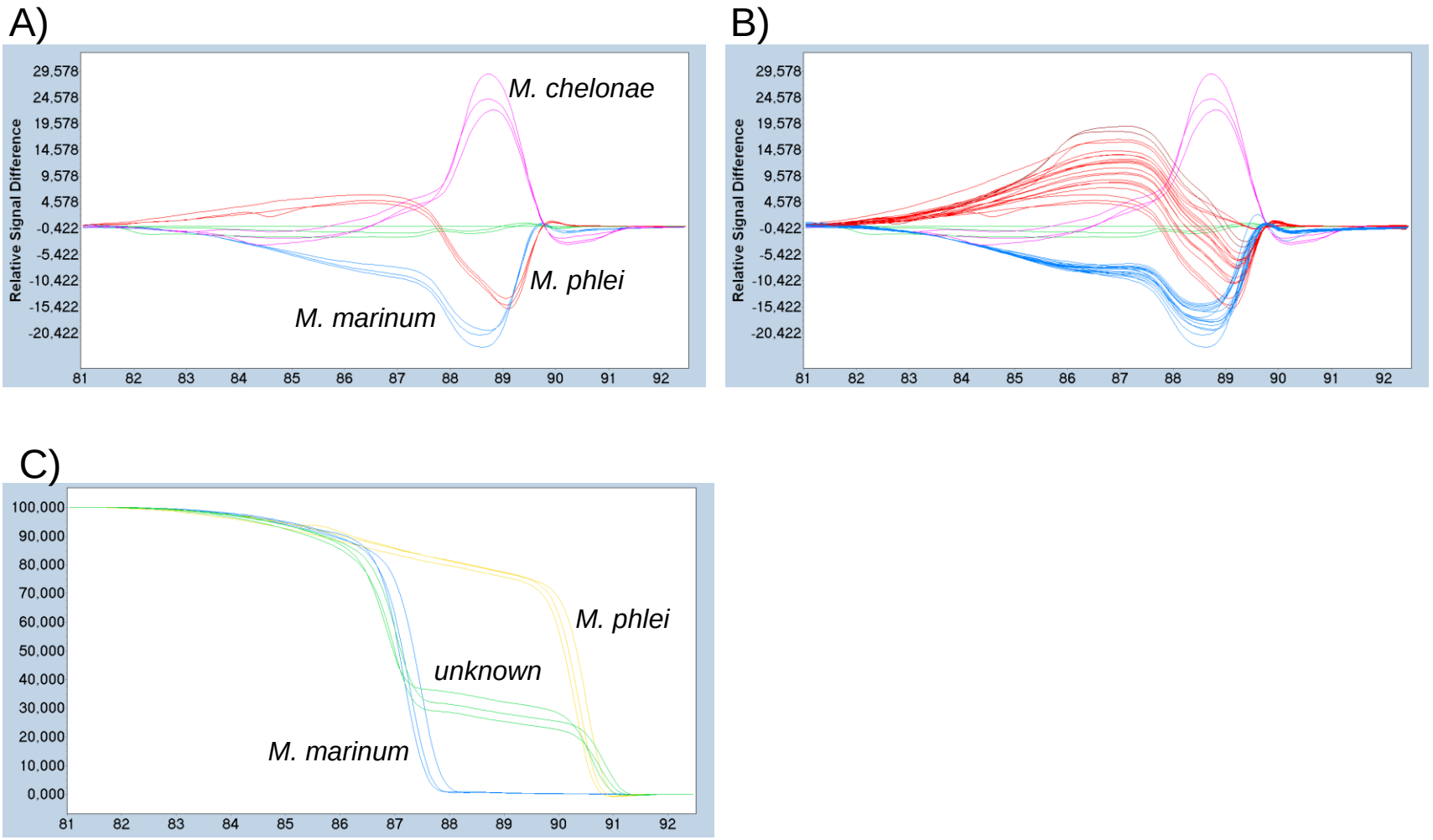

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