SEAFOOD TOMORROW

Nutritious, safe and sustainable seafood for consumers of tomorrow

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Optimized authenticity tools for seafood trade chain

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1. Summary

The authenticity of fish species is an important issue for both consumers, demanding authentic food and credible information in the moment of purchasing, and the seafood industry, concerned with the strong increment in seafood consumption and high rates of species substitution by fraudulent practice (Pardo et al., 2016). This is particularly relevant for species such as Atlantic and Pacific salmon, which are highly consumed nowadays. In fact, there are several bibliographic records of adulterations of both Atlantic and Pacific species with other species, most frequently of lower commercial value. This is the case of Atlantic salmon being substituted by rainbow trout and brown trout and the highly valued wild Pacific species substituted by farmed Atlantic salmon, particularly in certain seasons (Warner et al., 2015; Dalvin et al., 2010; Bénard-Capelle et al., 2015; Cutarelli et al., 2014), just to mention a few examples.

The European seafood industry needs more and more to cope with authenticity demands to have a competitive advantage and to provide a trustful image of the sector. For that, timely authenticity assessment using fast tools to unequivocal species identification is of paramount importance. Here we propose a fast and high throughput solution that relies on DNA barcode sequences for salmon species discrimination. The tool is based on the use of HRMA (High-Resolution Melting Analysis) of short amplified fragments from the main barcode genes used for fish identification, COI (Cytochrome oxidase c subunit I) and CytB (Cytochrome b) regions (Ward et al., 2005; Jiménez et al., 2007). The technique allows the discrimination of species accordingly to their melting profile and the subsequent identification when compared to profiles of standards. The further development and commercialization of the tool are expected to impact the industry as the trade chain can be more efficiently monitored, at a lower cost and in less time, enabling the delivery of higher quality and authentic products.

The tool optimization resulted in the design of two sets of primers as options for commercialization development. The majority of possible frauds in salmon species, in Europe, can be tested out using the primary primer of the final solution, since it can distinguish *S. salar* (Atlantic salmon), *O. mykiss* (rainbow trout) and *S. trutta* (brown trout), the last two species being the most frequent adulterants. Nevertheless, to achieve the initial objectives to discriminate 8 salmonid species a secondary set of primers was developed, which when used in combination with the primary set can discriminate all species with increased certainty. Thus, this solution not only covers the European market but also can be helpful globally, particularly in the North-American market, where *S. salar* is frequently the adulterant of the highly valued wild Pacific salmons.

2. Objective

The objective of the present deliverable is the development of a fast tool for high-throughput sequence-based identification of defined salmon species by using qPCR equipment, basic consumables and reagents. The tool essentially consists in the definition of two sets of primers and the optimal conditions/protocol for their use in the discrimination of the target species. Eight species were chosen according to their economic importance in the European and North-American markets, including consumers' preferences and demand. These are Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), chum salmon (*Oncorhynchus keta*), pink salmon (*Oncorhynchus gorbuscha)*, coho salmon (*Oncorhynchus kisutch)*, sockeye salmon (*Oncorhynchus nerka)* and chinook salmon (*Oncorhynchus tshawytscha)*. The developed primers, as well as the optimized protocol, can be used by SME partners to further develop a qPCR kit intended for commercialization.

3. Background

The fraudulent substitution of species in fish-based products exerts detrimental effects to the seafood industry, as it puts consumers at risk when purchasing potentially harmful and mislabeled products. It also affects the image of the sector and the economic activity, and reduces the effectiveness of conservation and management programs. For these reasons, the development of robust fish authentication methods are of paramount importance. Modern methods for fish species identification are based on the polymorphism detection either in constituent proteins or deoxyribonucleic acid (DNA), molecules that are unique to each species and possible to analyze even in samples with no detectable morphological features (Rasmussen & Morrissey, 2008). DNA-based methods are by far the most used mainly due to their high specificity and accuracy of results but also due to their applicability to target molecules even in highly processed food. Several methodologies have been developed by the scientific community and private companies using either nuclear or mitochondrial DNA. The most important ones are the forensically informative nucleotide sequencing (FINS), restriction fragment length polymorphism (RFLP), single-stranded conformational polymorphism (SSCP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and the loop mediated isothermal amplification (LAMP) assay (Aranishi et al., 2005, Asensio Gil, 2007, Clark, 2015, Rasmussen and Morrissey, 2008, Saull et al., 2016, Tomás et al., 2017).

Notwithstanding the efficacy and widespread use of sequencing methods, there is a strong need to use and make commercially available faster methods enabling the implementation of timely and efficient traceability schemes for monitoring purposes. Current DNA methods are time-consuming, and DNA is often found fragmented in very short pieces inhibiting the characterization by sequencing big fragments. So far, fast screening methods for fish identification using DNA are restricted to PCR-RFLP based kits (e.g. Agilent DNA Fish ID Ensemble; Qiagen Fish QIAxcel Advanced) and species specific qPCR assay (e.g. PCR Max fish speciation kit, R-Bioopharm SureFood Fish). These, however, require the use of expensive reagents, are usually of low throughput and applied to only one or a few species.

SEAFOOD^TOMORROW proposes a High Resolution Melting Analysis (HRMA) tool for Atlantic and Pacific salmon authentication based on a fast screening method for codfish authentication previously developed by ICETA (Tomás et al, 2017). HRMA is a powerful technique that enables the simultaneous analysis of a high number of samples in 96 well format, in a short time (1 hour, plus DNA extraction), targeting very short DNA regions (ca 100 bp or less) and at a low cost (less than €1/sample after extraction). The technique involves amplification of the target of interest in the presence of a saturation dye by qPCR and subsequent melting of the amplicons by gradually increasing the temperature. Considering that the melting profile depends on the sequence, length of the amplicon and strand complementarity, the technique is highly suitable for the detection of single-base variants in short DNA fragments (Druml et al., 2014).

4. Experimental design

4.1. Sampling, DNA extraction and PCR optimization

4.1.1. Reference samples acquisition

Whole specimens from Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) were acquired in local markets from farmed species. The brown trout (*Salmo trutta*) was kindly provided by ICNF (Instituto da Conservação da Natureza e Florestas) from nurseries located at Serra do Marão, Vila Real, Portugal. Pacific species (*O. keta, O. gorbuscha, O. kisutch, O. nerka, and O. tshawytscha*) were also gently provided by Fisheries and Oceans Canada, West Vancouver, Canada. Three specimens from each species were obtained.

4.1.2. DNA extraction and quantification

Muscle tissue from all samples was submitted to DNA extraction using the NucleoSpin® Tissue kit (Macherey-Nagel) according to the manufacturer's protocol. DNA purification was performed with GRS PCR & Gel Band Purification kit (Grisp). Following purification, the DNA concentration was determined using the Gen5TM Software and BioTek microplate readers by measuring the absorbance of the extracts at 260, 280 and 320 nm in 2 µl volume against a blank of ultrapure water. All samples were diluted to 2.5 ng/µl with ultrapure water.

4.1.3. Sanger sequencing of the barcode regions and sequence data collection

Barcode regions from COI and CytB genes (Ward et al., 2005; Jiménez et al., 2007) were amplified using primers FishF1 (TCA ACC AAC CAC AAA GAC ATT GGC AC) / FishR1 (TAG ACT TCT GGG TGG CCA AAG AAT CA) and FishCytB-F (ACC ACC GTT GTT ATT CAA CTA CAA GAA C) / cytBI-5R (GGT CTT TGT AGG AGA AGT ATG GGT GGA A), respectively. Sanger sequencing was performed at Eurofins Genomics. Sequences,

morphological vouchers and all metadata were uploaded to the SEAFOOD^{TOMORROW} database. Additionally, in order to cover all the known intraspecific sequence variation, available public sequences of the different salmon species for CytB and COI regions were downloaded from Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and BOLD Data systems (http://www.boldsystems.org/index.php), respectively. Representative sequences of the intraspecific variation were selected through phylogenetic analysis in order to reduce the number of entries to a minimal for further analysis.

4.1.4. Primer design for HRMA

Different approaches were theoretically planned to achieve the discrimination of the eight species with the minimal number of primer sets/reactions possible: (i) discrimination in a single step (using a unique reaction with one primer pair); (ii) discrimination in two-steps (using two sets of primers and the distribution of species in three different groups in each step in such a way to permit the discrimination of all species); (iii) and a three-step approach consisting in a first separation of the most relevant species and the further discrimination of the remaining species in the other two steps, as needed.

Using the representative reference sequences of each species, two different strategies were used to obtain the primer sequences: a free web-based tool (DECIPHER) and a manual design by comparing the aligned sequences. The DECIPHER software (http://www2.decipher.codes) (Wright et al., 2016) uses a file with unaligned DNA sequences distributed in groups corresponding to the desired output of group assignment after HRMA. Retrieved PCR primers will amplify all groups, by annealing to conserved regions, however, the inner sequence of the amplified fragment provides group differentiation. Based on this software, we tested *in silico* the different possible combinations, and selected the most promising ones relying on the software outputs: overall score, coverage, similar or missing signatures, the total of amplified sequences, and the percentage of amplification of each combination to select the options with the highest performance. For the manual primer design - the visual search of variable and conserved regions in alignments- sequences of other fish species were also included in the alignment to permit the identification of the most specific regions targeting our species. Manual primer design was supported by Primer3 software (http://bioinfo.ut.ee/primer3/) (Untergasser et al., 2012). In either case, the objective was the design of primers for the amplification of small fragments (50-200 bp). The oligonucleotide melting temperature, size, GC content, primer-dimer formation, and PCR product size were taken into account to select the most promising primers. Primer properties were checked using OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html). Moreover, the specificity of the primers was also tested *in silico* using Primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

After thoroughly studying all outputs considering the three scenarios for primer design the two-step approach revealed the most promising option to distinguish the eight salmon species. Theoretically, 280 different combinations of species assignment to groups (two groups with three species and one group with two species) were found possible, however the design of primers was not viable for many of the group combinations and, simultaneously, it was not feasible to manually test all possibilities since the

software does not automatically permit such approach. In this scenario, our option was to test the combinations that most probably will result in the possible design of primers based on species phylogeny. Finally, a shortlist of the most promising primers was achieved for each genomic regions CytB and COI. All primers were then synthesized at Eurofins Genomics in 10 nmol scales and at desalted purity.

4.1.5. qPCR and HRMA optimization

The qPCR and melting curves (HRMA) optimization was carried out for the primers previously selected, and involved three sequential phases:

1) Phase I: qPCR cycling conditions were established following the manufacturer instructions of the fluorophore mix (SsoFast Evagreen Supermix, Bio-Rad, USA) and using the optimal annealing temperature obtained by *in silico* design. Template DNA consisted of the three replicates of each of the eight species, in a total of 24 samples, using 10 ng of DNA in each reaction (see appendix for detailed qPCR protocol). The following default conditions (Figure 1) were used for HMRA on Precision Melt AnalysisTM V 1.0 Software from Bio-Rad. The default settings were kept in order to develop the tool with as less human interference as possible to increase the method robustness. Parameters used in primer selection were the amplification efficiency, discriminant power, primers compatibility in terms of annealing temperature for simultaneous amplification and specificity to salmonids.

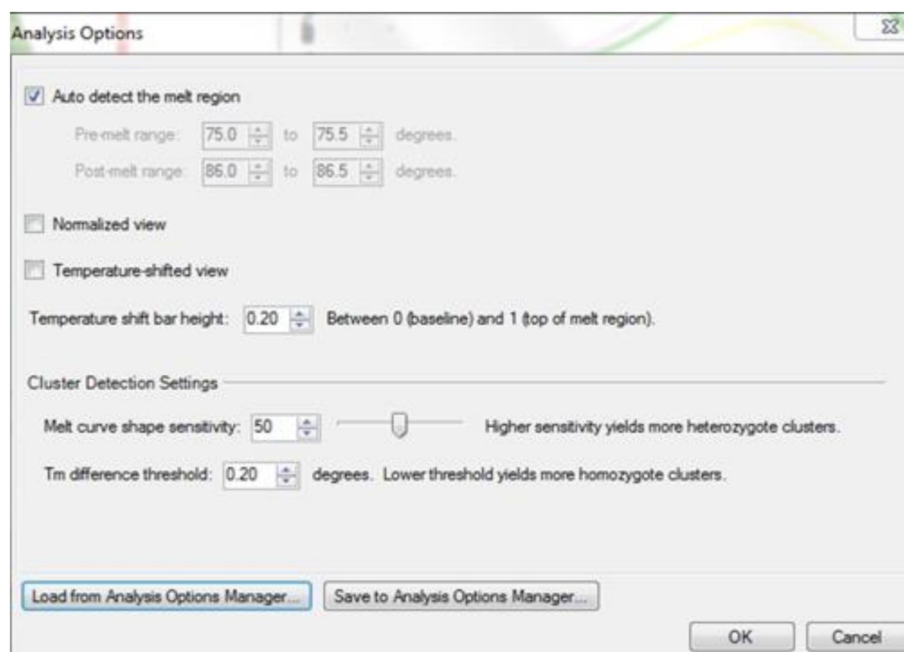

Figure 1. Default software conditions of HMRA.

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2) Phase II: Selected primers from phase I were carried-over to phase II, and some modifications were performed on PCR conditions such as annealing temperature and number of cycles to increase specificity and species discrimination. The amplification efficiency was recorded by lowering or increasing annealing temperature in two degrees steps. This information was essential to further optimize the primer combinations to use in subsequent steps. Moreover, in this phase, the specificity of the PCR was evaluated by amplifying DNA extracts from other fish species from the SEAFOOD^TOMORROW database, using the same conditions.

3) Phase III: Two of the most efficient primer pairs were selected and some modifications on the qPCR program (annealing temperature) were defined in order to enable the simultaneous amplification of both primers pairs. This final optimization resulted in the proposed final solution.

The optimized final protocol for salmonid identification using the proposed HRMA tool is described in annex 1. Primer sequences were omitted from this public deliverable as this information is subjected to intellectual protection.

5. Results and Discussion

5.1. Species sequencing and data collection

A total of 24 specimens of the eight salmonid species were acquired. A total of 2194 sequences, 1514 from the COI region (BOLD) and 652 sequences of CytB (GenBank) were gathered and aligned. Table 1 specifies the number of sequences that were obtained for each species and *loci*. Sequences were aligned and similar ones were excluded after phylogenetic analysis in order to enable a faster data treatment, but keeping the intraspecific variability represented.

Table 1. The number of sequences downloaded from GenBank and BOLD databases for each of the eight species under study.

Salmonid species	Number of COI sequences (from BOLD System)	Number of CytB sequences (from GenBank)
Salmo salar	218	28
Oncorhynchus mykiss	317	62
Oncorhynchus keta	138	73
Oncorhynchus tshawytscha	255	16
Oncorhynchus nerka	94	41
Oncorhynchus kisutch	169	30
Oncorhynchus gorbuscha	93	48
Salmo trutta	188	303
Total	**1514**	**652**

5.2. DNA extraction and quantification

It was possible to obtain DNA extracts from all samples using the kit in the conditions indicated by the manufacturer. The average DNA concentration obtained was 83.3 µg/ml and the average purity as determined by the ratio of absorbance at 260/280 nm was 2.1.

5.3. Primers selection

The qPCR and HRMA optimization was performed using the 25 most promising primers previously selected in phase I, 15 for the CytB region and 10 for the COI region (Table 2).

Table 2. Shortlist of pair primers obtained after selection in phase I.

Primer name (CytB region)	Primer name (COI region)
SFT_CYT_O3#2.1_f/r	SFT_COI_O3#2.1_f/r
SFT_CYT_O7#1.1_f/r	SFT_COI_O3#4.1_f/r
SFT_CYT_O7#1.2_f/r	SFT_COI_O7#1.1_f/r
SFT_CYT_MAN1_f/r	SFT_COI_MAN2_f/r
SFT_CYT_MAN2_f/r	SFT_COI_MAN7_f/r
SFT_CYT_MAN4_f/r	SFT_COI_MAN1_f/SFT_COI_MAN7_r
SFT_CYT_MAN1_f/SFT_CYT_MAN2_r	SFT_COI_MAN2_f/SFT_COI_O3#4.1_r
SFT_CYT_MAN1_f/SFT_CYT_O3#2.1_r	SFT_COI_MAN4_f/SFT_COI_MAN2_r
SFT_CYT_MAN1_f/ SFT_CYT_O3#2.1_r	SFT_COI_MAN7_f/SFT_COI_O3#4.1_r
SFT_CYT_MAN4_f/SFT_CYT_O7#1.2_r	
SFT_CYT_MAN4_f/ SFT_CYT_MAN5_r	
SFT_CYT_O3#1.1_f/ SFT_CYT_MAN3_r	
SFT_CYT_O3#2.1_f/ SFT_CYT_MAN2_r	
SFT_CYT_O7#1.1_f/ SFT_CYT_MAN3_r	
SFT_CYT_O7#1.2_f/SFT_CYT_MAN5_r	

During primer optimization in the subsequent phases II and III, primer pairs were selected to n=8 and n=2, respectively. The tool was optimized to a final solution comprising a primary primer pair, targeting the CytB region, and a secondary primer, targeting the COI region. The primary primer pair was the SFT_CYT_MAN1_f/r which amplifies a fragment of 116 bp from the CytB region, while the secondary primer pair was the SFT_COI_MAN1_f/SFT_COI_MAN7_r amplifying a 72 bp fragment from COI. The following table describes the main properties of the primers (Table 3).

Table 3. Oligonucleotide proposed for the tool and their main properties.

Primer name	Length	Tm (ºC)	GC-content	Product length
SFT_CYT_MAN1_f	21	54	38.10 %	116
SFT_CYT_MAN1_r	18	53.7	50 %	116
SFT_COI_MAN1_f	17	55.2	58.8 %	72
SFT_COI_MAN7_r	20	50.1	32.5 %	72

5.4. HRMA optimization and proposed identification scheme

HRMA optimization resulted in the selection of two sets of primers, as stated, targeting the two genetic regions COI and CytB, which showed the higher discrimination power in terms of species identification and, simultaneously, higher robustness in group assignment. Figure 2 presents the HRMA results obtained with the primary primer set SFT_CYT_MAN1_f/r in what concerns the difference curve melting profile and the corresponding automatic output group assignment.

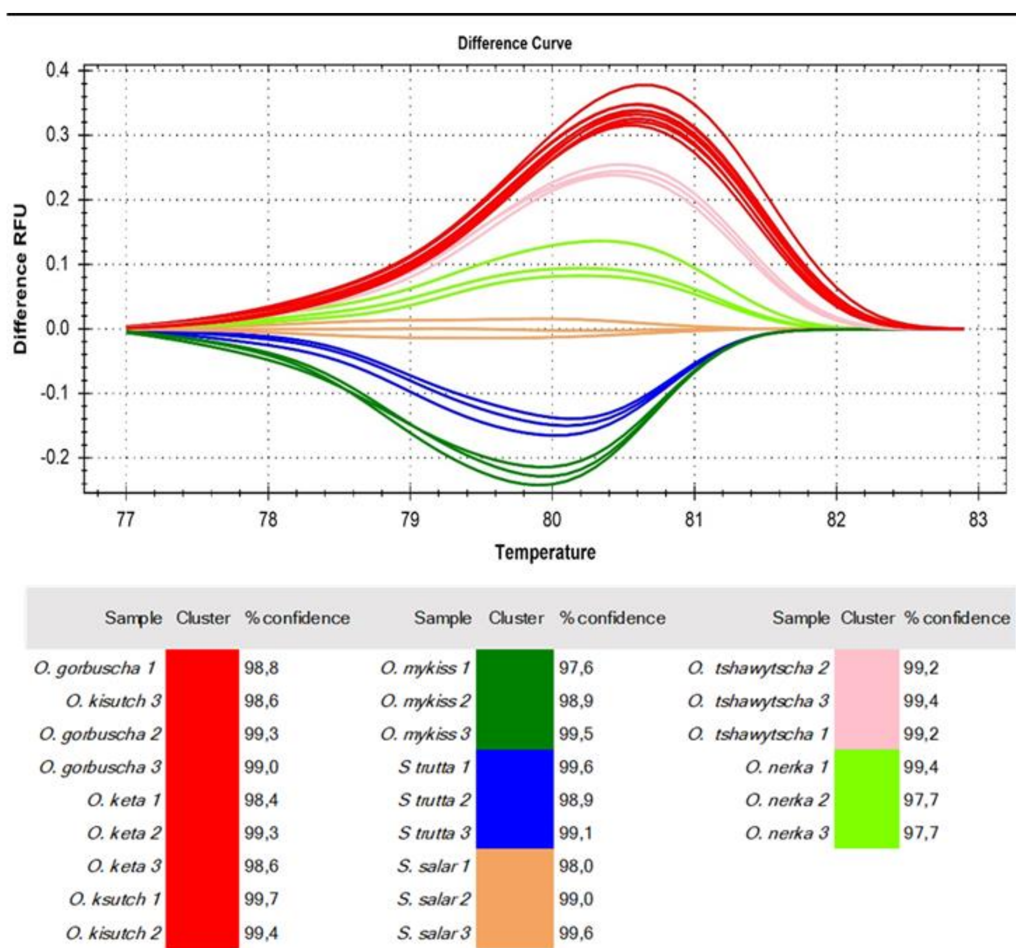
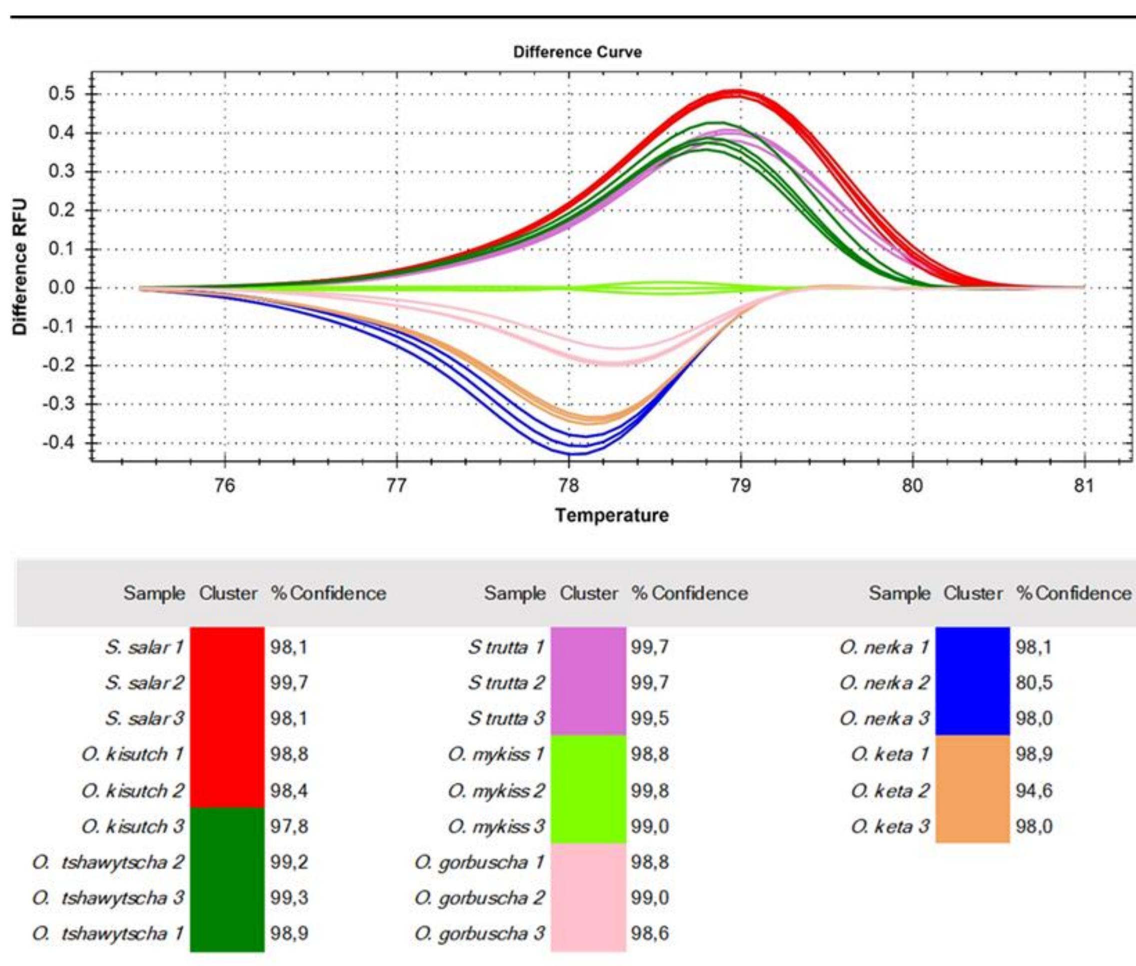


Figure 2. Difference melting curves of the amplified 118 bp fragments with the primer pair SFT_CYT_MAN1_f/r. Curves of the same color were automatically grouped in the same cluster. Percent confidence of samples group assignment are indicated.

With this primer set, the discrimination of the eight species in the study was not complete since *O. keta, O. gorbuscha* and *O. kisutch* were assigned into the same cluster (in red). Therefore, there was a need to optimize a secondary set of primers for complete discrimination (described below). Notwithstanding, the proposed primary set enables the identification of the species *S. salar, S. trutta* and *O. mykiss*, which will account for the majority of the market need in species identification. Most described authenticity issues are related to the substitution of *S. salar* (Atlantic salmon) by *O. mykiss* (rainbow trout) and to a lesser degree by *S. trutta* (brown trout), both situations can be clearly resolved by the described primary primer set.

A secondary set of primers was then selected from the previously identified regions. Results are presented in figure 3.



Sample	Cluster	% Confidence	Sample	Cluster	% Confidence	Sample	Cluster	% Confidence
S. salar 1		98,1	*S trutta 1*		99,7	*O. nerka 1*		98,1
S. salar 2		99,7	*S trutta 2*		99,7	*O. nerka 2*		80,5
S. salar 3		98,1	*S trutta 3*		99,5	*O. nerka 3*		98,0
O. kisutch 1		98,8	*O. mykiss 1*		98,8	*O. keta 1*		98,9
O. kisutch 2		98,4	*O. mykiss 2*		99,8	*O. keta 2*		94,6
O. kisutch 3		97,8	*O. mykiss 3*		99,0	*O. keta 3*		98,0
O. tshawytscha 2		99,2	*O. gorbuscha 1*		98,8			
O. tshawytscha 3		99,3	*O. gorbuscha 2*		99,0			
O. tshawytscha 1		98,9	*O. gorbuscha 3*		98,6			

Figure 3. Difference melting curves of the amplified 72 bp fragments with the primer pair SFT_COI_MAN1_f/MAN7_r. Curves of the same color were automatically grouped in the same cluster. Percent confidence of samples group assignment is indicated.

This time, the COI region was targeted in order to increase the robustness of the tool now amplifying two distinct genetic regions. This secondary set of primers can be used for the identification of samples

from the species *O. keta, O. gorbuscha,* and *O. kisutch* (not discriminated with the primary primers) and also as a confirmation of the identification resulted from the primary set. Although most species can be discriminated there was some group interchange between the species *S. salar, O. kisutch* and *O. tshawytscha* and for this reason we decided to consider all three species as belonging to the same group to increase the robustness of the method without losing its discriminatory power. The same applies to the species *O. keta* and *O. nerka*, included in the same group, as the melting profiles were quite similar although the software could discriminate them. The overall scheme for the identification of salmonids is shown in table 4.

Table 4. Distribution of species within groups discriminated using both primers and proposed identification scheme.

Group (6) CytB primary primer	Group (4) COI secondary primer	Identification
*S. salar**	*S. salar - O. kisutch - O. tshawytscha - S. trutta*	***S. salar***
*O. mykiss**	*O. mykiss*	***O. mykiss***
*S. trutta**	*S. salar - O. kisutch - O. tshawytscha - S. trutta*	***S. trutta***
*O. nerka**	*O. nerka - O. keta*	***O. nerka***
*O. tshawytscha**	*S. salar - O. kisutch - O. tshawytscha - S. trutta*	***O. tshawytscha***
O. gorbuscha - O. keta - O. kisutch	*O. gorbuscha*	***O. gorbuscha***
O. gorbuscha - O. keta - O. kisutch	*O. nerka - O. keta*	***O. keta***

* These species can be identified using only the primary primer.

The proposed tool can be used by analyzing only the results of the primary primer pair in which the identification of five species marked with * is achieved or by running the samples for both primary and secondary regions, in which case all species can be identified with higher robustness and confirmatory identification, despite reducing the number of samples per qPCR run. Notwithstanding, the total time of the assay (including the melting analysis) was 61 minutes which enables the analysis of several batches of samples per day.

The assay specificity was tested using other fish species included in the consortium database. Figure 4 presents the results of the amplification of salmonids, as well as other species (*Engraulis encrasicolus, Sparus aurata, Solea solea, Scomber scombrus, Scomber colias,* and *Sardina pilchardus*).

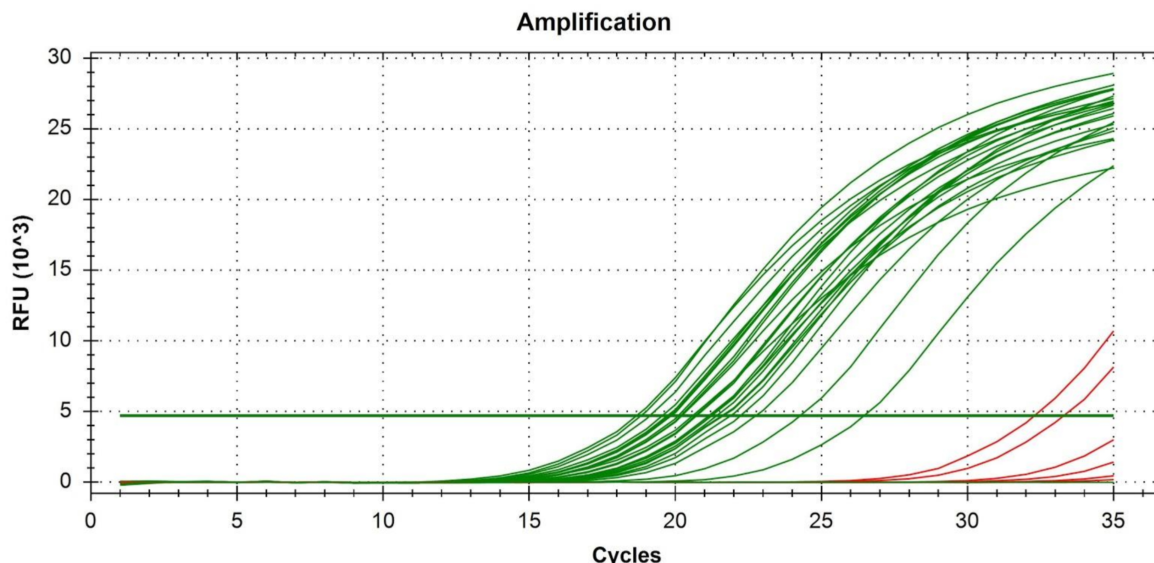


Figure 4. Amplification curves of the salmonids tested (green) and the other species used for specificity testing (red curves), using primers SFT_CYT_MAN1_f/r.

As it can be verified, the primer was overall specific, with amplification curves for all salmonid samples arising above the threshold well before 30 cycles and before 25 for most species. Some non-salmonid species however presented an amplification signal after cycle 30, but these can be excluded before the subsequent melting analysis simply by applying a cut-off Ct (cycle threshold) value. It should also be highlighted that the probability of adulteration of the eight targeted species with non-salmonid species or rare and unused salmonid species is very low since it is not interesting from an economic point of view and since the species fillets are morphologically very different, making the adulteration or substitution easily detectable by eye.

Specificity tests were also performed for the secondary primer set SFT_COI_MAN1_f/MAN7_r (Figure 5).

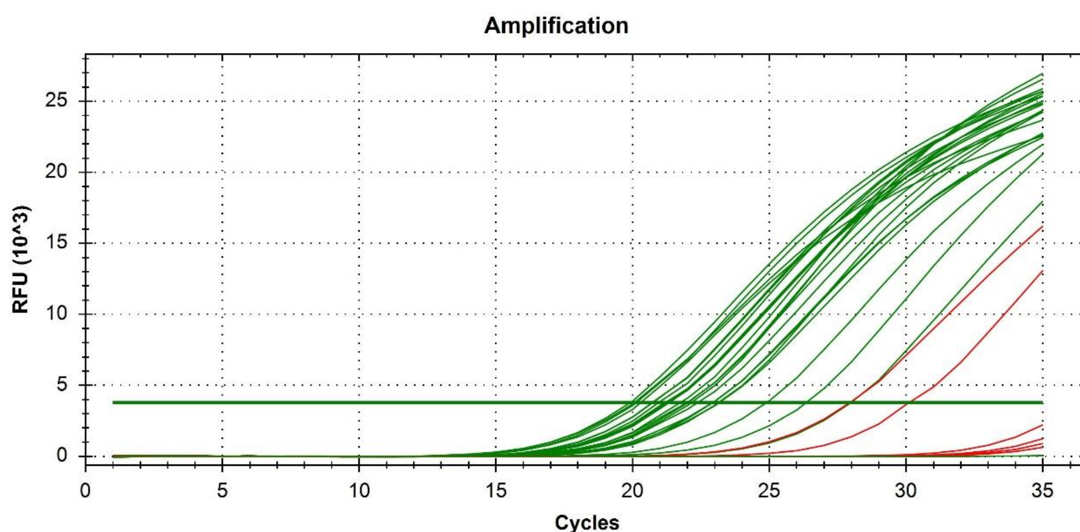


Figure 5. Amplification curves of the salmonids tested (green) and the other species used for specificity testing (red curves), using primers SFT_COI_MAN1_f/MAN7_r.

In this case, amplification curves for all salmonid samples arose above the threshold well before 30 cycles, and before 25 for most of the species, similarly to the primary primers. Some non-salmonid species also showed an amplification signal before cycle 25. However, these samples can simply be excluded by the primary primer set since they did not amplify before cycle 30 (Figure 4).

6. Conclusions

A demonstration of an optimized protocol for the simultaneous identification of eight salmonid species is presented using a fast qPCR/HRMA approach, which can run in 61 minutes after DNA extraction and qPCR reaction preparation. A protocol and identification scheme is proposed based on the amplification of two short fragments from the CytB and COI genes. An identification scheme is proposed based on the use of a single primer pair, with which the pacific species *O. gorbuscha*, *O. keta*, *O. kisutch* cannot be discriminated, or using two primer pairs, enabling the complete discrimination of the eight species with increased robustness and certainty.

The optimized tool is now ready to be studied for its stability using different qPCR equipment, different enzyme/fluorophore mixes, reproducibility in different laboratories (ICETA and ILVO), applicability in smoked samples, and use of artificial DNA standards, in order to further advance to a final commercial solution by consortium SME partners.

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8. Annex 1 - proposed final protocol

Protocol for salmonids identification using HRMA

1. Extract DNA from a representative piece of muscle tissue, preferably using a kit adapted to food matrices, and quantify it by UV at 260 nm. Evaluate the extract purity by determining the absorbance ratio at 260/280 nm. Exclude samples whose purity ratios are less than 1.8.

2. Dilute samples to 2.5 ng/µl, if necessary (all samples must have the same DNA concentration).

3. Prepare the PCR reaction cocktail by assembling all required components except the sample template. Reactions are run on a final volume of 10 µL. Adaptations to PCR mix specificity used should be considered. The example table presented is for the enzyme SsoFast EvaGreen Supermix from Bio-Rad.

qPCR mix components	Final Concentration	Volume per reaction
Supermix (2x)	1x	5 µl
Forward primer (10 µM)	0.4 µM	0.4 µl
Reverse primer (10 µM)	0.4 µM	0.4 µl
DNA template (2.5 ng/µl)	0.1 ng/µl	4 µl
Water		0.2 µl
Total volume per reaction		**10 µl**

4. Dispense 6 µl aliquots of PCR reaction cocktail into each reaction tube.

5. Add the sample template to each reaction tube (4 µl).

6. Centrifuge the reaction tubes and load to qPCR block.

7. Program cycling conditions for qPCR and HMR analysis accordingly to the table presented below. Initial enzyme activation times as well as recommended annealing/extension may vary, please adapt to the used mix.

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	Cycling step	Temperature	Time
1	Enzyme activation	98°C	2 min
2	Denaturation	98°C	5 sec
3	Annealing/extension	56ºC*	5 sec
4	Go to step 2, 34 more times		
5	Pre melt	98°C	1 min
6		60°C	1 min
8	Melt curve	65.0 to 95.0°C (increment 0.5°C)	5 sec
10	END		

*For both primer pairs: SFT_COI_MAN1_f/r, and SFT_COI_MAN1_f/SFT_COI_MAN7_r.

8. Analyze data using the software provided by the equipment in the HRM mode to obtain difference curve graphs. Take into consideration the threshold cycle amplifications for unspecific amplifications exclusion. Some optimization of the temperature windows can be necessary to increase the discriminatory power, depending on the specific software capabilities, keep the default setting as long as possible. Three replicates of each of the eight species should be used as standards. Apply the identification scheme provided in table 4 of the main document.