

PCR-RFLP Method to Identify Salmonid Species of Economic Importance

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Abstract

The identification of different fish species by molecular methods has become necessary to avoid both the incorrect labelling of individuals involved in repopulation programmes and the commercial frauds on the fish market. Different fish species of great economical importance, like the salmonids, which are very much requested for their meat, can be identified using molecular techniques such as PCR-RFLP. The method is based on the amplification of a target region from the genome by PCR reaction followed by endonucleases digestion to detect the polymorphism of restriction fragments. In our study we analysed the following salmonid species from Romania: *Salmo trutta fario*, *Salmo labrax*, *Salvelinus fontinalis*, *Onchorhynchus mykiss*, *Thymallus thymallus* and *Hucho hucho*. In order to discriminate between the analysed species we amplified a fragment of mitochondrial genome comprising tRNA^{Glu}/cytochrome b/ tRNA^{Thr}/ tRNA^{Pro}/ D-loop/ tRNA^{Phe}, followed by digestion with a specific restriction enzyme. The direct digestion of unpurified PCR products generated species-specific restriction patterns and proved to be a simple, reliable, inexpensive and fast method. Thus, it may be successfully utilized in specialized laboratories for the correct identification of the fish species for multiple purposes, including the traceability of fish food products.

Keywords: identification, PCR-RFLP, salmonids, traceability.

1. Introduction

Salmonid fish have long been of great interest due to the commercial and recreational value of some species. During the last decade significant progress has been made in the identification of salmonid species in international meat trade. The identification approaches for these species are focusing on polymerase chain reaction based on amplification of sequences belonging to the mitochondrial genome.

Salmonid identification has become important for two main reasons. Firstly, in order to expose the commercial frauds through species substitutions it was necessary to develop new methods to confirm the origin of different fish products on the market.

Secondly, it is necessary to correctly identify these species in order to develop efficient

restocking programs. If the adults are relatively easily to identify based on morphological characters, the correct identification of juveniles is very difficult and imposes an accurate molecular method for this. Recently, it was observed that release of non-native fish in the natural environment resulted in an admixture of stocks [1] which does limit the application of non-genetic methods used for species identification.

Economically important species like salmonids, which are requested for their meat, can be easily identified using molecular methods such as PCR-RFLP. The technique was shown to detect interspecies and subspecies variations in several fish groups such as sturgeon [2], tuna [3] or salmonids [2, 4].

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2. Materials and methods

DNA extraction

Fin samples from four salmonids species (*Salmo trutta fario*, *Salvelinus fontinalis*, *Onchorhynchus mykiss*, *Thymallus thymallus*) collected in 96% ethanol were used for DNA extraction. The total DNA was extracted using a specific method [5] and the concentration and quality were assessed spectrophotometrically at 260/280 nm.

PCR amplification

We have used a pair of primers (forward: 5'-TGACYTGAAAARCCACCGTTGTTA -3'; reverse: 5'-GTGTTATGTCYTTAGTTRAGC -3') in order to amplify a fragment from mitochondrial genome, including tRNA^{Glu}/cytochrome b/tRNA^{Thr}/tRNA^{Pro}/ D-loop/ tRNA^{Phe}. Initially, the PCR conditions were optimized by varying the annealing temperature between 51-62°C on a gradient thermocycler IQCycler (BioRad) and the optimum annealing temperature we selected was 56°C for *Salmo trutta fario* and *Onchorhynchus mykiss*, 53°C for *Salvelinus fontinalis*, and 58°C for *Thymallus thymallus*.

Amplification reactions were carried out in 25 µL final volume and contained 1X PCR Buffer, 1.5 mM of MgCl₂, 200 µM of each nucleotide, 10 µM of each primer, 1 unit of AmpliTaq Gold DNA polymerase (AppliedBiosystems), nuclease free water and 50 ng of DNA template. PCR amplifications were performed using a program with 45 cycles on GeneAmp 9700 PCR System (AppliedBiosystems) under the following conditions: denaturation at 95°C (30 seconds), annealing at specified temperatures for each species (45 seconds) and extension at 72°C (2 minutes). The first denaturation step was of 10 minutes at 95°C and the final extension was of 20 minutes at 72°C. The PCR products were analysed by electrophoresis on 2% agarose gel.

Restriction reaction

The RFLP reaction was carried out in 20 µL final volumes, containing 2 µL of Restriction Buffer 10X, 0.5 mg/ml BSA, 16 µL of each PCR product and 10 units of *HinfI* (Promega). The digestion was setup for three hours at 37°C. The restriction fragments were directly analysed by electrophoresis in 2.5% agarose gels, stained with ethidium bromide. The genotypes of the analysed individuals were established using the restriction fragments observed in the gel.

3. Results and discussion

In this study, we have developed a fast, simple, and accurate DNA-based screening method to identify the salmonid species. This versatile method employs PCR amplification of mitochondrial DNA extracted from blood, fin or tissue samples, followed by restriction fragment length polymorphism (RFLP) analysis to generate fragment patterns that can be resolved on agarose gel electrophoresis.

Initially, to discriminate between salmonid species we used PCR amplification. The size of the amplified fragment may vary slightly depending on the species: 2354 bp for *Onchorhynchus mykiss*, 2364 bp for *Salmo trutta*, 2315 bp for *Salvelinus fontinalis* and 2352 bp for *Thymallus thymallus* (Figures 1-2). This length variation is due to the higher variability of the non-coding control region (D-loop).

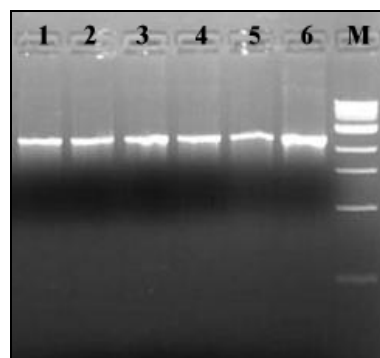


Figure 1: PCR amplification for a fragment from the mitochondrial genome. 1-3: *Salvelinus fontinalis*, 4-6: *Thymallus thymallus*, M: 1 Kbp molecular weight marker (NewEngland BioLabs).

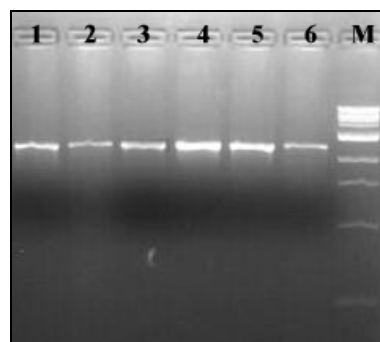


Figure 2: PCR amplification for a fragment from the mitochondrial genome. 1-3: *Salmo trutta*, 4-6: *Onchorhynchus mykiss*, M: 1 Kbp molecular weight marker (NewEngland BioLabs).

In order to identify different salmonid species, the amplified products were subjected to enzymatic digestion using the *HinfI* restriction endonuclease. The polymorphisms will generate distinct restriction sites of this enzyme and thus we will be able to differentiate between species. The restriction products obtained were separated on 2.5% agarose gel and the results showed in Figures 3-4.

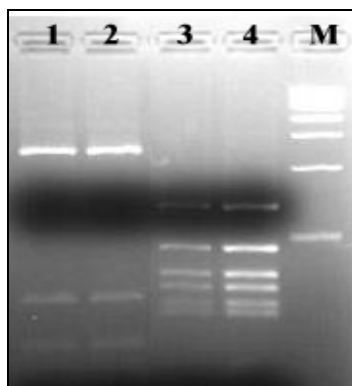


Figure 3. Results after *HinfI* digestion of a fragment from the mitochondrial genome. 1-2: *Salmo trutta*; 3-4: *Onchorhynchus mykiss*; M: 1 Kbp molecular weight marker (NewEngland BioLabs).

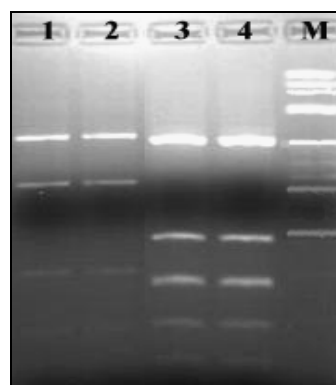


Figure 4. Results after *HinfI* digestion of a fragment from the mitochondrial genome. 1-2: *Salvelinus fontinalis*; 3-4: *Thymallus thymallus*; M: 1 Kbp molecular weight marker (NewEngland BioLabs).

After the digestion of the tRNA^{Glu}/cytochrome b/tRNA^{Thr}/tRNA^{Pro}/D-loop/tRNA^{Phe} fragment with *HinfI* the length of the restriction fragments for each salmonid species that we analysed in our study is shown in Table 1. The fragments smaller than 150bp were not visualised due to the limited resolution of agarose electrophoresis. The fragments with similar size co-migrated, thus only one band was present in the gel.

Table 1. Restriction fragment length polymorphism of the tRNA^{Glu}/cytochrome b/tRNA^{Thr}/tRNA^{Pro}/ D-loop/ tRNA^{Phe} fragment in four salmonid species.

Species	Enzyme	Restriction fragments size (bp)
<i>Salvelinus fontinalis</i>	<i>HinfI</i>	1117, 693, 323, 182
<i>Thymallus thymallus</i>	<i>HinfI</i>	1030, 400, 280, 269, 189, 124, 60
<i>Onchorhynchus mykiss</i>	<i>HinfI</i>	693, 462, 362, 318, 269, 250
<i>Salmo trutta</i>	<i>HinfI</i>	1260, 298, 192, 182, 153, 87, 75, 72, 45

Analysis of Figures 3-4 shows that cleavage of amplicons with restriction endonuclease *HinfI*, followed by agarose gel electrophoresis can easily discriminate between different salmonid species based on species specific restriction patterns. Thus, the proposed molecular technique can be used successfully to identify salmonids and hybrids between different species. Detecting these hybrids in the wild would be a major achievement in support of the conservation efforts. Also, this technique can be successfully applied for traceability of food products like salmon meat.

4. Conclusions

We demonstrate a simple, fast, and accurate mtDNA-based screening method for the identification of salmonid species. This powerful method provides reproducible, precise results in

less than one working day and it can be implemented in specialized laboratories for the correct identification of the fish species from which different food products are derived. The results confirmed that the PCR-RFLP technique can be successfully utilized in salmonids for discrimination between different species. In the future we propose to extend our study by PCR-RFLP to a higher number of salmonid species.

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References

1. Ludwig, A., Identification of Acipenseriformes species in trade, *Journal of Applied Ichthyology*, 2008, 24, 2-19
2. Wolf, C., Rentsch, J., Hubner, P., PCR-RFLP analysis of mitochondrial DNA: A reliable method for species identification, *Journal of Agricultural and Food Chemistry*, 1999, 47, 1350-1355
3. Quinteiro, J., Sotelo, C. G., Rehbein, H., Pryde, S. E., Medina, I., Ped Rez-Martidn, R. I., Rey-Mended Z. M., Machie, I. M., Use of mtDNA direct polymerase chain reaction (PCR) sequencing and PCR-restriction fragment length polymorphism methodologies in species identification of canned tuna, *Journal of Agricultural and Food Chemistry*, 1998, 46, 1662-1669
4. Nielsen, E., Hansen, M., Loeschcke, V., - Improved primer sequences for the mitochondrial ND1, ND3/4 and ND5/6 segments in salmonid fishes: application to RFLP analysis of Atlantic salmon, *Journal of Fish Biology*, 1998, 53, 216-220
5. Taggart, J. B., Hynes, R. A., Prodohol P. A., Ferguson A., A simplified protocol for routine total DNA isolation from salmonid fishes, *Journal of Fish Biology*, 1992, 40, 963-965