DNA-Based Methods for the Identification of Huchen (*Hucho hucho*, Linnaeus, 1758)

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Abstract
The Danube salmon or huchen (*Hucho hucho*, Linnaeus, 1758) is an endemic species in the Danube basin, being almost extinct in particular because of anthropogenic activities. The huchen has a special status throughout Europe, being listed on Appendix III of the Bern Convention and on Annexes II and V of the EU Habitat Directive and is a protected species in Romania. Molecular methods that use nuclear and mitochondrial DNA markers are recommended in order to contribute for a better management of aquaculture activities and also to improve the measures for conservation of endangered species such huchen. Our study aims to evaluate several DNA-based methods for species identification of *Hucho hucho* from the wild and aquaculture. We proposed two methods focused on the mitochondrial genome (DNA barcoding and PCR-RFLP), and one on the nuclear markers (genotyping). Methods that analyse the mitochondrial genome are focused on the COI gene and D-loop/Cytb regions, while those that analyse nuclear markers are focused on microsatellites. All three methods were tested on 10 huchen individuals from wild and aquaculture. Therefore, techniques such as PCR-RFLP, sequencing and genotyping gave results that were easily interpretable and could be recommended as powerful species detection methods with applicability in aquaculture and for restocking purposes.

Keywords: huchen, molecular identification, PCR-RFLP, sequencing, genotyping.

1. Introduction

The Danube salmon or huchen (*Hucho hucho* Linnaeus, 1758*)* is one of the four species of the genus *Hucho,* representatives of *Salmonidae* fish family. In the last century, the huchen populations have undergone a massive fragmentation so that its status is currently listed as endangered, the major threats being poaching or river damming [1, 2]. According to IUCN [1], various restocking activities were applied and several fishing regulations were approved in the last years. Nowadays, the huchen is listed on Appendix III of the Bern Convention and on Annexes II and V of the EU Habitat Directive [3].

Nowadays, in Romania its presence is reported in Vișeu, Tisa, and Bistriţa rivers, and also in Bicaz Lake, although historically it had a wide geographic distribution [4]. For protecting the wild species, the Annex I of the order No. 642 of 15 July 2005 prohibits the recreational fishing of *Hucho hucho.*

Molecular studies regarding the genetic diversity of huchen in Europe are limited [5] and revealed introgressive hybridization and extinction of autochthonous alleles in some cases [6]. Most often, the molecular methods involve the use of nuclear and mitochondrial markers in order to

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contribute to better management and conservational methods.

The salmoniculture is one of the most important branches in fish rearing, with a spectacular development in our country. The process of upgrading salmonids farms is in continuous progress and the main directions are species amelioration, disease control and finding better solutions regarding marketing and environmental protection.

In Romania, the molecular identification of reared salmonids was done in few studies and was focused mainly on *Oncorhynchus mykiss* (Rainbow Trout) [7-9]. The main aspects that encourage the molecular species identification in aquaculture are fish fraud prevention and correct labelling for fish and fish-derived products on the market. Also, in order to avoid restocking activities with hybrid species, correct molecular species identification is recommended.

Our study aims to evaluate several DNA-based methods for species identification of huchen from wild and aquaculture.

2. Materials and methods

DNA extraction

Fin clips from 10 huchen individuals (3 from the wild and 7 from aquaculture) were collected in 2 ml tubes with 96% ethanol and preserved until DNA extraction. Classic phenol-chloroform protocol was used for total DNA extraction.

PCR amplification

For DNA-barcoding method we amplified a fragment of the subunit I of the cytochrome oxidase gene (COI) using the following primers: StrSfo F: 5'–tccaccgcttaaactctcag-3', StrSfo R: 5'–ccgggtcaaagaaagtggta-3'.

For the amplification step of the PCR-RFLP reaction we used the pair of primers reported by [7], in order to amplify a fragment from the mitochondrial genome, including $tRNA^{Glu}$/ cytochrome b/$tRNA^{Thr}$/ tRNAPro/D-loop/$tRNA^{Phe}$.

For the microsatellites genotyping we used the following primers pairs: Str543 [10], BS131 [11], OmyRGT17, OmyRGT19 [12] and Str73 [13].

Initially, all the PCR conditions were optimized by varying the annealing temperature between 51-62ºC on a gradient thermocycler (Bio-Rad). Four out of the five tested nuclear markers failed to amplify the *Hucho hucho* genome, namely Str543, BS131, OmyRGT17, and OmyRGT19. The optimum temperatures are 53ºC for Str73 and D-loop/Cytb region primer pairs, while for the COI region the optimum annealing temperature is 54ºC. All the amplification reactions were carried out in 25µL final volume and contained 1X PCR Buffer, 1.5 nM $MgCl_2$, 200 µM of each nucleotide, 10 µM of each primer, 1 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems), nuclease free water and 50 ng of DNA template. All PCR amplifications were performed using a program with 35 cycles on GeneAmp 9700 PCR System (Applied Biosystems) under the following conditions: denaturation at 95˚C (30 seconds), annealing at specified temperatures for each primer pairs (45 seconds) and extension at 72˚C (1 minute). The first denaturation step was of 10 minutes at 95˚C and the final extension was of 10 minutes at 72˚C. The PCR products were verified by agarose gel electrophoresis.

Restriction reaction

The RFLP reaction was carried out in 20µL final volumes, containing 2 µl of Restriction Buffer 10X, 0.5 mg/ml Bovine Serum Albumin, 16 µl PCR product and 10 units of *Hinf I* restriction enzyme (Promega). The digestion was setup for three hours at 37ºC. The restriction fragments were analyzed by electrophoresis in 2.5% agarose gels, stained with ethidium bromide.

Sequencing and genotyping

The partial COI gene sequences for each individual were obtained using BigDye Terminator v3.1 Kit (Applied Biosystems), followed by BigDye XTerminator Purification Kit (Applied Biosystems) and samples were loaded on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems). For the genotyping step, the amplified products were loaded with the GeneScan-500 LIZ Size Standard (Applied Biosystems) into ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Data analysis

The COI sequences were used for phylogenetic tree construction, along with other *Salmonidae* fish family representatives from Romania previously sequenced in our laboratory and the outgroup was represented by the GenBank entry KJ190025.1 - *Plecoglossus altivelis*. The DNA alignment was done with BioEdit [14], while the maximum likelihood phylogenetic tree was done with MEGA7 [15].

The genotypes resulted from the RFLP reactions were compared with previous results obtained in our laboratory, while the alleles sizes resulted from the genotyping process were compared with literature data.

3. Results and discussion

The COI sequences for *Hucho hucho* had 424 bp in size and along with previously sequenced salmonids (*Thymallus thymallus*, *Salvelinus fontinalis*, *Salmo salar*, *Salmo trutta fario* and *Oncorhynchus mykiss*) were used to depict the relationship with other family representatives (Figure 1). The phylogenetic tree suggests that all 10 individuals share the same haplotype, with no genetic variation in the COI mitochondrial region.
The *Hucho hucho* samples were placed in a separate clade from the *Thymallus thymallus* samples and in the same clade as other *Salmoninae* subfamily members, supporting the known phylogenetic relationships [16]. Thus, the COI region, commonly used in barcoding studies for marine or freshwater species identification [17], and not only, could be successfully used in molecular identification of huchen.

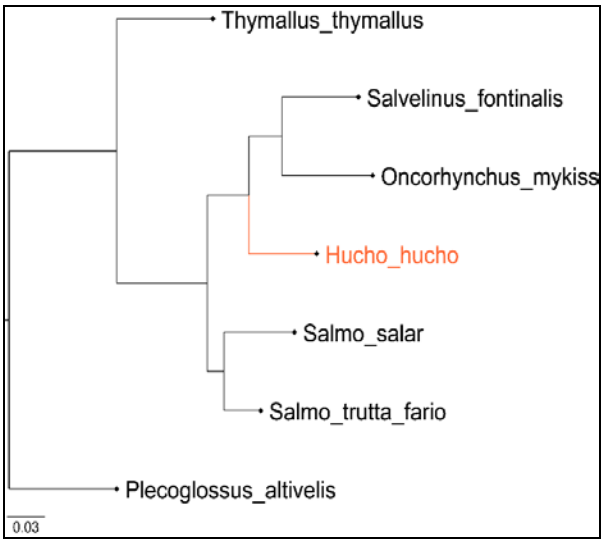

Figure 1. The phylogenetic tree resulted by applying the Maximum Likelihood (ML) method on *Salmonidae* family representatives, including the *Hucho hucho* cluster. The measurement unit is in number of substitutions per site.

The alleles sizes resulted from the PCR-RFLP reactions were ranging from 369 bp to 1147 bp and are presented in table 1, along with the data obtained from previous screening studies [4, 8]. A restriction profile for two huchen individuals after the digestion with *Hinf I* is shown in figure 2.

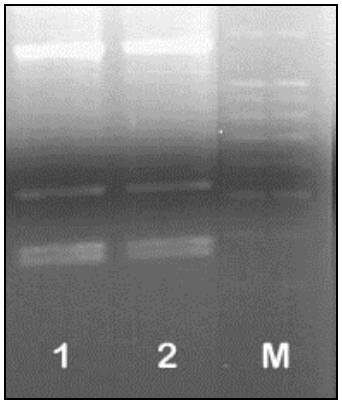

Figure 2. The restriction profile after digestion with *Hinf I* endonuclease. 1, 2 – *Hucho hucho*; M – molecular weight marker 100 bp (Promega).

These results suggest that the targeted mitochondrial region, namely the D-loop/Cytb region, could easily represent a molecular method for species identification.
Among the five microsatellites amplified only Str73 locus provided results that were useful to identify the huchen. The alleles obtained through genotyping, depicted in figure 3, were all fixed with a size of 136 bp in all analyzed individuals. The status of this locus as a monomorphic one was also reported by [5, 18] for various huchen individuals from Europe, thus the Str73 nuclear marker could be used in molecular identification of *Hucho hucho*.

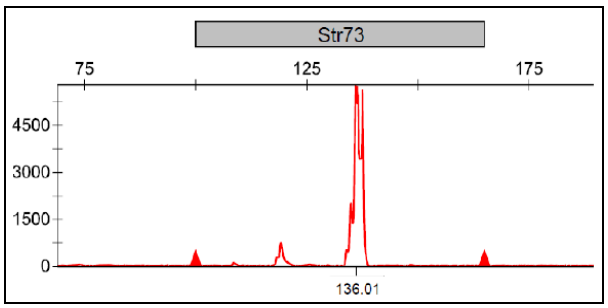

Figure 3. The genotype for Str73 microsatellite in huchen. The vertical scale represents the intensity of the signal, while the horizontal one represents the allele size in base pairs.

Table 1. Restriction fragments obtained by PCR-RFLP in several salmonids species, using *Hinf I* endonuclease.

Species name	Alleles sizes (base pairs)
Hucho hucho	1147, 542, 390, 369
**Salvelinus fontinalis*	1117, 693, 323, 182
**Thymallus thymallus*	1030, 400, 280, 269, 189, 124, 60
**Oncorhynchus mykiss*	693, 462, 362, 318, 269, 250
**Salmo trutta*	1260, 298, 192, 182, 153, 87, 75, 72, 45

*Data obtained from previous studies [4, 8]

4. Conclusions

We presented three molecular DNA-based methods that could easily be used for species identification of *Hucho hucho*, one of the most endangered salmonid: two are based on mitochondrial DNA and one on nuclear genome.
The techniques were proven powerful and they could successfully be applied on a large scale of fishes, not only on salmonids.
All three methods gave results that were easily interpretable thus they could be recommended as powerful species detection methods and could prove useful in proper identification of possibly mislabelled huchen derived products. Considering special conservative status of this species throughout Europe, the methods could also be useful in correct identification of individuals use for restocking purposes.

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