Phylogeny of *Salmonidae* Family Inffered from D-loop Mitochondrial Marker

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Abstract

Salmonids are a heterogeneous group of fish classified in one family – *Salmonidae*. These species are natives of the northern hemisphere, but currently have been introduced in many countries all over the world for sport fishing and aquaculture. Despite the considerable importance of salmonids, their evolutive history is still a controversial issue.

The aim of the current study is to infer the phylogeny within the *Salmonidae* family using as phylogenetic marker the D-loop region. For this purpose, an PCR amplification of D-loop region, followed by direct sequencing and phylogenetic analysis have been performed in four salmonids species from Romanian fauna - *Salmo trutta fario, Salvelinus fontinalis, Thymallus thymallus* and *Oncorhynchus mykiss*. For a more accurate phylogenetic classification of these species within *Salmonidae* family the analysis included similar sequences from GenBank belonging to 17 salmonid species and an osmerid species used as outgroup. The the phylogenetic trees illustrating the evolutive relationships within Salmonidae were constructed by using Neighbor Joining and Maximum Parsimony methodologies implemented in MEGA5.The phylogenetic analysis using mitochondrial control region as marker has allowed an overview about the positions occupied by Romanian salmonids within the *Salmonidae* family.

Keywords: D-loop, mitochondrial, molecular phylogeny, *Salmonidae*.

1. Introduction

Salmonid fishes are reunited in order Salmoniformes, with one family – *Salmonidae* and three subfamilies, *Salmoninae*, *Coregoninae* and *Thymallinae*. The most numerous of the subfamilies, *Salmoninae*, includes five genera *Hucho, Salmo, Oncorhynchus, Brachymystax* and *Salvelinus*, with a wide distribution in the Northern hemisphere.

Despite their socio-economical and scientific importance, the evolutive history of salmonids is still controversial. The phylogenetic studies at genus/ species level offered essential information about the relationships between *Salmonidae* family representatives, but there are still pending questions regarding the species within *Oncorhynchus* and *Salvelinus* genra [1-3].

In Romanian fauna six salmonid species are found: *Salmo trutta fario* (Brown trout), *Salmo labrax* (Black Sea salmon), *Salvellinus fontinalis* (brook trout), *Thymallus thymallus* (Grayling), *Hucho hucho* (Danube salmon) and *Onchorynchus mykiss* (Rainbow trout). Apart these six species, representatives of *Coregonus* genus (*Coregonus albula ladogensis* and *Coregonus lavaraetus maraenoides*) have been introduced in different lakes from Romania, but there are no data regarding the adaptation. Currently, *Salmo trutta fario* is the most outspread species in the Romanian mountain streams, while *Hucho hucho* and *Salmo labrax* are considered to be endangered [4].

Even if the native salmonid species from Romania have been well characterized from morphological point of view, the molecular studies are still at the beginning. Until now is available only one study

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regarding the phylogenetic classification of salmonid species from Romania based on 12S and 16S rRNA mitochondrial genes sequences [5].

Due to its compact organization, the mitochondrial genome has been sequenced in many organisms, including salmonids. Among other characteristics, based on the fact that presents a mutation rate up to 10 times higher than nuclear single copy coding genes [6] the mitochondrial genome has been used widely to investigate phylogenetic relationships.

The mitochondrial control region, also called the displacement loop or D-loop, possesses several sequences with a high degree of variation between populations of the same species, but D-loop variability is not uniform and the region shows several well-conserved sequences among vertebrate species [7, 8].

The aim of our study was to analyze the D-loop region in four salmonid species present in Romania and by using this mitochondrial marker to infer the phylogeny within *Salmonidae* family.

2. Materials and methods

Sample collection and DNA extraction

Samples from four salmonid species (*Salmo trutta fario, Salvelinus fontinalis, Thymallus thymallus* and *Oncorhynchus mykiss*) representing small fin fragments were collected from different Romanian rivers and salmonid farms.

Genomic DNA was extracted by a standard phenol/ chloroform / isoamyl alcohol method. The quality and concentration of DNA was assessed using the spectrophotometer.

Amplification and sequencing

The amplification of the entire D-loop region by PCR was done using the primers PST 5'cccaaagctaaaattctaaat3' and FST 5'gctttagttaagctacgc3' [9] for *S. trutta fario*, Tt/St F 5'cccaaagctaagattctaaat3'and Tt/St R 5'gctttagttaagctacgc3' for *T. thymallus* and *S. fontinalis*, Om F 5'cccaaagctaagattctaaat3'and Om R 5'gctttagttaagctacgt3' for *O. mykiss*. Amplifications were performed in a GenAmp PCR System 9700 (*Applied Biosystems*) thermocycler with a 25 µL reaction volume containing 50 ng of DNA template, 1X PCR Buffer, 1.5 mM of $MgCl_2$, 0.4 µM of each primer, 0.8 mM of each dNTP and 1 U of AmpliTaq Gold Polymerase (*Applied Biosystems*). The thermal cycling conditions were set as follows: initial denaturation (95°C, 10 min); 35 cycles of denaturation (95°C, 30 s), annealing (52°C, 30 s) and extension (1 min, 72°C) and then a final extension (72°C, 10 min). The amplified fragments were run on a 2% agarose gel to verify the amplification efficiency. The PCR products were purified with Wizard SV Gel and PCR Clean-Up System (*Promega*), sequenced in both directions using BigDye Terminator v3.1 kit (*Applied Biosystems*) and analyzed on ABI3130 DNA Genetic Analyzer (*Applied Biosystems*).

Sequence alignment and phylogenetic analyses

DNA sequences were edited using BioEdit Sequence Alignment Editor [10] and the alignment was performed with Clustal X. Gaps from the sequences represented indel mutations and were included in the phylogenetic analysis like a fifth character.

For a more comprehensive phylogenetic analysis, apart from our sequences for the Romanian salmonid specimens, we included D-loop sequences from GenBank for different salmonid species and an osmerid, *Plecoglossus altivelis,* used as outgroup (Table 1).

Table 1. D-loop GenBank sequences used in the phylogenetic analysis.

Species	***GenBank accession number***
Brachymystax lenok	EU760491
Coregonus lavaraetus	NC_002646
Hucho hucho	EU729361
Hucho taimen	EU760489
Oncorhynchus clarkii	AY886762
Oncorhynchus keta	NC_009261
Oncorhynchus kisutch	NC_009263
Oncorhynchus masou	NC_008747
Oncorhynchus mykiss	L29771
Oncorhynchus nerka	NC_008615
Oncorhynchus tshawytscha	NC_009263
Plecoglossus altivelis (outgroup species)	NC_002734
Salmo trutta (North Atlantic haplotype)	AF253559
Salmo trutta (South Atlantic haplotype)	AF253557
Salmo trutta (Adriatic haplotype)	AF253552
Salmo trutta (Duero haplotype)	AF253544
Salmo trutta (Danubian haplotype)	GQ284837
Salmo salar	AF133701
Salvelinus alpinus	NC_000861
Thymallus thymallus	DQ439975
Thymallus arcticus	NC_002734

The best-fitting model of molecular evolution for the aligned sequences was estimated based on AIC value in ModelTest [11] and the Tamura-Nei plus Gamma model was selected.
Two methodologies, Neighbor Joining and Maximum Parsimony implemented in MEGA5 [12] were used for phylogenetic reconstruction. The bootstrap values were calculated for 1000 bootstrap replicates.

3. Results and discussion

In this study we determined the entire D-loop sequence for the specimens of different salmonid species sampled in Romania. The variation of control region in term of length is relatively high in the analyzed species, ranging from 964bp in *S. fontinalis* to 1012bp in *S. trutta fario*, with intermediate values of 1001bp for *T. thymallus* and 1003bp for *O. mykiss.*
In order to determine the intraspectific nucleotide variation, our sequences were aligned and compare with similar sequences from GenBank belonging to the same salmonid species (Table 2). The highest number of polymorphic sites (18) was identified in *S. trutta fario*, while the lowest number of variations (7) was observed in *O. mykiss.*

Table 2. The analysis of four salmonid species from Romania concerning the number of polymorphic sites.

Species	***No. of polymorphic sites***
S. trutta fario	18
O. mykiss	7
S. fontinalis	8
T. thymallus	8

The consensus trees resulted by the Neighbor Joining (NJ) and Maximum Parsimony (MP) methodologies are shown in Figure 1 and 2.
The trees present a very similar topology, except the position occupied to *Salvelinus* genus related to *Oncorhynchus* and *Salmo*.
The phylogentic trees inferred from the D-loop analysis highlighted three main clades corresponding to the three subfamilies *Thymallinae*, *Coregoninae* and *Salmoninae.* Primitive species of salmonids like *T. thymallus, T. arcticus* and *C. lavaraetus*, belonging to *Thymallinae* and, respectively, *Coregoninae* subfamilies occupied basal position in the trees. In this case, the molecular phylogeny confirm the classical phylogenetic classification [13,14] that sustain the earlier arising from a common ancestor of these subfamilies reported to *Salmoninae.*
Salmoninae subfamily comprises the *Salmo, Oncorhynchus, Hucho, Brachymistax* and *Salvelinus* genera and according to our analysis these represent monophyletic groups.
Among *Salmoninae*, *Brachymistax* and *Hucho* genera are placed by both NJ and MP analysis in basal divergence. The analyzed species of *Salmo* genus form a distinct clade, in which *S. salar* occupies a basal position. The North Atlantic and South Atlantic lineages haplotypes and the Duero haplotypes form a monophyletic group related to the monophyletic group produced by the Adriatic and Mediterranean lineages haplotypes. The haplotypes that we obtained for Romanian *S. trutta fario* and the haplotypes from GenBank of Danubian lineage origin form distinct monophyletic groups within *Salmo* genus.
As we expected, in Romania *S. trutta fario* specimens that we analyzed belong to the Danubian lineage, this being an indication that non-native individuals have not being introduced to the water Romanian water streams.
Our analyses showed that the *Oncorhynchus* genus species form a distinct clade. Our haplotypes clustered with the similar haplotypes of *O. mykiss* from GenBank within this clade. *O. mykiss* and O. *clarki* form a monophyletic group, these species originating from Pacific Ocean and represent a distinct evolutionary lineage (Pacific Trout Lineage). *O. tshawytscha* and *O. keta* form a monophyletic group and represent one of the evolutive lineages of the Pacific salmon group.
O. masou masou, *O. nerka* and *O. kisutch* are placed in monophyletic groups and represent the second lineage of the Pacific salmon group. Our data confirm previous studies [15, 16].
The monophyly of *Salvelinus* genus within *Salmoninae* subfamily was supported by both NJ and MP analysis. According to the tree constructed by NJ methodology the group of (*S. fontinalis* and *S. alpinus*) occupies an intermediate position between *Salmo* and *Oncorhynchus*.
Thus, the classification of *Salvelinus* is in opposition with the classical phylogeny hypothesis according to which *Salmo* and *Oncorhynchus* are closely related and support other phylogenetic analyses based on molecular markers that sustain that there is a closer relationship between *Salvelinus* and *Onchorhyncus* [2,3].

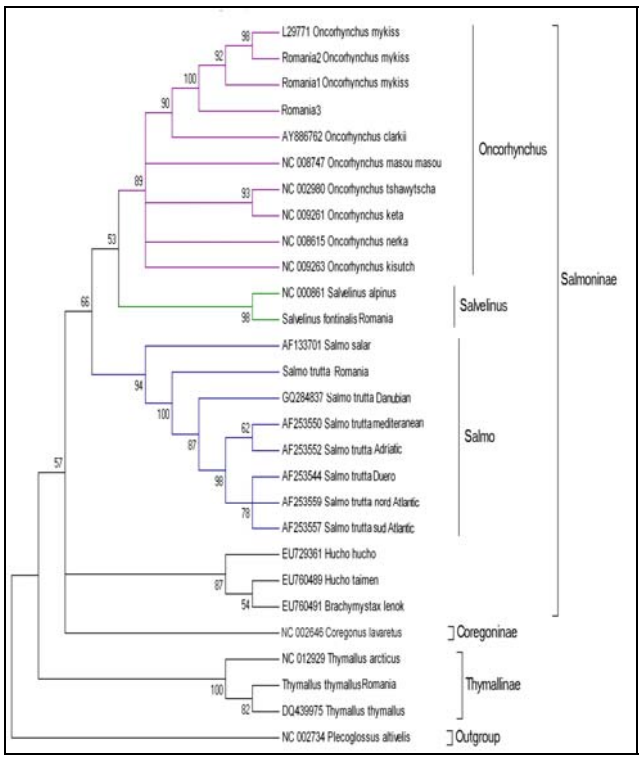

Figure 1. Majority NJ consensus tree for D-loop data (Bootstrap – 1000 replications)

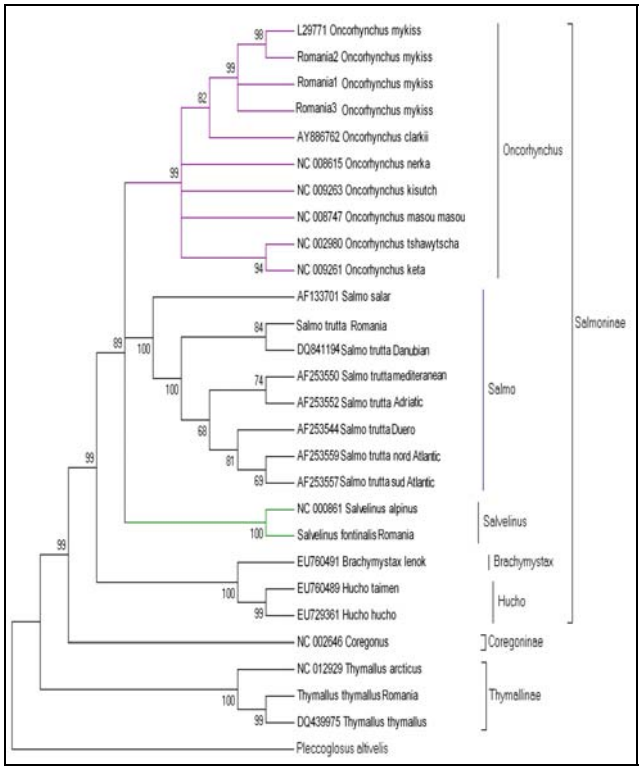

Figure 2. Majority MP consensus tree for D-loop data (Bootstrap – 1000 replications)

Instead, the MP analysis confirms the classical phylogeny hypothesis that sustain that *Salmo* and *Oncorhynchus* represent closely related taxa. The difference regarding the classification of *Salvelinus* in relation with *Salmo* and *Oncorhynchus* depending on the molecular marker and/ or the methodology of analysis was highlighted also by previous studies [3,5].

This finding state that the selection of phylogenetic marker and methodology should be treated with a high concern in order to obtain robust results.

4. Conclusions

The phylogenetic analysis using mitochondrial D-loop sequence as marker has allowed the classification of salmonid species from Romania within the *Salmonidae* family. Thus, Romanian *S. trutta fario* belong to Danubian evolutive lineage and this indicates that no restocking programs with non-indigenous individuals of brown trout have been yet performed on the water streams from which sampling was performed. *T. thymallus* appear to be the most primitive species among the Romanian salmonids that we analyzed in this study. The classification of S*alvelinus* genus remains controversial and appear to be variable based on the selected methodology of analysis.
Therefore is necessary to develop further studies increasing the number of mitochondrial markers and including nuclear markers also.
In the future, we propose to include in a phylogenetic analysis with a higher number of molecular markers, endangered species from Romanian fauna like *Hucho hucho* and *Salmo labrax.*

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