

MITOCHONDRIAL 16S AND 12S rRNA SEQUENCE ANALYSIS IN FOUR SALMONID SPECIES FROM ROMANIA

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In this study we proposed a phylogenetic analysis based on molecular markers of 16S rRNA and 12S rRNA mitochondrial genes in four salmonid species from Romania. For this purpose, a PCR amplification of one fragment from each mitochondrial gene mentioned above was performed, followed by direct sequencing, the analysis of nucleotide variation and a phylogenetic analysis of 4 species. The analyzed species are *Salmo trutta fario*, *S. labrax*, *Salvelinus fontinalis* and *Thymallus thymallus*. For a more accurate phylogenetic classification of these species within the Salmonidae family, the analysis was performed using similar sequences from GenBank Database from 14 salmonids and one osmerid species used as an outgroup. Three methodologies namely neighbor joining, maximum parsimony and maximum likelihood were used for phylogenetic reconstruction by each gene separately and the mitochondrial data combined. The phylogenetic analysis using mitochondrial rRNA genes as markers has allowed an overview about the positions occupied by Romanian salmonids within the Salmonidae family. This study has interesting implications for understanding the evolution and diversification of this group of fish and is the first molecular study on salmonid species from Romania.

Key words: salmonids, mitochondrial, rRNA, molecular phylogeny

INTRODUCTION

Salmonids are a heterogeneous group of fish, reunited in the Salmonidae family, that includes three subfamilies (Coregoninae, Thymallinae and Salmoninae) (NELSON 2006) classified into nine genera and sixty-eight species spread longitudinally from Iceland to Aral Sea and latitudinally from northern Scandinavia and northwestern Russia to the Island of Crete and the Atlas Mountains of North Africa.

Salmo trutta comprises several distinct ecological and geographical forms and with respect to this there is still controversy as far as their classification as species or subspecies is concerned (BERG 1948, GIUFFRA *et al.* 1994, PATARNELLO *et al.* 1994, OSINOV & BRENATCHEZ 1995). Based on morphological and ecological variations, the existing populations of *Salmo trutta* from distinct areas are grouped into different taxa (BERG 1948): i) Black Sea populations – *Salmo labrax*, ii) Caspian Sea populations – *Salmo caspius*, iii) Aral Sea populations – *Salmo oxianus* and iv) Mediterranean Sea populations – *Salmo macrostigma*.

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The brown (common) trout (*Salmo trutta* morpho *fario* and *Salmo trutta* morpho *lacustris*) and the sea trout (*Salmo trutta* morpho *trutta*) are fish of the same species, considered by some taxonomists different subspecies in order to distinguish the anadromous *Salmo trutta trutta*, living in the sea and migrating in freshwater only to spawn, from *Salmo trutta fario*, residing in freshwater and the lake dwelling form *Salmo trutta lacustris*. According to other authors (RYMAN 1983, HINDAR *et al.* 1991, CROSS *et al.* 1992) these do not necessarily represent monophyletic groups.

In Romania, *Salmo trutta fario* (LINNAEUS, 1758) is widely spread in a large number of water streams from the mountain area, whereas the Black Sea trout, *Salmo labrax* (PALLAS, 1814) is endemic to the Black Sea area and migrates for reproduction in the Danube River and its tributaries.

Salvelinus fontinalis (MITCHILL, 1815) is predominantly raised in fish farms for food consumption, but a low number of wild populations are still present in the Romanian mountain waters.

Thymallus thymallus (LINNAEUS, 1758) is the only native salmonid species for which no imports of biological material and restocking programs were completed in Romania.

Even if the salmonids are a well-studied group of fish, there are still a number of questions pending with regard to their phylogeny and evolution. So, despite the fact that a large number of studies based on both morphological (NORDEN 1961, STEARLEY 1992, 1993) and molecular data (PHILLIPS *et al.* 1995, 1997, KITANO *et al.* 1997, OOHARA *et al.* 1997, CRESPI *et al.* 2003) were performed, there are still different opinions concerning genus-level relationships.

The native salmonid species from Romania have been characterized only from a morphological point of view (BĂNĂRESCU *et al.* 1964), but the molecular aspects have never been analyzed before the present study.

Due to characteristics such as increased level of nucleotide sequence variation, fast rate of evolution, compact genome and lack of recombination, its maternal inheritance and higher mutation rates compared to those of nuclear genes, the mitochondrial DNA (mtDNA) has proved to be valuable in molecular phylogenetic studies (AVISE 2004). The mitochondrial 16S rRNA gene has been used to explore the phylogenetic relationships of fishes at different taxonomic levels (ORTI & MEYER 1997, MOYER *et al.* 2004, FENG *et al.* 2005, LI *et al.* 2008), mainly due to the fact that it is highly conserved and has a slow evolution (PAGE & HOLMES 1998). At the same time, the 12S rRNA gene is considered a promising tool for tracing the history of more recent evolutionary events (HILLIS & DIXON 1991) and it has been widely used to study the phylogenetic relationships among different levels of taxa such as families (ALVES-GOMES *et al.* 1995, DOUZERY & CATZE-

FLIS 1995, LEDJE & ARNASON 1996), genera (GATESY *et al.* 1997, MURPHY & COLLIER 1997, WANG *et al.* 2001, 2003), and species (MURPHY & COLLIER 1996, HALANYCH & ROBINSON 1997).

Although mtDNA has been proven to be a very useful marker, its use is not without complications. Due to the exclusively maternal inheritance, mtDNA represents a marker strictly for historical processes in females and does not reflect necessarily the history of species as a whole but that of the female portion (HURST & JIGGINS 2005). Furthermore, BALLARD and WHITLOCK (2004) have argued that mtDNA evolution is non-neutral with sufficient regularity to question its utility as a marker for genomic history and is not a sufficient marker for phylogeographical studies if the focus of the investigation is the species and not the organelle. Despite these known difficulties, mtDNA is still used as phylogenetic marker in an important number of studies.

The aim of this study was to analyze 16S rRNA and 12S rRNA in four salmonids species present in Romania and to position them within the Salmonidae family.

MATERIALS AND METHODS

Sample collection and DNA extraction

Samples from four salmonid species (*Salmo trutta fario*, *Salmo labrax*, *Salvelinus fontinalis* and *Thymallus thymallus*) were collected from different Romanian rivers (Table 1). Upon field collection, the specimens representing fragments of fins were fixed in 96% ethanol. Total DNA was extracted from fin tissue using the method described by TAGGART *et al.* (1992), with minor modifications.

Table 1. Collecting data of the Romanian samples used in this study.

Species	Number of samples	Collecting locality
<i>Salmo trutta fario</i>	4	Dambovita River
	5	Bratia River
	4	Gilau River
	6	Latorita River
	6	Cerna River
	5	Nera River
<i>Salmo labrax</i>	6	Danube Delta
<i>Salvelinus fontinalis</i>	8	Bratia River
	7	Gilau River
<i>Thymallus thymallus</i>	10	Cerna River

Table 2. Primers sequences for 16S rRNA and 12S rRNA amplification and sequencing. Primers were designed with Primer3 software (ROZEN & SKALETSKY 2000).

Species	Gene	Primer name	Primer sequence (5' to 3')
<i>Salmo trutta fario</i>	16S rRNA	16S rRNA F	cacctcccttacaccgagaa
<i>Salmo labrax</i>		16S rRNA R	gccgagttcctctcttct
<i>Salvelinus fontinalis</i>			
<i>Thymallus thymallus</i>			
<i>Salmo trutta fario</i>	12S rRNA	St12S rRNA F	ctagaaggtcccgagca
<i>Salmo labrax</i>		St12S rRNA R	tttcacagcgtggtcgtag
<i>Salvelinus fontinalis</i>			
<i>Thymallus thymallus</i>	12S rRNA	Tt12S rRNA F	cacctcccttacaccgagaa
		Tt12S rRNA R	gccgagttcctctcttct

Amplification and sequencing

The nucleotide sequences of primers for the amplification of 16S rRNA and 12S rRNA fragments are listed in Table 2. The primers were designed using Primer3 software (ROZEN & SKALETSKY 2000). Amplifications were carried out in a 25 µL final volume with 50 ng DNA, 10 pmols of each primer, 100 µM of each dNTP, MgCl₂ and 1 unit AmpliTaq Gold DNA Polymerase (Applied Biosystems). The following amplification program was used: a 10 minutes denaturation step at 95 °C, 35 cycles of 30 seconds of denaturation at 95 °C, 40 seconds annealing at 56 °C, 90 seconds of extension at 72 °C and a final polymerization step at 72 °C for 10 minutes.

The PCR products were purified with Wizard SV Gel and PCR Clean-Up System (Promega), sequenced using Big Dye Terminator v3.1 kit (Applied Biosystems) and analyzed on ABI 3130 DNA Genetic Analyzer (Applied Biosystems). Partial sequences for 16S rRNA and 12S rRNA were deposited in GenBank under the following accession numbers: GU213390 – GU213392 (tRNA^{Val}/16S rRNA gene) and GU233801–GU233803 (tRNA^{Phe}/12S rRNA gene).

Sequence alignment and molecular phylogenetic analyses

DNA sequences were primarily aligned with the default parameters of CLUSTAL W (THOMPSON *et al.* 1994) and edited using the BioEdit Sequence Alignment Editor (HALL 1999). Gaps from the sequence alignment represent indel mutations occurred in 16S rRNA and 12S rRNA genes and are treated as phylogenetic signal and not as missing data, so in consequence they were included in the phylogenetic analysis.

For a more complex phylogenetic evaluation beside the sequences determined from salmonid specimens from Romania, 14 salmonid and 1 osmerid sequences from GenBank were also included in the analysis as follows: *Brachymystax lenok* – AF125513; *Coregonus lavaretus* – NC_002646 (MIYA & NISHIDA 2000); *Oncorhynchus clarki henshawi* – AY886762 (BROWN *et al.* 2006); *Oncorhynchus gorbuscha* – NC_010959; *Oncorhynchus keta* – NC_009261; *Oncorhynchus kisutch* – NC_009263; *Oncorhynchus masou* – NC_008747; *Oncorhynchus mykiss* – L29771 (ZARDOYA *et al.* 1995); *Oncorhynchus nerka* – NC_008615; *Oncorhynchus tshawytscha* – NC_009263; *Plecoglossus altivelis* – NC_002734 (ISHIGURO *et al.* 2001); *Salmo salar* – AF133701; *Salmo trutta trutta* – NC_010007; *Salvelinus alpinus* – NC_000861 (DOIRON *et al.* 2002); *Thymallus articus* – FJ872559

(YASUIKE *et al.* 2010). *Pleccoglossus altivelis* belonging to Osmeridae family was selected as outgroup. Osmerids are basal euteleosts related to salmonids, with which are widely believed to share an ancient common ancestry.

Estimation of phylogenetic relationships was achieved using 16S and 12S rRNAs gene sequences and the concatenated data set.

Three methodologies – maximum parsimony (MP) (CAVALLI-SFORZA & EDWARDS 1967, FITCH 1971), maximum likelihood (ML) (FELSENSTEIN 1981) and Neighbour-joining (NJ) (SAITOU & NEI 1987) implemented in PHYLIP software, version 3.68 (FELSENSTEIN 2004) were used for phylogenetic reconstructions, in order to compare the consistency of the results produced by different methods.

In order to select the method for NJ tree reconstruction, sequence divergences were calculated with DNADIST under the following nucleotide substitution models: log-determinant (LogDet; LAKE 1994), F84 (FELSENSTEIN & CHURCHILL 1996), Kimura2P (KIMURA 1980), Jukes-Cantor (JC, JUKES-CANTOR 1969).

The MP algorithm was that of the program DNAPARS, using ordinary parsimony, with no sites weighted, the more thorough search option (default) and randomizing the sequences input order. A ML procedure using the DNAML program was performed using the calculated transition/transversion ratio for each of the analyzed data. The ML analyze was performed under a constant rate of variation among sites and randomizing the sequences input order.

Bootstrap values based on the analysis of 1000 bootstrap replicates were calculated using the SEQBOOT program and the 1000 resulting trees were combined using majority-rule consensus tree analysis (CONSENSE program). For the graphical representations of tree topologies, the Treeview program, version 1.6.5 (PAGE 1996), was applied.

RESULTS

In this study, partial sequences for the mitochondrial rRNA genes in *Salmo trutta fario*, *Salmo labrax*, *Salvelinus fontinalis*, *Thymallus thymallus* salmonids from Romania were determined. A number of 864 nucleotide sites for the 16S rRNA gene, 745 nucleotide sites for the 12S rRNA gene and 1609 nucleotide sites for the two mitochondrial genes concatenated were analyzed. No polymorphism was found within the species.

Distinctive pairwise sequence differences among species were uncovered for each of these salmonids (Fig. 1). From the 864 nucleotide sites analyzed for 16S rRNA, 66 are variable and thirteen are parsimony informative. Transitions were the most common substitutions detected for all analyzed species. The number of transitions (s_i) and transversions (s_v) in 16S rRNA is 26 and 12, respectively, with a ratio of 2.2 ($R = s_i/s_v$). For the 16S rRNA gene sequences from *S. trutta fario*, *S. labrax*, *S. fontinalis*, *T. thymallus* the percentage of nucleotide variation is about 7.64%. The lowest nucleotide variation (0.93%) was observed between *Salmo trutta fario* and *Salmo labrax*, suggesting the close relationship among these two taxonomic forms with an ambiguous classification (BERG 1948, ELLIOT 1994,

Table 3. Variation and information content of DNA sequence in 18 salmonid species (four from Romania and 14 from GenBank).

Gene region	No. sites	No. variable sites (%)	No. parsimony informative sites (%)
16S rRNA	869	152 (17.5)	92 (10.6)
12S rRNA	748	87 (11.6)	57 (7.6)
Two genes combined	1616	240 (14.9)	155 (9.6)

KOTTELAT 1997, LELEK 1987). The highest percentage of sequence divergence (about 5%) was found between the species of genus *Salmo* (*S. trutta fario* and *S. labrax*) and the representative of the genus *Thymallus* (*T. thymallus*).

For the 12S rRNA gene we analyzed 745 nucleotide sites, of which 42 sites are variable and 6 are parsimony informative ones. In the 12S rRNA sequences analyzed, 17 transitional (s_i) and 6 (s_v) transversional sites were identified with a determined ratio between them of about 2.9 ($R = s_i/s_v$). The percentage of pairwise sequence divergence between the studied species is about 5.64. Similar to 16S rRNA, the lowest nucleotide variation (0.84%) was noticed between *Salmo trutta fario* and *Salmo labrax*. Using the analysis of the mitochondrial combined data (16S rRNA and 12S rRNA), we identified 108 variable and 19 parsimony informative sites from a total of 1609 sites.

For a more complex analysis in terms of nucleotide variation and number of parsimony informative sites the 16S rRNA and 12S rRNA sequences were aligned and compared with 14 similar GenBank sequences from other salmonid species (Table 3).

1	111 1111122334 4444444555 5556666666 6667777777 7777888888 8888888
	4455689011 1123646051 3333567145 5881122456 6781112557 8999001122 223555
	4824398645 7912302096 0456308940 1673767144 5441597248 8035237936 780789
	S_t_fario CTTTAACGAA CTCAATAGTT GCTCCAGACC GGTAGCCTAG TCCACTACAA ACTAATGAGT CTTTTA
	S_t_labrax AC.T.A... .A.G....G.G...
S_fontinalis AACC...A.G TAT..A.A.C A.C.T.ATAT AAC.A..CG. CAA.T...G ...GG.A.AA A-CCC.	
T_thymallus TCA.GCTAG. .-TGCCTA.C AA.AAG...T AA..ATT.GA .TAGTC.TC. TACG.AAGAC .A.AAG	
2	111122 2233333333 3333333344 4444666666 77
	2236122766 7700011135 6668999911 6667044466 33
	7823834959 6913603484 4577014814 2378023435 08
	S_t_fario TCCT.C.AAT TCC.A.CAC. A.TCTG..CC ..C.CCCTCA TC
	S_labrax ..CT.C.AAT TCC.A.CAC. A.TTTA..CC ..C.CCCTCA TC
S_fontinalis ...GCA..T TACAAG.ACA ACTTTACTCC AGAT.CCCCA TC	
T_thymallus CATCATCGGA CTTGTATGTG CTC-CCTCTA GATCCTTATT AT	

Fig. 1. Variable sites in 16S rRNA (1) and 12S rRNA (2). The numbers represent the position occupied in the 16S rRNA, and 12S rRNA respectively. Identical sites are indicated by the symbol “.” and gaps by “-”

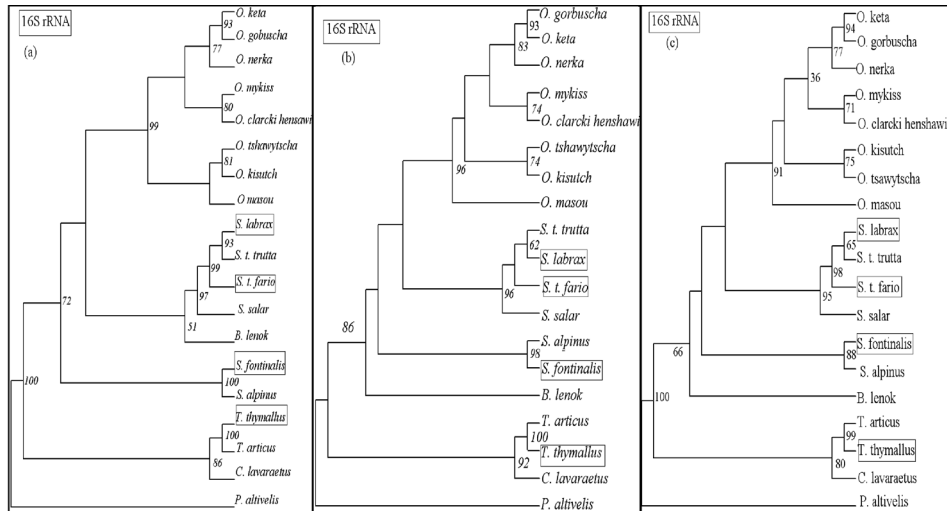


Fig. 2. Majority with bootstrap support consensus trees for 16S rRNA. (a) 16S rRNA Neighbor Joining tree, distance model Kimura 2 Parameters, transition/transversion ratio 2.3; (b) 16S rRNA Maximum Parsimony tree; (c) 16S rRNA Maximum Likelihood tree

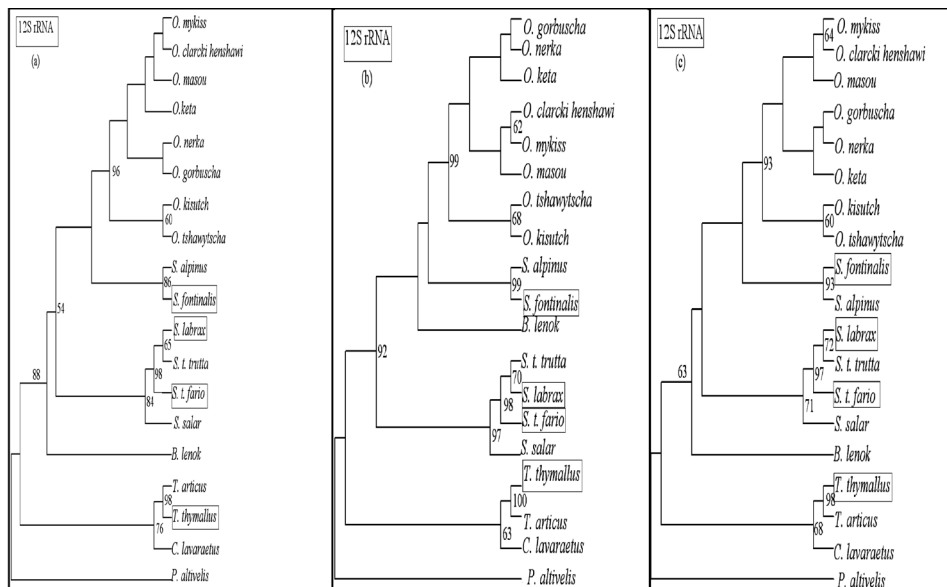


Fig. 3. Majority with bootstrap support consensus trees for 12S rRNA. (a) 12S rRNA Maximum Parsimony tree; (b) 12S rRNA Neighbor Joining tree, distance model Kimura 2 Parameters, transition/transversion ratio 2.3; (c) 12S rRNA Maximum Likelihood tree

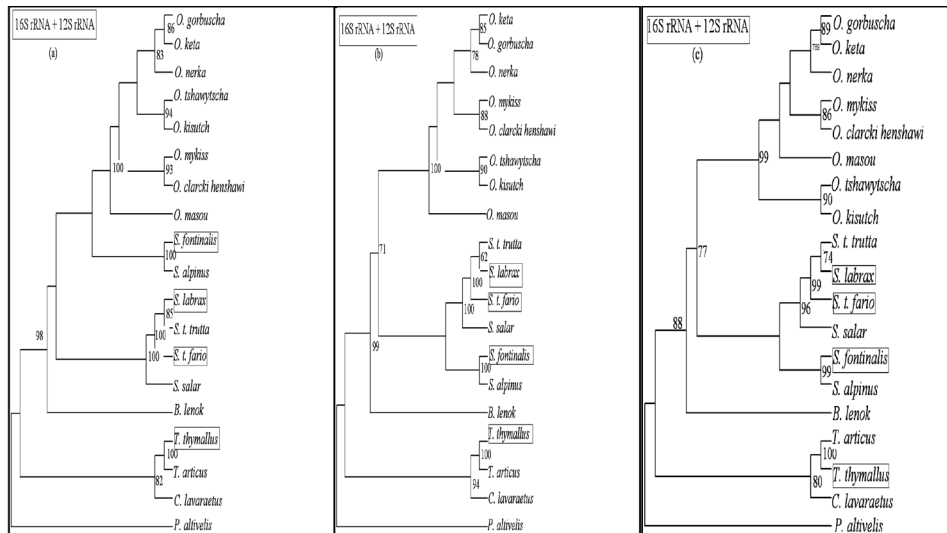


Fig. 4. Majority with bootstrap support consensus trees for combined data (16S rRNA and 12S rRNA). (a) Combined data Neighbor Joining tree, distance model Kimura 2 Parameters, transition/transversion ratio 2.3; (b) combined data Maximum Parsimony tree; (c) combined data Maximum Likelihood tree

The consensus trees resulted by the Neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods for individual genes data and for the combined data set (16S rRNA + 12S rRNA) are shown in Figures 2–4. Before the construction of the NJ tree we have tested different substitution models (F84, K2P, JC, LogDet) using DNADIST application in order to select the most appropriate model of sequence evolution.

The distances estimated by different methods were very similar (Supplementary 1) and the preliminary test with these models built up similar topologies of NJ trees. For further analysis we selected the K2P model with a corresponding transition/transversion ratio.

DISCUSSION

We assessed the degree of support by bootstrap values for each of the clades recovered by NJ, MP and ML clades. The bootstrap values obtained for the main clades (*Salmoninae*: *Brachymistax*, *Salmo*, *Salvelinus* and *Onchorhynchus*), *Thymallinae* and *Coregoninae* are above 60 (Figs 2–4) and sustain the reliability of our results.

Our data reveal that primitive salmonid species such as *Coregonus lavaretus* and the representatives of genus *Thymallus*, *T. thymallus* and *T. articus* occupy basal divergence in the tree topology confirming that the Coregoninae and Thymallinae subfamilies arise from a common ancestry before Salmoninae (with the genera *Salmo*, *Oncorhynchus*, *Hucho*, *Brachymystax* and *Salvelinus*). Based on morphological and molecular data, Coregoninae and Thymallinae were thought to be the earliest branches within the Salmonidae family (NRDEN 1961, DOROFEEVA 1989, STEARLEY & SMITH 1993, CRESPI 2004).

Depending on the phylogenetic method which has been used, the position of *Brachymystax lenok* within the Salmoninae subfamily it cannot be estimated reliably. MP and ML methods place *Brachymystax lenok* in a basal divergence within the Salmoninae, while in case of NJ tree this species seems to occupy a basal divergence within the group of *Salmo* species. The species of the *Salmo* genus form a distinct clade, in which the Atlantic salmon, *Salmo salar* occupies a basal divergence. Our data reveal a close relationship between *Salmo trutta fario* and the clade formed by sea trout *Salmo trutta trutta* and the Black Sea trout *S. labrax*. The resulting clade (*Salmo trutta trutta*, *S. labrax*) is not surprising, taking into consideration some characteristics of the life history and reproductive behavior of these species (BENKE 1965, 1968, BĂNĂRESCU *et al.* 1971, ECONOMIDIS & BANARESCU 1991, DOROFEEVA 1998, OTEL 2007).

The monophyly of *Salvelinus* was supported by 16S rRNA, 12S rRNA and combined data, but the position of the clade formed by (*S. alpinus*, *S. fontinalis*) in relationship with *Salmo* and *Onchorhynchus* is dependent on the molecular marker selected for the phylogenetic analysis.

Despite the 16S gene based phylogeny showing a sister-taxon relationship between *Salmo* and *Oncorhynchus*, the 12S gene and combined analyses have demonstrated a closer relationship between *Salvelinus* and *Oncorhynchus* than between *Salmo* and *Oncorhynchus*. The relationship between *Salvelinus* and *Oncorhynchus* was also supported by previous molecular phylogeny studies based on three other genes (GH1C, VIT and ND3) (OAKLEY & PHILLIPS 1998, CRESPI & FULTON 2004).

In our study the monophyly of *Oncorhynchus* is supported by the individual genes and by the combined data set. Phylogenetic analysis of 16S rRNA shows that *Oncorhynchus* genus comprises three clades: ((*O. masou*, (*O. kisutch*, *O. tshawytscha*)), (*O. mykiss*, *O. clarcki henshawi*) and (*O. nerka*, (*O. gorbuscha*, *O. keta*)), showing some differences compared with previous findings based on the same marker (CRESPI *et al.* 2004). The positions that the *Oncorhynchus* species occupy in our trees are similar with those found in the previous studies of KITANO *et al.*

1997, MCKAY *et al.* 1996, MURATA *et al.* 1996, OOHARA *et al.* 1997, 1999, OLEINIK 2000, PHILLIPS *et al.* 1994, 1995, WESTRICH *et al.* 2002.

The phylogenetic analysis using mitochondrial ribosomal genes as markers has allowed for the classification of salmonid species from Romania within the Salmonidae family. Thus, Romanian *Salmo trutta fario* and *Salmo labrax* are placed together within the *Salmo* genus. The *Salmo labrax*, endemic in the Black Sea, appears to be the sister taxa of the sea trout *Salmo trutta trutta* from the northwest of Europe (Atlantic coast) and Baltic Sea.

The basal divergence in phylogenetic trees occupied by *Thymallus thymallus*, a primitive species, is in agreement with the taxonomic and evolutionary data. Unfortunately, the position of the *Salvelinus* genus relative to the *Salmo* and *Oncorhynchus* genera remains controversial. Our data reveal a possible sister-taxon relationship between *Oncorhynchus* and *Salvelinus* despite the fact that morphological data support a closer relationship between *Salmo* and *Oncorhynchus*, thus confirming earlier findings of OAKLEY and PHILLIPS (1998) and CRESPI *et al.* (2004). These findings might have interesting implications for understanding the evolution of salmonid life history, behavior and diversification. Therefore it is necessary to develop further investigations using other phylogenetic markers.

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