

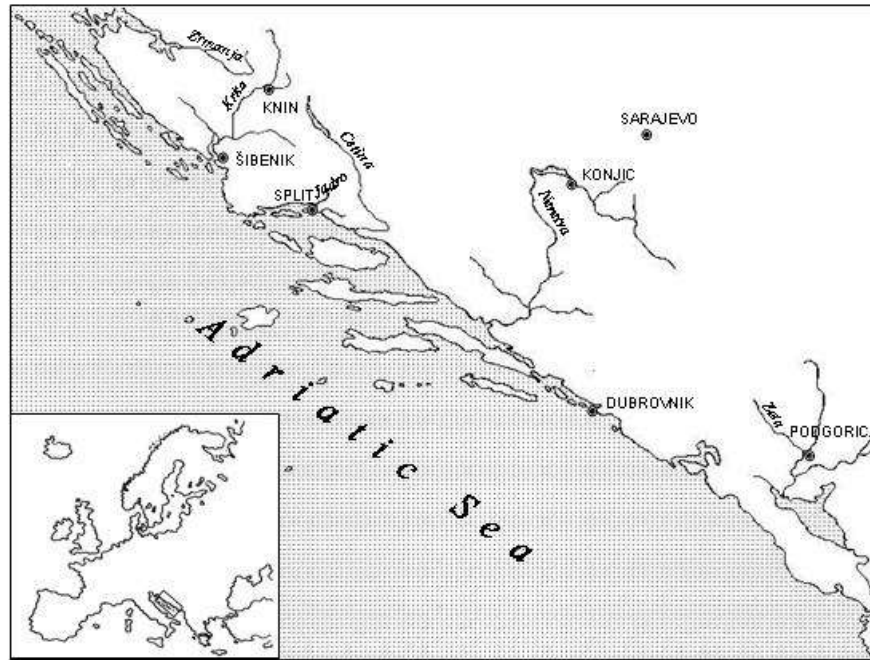


# The research on endemic soft-muzzled trout (*Salmo obtusirostris*)

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- the softmouth trout is endemic to the Adriatic river system of the western Balkans
- first described from the Rivers Zrmanja, Jadro and Vrljika as *Salar obtusirostris* (Heckel, 1851)





- the Balkan Peninsula has never been seriously affected by the Pleistocene glaciations and the climate impact on the living world survival was consequently low or null
- for this reason, it served as one of major refuges for diverse flora and fauna of central and northern Europe during the ice age
- this region still exhibits an extraordinary level of biodiversity constituted by both, incipient residents and fugitive newcomers
- the Adriatic river system, comprising the south-eastern part of the Balkan Peninsula, is extremely rich in fish fauna that includes genera with numerous important unresolved taxonomic problems, many endemic subspecies and species with little information on distribution and conservation





> In their external appearance, the Jadro softmouth trout show some resemblance to the brown trout.

- one of the most enigmatic fish species inhabiting the Adriatic river system seems to be the softmouth trout or soft-muzzled trout (*Salmo (Salmothymus) obtusirostris* Heckel, 1851)
- it is endemic only to few rivers of Balkan middle and southern part
- due to its extraordinary appearance, which makes it much different from other *Salmo* members, the softmouth trout was placed in a separate genus *Salmothymus*



- morphological differences characteristic for different softmouth trout populations gave rise to the description of three additional putative subspecies:
- *Salmo obtusirostris oxyrhynchus* (Steindachner, 1882) from the River [Neretva](#), Bosnia and Herzegovina
- *Salmo obtusirostris salonitana* (Karaman, 1927) from the river [Jadro](#), Croatia
- *Salmo obtusirostris krkensis* (Karaman, 1927) from the River [Krka](#), Croatia
- Softmouth trout-like salmonids from the River Zeta, Montenegro are also sometimes regarded as a subspecies of *Salmo obtusirostris* as *Salmo obtusirostris zetensis* (Hadžišće, 1961)



# Softmouth Trout From The River Krka – *Salmo obtusirostris krkensis*

- The main morphological trait, typical of the Krka softmouth trout is blunt snout, which gives it “unkind look”
- That’s why natives call it *zlousta* or “evil mouth” trout





› Krka softmouth trout – *S. obtusirostris* *krkensis* – male (above) and female (below).



- the Krka softmouth trout inhabit very restricted area of the upper part of the River Krka at the town of Knin, Croatia
- according to our observation, it is the most scarce and endangered taxon of the softmouth trout
- they sympatrically co-exist with brown trout





→ The mighty origin of the River Krka.





Typical Krka softmouth trout habitat now restricted to only few hundred meters

# Softmouth trout from the River Vrljika

## – *Salmo obtusirostris ssp.*

- The softmouth trout from the [River Vrljika](#) near the town of Imotski (Croatia) was first described by Heckel in 1851
- This was, as a matter of fact the only relevant information referring to the Vrljika softmouth trout
- According to Mrakovčić and Mišetić (1990) this population was considered extinct



› Vrljika softmouth trout – all the specimens caught exhibited characteristic golden shine, not observed in the other softmouth trout populations.





› Vrljika softmouth trout – *S. obtusirostris* ssp.  
Some individuals reach the weight of two kg  
and more.



› Vrljika softmouth trout – a close-up.

- inferred from molecular genetic data, the Vrljika softmouth trout seems to be most similar to the subspecies from the River Neretva although some autapomorphies do exist in the Vrljika population



# River Vrljika



› A fish-trap used by local poachers.

- › Clear calm water, sandy bottom and a lot of water plants make a perfect habitat for the softmouth trout.



# Softmouth Trout From The River Neretva - (*Salmo obtusirostris oxyrhynchus*)

- The [Neretva](#) softmouth trout shows the most pronounced morphological characteristics featuring the softmouth trout; all the other subspecies are more brown trout-like.



› Neretva softmouth trout – *S. obtusirostris oxyrhynchus*



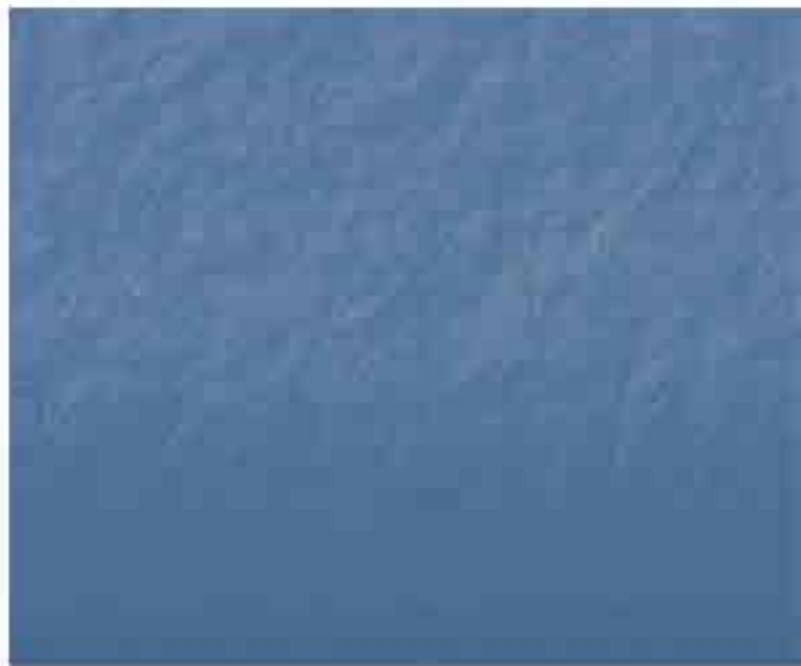
- On the basis of DNA analysis of several salmonid genera a close relationship between the softmouth and brown trout was established





1. Searching for "glavatica"...

Remote and hardly accessible part of the River Rakonca, the right tributary to the River Neretva.



# Softmouth Trout From The River Zeta –

## *Salmo obtusirostris zetenzis*

- This taxon has been less studied
- The general external features of the Zeta softmouth are more brown trout-like than any other subspecies of the softmouth trout
- Even its spawning time that is allegedly in December is different from other softmouth trout that spawn in spring
- It also shares some morphological similarities with grayling. Interestingly, local people call it “lipjen”, which is a derivate form of the word “lipljen” meaning grayling
- From the above mentioned it is evident that the taxonomy of this taxon is puzzling and still needs to be resolved
- These fish used to be abundant, however, due to intense poaching, their number has drastically declined, and according to some ichthyologists, the taxon is considered extinct





# Softmouth Trout From The River Jadro

## - *Salmo obtusirostris salonitana*

- There were raised doubts concerning the evolutionary origin and taxonomic identity of the Jadro softmouth trout
- In their external appearance, they show some resemblance to the brown trout from the Adriatic river basin
- In the light of these observations, we sought to evaluate the evolutionary origin of the softmouth trout from the River Jadro



> Jadro softmouth trout – *S. obtusirostris salonitana*.

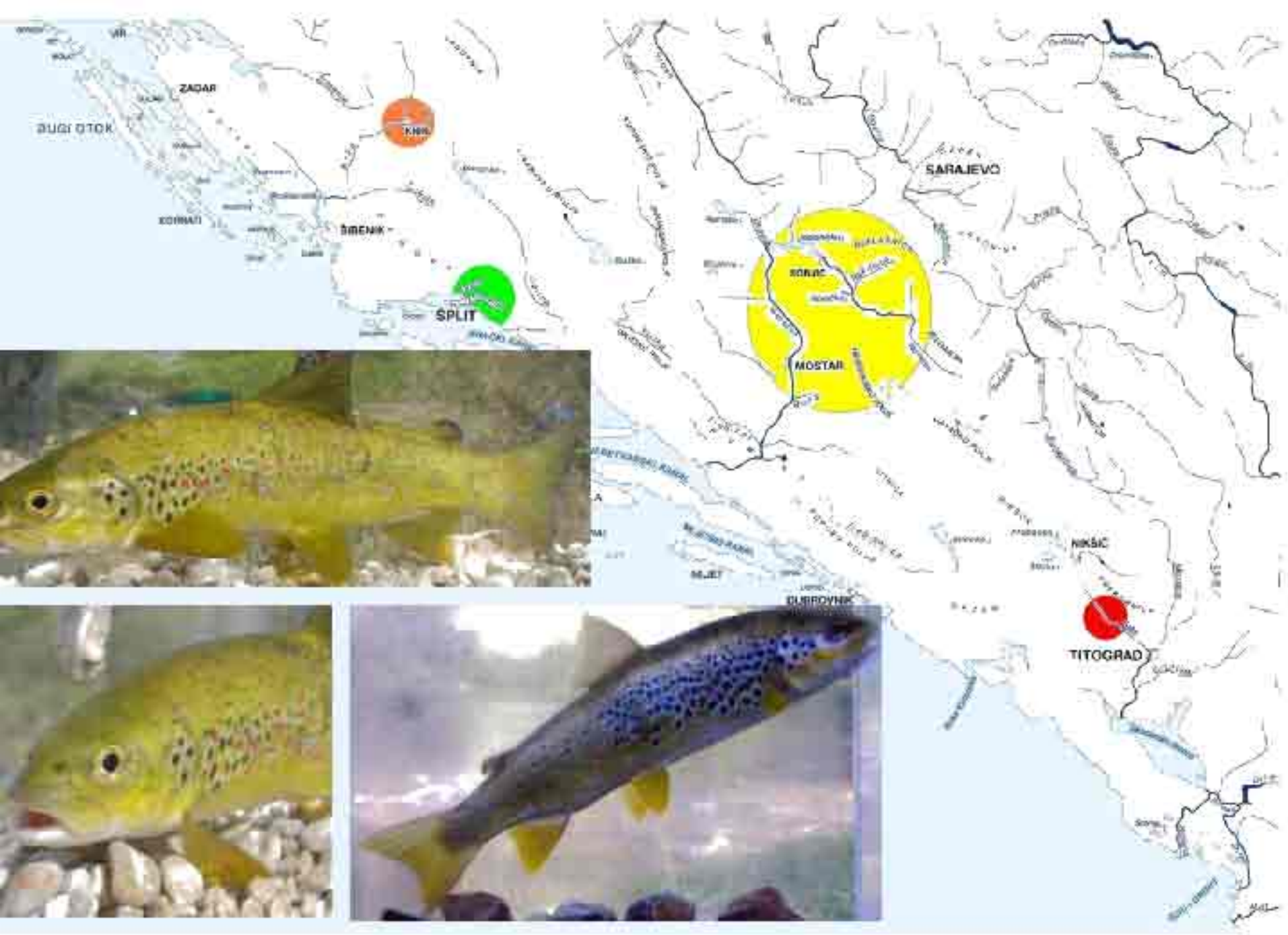


> Jadro softmouth trout – a close-up.

- small mouth with big lips
- tiny teeth
- small scales
- short jaws
- golden shine of a body
- tight dark dots behind gills
- light red dots till the end of the body
- unpigmented fins, except dorsal fin
- spring spawn







# The River Jadro

- about five kilometres long
- emptying into the Adriatic Sea near the ancient Roman town of Solin (formerly know as Salona) near Split
- The lower and middle part of the river is practically destroyed due to an operating cement factory and severe river bank regulation
- In contrast, the upper part of the river is well preserved and offers suitable habitat for a relatively large population of softmouth trout



> Upper part of the River Jadro is still densely inhabited by the softmouth trout.









# f

- electro-fishing for softmouth trout in the River Jadro was successful since fish were hiding in the grass and beneath rocks, where they could be easily reached with the anode of our electro-fishing apparatus
- this unexpected characteristic that is otherwise more typical for brown trout, along with some morphological resemblance with brown trout, raised some doubts concerning evolutionary origin and taxonomic identity of the Jadro softmouth trout



# The River Žrnovnica



- soft-muzzled trout was transferred into the Žrnovnica river about 30 years ago
- this river was temporal and so unsuitable for fish
- after building the Prančeviča dam at the other side of the karst mountain at which this river originates, it started to receive permanent water



# The aim of our work was:

- to apply mitochondrial and genomic DNA markers to molecularly define the subspecies *salonitana* in order to examine
  - (1) its evolutionary history
  - (2) its relationship to the subspecies *oxyrhynchus* and other adjacently located populations of the brown trout
- Clarification of these issues would in broader sense, help to understand better genealogy of the genus *Salmo*, especially in terms of impacts that historical events have had on it.

# Material and Methods

## *Samples and DNA extraction*

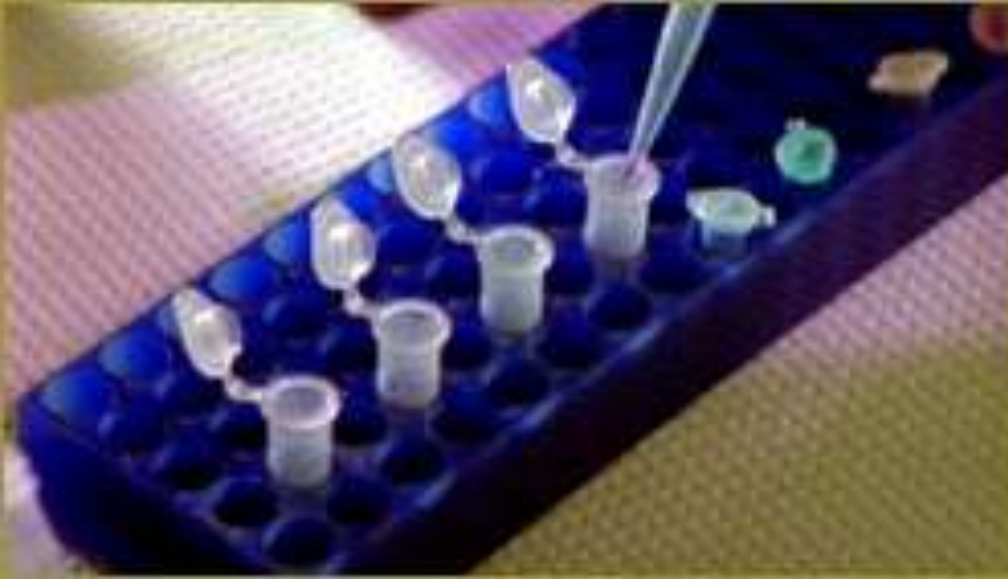
- Fishes were caught by electrofishing performed during three sampling campaigns in 1999, 2001 and 2002
- *S. o. salonitana* – Jadro (19)
- *S. o. salonitana* - Žrnovnica (29)
- *S. o. oxyrhynchus* – Neretva (7)
- *Salmo trutta* - Zrmanja (5)
- *Salmo trutta* - Krka (19)
- DNA sequences of other salmonid taxa (*S. salar* and different *S. trutta* haplotypes) used as a reference material were also included in the investigation was found in GenBank.





- Total DNA was isolated from fin clips, preserved in ethanol



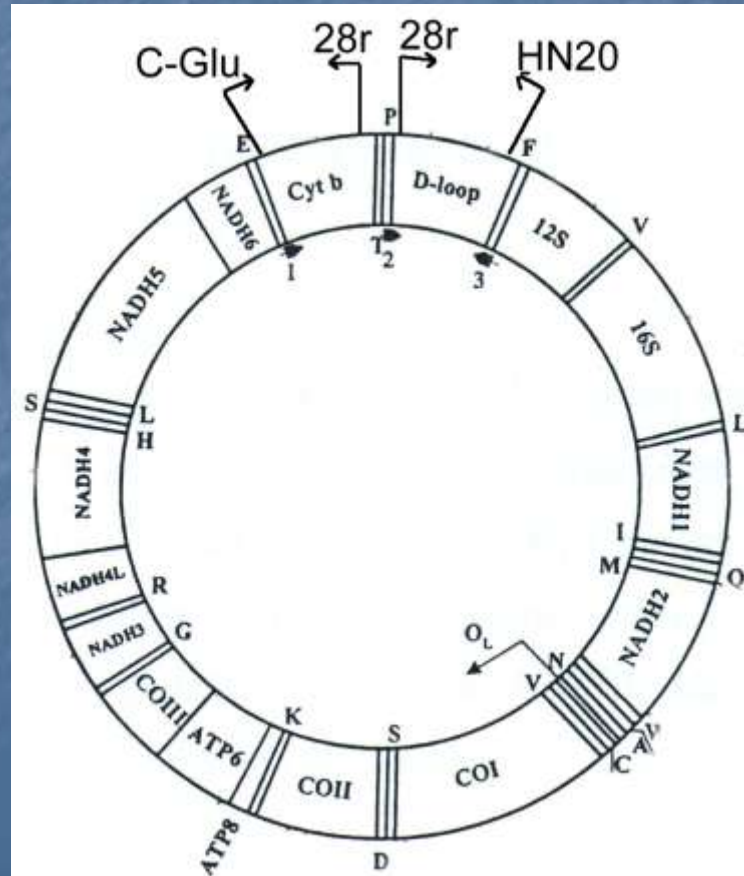


- For isolation of total DNA Wizard Genomic DNA Purification Kit (Promega) was used



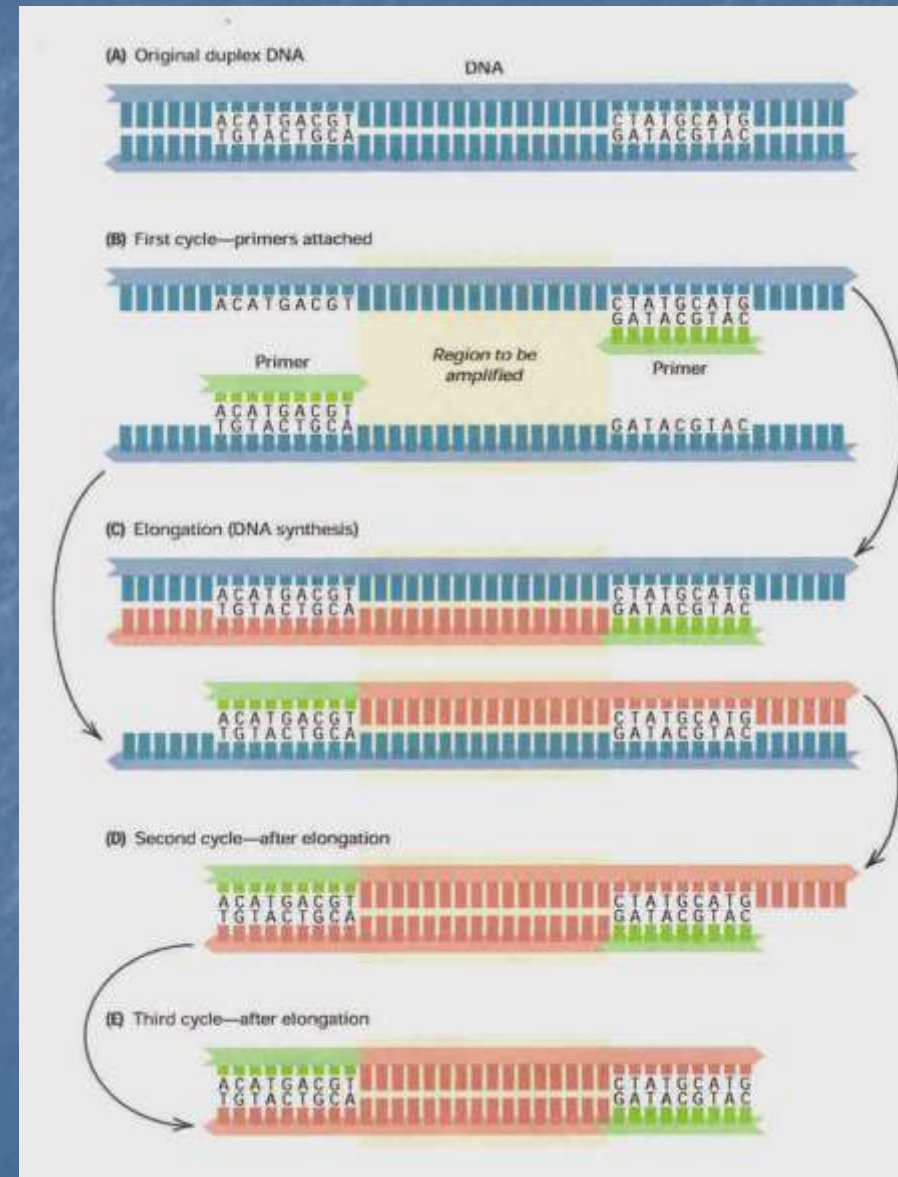
# *DNA amplification and sequencing*

- Comparative nucleotide analyses included the regions recognized as informative
  - mtDNA between the tRNA<sup>Pro</sup> gene and poly T block
  - mtDNA cytochrome *b* gene
  - lactate dehydrogenase gene

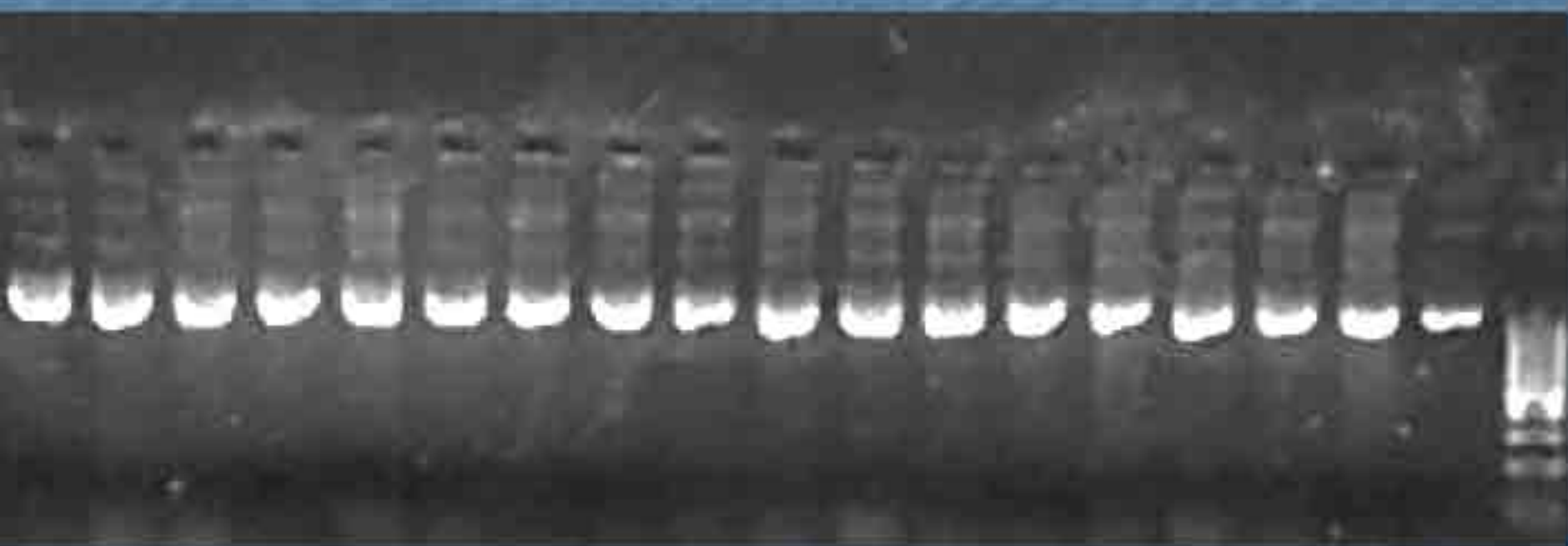


# PCR amplification

- PCR amplification of an ca. 2400 bp mtDNA fragment spanning the entire **cytochrome *b*** (Cyt *b*) gene and control region (CR) was performed using primers HN20 and C-Glu
- The **PCR conditions** were: initial DNA denaturation (95°C, 3 min) and 30 successive cycles of strand denaturation (94°C, 45 s), primer annealing (52°C, 45 s) and DNA extension (72°C, 2 min)
- Primers Ldhxon3F and Ldhxon4R were used for amplifying ca. 440 bp of the **LDH-C1\* gene**
- All DNA-amplifications were performed in a programmable thermocycler GeneAmp® PCR System 9700 (AB Applied Biosystems)

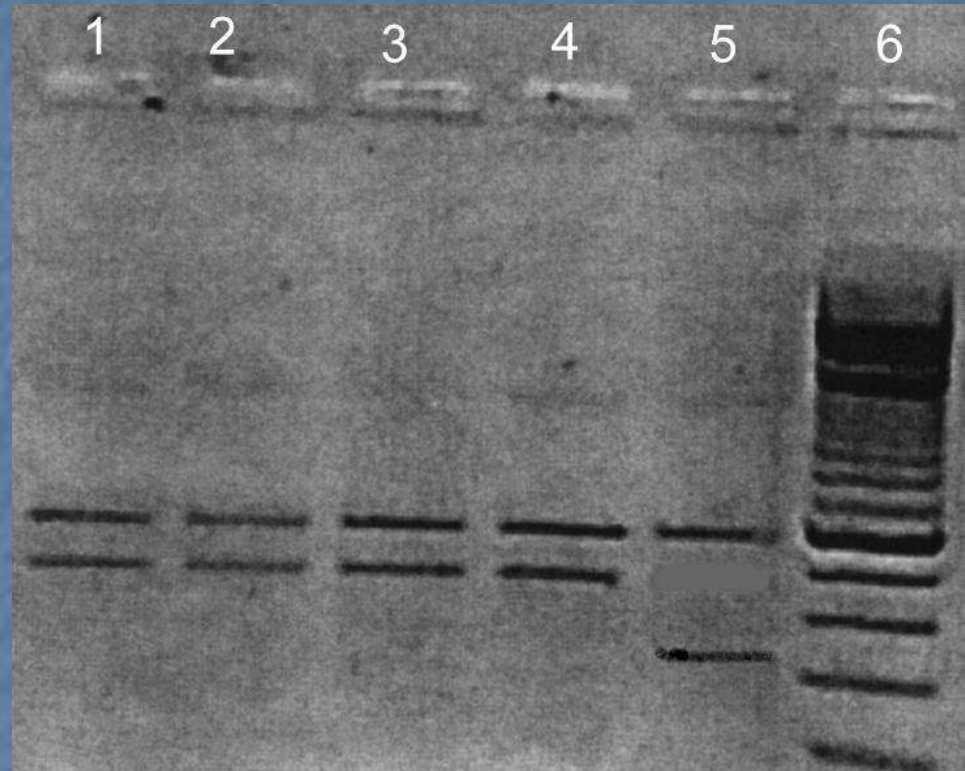






# PCR-RFLP

- Control region(1200 bp) – *A/ul*
- On the basis of nucleotide sequences obtained, restriction sites enabling differentiation between the softmouth and brown trout were established
- Restriction analysis of PCR-products was performed according to the enzyme supplier's instructions.



- 1 - *S. o. salonitana* – Jadro
- 2 - *S. o. salonitana* - Žrnovnica
- 3 - *Salmo trutta* - Krka
- 4 - *Salmo trutta* - Zrmanja
- 5 - *S. o. oxyrhynchus* – Neretva
- 6 - 100 bp DNA ladder, Gibco BRL



# Sequencing of control region

ZRM1  
ZRM2AD4  
Krka  
Ž. .

TTTTTCAGCTATGTACAATAACAACTGTTGTACCTTGCTAACCCAATGTTATACTACATC  
TTTTTCAGCTATGTACAATAACAACTGTTGTACCTTGCTAACCCAATGTTATACTACATC  
TTTTTCAGCTATGTACAATAACAACTGTTGTACCTTGCTAACCCAATGTTATACTACATC

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tranzicion T → C

	varijabilna mjesta												
	1	2	3	4	5	6	7	8	9	10	11	12	13
ZRM1	C	C	A	T	A	C	C	C	A	G	T	C	C
ZRM2AD4		T											
Krka	T	T						T					
ZRN		T		C			T	T					
Soxy		T	G		C	G	G	T	T	-	C	T	A

# Variable nucleotide positions of mtDNA control region

<i>Species/ lineage</i>		1	2	(3	3a	3a	4	(5	(6	(7	10	11	12	13	14	15	15	16	17	18
<i>(haplotypes)</i>				)	1			)	)	)		a		a			a			
<b>S. salonitana</b>	<b>Ad11</b>	<b>T</b>	<b>C</b>	<b>C</b>	<b>T</b>	<b>A</b>	<b>/</b>	<b>G</b>	<b>C</b>	<b>A</b>	<b>T</b>	<b>A</b>	<b>T</b>	<b>T</b>	<b>A</b>	<b>G</b>	<b>T</b>	<b>C</b>	<b>C</b>	<b>C</b>
<b>S. trutta</b>	<b>Ad1</b>	-	-	-	-	-	/	-	T	-	-	-	C	-	-	-	-	-	-	-
	<b>Ad3</b>	-	-	T	-	-	/	-	T	-	-	-	C	-	-	-	-	-	-	-
	<b>Ad4</b>	-	-	-	-	-	/	-	T	-	-	-	C	C	-	-	-	-	-	-
	<b>Ad12</b>	-	-	-	C	-	/	-	T	-	-	-	C	C	-	-	-	-	-	-
	<b>Ad13</b>	-	T	-	-	-	/	-	T	-	-	-	C	-	-	-	-	-	-	-
	<b>Ma1</b>	-	-	-	-	-	A	-	T	-	-	-	A	-	-	-	-	-	-	-
	<b>Me1</b>	-	-	-	-	-	/	A	T	C	-	-	G	-	-	-	-	-	-	-
	<b>Da1</b>	C	A	-	-	-	/	-	T	-	G	-	G	-	-	-	-	-	-	T
	<b>At1</b>	-	T	-	-	-	/	-	T	-	-	-	G	-	-	-	C	T	-	T
<b>S. obtusirostris</b>		-	-	-	-	G	/	-	T	-	-	C	G	-	T	C	-	/	A	-



- Mitochondrial DNA (410 bp of the control region) sequence analysis of the softmouth trout from the River Jadro revealed a new haplotype, which has not been described before
- HAPLOTYPE **ZRN**

TTTTTCAGCT	ATGTACAATA	CAACTGTTG	TACCTTGCTA	ACCCAATGTT	ATACTACATC
TATGTATAAT	ATTACATAAT	ATGTATTTAC	CCATATATAT	AATATAGCAT	G-TGAGTAGT
ACATCATATG	TATTATCAAC	ATTAGTGAAT	TTAACCCCTC	ATACATCAGC	ACTAACCCAA
GGTTTACATA	AAGCAAAACA	CGTGATAATA	ACCAACTAAG	TTGCTTAAAC	CCGATTAATT
GTATATCAA	TAAAACTCCA	<u>T</u> CTAACACGG	GCTCCGTCTT	TACCCACCAA	CT-TTCAGCA
TCAGTCCTGC	TTAATGTAGT	AAGAACCGAC	CAACGATATA	TCAGTAGGCA	TACTCTTATT
GATGGTCAGG	GACAGATATC	GTATTAGGTC	GCATCTCGTG	AACTATTCCT	GG

- On the basis of novel mutation at the position 278 an appropriate restriction enzyme (*Eco130I*) was selected and used for diagnostic PCR-RFLP analysis
- All the samples surveyed from the River Jadro, either by sequencing or by restriction analysis, exhibited the same haplotype



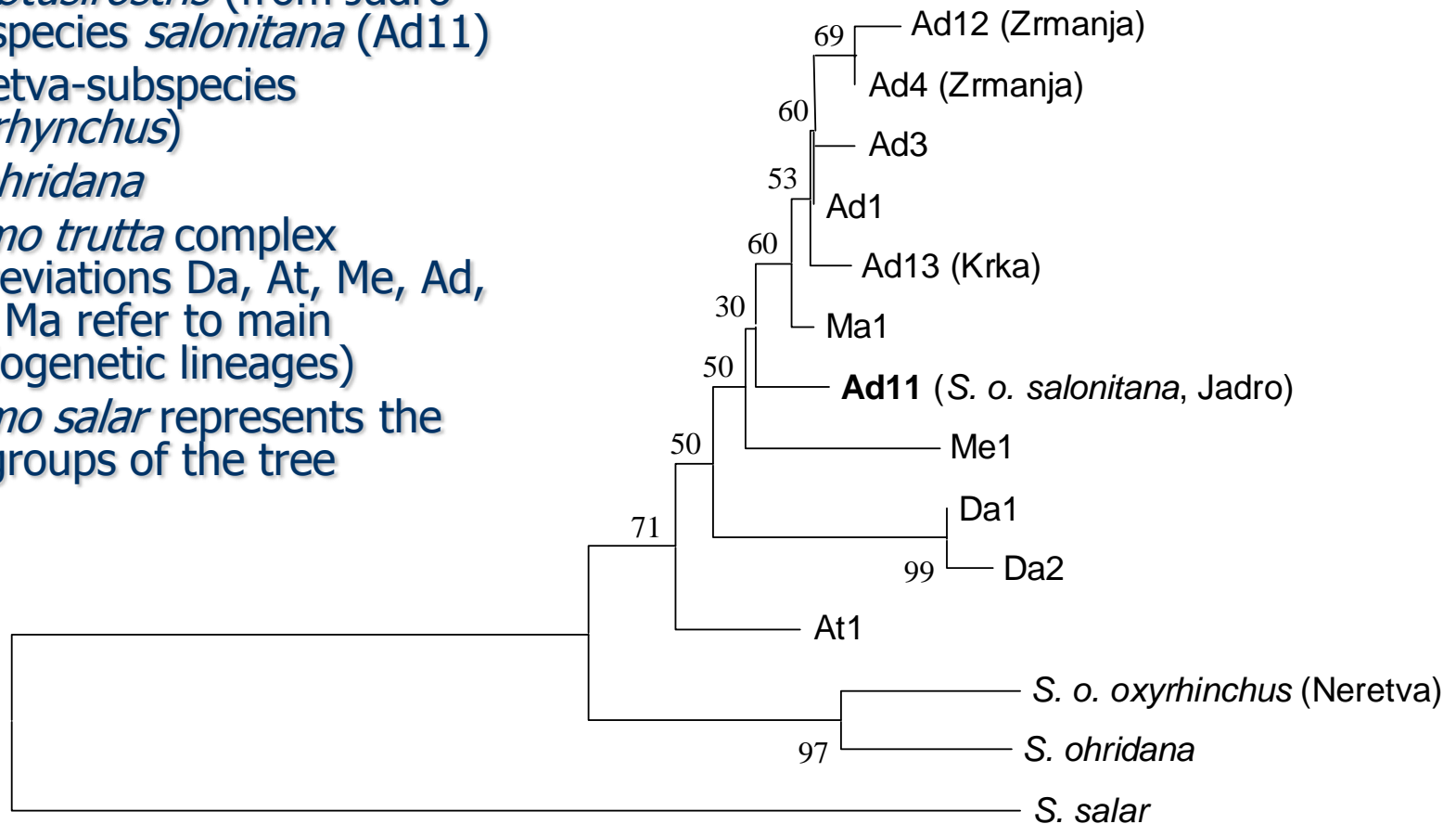
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- 2 - *S. o. salonitana* - Žrnovnica
- 3 - *Salmo trutta* - Krka
- 4 - *Salmo trutta* - Zrmanja
- 5 - *S. o. oxyrhynchus* – Neretva
- 6 - 1 kb DNA ladder, Gibco BRL



- In the River Zrmanja, two mtDNA haplotypes were detected in the brown trout:
  - Ad4, previously described in Bernatchez (2001)
  - and a newly discovered haplotype Ad12
- In the River Krka, a single haplotype, not yet described in the brown trout, was found Ad13

- Neighbour-Joining tree, based on combined data set of mtDNA control region and cytochrome *b* gene

- *S. obtusirostris* (from Jadro-subspecies *salonitana* (Ad11))
- Neretva-subspecies *oxyrhynchus*)
- *S. ohridana*
- *Salmo trutta* complex (abbreviations Da, At, Me, Ad, and Ma refer to main phylogenetic lineages)
- *Salmo salar* represents the outgroups of the tree

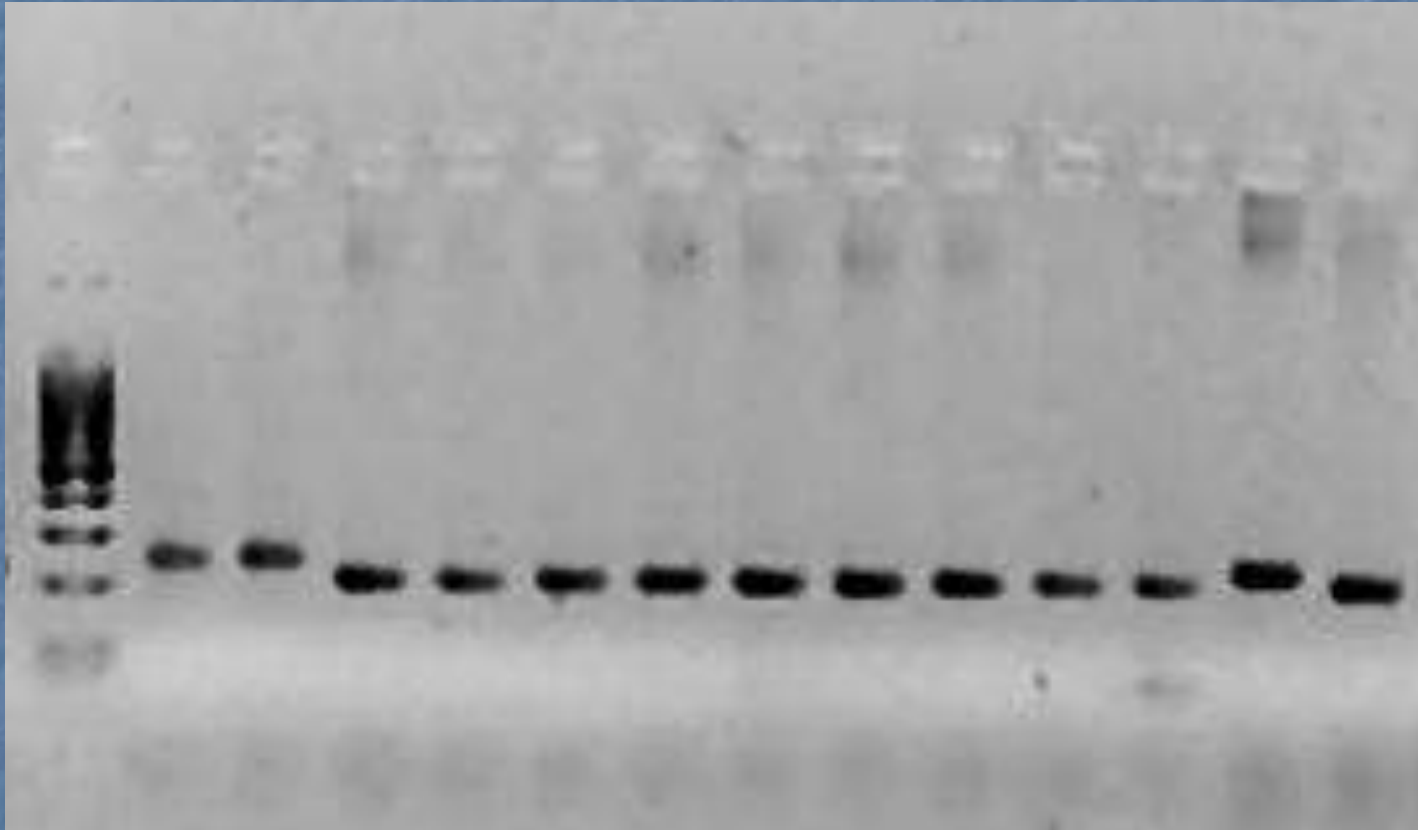




# Microsatellites

Ime lokusa	Ponavljajući	.....	.....	.....
BFRO 001	(TG) <sub>13</sub> (AG) <sub>4</sub> (TG) <sub>2</sub> - CATGTGCGAC- (TG) <sub>12</sub>	22	200-256	5' ATGTTTTTGGACTGCACTATGTATT 3' 5' CTTACAGCCACCTTTATGCG 3'
BFRO 002	(TG) <sub>14</sub>	3	116-124	5' ATGTTTTTGGACTGCACTATGTATT 3' 5'GGAGATAAGAGTCAACGAGGC 3'
Ssa 197	(GTGA)+(GT)	18	107-177	5' GGGTTGAGTAGGGAGGCTTA 3' 5' TGGCAGGGATTTGACATAAC 3'
Strutta 58	GT	38	102-190	5' AACAATGACTTTTCTCTGAC 3' 5' AAGGACTTGAAGGACGAC 3'
Str 591 INRA	CT	22	146-198	5' CTGGTGGCAGGATTTGA 3' 5' CACTGTCTTTCGTTCTT 3'
Strutta 24	GT	20	171-237	5' ATGTTTTTGGACTGCACTATGTATT 3' 5'GGAGATAAGAGTCAACGAGGC 3'
OmyFgt1TUF	GT	27	187-263	5'-AGATTTACCCAGCCAGGTAG-3' 5'-CATAGTCTGAACAGGGACAG-3'
SsoSL438	GT	7	103-115	5' GACAACACACAACCAAGGCAC 3' 5' TTATGCTAGGTCTTTATGCATTGT 3'
StMS-LDH4	GT	9	102-127	5' TCATCAAACACTCCCCCAACTGC 3' 5'-CATAGTCTGAACAGGGACAG-3'

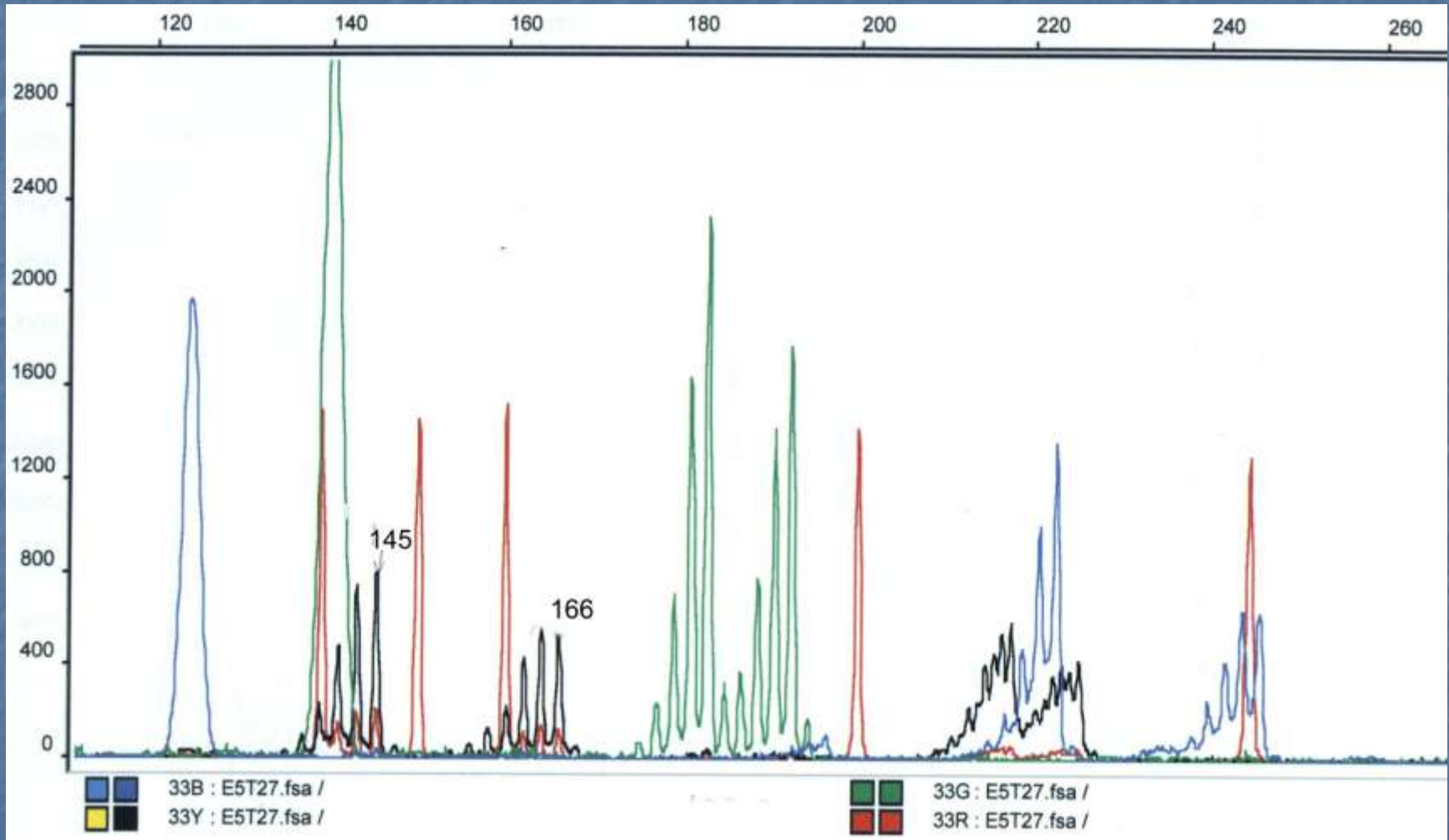
# PCR



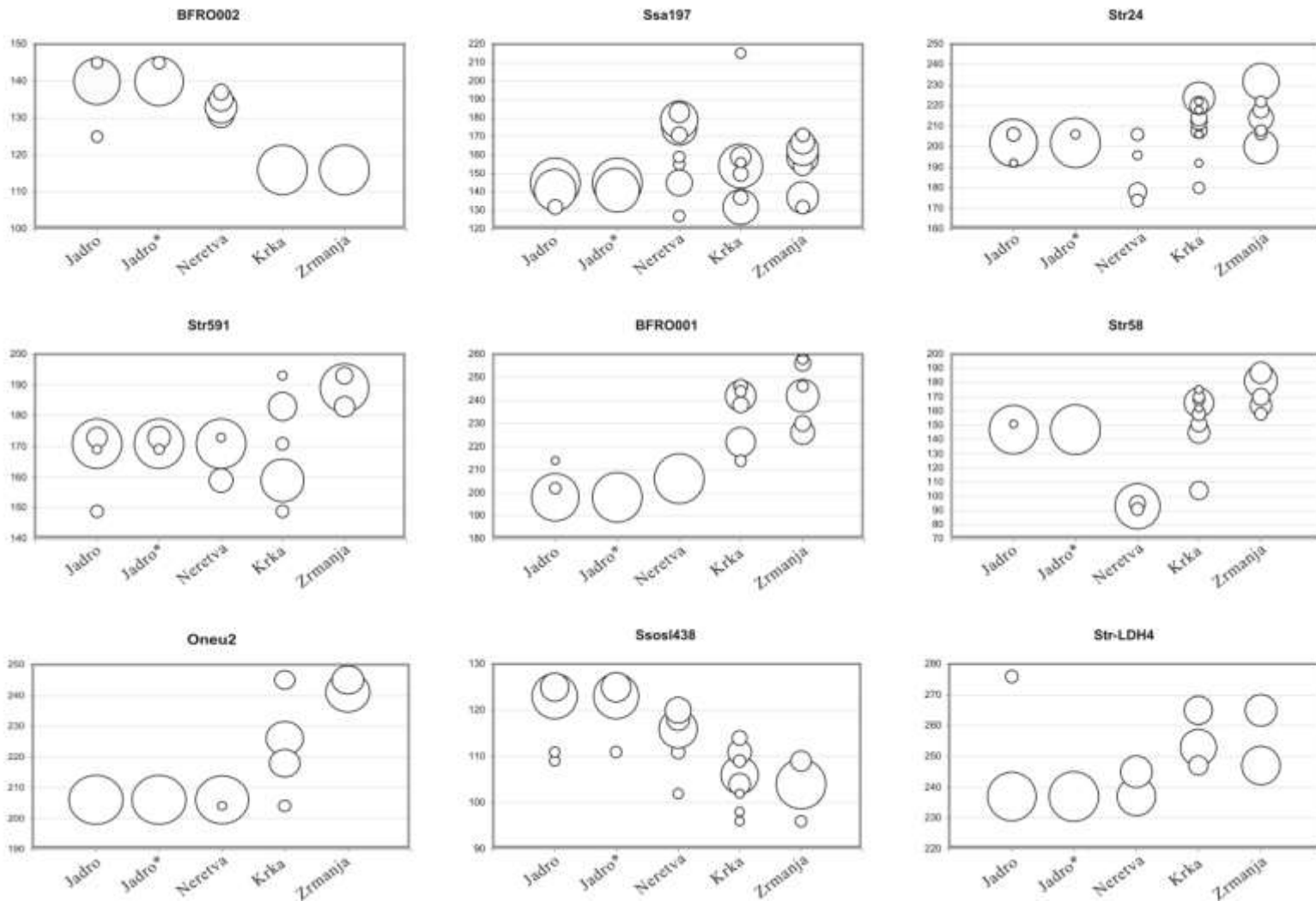
Microsatellite OmyFgt1TUF



# Analysis of microsatellites loci on ABI Prism 310

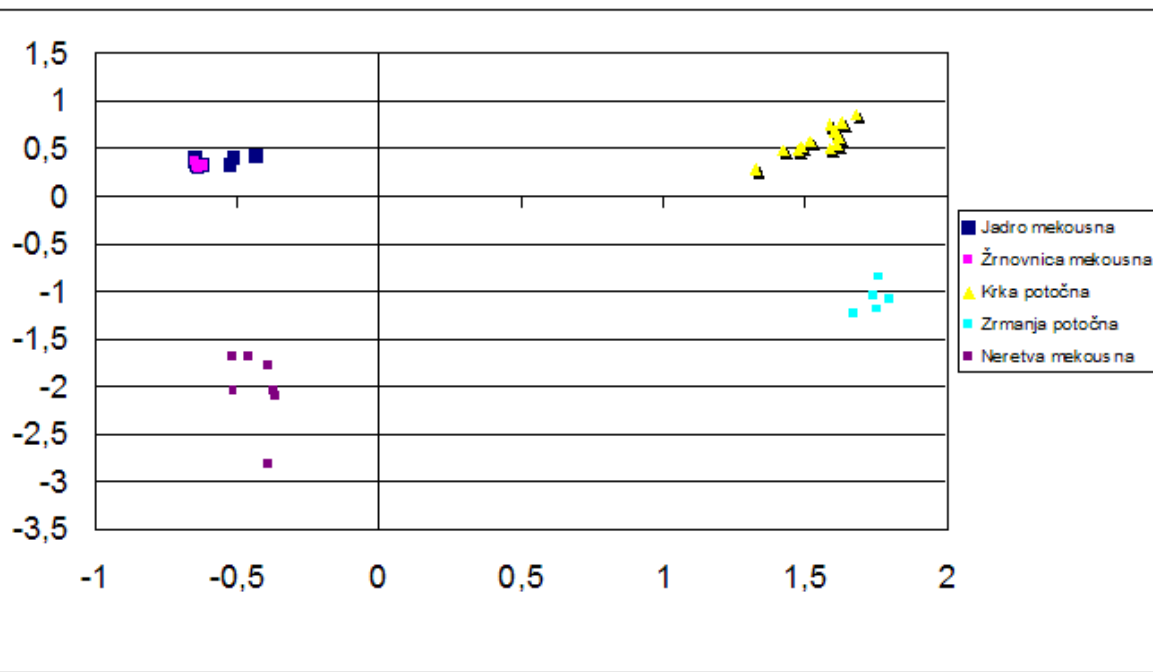


- Allele frequencies and size distribution of nine microsatellite loci in *Salmo sp.*
- Area of bubbles correspond to frequencies of the respective alleles in given population





- Diagram showing the genetic relationships among *S. obtusirostris* (from Jadro-subspecies *salonitana*, Neretva-subspecies *oxyrhynchus*) and *S. trutta* (from Krka and Zrmanja) individuals, based on **factorial correspondence analysis**



FCA clearly demonstrates four genetic groupings, corresponding to the four taxa analyzed:

- (1) *salonitana*
- (2) *oxyrhynchus*
- brown trout from the Rivers (3) Krka
- brown trout (4) Zrmanja



## **Origin of *salonitana***

- our results indicate that *S. o. salonitana* is likely to be a **hybrid taxon** that evolved from natural hybridization between the brown trout and the softmouth trout
- this view is based on the data revealing that the subspecies *salonitana* exhibits mtDNA haplotype, which is indicative of the Adriatic phylogeographic lineage of the brown trout
- on the other hand, several nuclear genes, that have been examined in *salonitana* (i.e., microsatellites, LDH), were recognized as characteristic of the softmouth trout subspecies *oxyrhynchus*



- a great deal of common alleles between the specimens originating from the River Jadro and the brown trout specimens originating from the River Krka was found as well
- this allele sharing was in the River Jadro entirely constrained to only three specimens (J13, J15 and J17) and was observed on eight microsatellite loci out of nine examined
- the common alleles always appeared in a heterozygous state together with the alleles, characteristic of *salonitana*
- these evidences indicate that the three specimens probably resulted from **recent hybridization** between *salonitana* and the brown trout
- no alleles were actually found to be in common with *salonitana* and the brown trout

# *Paleoenvironmental background*

- During the glacial periods, sea-level was about 120 m below the current level
- This resulted in novel freshwater connections between the rivers that had previously discharged directly into the Adriatic Sea creating the so-called “freshwater bridge” between freshwater allopatric taxa
- We presume that this paleohistorical change of hydrographic network of the Adriatic river system caused a break down of geographic isolation between *S. obtusirostris* and *S. trutta* and enabled their temporary contact and gene flow in the River Jadro



# *Hybridisation*

- Physiologically and genetically successful hybridization between *S. trutta* and *S. obtusirostris* has been already proved
- The three recent hybrids (J13, J15 in J 17) detected in the River Jadro using molecular approach, indicate that natural hybridization between *S. obtusirostris* and *S. trutta* also takes place
- The gap between the spawning seasons of both species obviously does not represent an efficient inter-specific reproductive barrier
- It can be speculated therefore, that in natural habitats of *salonitana*, a certain selective pressure against *S. trutta* and the hybrids might exist

- two main issues can be inferred from the evidence indicating a recent hybridization between *salonitana* and *S. trutta*
  - this is a practical proof indicating that such or similar hybridization event could have readily occurred also in the past and might have represented a decisive evolutionary process that caused a formation of the new taxon
  - the possibility of hybridization between *salonitana* and *S. trutta* must be seriously considered



# Future management:

- any stocking activities of non-native fishes into the *salonitana* habitats must be strictly avoided
- catch and relise fishing
- conservation of natural area
- control breeding of softmuzzled trout and put beck the offspring
- translocation part of population