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DNA phylogeny supports revised classification of *Salmothymus obtusirostris*

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Salmothymus obtusirostris (soft-muzzled trout) is endemic to the South Adriatic drainage. Owing to its unusual appearance, which resembles both trout and grayling, it has been initially classified as a separate genus. However, this classification is ambiguous and has never been firmly established. We have studied mtDNA (control region and cytochrome *b* gene) and nuclear DNA (a part of LDH C*1 gene) variation between soft-muzzled trout from the upper part of the River Neretva, Bosnia and Herzegovina, and other salmonid representatives in order to examine how the current classification is congruent with molecular data. On the basis of sequence identity of mtDNA control region among several genera (i.e. *Salmo*, *Oncorhynchus*, *Salvelinus*, *Acantholingua*, *Brachymystax*, *Thymallus* and *Coregonus*) a close relationship between *Salmothymus*, *Salmo* and *Acantholingua* was established. Phylogenetic analysis on a combined data set of mitochondrial and nuclear DNA, supported by 100% bootstraping, indicated that *S. obtusirostris* and *A. ohridana* are sister taxa which exhibit a closer relationship to *S. trutta* than to *S. salar*. This finding refutes the current classification, which recognizes *S. obtusirostris* as separate genus, and instead suggests its reclassification on the species level as *Salmo obtusirostris*. *Biological Journal of the Linnean Society*, 2002, **77**, 399–411.

ADDITIONAL KEYWORDS: Acantholingua – archaic trout – brown trout – evolution – River Neretva – Salmo – soft-muzzled trout – taxonomy.

INTRODUCTION

The classification of the Salmonidae family is only partially established and the general consensus regarding the taxonomic status, recently confirmed by molecular data analyses (Oakley & Phillips, 1999; Shed'ko, 2002), has been reached only for well-studied genera (i.e. *Oncorhynchus*, *Salmo*, *Salvelinus*, *Brachymystax* and *Hucho*; Phillips & Oakley, 1997). Other genera, especially those inhabiting remote places in Asia and the Balkan Peninsula (i.e. *Platysalmo*, *Acantholingua*, *Salmothymus*), have been poorly studied and the available data are limited. This makes their classification and phylogenetic positions unclear (Stearley & Smith, 1993; Shed'ko *et al*., 1996; Oakley & Phillips, 1999; Osinov, 1999; Osinov & Lebedev, 2000).

Salmothymus obtusirosrtis Heckel, 1851 (Crivelli, 1996; Kottelat, 1997) is certainly one of the most intriguing genera whose classification has been controversial since the pioneering studies on systematics of Balkan salmonids in the mid nineteenth century, up to modern times. *S. obtusirostris* is characterized by its striking similarity in appearance to both trout and grayling (Fig. 1). It is morphologically primitive and

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as such considered as 'archaic' trout (Stearley & Smith, 1993). It exhibits a range of morphological particularities (Fig. 1) among which the most evident are the elongated muzzle, small, fleshy mouth and the cranial bone convex in the orbital region (for a detailed morphological description see Karaman, 1927; Janković, 1961; Schöffmann, 1991; Stearley & Smith, 1993; Wilson & Li, 1999).

Its native range is restricted to few Adriatic rivers, the Krka, Jadro, Žrnovnica and Cetina in Croatia (Mrakovčić, Mietić, & Povž, 1995; T. Treer, Faculty of Agriculture University of Zagreb, personal communication; authors' personal observation), the Neretva with its tributaries in Bosnia and Herzegovina (Mrakovčić *et al*., 1995; authors' personal observation) and the Zeta in Montenegro (Marić, 1995; Fig. 2). It is worth mentioning that several local ichthyologists precisely surveyed and described spatially separated populations. On the basis of their observations, they identified morphologically different, but doubtfully valid subspecies called *salonitana*, *krkensis*, *oxyrhynchus* and *zetensis* (Mrakovčić & Mišetić, 1990).

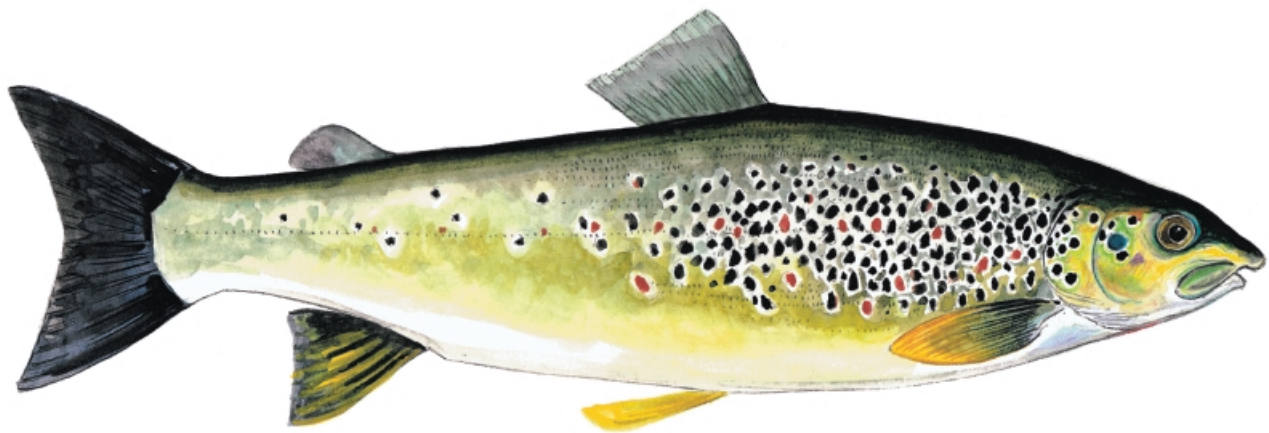

Figure 1. Soft-muzzled trout *Salmothymus obtusirostris oxyrhynchus* from the River Neretva; the mature fish, length approximately 36 cm. (Painted by James Prosek.)

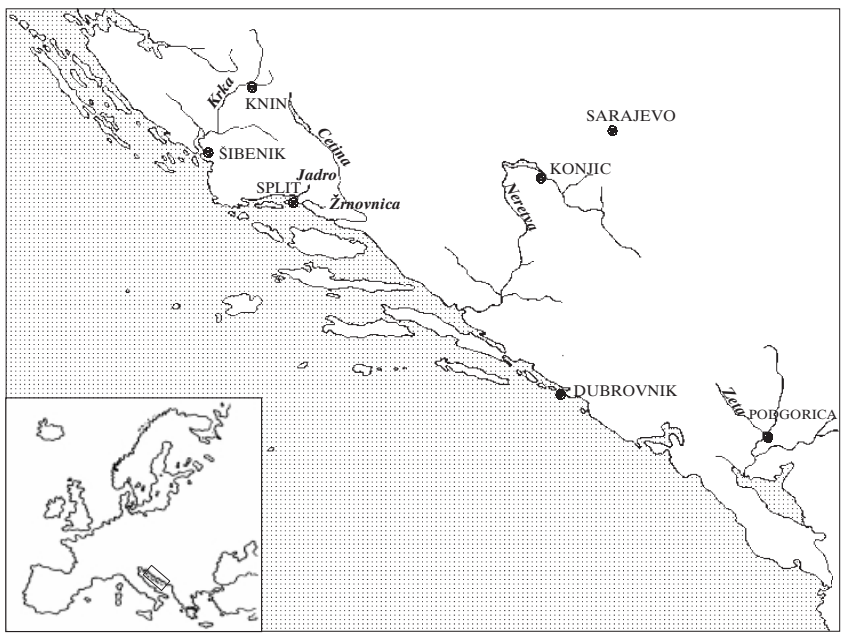

Figure 2. The native range of *Salmothymus obtusirostris* is restricted to the rivers Krka, Jadro and Žrnovnica, Cetina (Croatia), Neretva (Bosnia and Hercegovina) and Zeta (Montenegro).

There is no consensus regarding the common name of *S. obtusirostris* in English; sometimes it is designated as Adriatic salmon or Adriatic trout (FishBase, 1997), Dalmatian trout (Stearley & Smith, 1993) or soft-muzzled trout (Janković, 1961). The later designation is a direct translation from Croatian original (= mekousna pastrva), which reflects its main morphological characteristic. That is why we feel this name is most appropriate and we use it throughout the text.

Soft-muzzled trout was first described by Heckel (1851) and named as *Salar obtusirostris*. Owing to morphological peculiarities, its position was later rearranged on numerous occasions. According to Steindachner (1874), it was first regarded as a member of *Thymallus* (i.e. *Thymallus microlepis*) but later placed in *Salmo*, designated as *Salmo obtusirostris* (Steindachner, 1882; Behnke, 1968). A generic name *Salmothymus* was first introduced by Berg (1908). This genus was primarily created not only for the soft-muzzled trout but also for an apparently related salmonid, endemic to Ohrid Lake, called *belvica* (pronounced: bealvitsah) in the Macedonian language (*Acantholingua* (*Salmothymus*) *ohridana*; Steindachner, 1892; Berg, 1908). Hadžišèe (1961) disagreed with this classification and on the basis of morphological evidence suggested that *belvica* might be recognized as a new genus, *Acantholingua*. This classification has been disputed by other investigators (reviewed by Phillips & Oakley, 1997). According to detailed morphological description and ecological studies performed by Janković (1961), clear differences in a whole range of characters analysed were observed between the soft-muzzled trout and *belvica*. In contrast, as inferred from cladistic analysis of osteological characters (Stearley & Smith, 1993; Wilson & Li, 1999), they were described as close relatives and classified as an intermediate clade between graylings and advanced salmonids. Regardless, all the arguments were based only on morphological analyses providing contradictory results, and the relationship between *S. obtusirostris* and *A. ohridana* is still uncertain. Nevertheless, phylogenetic analysis of *belvica* inferred from molecular data has already been performed and, as a result of these studies, this species has been included within *Salmo* (Oakley & Phillips, 1999; R. A. Duguid, Queen's University, Belfast, personal communication; Phillips *et al*., 2000).

On the basis of its apparent external and morphological resemblance to the Asiatic salmonid *Brachymystax lenok*, *S. obtusirostris* was considered also as an intermediate evolutionary form between *B. lenok* and *Salmo trutta* (Berg, 1908; Janković, 1961). According to the phylogenetic hypothesis proposed by Dorofeyeva (1989), the genus *Salmothymus* was not related to *Brachymystax* and was placed on the same branch with *Salmo*.

It is evident from previously performed studies that the taxonomic status of *S. obtusirostris* is not yet clear and that the morphology is neither a sufficient nor a reliable criterion for evaluating and appraising its phylogeny. Therefore, the main objective of our investigation was to study the molecular phylogeny of *S. obtusirostris* in order to examine how its current classification is congruent with molecular data and to determine its relationship with other taxa within the Salmonidae family. In this regard, we also intended to reconsider the question of relatedness between *S. obtusirostris* and *A. ohridana*.

We first focused on analysis of the mtDNA, a well-established and highly informative experimental genetic system for reconstructing phylogenetic history. However, the mtDNA molecule may not always reflect the evolution of the nuclear genome. Deviation from the real situation may be a consequence of homoplasy and a low effective population size caused by maternal pattern of inheritance. To avoid 'one-character taxonomy' and to confirm the credibility of the results obtained by mtDNA analysis, we included also a nuclear DNA marker. As an experimental system, we chose a genomic fragment of the lactate dehydrogenase (LDH) C1* gene containing parts of exons 3 and 4 with intermediate intron which has been, particularly in brown trout, already proven as an informative marker at the protein level (Allendorf *et al*., 1977) and for which a nucleotide sequence has been recently determined (McMeel, Hoey & Ferguson, 2001). For both experimental systems, a nucleotide sequence of *S. obtusirostris* was determined and compared to nucleotide sequences of the appropriate DNA fragments belonging to different taxa of the Salmonidae family.

MATERIAL AND METHODS

Sample collection and DNA extraction

Several salmonid genera and the main phylogenetic assemblages of *S. trutta* described in Bernatchez, Guyomard & Bonhomme (1992; Atlantic (At), Danubian (Da), *marmoratus* (Ma), Adriatic (Ad) and Mediterranean (Me)) were included in the investigation (Table 1).

Soft-muzzled trouts were sampled in the upper part of the River Neretva (Fig. 2). The origins of *S. trutta* and *S. marmoratus* populations have been described in Snoj *et al*. (2000); the additional samples, representing other salmonid genera, originate from different Slovenian hatcheries except for *belvica*, which derives from Ohrid Lake, Macedonia.

Total DNA was obtained from blood or fin tissue following the protocol of Medrano, Aasen & Sharrow (1990).

Table 1. Sample description: species name, number of individuals analysed (*N*) and GenBank Accession numbers for mtDNA control region, cytochrome b gene and LDH-C* gene

	mtDNA control region		LDH-C1* gene		Cytochrome *b* gene	
Species/population	*N*	Acc. No.	*N*	Acc. No.	*N*	Acc. No.
Salmo trutta						
Danubian (Da)	*	AF498758	6	AF488538	**	X76252
Adriatic (Ad)	*	AF498756	1	AF488541	**	X76251
Atlantic (At)	*	AF498757	3	AF488539	**	X76254
Mediterranean (Me)	**	AF25347	–		–	
S. marmoratus (Ma)	*	AF498755	6	AF488537	**	X76251
S. salar	**	U12143	1	AF488545	**	X76253
Salmothymus obtusirostris	22	AF488535	10	AF488540	10	AF488534
Acantholingua ohridana	2	AF488536	1	AF488546	1	AAF25872
Brachymystax lenok	**	AF125519	–		–	
Oncorhynchus mykiss	**	M81755	1	AF488542	**	L29771
O. keta	**	AB039890	–		–	
Salvelinus fontinalis	**	AF297987	1	AF488543	**	D58399
S. alpinus	**	AF298052	–		–	
Coregonus lavaretus	**	AB034824	–		–	
C. albula		AF192552	–		–	
Thymallus thymallus	*	AF329989	1	AF488544	–	
T. arcticus	*	AF329990	–		–	

*Data obtained previously in our laboratory; **data from the GenBank.

DNA AMPLIFICATION, SEQUENCING AND SEQUENCE ANALYSIS

PCR amplification of an approximately 2400 bp mtDNA fragment composed of cytochrome *b* gene and control region was performed using primers HN20 (Bernatchez & Danzmann, 1993) and C-Glu (Cronin, Spearman & Wilmot, 1993). The conditions of PCR were: initial denaturation (95°C, 3 min) followed by 30 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 (McMeel *et al*., 2001) were utilized for DNA amplification of an approximately 440-bp-long fragment of lactate dehydrogenase (LDH)-C1* gene, comprised of 43 bp of exon 3, 77 bp of exon 4 and intron 3 of variable length. The following thermal profile was used: denaturation (95°C, 3 min) followed by 30 cycles of strand denaturation (94°C, 30 s), primer annealing (62°C, 30 s) and DNA extension (72°C, 1 min).

All PCR amplifications were performed in a programmable thermocycler GeneAmp PCR System 9700 (AB Applied Biosystems). A total volume of 30 μL contained 1 μM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1 × PCR buffer, 1 U of *Taq* polymerase (PE Applied biosystems) and 100 ng of genomic DNA. Amplified DNA fragments were run on a 1.5% agarose gel and were isolated from the gel using QIAEX II Gel Extraction Kit (QIAGEN).

All sequencing reactions were prepared using BigDye Terminator Ready Reaction Mix (PE Applied Biosystems) according to the manufacturer's recommendations. The sequence of the entire mtDNA control region of *S. obtusirostris* was determined using seven primers (28RIBa (Sušnik, Snoj & Dovč, 2001), vHF-F: 5′-TCC GTC TTT ACC CAC CAA CT-3′, HF: 5′-CCT GAA GTA GGA ACC AGA TG-3′, vCSB3-F: 5′-AAG CCG GGC GTT CT(CT) TTA TA-3′, CSB3-F: 5′-CTT ATA TTC CTG TCA AAC C-3′, RCSB3-R: 5′-CGC TGA TTT GAG ACT TCC T-3′ and HN20; Appendix). The sequencing primer at the 5′-end of cytochrome *b* gene was C-Glu (Cronin *et al*., 1993).

Sequence of the LDH-C1* gene fragment was determined using Ldhxon3R primer (McMeel *et al*., 2001). Termination PCR reactions were performed on a programmable thermocycler GeneAmp PCR System 9700 (AB Applied Biosystems) under the following conditions: 10 s denaturation at 96°C, 5 s annealing at 50°C and 4 min extension at 60°C, repeated for 30 cycles. The amplified, fluorescently labelled and terminated DNA was salt-precipitated and analysed on the ABI Prism 310 automated sequencer.

For intergeneric phylogenetic analysis, the 5′-end of the control region and a fragment of the LDH-C1* gene were used considering sequences from several

Salmonidae genera obtained either in our laboratory or available in the GenBank. To establish phylogenetic relationship within the *Salmo* genus, a combined data set (approximately 1000 bp) of mtDNA (control region and cytochrome *b* gene) and nuclear DNA (part of LDH-C1* gene) was used. Since we were not able to obtain the LDH-C1* gene sequence of *S. trutta* Mediterranean phylogeographical lineage, we omitted this haplotype from the combined analysis. In order to appraise the statistical significance of the entire topology for this *Salmo* cluster, *Oncorhynchus mykiss* and *Salvelinus fontinalis* were included in the analysis as outgroups to root the cladograms.

The computer program ClustalX (Thompson, Higgins & Gibson, 1994) was used to align sequences. A phylogenetic tree was generated from the aligned sequences using the quartet-puzzling, maximum likelihood procedure in the PUZZLE program, version 5.0 (Strimmer & von Haeseler, 1996). It was performed under the HKY model of sequence evolution (Hasegawa, Kishino & Yano, 1985). Support values for each internal branch were obtained with the construction of 10 000 intermediate trees. A maximum likelihood procedure using the DNAML program of PHYLIP Phylogeny Inference Package (Version 3.6, Felsenstein, 1993) was also performed. For the graphical representations of tree topologies, the Treeview program, version 1.6.5 (Page, 1996), was applied.

Sequencing data were subjected to a distance analysis using the PHYLIP Phylogeny Inference Package (Version 3.6, Felsenstein, 1993). Sequence divergences were calculated with the DNADIST program, applying the Kimura two-parameter model (Kimura, 1980) and with the transition/transversion ratio of 2.

RESULTS

The nucleotide sequences of the entire mtDNA control region (1027 bp; see Appendix), a 276-bp 5′-end of cytochrome *b* gene and 381-bp-long fragment of LDH-C1* gene were determined for *S. obtusirostris* individuals (Table 1). No polymorphism was found within the species.

RELATIONSHIP AMONG SALMONID GENERA

The nucleotide-identity test of the 5′-end control region (400 bp), performed between the *Salmothymus* and representative genera of the Salmonidae family (Table 1), showed distinctive pair-wise sequence divergences, varying from 2 to 25%. The lowest divergence was found in a triangle between *Salmothymus*, *Salmo* and *Acantholingua*, varying from about 2% between *Salmothymus* and *S. trutta* or *Acantholingua* and 6% between *Salmothymus* and *S. salar*. Phylogenetic analysis, summarized in Figure 3, placed both *Salmothymus* and *Acantholingua* in the *Salmo* cluster, closer to *S. trutta* than to *S. salar*.

The placement of *Salmothymus* in the *Salmo* cluster was supported also by results obtained by LDH-C1* gene sequence analysis. A nucleotide sequence analysis revealed that point mutations prevailed in the first and in the last third of the fragment, whereas the central part was characterized by larger deletions and insertions, being more or less generically specific. The most distinctive was a 47-bp-long insertion, which was present only in *Salmo* representatives, *Salmothymus* and *Acantholingua* (Fig. 4). The intergeneric relationship, inferred from the entire LDH-C1* gene fragment, is shown in Figure 5. Because the statistical programs could not evaluate large and compound insertions or deletions, we weighted them as transitions or transversions. However, the results obtained with sequences evaluated in this way were very similar to the results obtained when insertions were excised and ignored from the analysis. The consensus tree (Fig. 5), based on these data, exhibits a pronounced differentiation between all the genera analysed, except for *Salmothymus* and *Acantholingua*, which fell into the *Salmo* cluster.

INTRA *SALMO* DIFFERENTIATION

As already shown, *S. obtusirostris* and *A. ohridana* are related more to *S. trutta* than to *S. salar*. Comparing nucleotide sequences of mtDNA control region between *S. obtusirostris*, *A. ohridana* and several haplotypes for all five phylogenetic assemblages of *S. trutta*, at least 19 variable positions were found (Table 2). Three of these represent private mutation events, strictly associated with the *S. obtusirostris* haplotype, whereas two were shared by *S. obtusirostris* and *A. ohridana*. There is a deletion within a variable nucleotide quartet (400–403; Table 2; Appendix) that prevents its exact location from being determined. In order to overcome this inconvenience, all possible positions of the deletion and also the truncated sequence without the variable quartet were taken into consideration for phylogenetic analysis. All the tree-diagrams revealed *S. salar* as basal taxon, and exhibited already known phylogeographical assemblages of *S. trutta*, clustering in a separate clade. Irrespective of whether the truncated or the entire sequence was analysed and which variant within the variable quartet was considered, a character state analysis has always separated *S. obtusirostris* from the existent clusters and put it together with *A. ohridana* as a separate clade (data not shown). However, the branching pattern was supported with rather low bootstrap values, ranging from 52 to 74%.

In order to increase the number of informative sites and to obtain statistically supported topology within

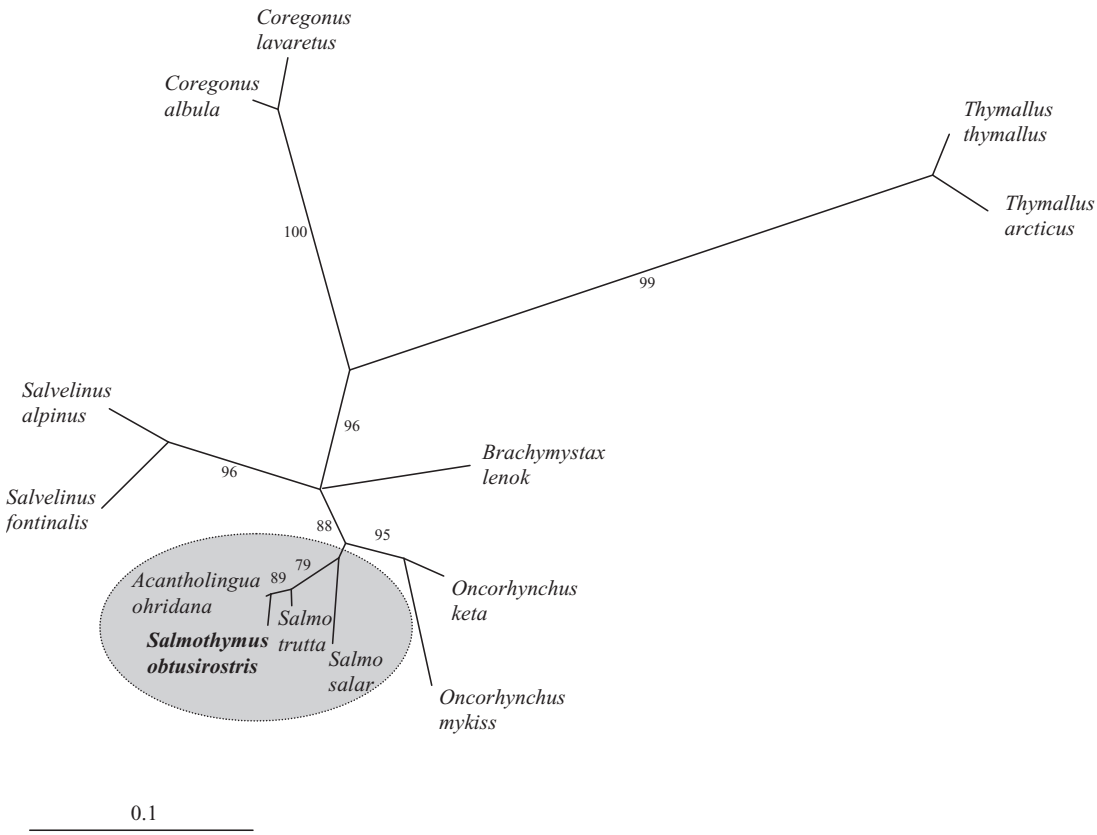

Figure 3. Unrooted maximum likelihood tree relating mtDNA control region haplotypes of eight Salmonidae genera. *S. trutta* is represented by the Danubian phylogeographical lineage. Confidence statements (in per cent) estimated from 10 000 puzzling steps are shown between the nodes. The *Salmo* clade is circled and shaded.

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                          121                                                                                  201
Salmo marmoratus Ma       TCATTAATAGATCTAATGGCAGGACTATTACATGTCAAAGTAGGATTTCAGAAATTGCTTTGAGAAACTTCATTCATACAT
Salmo trutta Ad           TCATTAATAGATCTAATGGCAGGACTATTACATGTCAAAGTGGGATTTCAGAAATTGCTTTGAGAAACTTCATTCATACAT
Salmo trutta Da           TCATTAATAGATCTAATGGCAGGACTATTACATGTCAAAGTGGGATTTCAGAAATTGCTTTGAGAAACTTCATTCATACAT
Salmo trutta At           TCATTAATAGATCTAATGGCAGGACTATTACATGTCAAAGTGGGATTTCAGAAATTGCTTTGAGAAACTTCATTCATACAT
Salmothymus obtusirostris TCATTAATAGATCTAATGGCAGGACTATTACATGTCAAAGTAGGATTTCAGAAATTGCTTTGAGAAACTTCATTCATACAT
Acantholingua ohridana    TCATTAATAGATCTAATGGCAGGACTATTACATGTCAAAGTGGGATTTCAGAAATTGCTTTGAGAAACTTCATTCATACAT
Salmo salar               TCATTAATAGATCTAATGGCAGGACTATTACATGTCAAAGTGGGATTTCAGAAATTGCTTTGAGAAACTTCATTCATACAT
                          **************************************** ****************************************
Thymallus thymallus       TCATTAATA----TAATAAT--G-----------------------------------------------CATTCATACAT
Oncorhynchus mykiss       TCATTAATAG-------AAT--G-----------------------------------------------CATTCATACAT
Salvelinus fontinalis     TCATTAATAG----AATAGT--G-----------------------------------------------CATTCATACAT
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Figure 4. An 80-bp-long fragment of LDH-C* gene. Shaded sequences specify an insertion characteristic for *Salmo* representatives, *Salmothymus obtusirostris* and *Acantholingua ohridana*.

in-group taxa, the sequences of the fragment of the LDH-C1* gene and the cytochrome *b* gene were additionally included in the phylogenetic analysis.

As inferred from LDH-C1* gene sequence analysis, a differentiation between *S. obtusirostris*, *A. ohridana* and *S. trutta* complex is predominantly based on single base substitutions. A total of nine mutations were found (Table 3a). Three were diagnostic for the Atlantic lineage, one for *S. obtusirostris* and four for *A. ohridana*. All the mutations were found in the intron with the only exception being a previously described mutation (McMeel *et al*., 2001), causing an

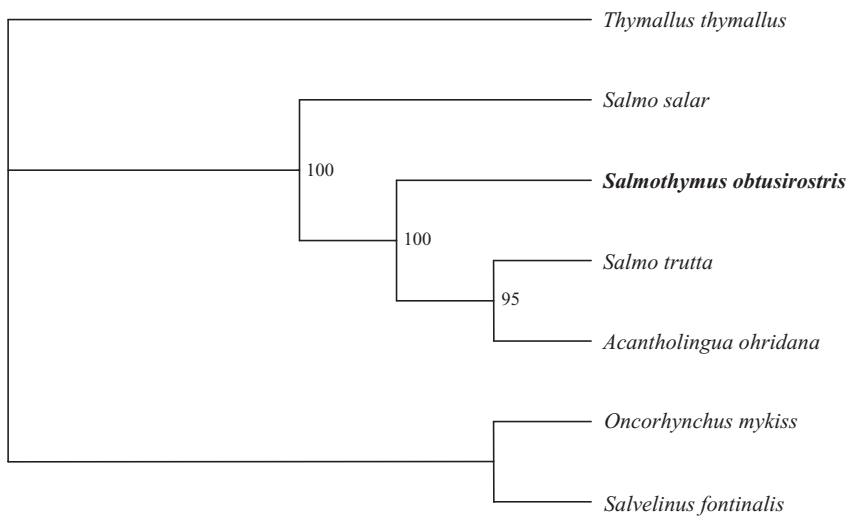

Figure 5. A consensus cladogram based on maximum likelihood analysis of molecular data from LDH-C* gene fragment sequences relating Salmonidae genera. *S. trutta* is represented by the Danubian phylogeographical lineage.

Table 2. Variable nucleotide positions of a 5′-end of the mtDNA control region. Numbers above the nucleotide refer to variable positions according to the successive nucleotide in sequences as given in the Appendix. The nucleotide for each position is given for *Salmothymus obtusirostris*; for other haplotypes variable nucleotides are entered; the identity is indicated by dashes and deletion with slashes

	Mutation sites																		
	15	39	57	120	126	159	191	209	239	248	249	269	275	351	401	402	403	410	416
S. obtusirostris	T	C	C	G	/	G	T	A	C	A	T	C	G	T	/*	C	T	A	C
A. ohridana	–	T	–	A	/	–	–	–	T	–	–	–	–	–	A	T	C	C	–
S. trutta																			
Ma1	–	–	–	A	A	–	–	–	–	–	–	A	A	A	G	T	C	C	–
Me1	–	–	–	A	/	A	–	C	–	–	–	A	–	A	G	T	C	C	–
Ad1	–	–	–	A	/	–	–	–	–	–	–	A	C	A	G	T	C	C	–
Da1	C	A	–	A	/	–	–	–	–	–	G	A	–	A	G	T	C	C	T
At1	–	T	–	A	/	–	–	–	–	–	–	A	–	A	G	–	–	C	T

*Deletion in the *Salmothymus obtusirostris* haplotype lies within a variable nucleotide quartet (from nucleotide positions 400–403 in the Appendix), and therefore its exact position cannot be determined.

amino acid change in the Atlantic lineage. Within the cytochrome *b* gene, a total of 10 variable sites were found; none of them caused an amino acid change. No diagnostic variable sites were found for *S. obtusirostris*, whereas five of them differentiated *S. obtusirostris* and *A. ohridana* from all the other taxa (Table 3b).

The combined sequence data set of the control region, cytochrome *b* gene and LDH-C1* gene revealed seven composite haplotypes, representing *S. salar*, *S. obtusirostris*, *A. ohridana* and four main phylogeographical lineages (Da, At, Ma, Ad) of *S. trutta*. A maximum likelihood analysis, performed either by the PUZZLE or DNAML programs, split the *Salmo* representatives, *S. obtusirostris* and *A. ohridana*, into three clades, represented by *S. salar* as a basal clade, *S. obtusirostris* + *A. ohridana* and *S. trutta* complex. (Fig. 6). The formation of each clade is supported by 100% bootstraping, whereas the branching pattern within the *S. trutta* group is less significant. Nevertheless, the tree topology of *S. trutta* complex reflects the relationship among the corresponding lineages, which has been already proposed in Bernatchez (2001).

Pair-wise nucleotide divergence estimates between *S. obtusirostris*, *A. ohridana* and *S. trutta* (Table 4) varied from 0.19 to 2.20% (1.44 ± 0.62%). Mean

Table 3. Variable nucleotide positions of a part of (a) the LDH-C* gene and (b) the cytochrome b gene. Numbers above the nucleotide refer to variable positions according to the successive nucleotide in sequences as given in the GenBank (see Table 1 for accession numbers). The nucleotide for each position is given for *Salmothymus obtusirostris*; for other haplotypes variable nucleotides are entered; the identity is indicated by dashes and deletion by slashes

	Mutation sites													
(a)	41	56	59	61	103	110	113	162	205	206	215–231	261	266	283	359
S. obtusirostris	T	A	A	C	C	A	T	A	C	C	TTTTCCCCCCC	A	C	C	G
A. ohridana	–	G	–	–	–	C	–	G	A	A	TTTCCCCCCCCCCCCCC	–	G	–	–
S. trutta															
Ma1	–	–	–	–	–	–	–	–	–	–	CCCC	–	G	–	–
Ad1	–	–	–	–	–	–	–	G	–	–	TTTCCCCCCCC	–	G	–	–
Da1	–	–	–	–	–	–	–	G	–	–	TTTCCCCCCCCCC	–	G	–	–
At1	C	–	–	–	–	–	G	G	–	–	TTTCCCCCCCCCC	–	G	G	–
S. salar	–	–	/	T	T	–	–	G	–	–	TTTTCCC	T	G	–	A

	Mutation sites																					
(b)	2	20	22	26	40	49	70	79	88	91	103	121	130	136	145	196	211	214	226	247	256	262
S. obtusirostris	T	T	G	T	T	G	C	C	A	C	C	T	C	A	A	T	C	C	C	C	C	A
A. ohridana	–	–	–	–	–	–	–	–	C	–	–	–	–	G	–	–	–	–	–	–	–	–
S. trutta																						
Ma1	–	–	A	C	–	A	–	T	–	–	T	C	–	–	–	–	–	–	–	T	–	G
Ad1	–	–	A	C	–	A	–	T	–	–	T	C	–	–	–	–	–	–	–	T	–	G
Da1	–	–	–	C	–	A	T	T	–	–	T	C	–	–	G	–	–	–	–	T	–	G
At1	–	–	A	C	–	A	–	T	–	–	T	–	–	–	–	–	–	–	–	T	–	G
S. salar	C	C	A	C	C	–	–	T	–	T	T	–	T	–	T	C	T	T	T	T	T	T

sequence divergence within *trutta* complex was 0.79%, between *S. obtusirostris* and *trutta* complex 1.79%, between *A. ohridana* and *S. trutta* complex 2.08%, and between *S. obtusirostris* and *A. ohridana* 1.42%. Genetic distances between *S. obtusirostris* + *A. ohridana* + *S. trutta* and *S. salar* ranged from 4.4 to 5.3% (Table 4).

DISCUSSION

PHYLOGENETIC STATUS OF *S. OBTUSIROSTRIS* WITHIN THE SALMONIDAE FAMILY

In this study we demonstrated that the current classification of *Salmothymus* is not congruent with molecular data, obtained from both mtDNA and nuclear DNA nucleotide sequence analysis. Despite its unusual and apparently primitive morphology, which places it within archaic representatives of the Salmoninae subfamily (Stearley & Smith, 1993), molecular phylogenetic analysis at the intrageneric level did not support this view, suggesting that the soft-muzzled trout is a more derived form. Furthermore, evolutionary changes in the combined data set of mitochondrial and nuclear DNA, assessed by likelihood method, indicated a close relationship between *Salmothymus*, *Salmo* and *Acantholingua*. This finding refutes the hypothesis proposed by Stearley & Smith (1993), who claimed that *Salmothymus* and *Acantholingua* are cladistically intermediate between graylings and advanced salmonids (i.e. *Hucho*, *Salvelinus*, *Salmo* and *Oncorhynchus*), and supports the inclusion of *A. ohridana* in the genus *Salmo*, as proposed recently (Oakley & Phillips, 1999; Phillips *et al*., 2000; R. A. Duguid, personal communication). The relatively close relationship between *Salmothymus* and *Salmo* is supported also by genetic distance data. Genetic distances between *Salmothymus* and two representatives of the genus *Salmo* were estimated to be 6% (*S. salar*) and ~2% (*S. trutta*). On the basis of mtDNA control region variation within genera *Oncorhynchus*, *Salvelinus* and *Thymallus*, maximum intrageneric genetic distances were 6.7% (Shedlock *et al*., 1992), 5.8% (Brunner *et al*., 2001) and 7.5% (Sušnik, *et al*., 2001), respectively, exhibiting the same or even lower values than those observed between *Salmothymus* and

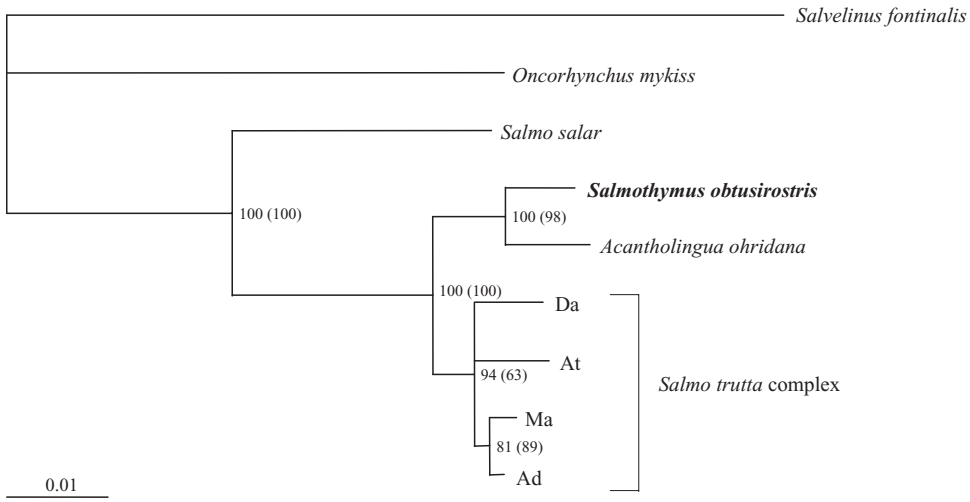

Figure 6. Maximum likelihood tree, based on the combined data set of mtDNA control region, cytochrome b gene and LDH-C* gene, relating *Salmo salar*, *Salmothymus obtusirostris*, *Acantholingua ohridana* and *Salmo trutta* complex (abbreviations Da, At, Ad, and Ma refer to the main phylogenetic lineages). *Oncorhynchus mykiss and Salvelinus fontinalis* represent outgroups of the tree. Confidence statements (in per cent) estimated from 10 000 puzzling steps (PUZZLE), and bootstrap values based on 100 replications (DNAML, in parentheses), are shown in the nodes. The analysis performed by algorithms in NEIGHBOUR and FITCH programs of the PHYLIP computer package resulted in the identical topology of the tree.

Table 4. Matrix of pair-wise distance estimates under the Kimura 2-parameter method for the mtDNA control region, cytochrome b gene and LDH-C* gene composite haplotypes of *Salmothymus obtusirostris, Acantholingua ohridana,* and representatives of four main phylogenetic lineages within *Salmo trutta* and *Salmo salar*

	Salmothymus obtusirostris	*Acantholingua ohridana*	*Salmo trutta*			
			Ma	Ad	Da	At
A. ohridana	1.42					
S. trutta						
Ma1	1.63	2.11				
Ad1	1.81	2.08	0.28			
Da1	2.10	2.19	0.95	0.85		
At1	1.81	2.19	1.05	0.94	1.13	
S. salar	4.88	5.27	4.39	4.37	4.56	4.67

Salmo. Considering genetic distances, it is, thus, unacceptable to classify *Salmothymus* as a separate genus.

Close relatedness between *Salmothymus* and *Salmo* has been confirmed also by LDH-C*1 gene analysis (Fig. 5). Studying the evolutionary tree, it could be recognized that the genus *Salmo* forms a statistically significant cluster, which is distinctly separated from the other genera and together with *S. salar* and *S. trutta* incorporates also *Salmothymus* and *Acantholingua*. Furthermore, it was observed that within intron 3, all the analysed representatives of this group share a 47-bp-long insertion (Fig. 4), which distinguishes them from the representatives of all other genera analysed, and should be therefore regarded as an apomorphic state, characteristic for members of the *Salmo* genus.

The findings, inferred from nuclear DNA, strongly corroborate and additionally support the previous observation, deduced from mtDNA analysis. Therefore, taking into account the molecular phylogeny, both ambiguous genera, *Salmothymus* and *Acantholingua*, do not represent taxonomic units above the species level but instead merely belong within the same genus, i.e. *Salmo*.

RELATIONSHIP BETWEEN *S. OBTUSIROSTRIS, A. OHRIDANA* AND *S. TRUTTA* COMPLEX

A genetic background of *Salmo trutta* has been intensively studied over the last 20 years and its phylogeographical structure is well established. A consensus

has already been reached that the recent *S. trutta* complex is composed of five major evolutionary lineages (Da, At, Ma, Ad and Me) and that the additional subdividing of similar deep divergence is very unlikely (Bernatchez, 2001). However, this assumption was made without taking into consideration soft-muzzled trout and *belvica*, which used to be regarded as non-*Salmo* representatives based on the current classification. In this paper we showed, however, that both taxa are very closely related to brown trout. This observation raises the question of their phylogenetic status within the genus, particularly with regard to the extant phylogeographical lineages of *S. trutta*. On the basis of different phylogenetic approaches (Puzzle, Fitch, N.J.) performed on a combined data set (mitochondrial and nuclear DNA), neither taxa fell into any of the *S. trutta* lineages; moreover, *S. obtusirostris* and *A. ohridana* always appeared in a separate clade as sister taxa. Accordingly, genetic distance between *S. obtusirostris* + *A. ohridana* and *S. trutta* are relatively high (1.79 and 2.08%, respectively) and exceed by more than two-fold the mean genetic distance within *S. trutta* lineages (0.79%). This observation implies that they must have diverged prior to the divergence of the different lineages of *S. trutta* complex, which does not warrant the inclusion of *S. obtusirostris* and *A. ohridana* in existent *S. trutta* cluster as additional phylogeographical lineage(s). We can therefore assume that the monophyletic clade, consisting of *S. obtusirostris* and *A. ohridana*, is phylogenetically intermediate between *S. salar* and *S. trutta* and may be in relation to *S. trutta* merely considered as an archaic branch.

Genetic data therefore imply that *S. obtusirostris* and *A. ohridana* evolved from a common ancestor. Besides the genetic data, there is other evidence suggesting their monophyletic origin. For instance, considering their close geographical distribution, and assuming that both taxa are endemic in this region, it would be reasonable to agree with Berg (1908), who proposed that their evolution took place in a spatially united area. However, it is evident from their mutual evolutionary change that they should have diverged a long time ago. According to the genetic distance (1.42%), they are, for instance, considerably less related than the lineages within *S. trutta* complex and the genetic distance between *S. obtusirostris* and *A. ohridana* is almost two-fold higher than the mean genetic distance within *S. trutta* complex. Assuming there is no relevant deviation from the molecular clock model and that the separation of the most ancient lineages (between At and Da) occurred between the early and upper mid-Pleistocene (Bernatchez, 2001), the divergence between soft-muzzled trout and *belvica* appears to have occurred 2 million years ago (early Pleistocene). This ancient divergence indicates their evolutionary distinctness, which is indeed reflected also by their distinctive morphology (Stearley & Smith, 1993; Wilson & Li, 1999), being a consequence of *A. ohridana*'s long lasting independent evolution and separation from *S. obtusirostris*.

An overall conclusion can be made that the mutual phylogenetic relationship among *S. obtusirostris*, *A. ohridana* and *Salmo trutta* is best classified as interspecific rather than conspecific or even intergeneric. Such classification of *A. ohridana* has already been proposed by Phillips *et al*. (2000), who suggested its reclassification to the species level as *Salmo ohridana*. Regarding *S. obtusirostris*, we strongly believe that such reclassification is needed. Therefore, we propose to recognize it as a distinctive species, called *Salmo obtusirostris*, as primarily proposed by Steindachner (1882).

Discrepancy between phenotypic characters and molecular data

Phenotypic plasticity is a very common and well known characteristic of Salmonids, which exhibit a large amount of variation in body coloration, meristic features, food preference, growth rate, growth potential, behaviour, reproduction, etc. The variation in such characters may be environmentally governed, as demonstrated by ecological morphs (anadromous, fluviatile and lacustrine) of *S. trutta*, depending on specialized mode of life, or genetically determined, expressing several unique morphotypes within the *Salmo* genus (e.g. *Salmo marmoratus*, *S. ferox*, *S. carpio*, *S. letnica, S. macrostigma*, etc., reviewed by Kottelat, 1997; Doubs trout, Largiadèr & Scholl, 1996; marmorated trout from Ottra river, Skaala & Solberg, 1997). This variation in morphology has raised a question regarding their taxonomic status. In *Salmothymus*, there is an obvious discrepancy between the evolution of the morphological characters studied and the evolution of independent molecules. Such a discrepancy between genetic and morphological differentiation has already been reported in fish; for example, within African cichlids, which are known to exhibit very little genetic differentiation (Meyer *et al*., 1990; Moran, Kornfield & Reinthal, 1994) although several morphologically, ecologically and ethologically distinct species are involved. Similarly, despite a close genetic relationship to *S. trutta*, *S. obtusirostris* closely resembles *Thymallus*, not only morphologically but also in terms of ecology (same habitat), nutrition (similar food preferences), ethology (living in flocks) and reproduction (spring spawner). However, grayling have never been native to soft-muzzled trout habitats and its recent appearance there is merely a consequence of stock-

ing. It seems likely that *S. obtusirostris* colonized an empty ecological niche and adapted to it by expressing the main phenotypic characteristics, exhibited also by unrelated salmonids (e.g. grayling) living in a similar environment. This is an indication therefore that these characteristics are non-monophyletic, probably heavily influenced by environment and consequently being under a strong selection pressure. As inferred from morphological observations, these characteristics may be regarded as plesiomorphies, which have independently evolved from different ancestors, and, in the case of *S. obtusirostris*, are based either on the absence of apomorphic characters or on the secondary loss of derived features, which results in the apparent reversion to the ancestral condition. Owing to the extensive range of soft-muzzled trout plesiomorphies, it is unlikely that it has redeveloped them as a response to environmental adaptation. The former view seems to be more realistic, and implies that a part of the ancestral trout would have split off and colonized a geographically restricted area of the west Balkan Peninsula, and further evolved the extent of its primary morphology. Further allopatric fragmentation of this ancient population may have led towards development of modern morphs of *S. obtusirostris* and *A. ohridana*. The other branch of ancestral trout would have remained bound to Paratethys and been evolving in accordance with rearrangement of their habitats, giving rise to five major evolutionary lineages of extant *S. trutta.* As revealed by this study, *S. obtusirostris* probably evolved before the ice age and therefore became adapted to relatively mild climatic conditions. Since the habitats of *S. obtusirostris*, which were restricted to the peri-Adriatic region of the west Balkan Peninsula, were only minimally affected by temperature changes during the glacial periods, this species may have retained unchanged the majority of its ecologically sensitive features (e.g. spawning period, freshwater dependence). In contrast, *S. trutta* habitats were more seriously influenced by temperature oscillation during the Pleistocene, which probably led to development of advanced and environmentally adapted phenotype of *S. trutta*. As suggested by Karaman (1927), it seems probable that different patterns of evolutionary history, mainly in terms of different environmental constraints, would have been one of the main reasons that governed such a distinctive morphological discrepancy between relatively closely related taxa, such as *S. salar*, *S. trutta*, *S. obtusirostris* and *A. ohridana*.

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APPENDIX

MtDNA control region sequence of *Salmothymus obtusirostris*. Variable nucleotide positions as given in Table 2 are in bold type, primers used for PCR amplification and sequencing are underlined and marked.

28RIBa-F →

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1
AAACTATCCT CTGATTTTTC AGCTATGTAC AATAACAACT GTTGTACCTT GCTAACCCAA
61
TGTTATACTA CATCTATGTA TAATATTACA TATTATGTAT TTACCCATAT ATATAATATG
121
GCATG-TGAG TAGTACATCA TATGTATTAT CAACATTAGT GAATTTAACC CCTCATACAT
181
CAGCACTAAC TCAAGGTTTA CATAAAGCAA AACACGTGAT AATAACCAAC TAAGTTGTCT
241                                                  vHF-F →
TAACCCGATT AATTGTTATA TCAATAAACC TCCAGCTAAC ACGGGCTCCG TCTTTACCCA
301
CCAACTTTCA GCATCAGTCC TGCTTAATGT AGTAAGAACC GACCAACGAT TTATCAGTAG
361
GCATACTCTT ATTGATGGTC AGGGACAGAT ATCGTATTAG -CTGCATCTA GTGAACTATT
421                          ← HF
CCTGGCATTT GGTTCCTATA TCAAGGGCTA TCCTTAAGAA ACCACCCCCT GAAGCCGAAT
481                                                  vCSB3-F →
GTAAAGCATC TGGTTAATGG TGTCAATCTT ATTGCCCGTT ACCCACCAAG CCGGGCGTTC
541
TCTTATATGC ATAGGGTTCC CTTTTTTTTT TTTCCTTTCA GCTTGCATAT ACAAGTGCAA
601
GCAAAGAAGT CTAACAAGGT CGAACTAGAT CTTGAATTCC AGAGAACCCA TGTATCATGG
661
TGAAATGATA TTCTATAAAG AATCACATAC TTGGATATCA AGTGCATAAG GTTAATATTT
721
CACTTCATAT ATCTCTAAGA TACCCCCGGC TTCTGCGCGG TAAACCCCCC TACCCCCCTA
781                CSB3-F →                                  ← RCSB3-R
CGCTGAAGGA TCCTTATATT CCTGTCAAAC CCCTAAACCA GGAAGTCTCA AATCAGCGCC
841
AATCTTTTTA TATACATTAA TGAACTTTTT TGCCAATTTT ATAGCATTTG GCACCGACTA
901
CTCTATCATT GGCACCACTT TTATAATTAA AGTATACATT AATAAACTTT TCGCTAAATT
961
TTATAACATT TAGCACCGAC TCCACTGTCA TTAGTACCCT GTCAATCAAA CATAAAAGGC
1021      1027      ← HN20
CTAGTGG
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