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Comparative analysis of IgM sub-variants in salmonid fish and identification of a residue in μ3 which is essential for MAb4C10 reactivity

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ABSTRACT

In rainbow trout (*Onchorhynchus mykiss*) it has been shown that high affinity IgM antibodies have a higher degree of disulfide polymerization and a longer half life time. In the present study, distinct IgM sub-variants related to ancestral tetraploidy in salmonid fish were analyzed to reveal possible characteristic differences between these. A monoclonal antibody (MAb4C10) which distinguishes between IgM-A and IgM-B in Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) was further characterized. It was shown that substitution of a proline located in the loop between the B and C beta strands of the third constant domain (μ3) of salmon μA eliminated MAb4C10 reactivity. Accordingly, the reverse substitution in salmon μB restored MAb4C10 reactivity. Molecular cloning of μ cDNA from arctic char (*Salvelinus alpinus*) revealed two sub-variants (μA-1 and μA-2), i.e. a similar situation as in Atlantic salmon and brown trout. However, arctic char IgM eluted in one peak by anion exchange chromatography, in contrast to salmon and brown trout IgM that are eluted in two peaks. The only characteristic residue of salmon and brown trout μB is an additional cysteine in the C-terminal part of μ4. Most likely, this cysteine is involved in inter-chain disulfide bonding and influences the elution profiles of IgM-A and IgM-B on anion exchange chromatography. Neither of the μ sub-variants in arctic char have the additional cysteine, and char IgM, as well as salmon and brown trout IgM-A, showed a lower degree of inter-chain disulfide bonding than IgM-B when subjected to denaturation and gel electrophoresis under non-reducing conditions. Hybrids of char/salmon expressed μA-1, μA-2, μA and μB, indicating that there are two paralogous Ig heavy chain gene complexes in the haploid genome of char, like in Atlantic salmon. A comparison of salmonid μ sequences is presented, including representatives of *Salmoninae* (trout, salmon and char), *Thymallinae* (grayling) and *Coregoninae* (whitefish).

1. Introduction

The salmonid fish family (*Salmonidae*) comprises three subfamilies: *Salmoninae* (trout, salmon and char), *Coregoninae* (ciscos and whitefish) and *Thymallinae* (grayling). Salmonid fish are in a pseudotetraploid state as a result of a whole genome duplication event that occurred in the common ancestor of salmonids. It has been suggested that the whole genome duplication occurred through autotetraploidization about 25–100 million years ago [1]. Based on comparative analysis of partial IgM genomic sequences it was estimated that the three genera *Salvelinus*, *Salmo* and *Onchorhynchus* radiated in short successions 10–18 million years ago [2].

Different *Salmoninae* species can be crossed to give viable offspring. Hybrids between salmon and brown trout are known to occur naturally [3], while hybrids between salmon and char, and char and brown trout have been produced artificially [4]. The first attempt to make inter species hybrids within the *Salmoninae* subfamily dates back to 1865 [5].

As a result of ancestral tetraploidy, the genome of Atlantic salmon (*Salmo salar*) contains two paralogous gene complexes A and B encoding IgM, IgD and IgT heavy chains [6–9]. Two subpopulations of IgM which are separable by anion exchange chromatography [10] correspond to the IgM heavy chain sub-variants μA and μB [11–13]. The situation in brown trout (*Salmo*

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trutta) is equivalent to that in Atlantic salmon, and the molecules have been named μA and μB in both species [12]. Comparison of the translated cDNA sequences and mass spectrometry analysis of the native proteins has shown that only a single amino acid position is characteristic for the IgM-B subtype in Atlantic salmon and brown trout; namely an additional cysteine near the C-terminus of μB [12,13].

In rainbow trout (*Onchorhynchus mykiss*) only one μ gene (and allotypes of this) appears to be expressed, although the Ig heavy chain locus might be duplicated in this species as well [14]. A partial genomic sequence comprising μ1–μ4 has been reported from arctic char (*Salvelinus alpinus*), but questions regarding sub-variants were not addressed [2]. To our knowledge IgM sequences from grayling and whitefish have not been characterized.

Fish immunoglobulin responses differ from higher vertebrates by the lack of a class switch mechanism. It has been suggested that post translational diversity of IgM in teleosts might compensate to some degree for the absence of for example IgG during secondary immune responses [15]. In rainbow trout it has been shown that high affinity IgM antibodies have a higher degree of disulfide polymerization and a longer half life time [16].

A monoclonal antibody (MAb4C10) raised against rainbow trout IgM [17], showed to react with salmon IgM-A and brown trout IgM-B, but not with salmon IgM-B and brown trout IgM-A [13]. It is plausible to assume that this pattern of reactivity is a result of crossover events between the A and B loci during evolution since parts of the sequences show contradictory relationships; for example that salmon μA is most similar to brown trout μB in a defined region [12]. However, both sub-variants, with and without the additional cysteine, have been maintained for a long period of evolutionary time since salmon and brown trout radiated and appear to be present in every individual of salmon and brown trout (Hordvik, personal observations).

Based on comparative analysis we postulated that μ3 is involved in MAb4C10 reactivity. This hypothesis was confirmed by transfection of a series of tagged plasmid constructs into eukaryotic cells, followed by immunostaining with MAb4C10 (and an anti-tag antibody as control). MAb4C10 reacts in Western blots, implying that it recognizes a linear epitope [13]. Alignment of amino acid sequences from rainbow trout, salmon and brown trout indicated that a defined region of μA3 might be involved in the interaction with MAb4C10 [13]. In contrast to MAb4C10, three newly published monoclonal antibodies showed reaction with both salmon μA3 and salmon μB3 [18].

In the present work, site directed mutagenesis of salmon μA3 and μB3 was performed to identify essential residues in the interaction with MAb4C10. The reactivity between char IgM and MAb4C10 was tested, and the degree of inter-chain disulfide bonding was examined for IgM-A and IgM-B of salmon and brown trout, and IgM of char. Furthermore, we cloned μ cDNAs from arctic char, performed an *in silico* assembly of corresponding ESTs from grayling and whitefish, and carried out a comparative analysis with previously characterized μ sequences from salmon, brown trout and rainbow trout. Hybrids of char/salmon were used to reveal possible duplicated μ genes in the haploid genome of char.

2. Materials and methods

2.1. Fish

Atlantic salmon (*S. salar*) were obtained from The Industrial and Aquatic Laboratory at the High Technology Center in Bergen. Brown trout (*S. trutta*) and arctic char (*S. alpinus*) were caught in a mountain lake near Bergen (Bergsdalen). In addition, samples were taken from arctic char caught in Skogseidsvatnet near Bergen and reared in tanks at Institute of Marine Research, Matre Research Station, and salmon (female, Aquagen strain) – char (male, caught in Hopsvatnet in Masfjorden western Norway) hybrids made and raised at Institute of Marine Research, Matre Research Station.

2.2. Purification of IgM

Blood was sampled from Atlantic salmon, brown trout, rainbow trout, arctic char and salmon/char hybrids, respectively, and kept for 2–15 h at 4 °C before centrifugation. 1–3 ml of serum (fresh or stored at −80 °C) was purified by gel filtration followed by anion-exchange chromatography [10,13].

2.3. Mutagenesis of μ3 transfection constructs

The QuikChange Lightening Site-Directed Mutagenesis Kit (Stratagene) was utilized to substitute amino acids in μ3 transfected plasmids described previously [13]. For μA3 mutants the following primers were used; SalA-F1/SalA-R1 (E247K), SalA-F2/SalA-R2 (K225N) and SalA-F3/SalA-R3 (P251T). For μB3 mutants the following primers were used: SalB-F1/SalB-R1 (K247E), SalB-F2/SalB-R2 (N225K) and SalB-F3/SalB-R3 (T251P). Primer sequences are listed in Table 1.

2.4. Transfection and immunostaining

HEK293 cells were grown in DMEM media (Sigma–Aldrich) and transfected using CalPhos Mammalian transfection kit (Clontech) according to the manufacturer's protocol at approximately 35% confluency. After 6 h the medium was removed and cells were washed once with PBS before adding DMEM containing ampicillin/streptomycin and 10% serum and thereafter incubated for further 24 h. When the cells were approximately 80% confluent they were fixed on cover slips by incubation for 30 min in 4% formaldehyde solution at RT. The cells were washed after each step with 1× TBS. Cells were permeabilized with 0.2% Triton X-100 for 10 min. Blocking was performed for 1 h at RT with 10% BSA. Cells were immunostained overnight at 4 °C using mouse-monoclonal anti-Flag (1:1000) or MAb4C10 (1:40) with 3% BSA. Thereafter cells were incubated with FITC anti-mouse (1:500) with 3% BSA for 1 h in dark at RT. The cover slips were mounted on an object glass with a drop of mounting solution ProLong® Gold antifade with DAPI (Invitrogen).

2.5. SDS-PAGE, western blotting and immunodetection

A 4–12.5% denaturing and reducing SDS-PAGE was performed according to Laemmli [19]. Denaturing, but non-reducing, native-PAGE was performed using NativePAGE™ Novex® 4-16 Bis-Tris gels (Invitrogen) with slight modifications. The Laemmli buffer without reducing agent was added to protein samples and boiled at 95 °C

Table 1
Primers used for site directed mutagenesis.

SalA-F1	CTTGTGTGCGATGTCAAGGAACTAGTTCCTGGC
SalA-R1	GCCAGGAACTAGTTCCTTGACATCGCACACAAG
SalB-F1	CTTGTGTGCGATGTCGAAGAACTAGTTACTGGC
SalB-R1	GCCAGTAACTAGTTCTTCGACATCGCACACAAG
SalA-F2	CATTCAGTAGTCATTAACATCACCCCGCCGTCT
SalA-R2	AGACGGCGGGGTGATGTTAATGACTACTGAATG)
SalB-F2	CATTCAGTGGTCATTAAGATCATCCCGCCGTCT
SalB-R2	AGACGGCGGGATGATCTTAATGACCACTGAATG
SalA-F3	GTCGAGGAACTAGTTACTGGCTTCATGAGTGTC
SalA-R3	GACACTCATGAAGCCAGTAACTAGTTCCTCGAC
SalB-F3	GTCAAAGAACTAGTTCCTGGCTTCATGAGTGTC
SalB-R3	GACACTCATGAAGCCAGGAACTAGTTCTTTGAC

for 20 min and 40 min, respectively. The gel was run at 150 V for 4 h at 4 °C. Western blotting was performed at 50 V or 25 V for 1 h at 4 °C (BioRad system and Amersham HybondTMP PVDF Membrane). Thereafter the PVDF membrane was blocked at RT for 1 h in 0.5% dry milk and incubated overnight with either mouse monoclonal MAb4C10 (1:200) or rabbit polyclonal IgM (1:1000) at 4 °C. The membrane was washed 4× with 1× TBST, each for 5 min at RT on a rocker before and after incubating with either HRP-conjugated anti mouse IgG or HRP-conjugated anti rabbit IgG (1:5000) for 1 h at RT. The membrane was developed using ECL reagents as described by the manufacturer (ECL Plus Western Blot Detection, GE Healthcare Life Sciences).

2.6. Isolation of RNA and synthesis of cDNA

RNA was isolated by use of Trizol Reagent (Life Technologies, USA). First strand cDNA was synthesized by use of MMLV reverse transcriptase (Promega, Madison, USA) and an oligo-dT primer.

2.7. Polymerase chain reaction (PCR)

PCR was performed with Accuprime (Invitrogen). Following profile was repeated 25–40 cycles: 94 °C, 30 s, 55 °C, 30 s, and 72 °C, approximately 1 min per kb of the expected length of the PCR-product.

2.8. Sequencing and analysis of DNA

DNA sequencing was performed by use of BigDye Sequencing kit (Amersham Life Science, Cleveland, USA). DNA and peptide sequences were analyzed with BLAST (www.ncbi.nih.nlm.gov) and CLUSTAL (www.ebi.ac.uk/services). 3D structures of polypeptide sequences were predicted by use of PHYRE software (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

2.9. Molecular cloning of arctic char μ cDNA

RNA was purified from spleen and head kidney of arctic char and reverse transcribed. cDNA fragments were amplified by different combinations of primers previously used for PCR on salmon and trout (Table 2). Selected PCR-fragments were cloned into TOPO cloning vector (Invitrogen). Products generated by following primer combinations were sequenced: J-sense1/μ3-anti, J-sense2/μ3-anti, J-sense1/μ4-anti, μ2-sense/TM2-anti, J-sense1/TM2-anti. Sequencing of a number of clones revealed two distinct μ sequences, named μA-1 and μA-2. cDNA fragments encoding the constant part of secreted and membrane form of each variant were reported to GenBank (Acc. nos. KC012596, KC012597, KC012598 and KC012599).

2.10. In silico assembly of grayling and whitefish μ sequences

Grayling (*Thymallus thymallus*) and whitefish (*Coregonus clupeaformis*) ESTs encoding the IgM heavy chain were identified by BLAST searches in GenBank using char μ peptides as queries. Eight ESTs were identified among grayling ESTs whereas only one was found for whitefish (Accession no. EV368531). The grayling ESTs were assembled to a full-length μ cDNA sequence encoding the secreted form of IgM (Fig. 1).

Table 2
Primers used for PCR of char μ cDNA.

J-sense1	TTTGACTACTGGGGGAAAGG
J-sense2	TGGGGGAAAGGNACMATGG
μ3-anti	CCCATTGCTCCAGTCCTCAT
μ2-sense	TAATGACCCCCTCTAAAGAG
μ4-anti	ACACAACACAACCTCTACTG
TM2-anti	GATATCATCATTTCACCTTGATGGCAGT

3. Results

3.1. A proline residue in salmon μA3 is essential for MAb4C10 binding

To test positions that were essential for binding between MAb4C10 and μ3, a series of transfection mutants were constructed by site-directed mutagenesis of plasmids previously used to confirm reactivity with salmon μA3 and lack of reactivity with salmon μB3. The mutants are aligned with relevant μ3 sequences in Fig. 2. Substitution of two charged amino acids (K-225 and E-247) that we previously suspected to have impact on MAb4C10 reactivity [13] did not appear to have any effect (mutants 1, 2, 3, 5, 6, 7). However, MAb4C10 reactivity with μA3 was abolished when P was substituted with a T in position 251 (mutant 4). Accordingly, reactivity between MAb4C10 and μB3 was restored by substituting T with a P in position 251 (mutant 8).

3.2. IgM-B exhibits a greater degree of disulfide bonding than IgM-A

The degree of inter-chain disulfide bonding in IgM populations purified from salmon, brown trout and char was analyzed by denaturation (heating), followed by SDS-PAGE analysis without reducing agents. After denaturation, one major band corresponding to a tetramer (800 kDa), one band corresponding to a monomer (200 kDa), one band corresponding to a trimer (and some very weak bands presumably corresponding to dimers and half-mers), could be seen in salmon and brown trout IgM-A, and char IgM, whereas a single 800 kDa band was dominant in salmon and brown trout IgM-B samples (Fig. 3).

3.3. Arctic char μ sub-variants

Molecular cloning of IgM heavy chain cDNA from arctic char revealed two distinct sub-variants, named μA-1 and μA-2. IgM heavy chain cDNA amplified from different strains of arctic char ("Bergsdalen" and "Skogseidvatnet") showed some allelic differences, but μA-1 and μA-2 were present in all fish examined. Analysis of char/salmon hybrids showed that these fish express four μ sub-variants: char μA-1, char μA-2, salmon μA and salmon μB. Char IgM was eluted in a single peak by anion exchange chromatography, as previously shown [12]. The heavy chain of char IgM reacted in Western blots with MAb4C10 (Fig. 3).

3.4. Grayling and whitefish μ sequences

In silico assembly of grayling ESTs revealed two different μ sequences. The identity index of the two polypeptides is 85%. Only a partial μ sequence of whitefish (*Coregoninae*) was identified in the

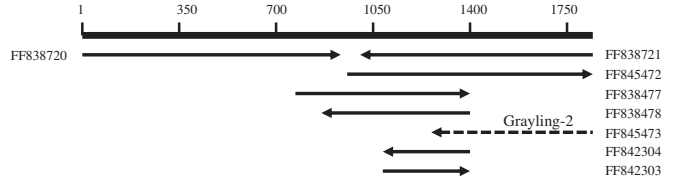

Fig. 1. Assembly of μ ESTs from grayling. A slightly different μ EST is indicated with a dotted line.

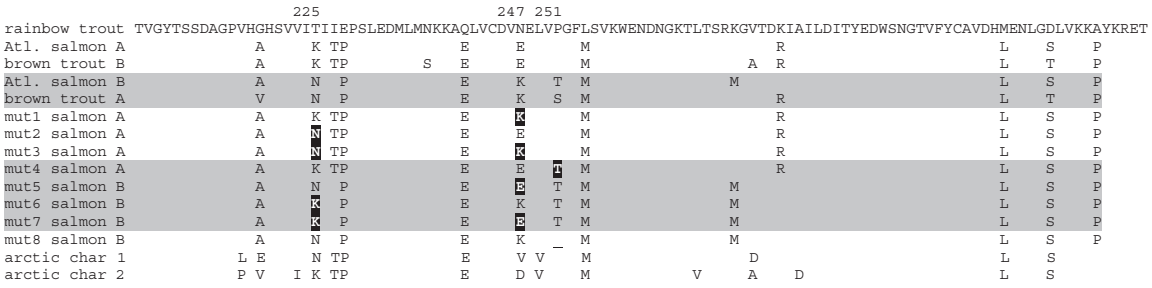

Fig. 2. Salmon μ3 mutants. Residues in Atlantic salmon, brown trout and arctic char μ sequences which are different from rainbow trout are indicated, and substituted residues in the mutants are shown with white letters on black background (the T251P substitution in mutant 8 is underlined). Sequences that do not react with MAb4C10 are indicated with grey.

databanks. Table 3 shows identity scores between representatives of salmonid fishes based on comparison of fragments comprising a part of μ3, plus μ4 (the aligned fragments are indicated on Fig. 4).

4. Discussion

The objectives of this study were (1) to discover how the monoclonal antibody MAb4C10 distinguishes between IgM-A and IgM-B in Atlantic salmon and brown trout, and (2) to gain further insight into IgM heterogeneity related to pseudotetraploidy in salmonid fish. The interaction between MAb4C10 and μ3 was narrowed down to a proline residue which is located in the loop between the B and C beta strands of the Ig domain [20]. In accordance with this, char IgM (with a proline in the comparable position of *S. salar*) reacted with MAb4C10 in Western blots. Prior to the present study, the proline residue was ignored since one of two brown trout μB cDNAs reported to GenBank had a T instead of P in this position. It was regarded as an allotypic difference, but in the course of the present work this discrepancy was found to be attributed to "PCR jumping", i.e. formation of μA/μB hybrids by partial extension on one sub-variant followed by annealing and continued extension on the opposite sub-variant.

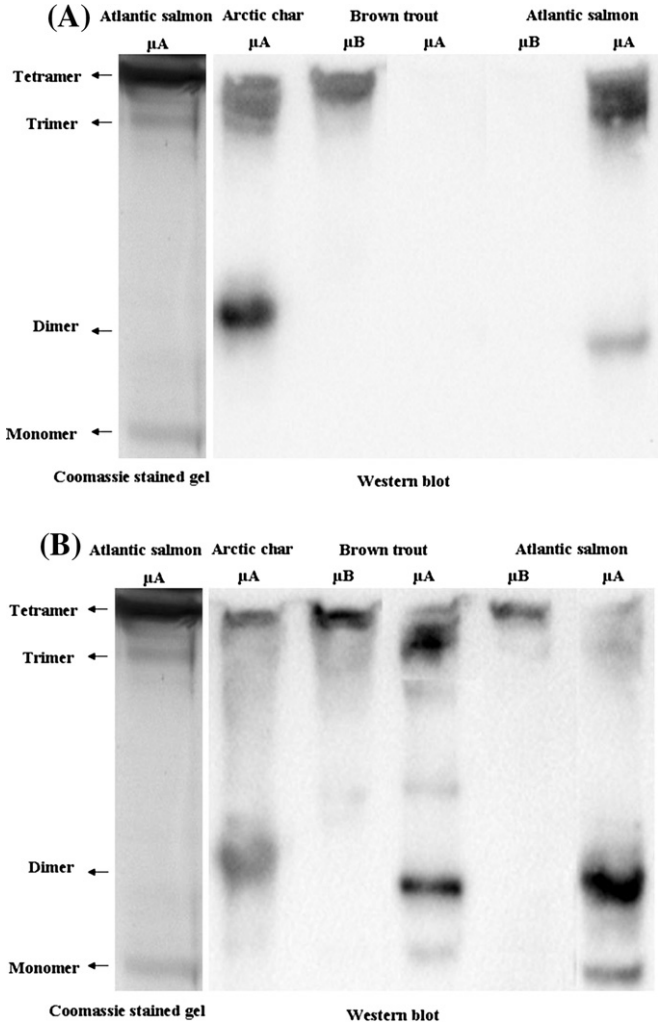

Fig. 3. Non-reducing SDS-PAGE of IgM samples from Atlantic salmon, brown trout and arctic char. Stained protein is shown only for Atlantic salmon. (A) Reactivity with MAb4C10. (B) Polyclonal antiserum against salmon IgM (the membrane was first incubated with MAb4C10 and then with the polyclonal antibody without stripping between).

Molecular cloning revealed two distinct cDNAs encoding IgM heavy chains in arctic char (μA-1 and μA-2) although the protein is eluted in one peak by anion exchange chromatography [12 and present study]. As shown in Fig. 4, neither of the two char μ sub-variants has the additional cysteine residue near the C-terminus which characterizes the μB type in Atlantic salmon and brown trout. Thus, the present study support our previous hypothesis that IgM-A and IgM-B of Atlantic salmon and brown trout are eluted in distinct peaks by anion exchange chromatography as a result of structural features related to inter-chain disulfide bonding [12,13]. Accordingly, it was shown that the IgM-B exhibits a greater degree of disulfide bonding than IgM-A (Fig. 3). As estimated by non-reducing SDS-PAGE, the size of IgM-A is comparable to IgM-B, in agreement with the tetramer structure in teleost fish [21,22]. Many different approaches were attempted to reveal the "building blocks" of the IgM samples, i.e. tetramers, trimers, dimers, monomers and halfmers (results not shown). In agreement with a study of halibut IgM [23], protein staining showed that the majority of the IgM molecules were covalently linked tetramers, whereas immune-staining could give a somewhat misrepresenting picture with a disproportionately strong dimer band (Fig. 3).

The finding of four μ sub-variants in hybrids of char/salmon strongly indicates that there are two paralogous Ig heavy chain

Table 3
Identity scores obtained by multiple alignment of μ sequences from grayling (g), whitefish (wf), rainbow trout (rt), salmon (sal), brown trout (bt) and char.

	g-1	g-2	rt	sal-A	sal-B	bt-A	bt-B	char-1	char-2
wf	89	79	61	61	61	60	61	61	61
g-1		85	62	64	64	64	63	64	64
g-2			54	54	54	55	54	55	55
rt				93	94	93	95	94	93
sal-A					98	95	97	93	93
sal-B						96	98	94	93
bt-A							97	94	93
bt-B								94	93
char-1									98

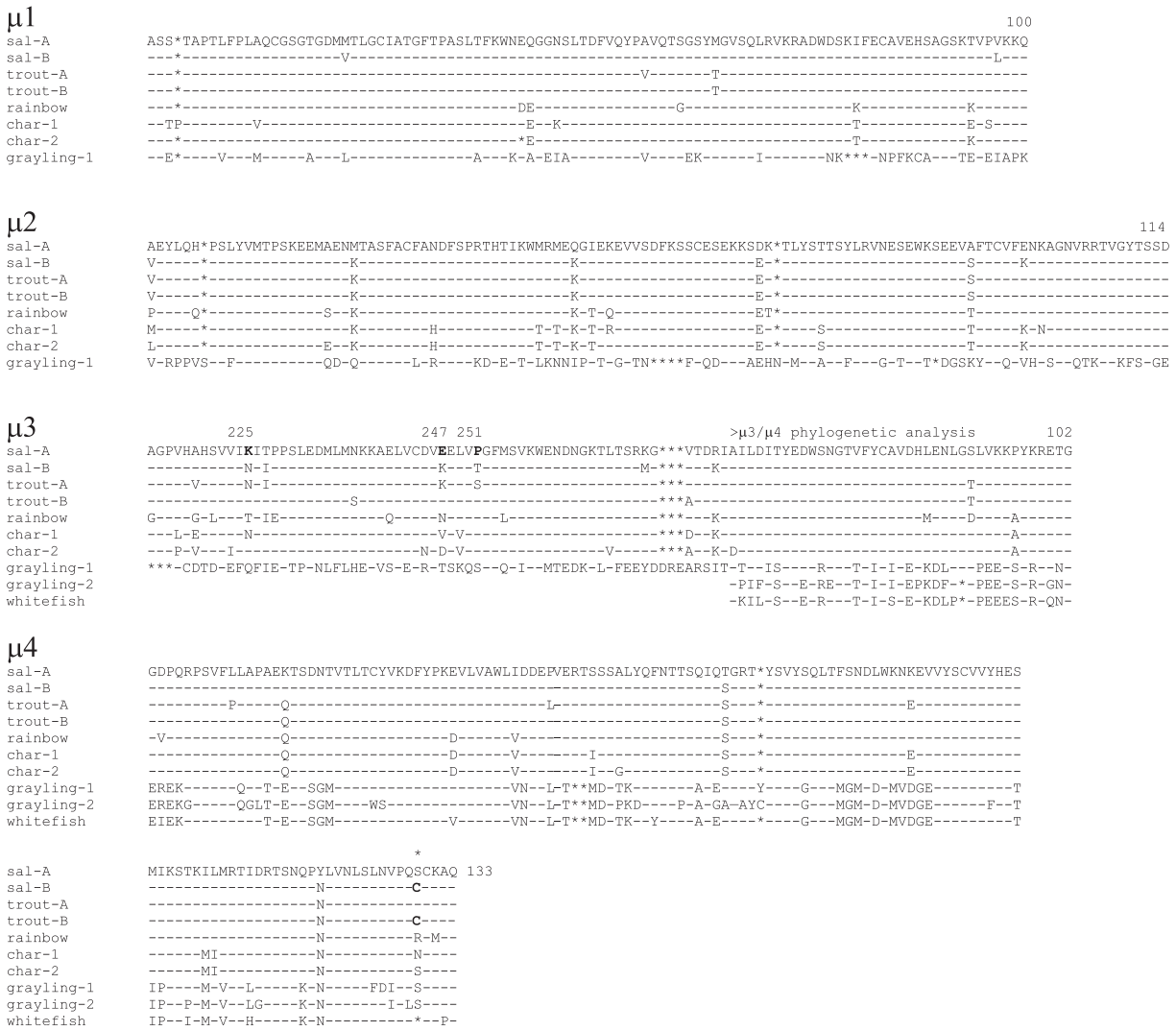

Fig. 4. Alignment of μ sequences from salmonid fish (GenBank acc. nos. in brackets): rainbow trout (AY870258), Atlantic salmon (Y12456, Y12457, Y12392), brown trout (AF228580, AF228581), arctic char (KC012596 and KC012598), grayling (Fig. 1) and whitefish (acc. no. EV368531). Residues that are identical to salmon μA are indicated by hyphens. Positions which were mutated are in bold (and with numbers above the sequences). The additional cysteine in Atlantic salmon and brown trout μB4 is indicated with an asterisk above the sequences.

gene complexes in arctic char, like in Atlantic salmon [6,9]. The presence of two types of grayling μ ESTs in GenBank might be attributed to ancestral tetraploidy as well (Fig. 1). However, the variant named grayling-2 (represented by one EST) shows significantly lower identity indices than grayling-1 (represented by 7 ESTs) when compared to other salmonid species (Table 3). Evolution can act asymmetrically on paralogs, allowing one of the pair to diverge at a faster rate [24]. Thus, grayling Ig heavy chain genes might be in the process of re-establishing a situation with one functional μ gene. In Atlantic salmon and brown trout cross-over events between A and B loci might have homogenized the μ genes to some degree, and the paralogous gene complexes are still very similar in salmon [9,13].

Typically, the fourth constant domain (μ4) show the highest degree of conservation. The first constant domain (μ1) is also relatively highly conserved, whereas the third constant domain (μ3) diverges more rapidly. The identity indices between grayling-1 and rainbow trout, for example, are: μ1: 62%, μ2: 46%, μ3: 32% and μ4: 67%. Comparison of μ sequences (part of μ3, plus μ4: indicated on Fig. 4) from grayling (subfamily *Thymallinae*) and whitefish (subfamily *Coregoninae*) with those from trout, salmon and char, respectively (subfamily *Salmoninae*), showed similar identity indices (60–64%), supporting previous analyses which indicated that the genera *Salmo*, *Oncorhynchus* and *Salvelinus* radiated in relatively short successions [2]. The corresponding identity score of grayling-1 and whitefish is 89%, whereas the identity indices within *Salmoninae* are above 93%. The present study illustrates that both *Coregoninae* and *Thymallinae* are distantly related to *Salmoninae*. The relationship of the sequences is indicated in Fig. 5. Comprehensive studies of salmonid phylogeny have been published previously, e.g [25–27].

The present study strongly indicates that the IgM-B sub-variant is present only in salmonid fish belonging to the genus *Salmo* (i.e. Atlantic salmon and brown trout). However, an IgM heavy chain with an additional cysteine near the C-terminus (and with proven effect on the inter-heavy chain bonding of the IgM tetramer) has evolved independently in catfish [22]. A connection between antibody affinity and increased disulfide bonding of the IgM molecules has been documented in rainbow trout [16]. Accordingly, it is plausible to assume that the emergence of the μB variant in Atlantic salmon and brown trout has functional implications.

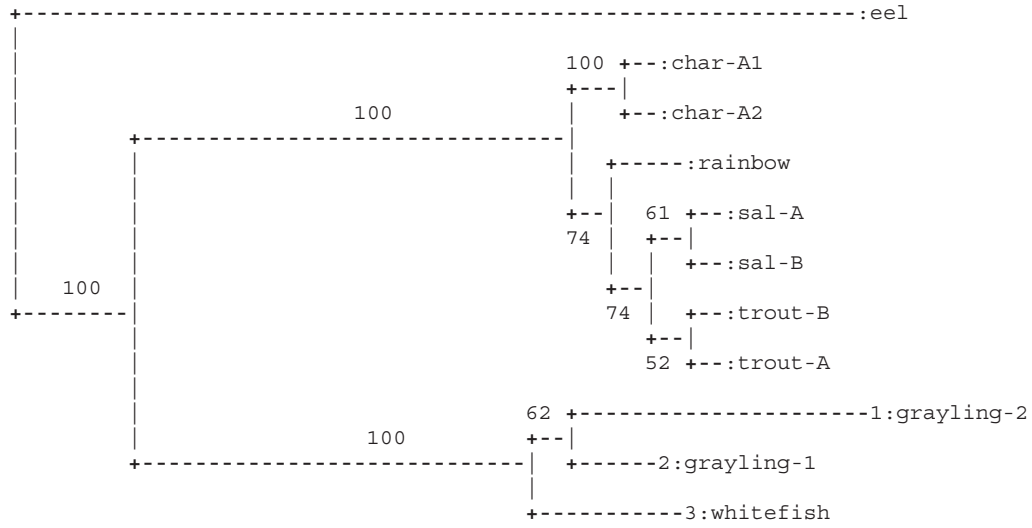

Fig. 5. Phylogenetic relationships of salmonid μ sequences based on comparison of polypeptides comprising a part of μ3, plus μ4 (accession numbers and the aligned polypeptides are shown in Fig. 4, except eel: EU551243). The neighbor joining tree generated by ClustalW (default parameters) was bootstrapped 100 times.

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