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Sequence diversity of *Mhc* genes in lake whitefish

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The sequence variation of three exons of the major histocompatibility complex (*Mhc*) was examined in a lake whitefish *Coregonus* sp., population from the Swiss lake of Hallwil. DNA sequences from the *Mhc* class I *A1*, *A2* and class II *B1* exons, corresponding to the $\alpha 1$, $\alpha 2$ and $\beta 1$ domains of the *Mhc* glycoproteins, were obtained by the polymerase chain reaction followed by cloning and sequencing. The numbers of variable sequences detected for each exon were 15 (*A1*), 11 (*A2*) and 20 (*B1*). Levels of nucleotide similarity ranged from 82 to 99% for the *A1* exon, 58–96% for the *A2* and 88–99% for the *B1* exon. At the *A1* and *B1* exons, the nonsynonymous substitution rates (*dn*) exceeded synonymous substitution rates (*ds*) greatly within the peptide binding regions, indicating the effect of balancing selection. Sequence diversity at the *A2* exon did not seem to be maintained by balancing selection ($ds > dn$). Phylogenetic comparison of whitefish *Mhc* sequences with sequences from other salmonid species and more distantly related teleosts indicated shared ancestral (trans-species) polymorphism.

Key words: major histocompatibility complex; Coregonidae; whitefish; sequence diversity; polymorphism.

INTRODUCTION

Major histocompatibility complex (*Mhc*) loci encode glycoproteins which bind foreign peptides and thus initiate immune responses through the interaction with T-cells. There are two classes of glycoproteins which differ in structure, peptide binding specificity and the sub-set of T-cells they activate (Klein, 1986). In mammals, class I and class II genes are linked together in a single gene complex (Hughes & Yeager, 1998). Within the last decade, both class I and class II *Mhc* loci have been identified in a number of teleost species such as carp *Cyprinus carpio* L. (Hashimoto *et al.*, 1990), zebrafish *Brachydanio rerio* Hamilton Buchanan (Takeuchi *et al.*, 1995), threespine stickleback *Gasterosteus aculeatus* L. (Sato *et al.*, 1998) and channel catfish *Ictalurus punctatus* (Rafinesque) (Antao *et al.*, 1999). Some recent studies demonstrate that class I and II genes in teleosts are not localized on the same linkage group (Bingulac-Popovic *et al.*, 1997; Hansen *et al.*, 1999*a*).

Mhc genes are characterized by their high levels of polymorphism, both in terms of the large amount of alleles present in populations and the high sequence

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variation between alleles. In some fish species within the cichlid family, one single individual may display up to 17 polymorphic class I loci (Malaga-Trillo *et al.*, 1998) whereas in salmonid fish, only three class I loci have been characterized so far (Grimholt *et al.*, 1994; Miller & Withler, 1998). The high variability of *Mhc* genes is probably maintained through some kind of balancing selection which favours the diversity in domains containing peptide binding regions (PBRs). However, it is still unclear whether the selection is overdominant (heterozygote advantage hypothesis), frequency-dependent (rare-allele advantage hypothesis), or a combination of both (Hughes & Yeager, 1998; Penn & Potts, 1999). The forces driving balancing selection are thought to be parasite resistance and reproductive mechanisms that increase the survival of the offspring or avoid inbreeding (Potts *et al.*, 1991; Hedrick, 1994).

Coregonid fishes (whitefish and allied species) are widely distributed across the northern hemisphere (Hansen *et al.*, 1999*b*). The observation of pronounced morphological variation within the Coregonidae of Europe has resulted in the description of various species (Himberg & Lehtonen, 1995). A recent study performed in the central alpine region of Europe using molecular markers showed that the extensive phenotypic variation of sympatric populations probably originates from the reproductive isolation of species flocks within the lakes (Douglas *et al.*, 1999). However, the complex taxonomy of the central alpine *Coregonus* and the postulated particular mode of evolution remains unresolved (Douglas *et al.*, 1999).

In many Swiss lakes, whitefish are the most important fish in terms of yield and biomass (Müller & Bia, 1998). Due to their substantial economic importance, these populations are subjected to supportive breeding programmes to compensate for insufficient natural reproduction which is caused presumably by eutrophication (Ryman & Laikre, 1991). The progeny resulting from such breeding efforts is generated by random mating of captured fish stocks. However, in natural populations, mating is assumed to be non-random, for example to avoid inbreeding or to increase heterozygosity at loci implicated in parasite resistance, especially *Mhc* genes (Andersson, 1994; Penn & Potts, 1999). Recently, C. Wedekind *et al.* (unpubl. data) found that appropriate mate choice in *Coregonus* would increase fitness by enhancing resistance against the bacterial pathogen *Pseudomonas fluorescens*, causative agent of bacterial furunculosis.

It is the high polymorphism and the central role in the immune response that makes *Mhc* genes highly suitable as markers in population and disease studies. Therefore, a number of whitefish *Mhc* genes have been cloned and sequenced. This study reports on the levels of DNA polymorphism in three *Mhc* domains in a *Coregonus* population from the Lake Hallwil, Switzerland. Allelic variation was assessed by analyses of nucleotide sequences within the *Mhc* class I *A1* and *A2* exons and a class II *B1* exon, all putatively encoding peptide binding regions.

MATERIALS AND METHODS

ORGANISMS AND DNA EXTRACTION

Eight male and seven female adult whitefish (type ballen) from a small Swiss lake (Lake Hallwil) were provided by a local fisherman during the breeding season. A small piece of muscle tissue was removed, stored at -70° C and later homogenized in liquid nitrogen

TABLE I. The amplification primers, annealing temperatures and PCR fragment length for the class I *A1* and *A2* and the class II *B1* whitefish exons. Primer sequences are from Miller *et al.* (1997) and Miller & Withler (1998)

MHC class/Exon	Name	Annealing temperature (° C)	Fragment length (bp)	Sequence
I *A1*	A1Ar A1eF	51	260–266	5′ TGA CTC ACG CCC TGA AGT A 3′ 5′ CTC CAC TTT GGT TAA AAC G 3′
I *A2*	A2GAR A2GF	55	215	5′ GAG TGG GAT GAT GAG RCT GGA K 3′ 5′ CTT GCT TCT TCA GCC AAT CAA TGC A 3′
II *B1*	B1RA B1FA	55	293–296	5′ CCG ATA CTC CTC AAA GGA CCT GCA 3′ 5′ GGT CTT GAC TTG MTC AGT CA 3′

TABLE II. Species, sequence name, Genbank accession numbers and references of the *Mhc* amino acid sequences shown in Figs 1 and 2

Latin and common name	Sequence name	Genbank accession numbers	Reference
Coregonus (whitefish)	*A1*-Cosp-H-1 to 15,	AF213287-AF213301	This study
	A2-Cosp-H-1 to 11,	AF213302-AF213312	This study
	B1-Cosp-H-1 to 20	AF213313-AF213332	This study
Aulonocara hansbaenschi	Auha-705, Auha-706	AF038551	Sato *et al.*, 1997
		AF038552	Sato *et al.*, 1997
Homo sapiens (human)	*A1*-HLA-G	U65241	Ober *et al.*, 1996
	HLA-A*0231	AF113923	Unpubl. data
	HLA-DRB1 O4S	L14481	Zhang *et al.*, 1993
Brachydanio rerio (zebrafish)	Brre-DEB	U08874	Sültmann *et al.*, 1994
Oncorhynchus tshawytscha (chinook salmon)	*B1*-Onts-H-1	U80299	Miller *et al.*, 1997
	Onts-B*1α1,	AF104543,	Miller & Withler, 1998
	Onts-B*1α2,	AF104583,	Miller & Withler, 1998
	Onts-UA*6	AF104594	Miller & Withler, 1998
	Onts-A*1	AF162871	Miller *et al.*, 1999
	Onts-Wr2	AF041010	Kim *et al.*, 1999
Cyprinus carpio (common carp)	Cyca-D(me2)B,	Z47757,	Van Erp *et al.*, 1996
	Cyca-UAw1	X91022	Van Erp *et al.*, 1996
Danio rerio (zebrafish)	Dare137534	AF137534	Unpubl. data

Table II. Continued

Latin and common name	Sequence name	Genbank accession numbers	Reference
Oncorhynchus kisutch (coho salmon)	Onki-A*1α1,	AF104503,	Miller & Withler, 1998
	Onki-A*1α2,	AF104554,	Miller & Withler, 1998
	Onki-B*1α1,	AF104521,	Miller & Withler, 1998
	Onki-B*1α2	AF104577	Miller & Withler, 1998
Oncorhynchus mykiss (steelhead trout)	Onmy-A*1α1,	AF104523,	Miller & Withler, 1998
	Onmy-A*1α2,	AF104579,	Miller & Withler, 1998
	Onmy-UA*1	AF104582	Miller & Withler, 1998
	Onmy-DAB,	U20947,	Glamann, 1995
	Onmy.race1	U20945	Glamann, 1995
Melanochromis auratus	Meau 236C	L17477	Ono *et al.*, 1993
Morone saxatilis (striped bass)	Mosa S-2	L33967	Walker *et al.*, 1994
Poecilia reticulata (guppy)	PRUAA3,	Z54084,	Sato *et al.*, 1995
	PRUAA30	Z54085	Sato *et al.*, 1995
Salvelinus namaycush (lake trout)	Sana561G	AF129988	Unpubl. data
Salmo salar (Atlantic salmon)	Sasa-a*1,	AF104605,	Miller & Withler, 1998
	Sasa-A*1,	AF104548,	Miller & Withler, 1998
	Sasa-A*6,	AF104600,	Miller & Withler, 1998
	Sasa-B*1α1,	AF104553,	Miller & Withler, 1998
	Sasa-B*1α2	AF104603	Miller & Withler, 1998
	Sasa c144	X70165	Hordvik *et al.*, 1993

with a mortar and pestle. Then, the sample was freeze-dried for 3 days under vacuum at − 50° C. Twenty-five mg of the powder were used routinely for the extraction of DNA. Genomic DNA was isolated using the Dneasy tissue kit from Qiagen (Basel, Switzerland).

PCR AMPLIFICATIONS

PCR was conducted in a Perkin-Elmer model GeneAmp PCR System 2400 (PE Biosystems, Rotkreuz, Switzerland). A reaction mixture of 50 µl contained 50 mM KCl, 10 mM Tris . HCl (pH 8·3), 2·5 mM $MgCl_2$, 0·01% gelatin, all four dNTPs (each at 0·2 mM), 1·5 µM of each primer (Table I), 100 ng of isolated genomic DNA and 1·25 units of Amplitaq Gold polymerase (PE Biosystems). Primers were derived from class I and II cDNA sequences as described for the isolation of Atlantic and chinook salmon genes (Grimholt *et al.*, 1993; Hordvik *et al.*, 1993; Miller *et al.*, 1997). Amplification started with 10 min at 95° C, followed by 40 cycles of 30 s at 95° C (DNA denaturation), 30 s at the respective annealing temperature (Table I), and 75 s at 72° C (primer extension). The reaction was terminated after a 7 min final primer extension at 72° C. PCR products were separated in a 2% agarose gel containing ethidium bromide (0·5 µg ml^{-1}). Separated PCR products were visualized under UV light and photographed to examine the banding patterns.

CLONING OF PCR PRODUCTS AND DNA SEQUENCING

After agarose gel electrophoresis, the PCR-products of the appropriate size (Table I) were removed, purified and concentrated with the help of the QIAEX gel extraction kit (Qiagen). The DNA was blunt-ended, phosphorylated, and ligated to *SmaI*-digested pUC18 plasmid vector using the SureClone ligation kit purchased from Pharmacia Biotech (Dübendorf, Switzerland). DNA sequences of both ends of cloned fragments were obtained by dye terminator cycle sequencing using AmpliTaq DNA Polymerase (PE Biosystems). The primers used to determine the sequences were the M13 forward primer (5′-GTAAAACGACGGCCAGT-3′) and the M13 reverse primer (5′-AAACAGCTA TGACCATG-3′). Sequence determination used a Perkin-Elmer/Applied Biosystems Model 377 DNA Sequencer.

DATA ANALYSIS

Nucleotide similarity was calculated on a pairwise basis between sequences as the sum of the transitional and transversional point mutations divided by the total length of the sequences and converted to a measure of nucleotide similarity by subtraction from 1. Indels were excluded from analysis. Percentage similarity of amino acid sequences was calculated by subtracting the proportion of different amino acids in pairwise sequence comparisons from 1 and multiplying by 100. The number of synonymous and nonsynonymous substitutions per site were calculated using Nei & Gojobori's (1986) method with the help of the computer package MEGA (Kumar *et al.*, 1993). Significance of the difference between nonsynonymous and synonymous nucleotide substitution rates was tested using a paired Student's *t*-test.

Amino acid sequences were aligned by eye. The relationships among alleles was assessed by employing the Neighbour Joining algorithm (NJ) (Saitou & Nei, 1987) on the pairwise genetic distance matrix (mean character differences) using the PAUP* 4.0b2 program package (Swofford, 1998). Confidence in the resulting relationships was assessed using the bootstrap procedure in PAUP* (1000 replicates).

All the *Mhc* sequences from whitefish have been deposited in Genbank (Table II). Additional DNA sequence information retrieved from Genbank is listed in Table II.

RESULTS

POLYMORPHISM OF *Mhc* CLASS I GENES

In 81 of 86 cases, for each exon, two clones per individual were sequenced. For the *Mhc* class I genes, 14 individuals were analysed. The *A1* and *A2* exons

TABLE III. Mean and range of nucleotide and amino acid similarities, shown as percentages, for pairwise comparisons of whitefish *Mhc* class I *A1*, *A2* and class II *B1* alleles

Exon	*n*	Nucleotide			Amino acid		
		bp	Range	Mean ± S.E.	Codons	Range	Mean ± S.E.
Class I							
A1	15	222–228	82–99	89·1 (4·56)	74–76	67–99	78·8 (8·82)
A2	11	168	58–96	73·9 (10·91)	56	39–95	60·7 (14·77)
Class II							
B1	20	249–252	88–99	93·1 (2·40)	83–84	75–98	85·5 (4·55)

n, Number of alleles compared; bp, nucleotide length in base pairs; Codons, number of codons amplified; S.E., standard error.

displayed 15 and 11 distinct sequences among 26 and 25 analysed, respectively. The *Mhc* class II *B1* exon was the most polymorphic, displaying 20 different sequences out of 30 clones from 15 individuals. The highest mean pairwise nucleotide similarity was 93·1% (S.E. 2·4) for the *B1* sequences (Table III) and the lowest was 73·9% (S.E. 10·9) for the *A2* exon. The deduced amino acid sequences for all exons also differed considerably with mean pairwise similarities of 78·8% (S.E. 8·8), 60·7% (S.E. 14·77) and 85·5% (4·55) for the *A1*, *A2* and *B1* exons, respectively.

For the *A1* exon, which was 222–228 bp in length, in all, 47% of the 76 amino acids were variable (Table III, Fig. 1). Nine of the ten residues found polymorphic in humans (Bjorkman *et al.*, 1987) were variable in whitefish (residue 74 was not, Fig. 1). All of the 26 additional residues polymorphic in whitefish are also variable in other fish species with 13 only in the PBR (Fig. 1). An insertion of two codons in the *A1* exon (position 58 and 59), as observed for the whitefish class I sequences *A1*-Cosp-H-14 and 15, has been reported previously for *Mhc* genes of pink salmon *Oncorhynchus gorbuscha* (Walbaum) and rainbow trout *O. mykiss* (Walbaum) (Fig. 1; Miller *et al.*, 1997). The *A1*-Cosp-H-1 to *A1*-Cosp-H-12 sequences exhibited a closer relationship to the *A1* exons from Atlantic salmon, *Salmo salar* L. (Sasa-A*1, Sasa-A*2, Sasa-AB*1) and rainbow trout (Onmy-A*1, Onmy-A*4) [Fig. 2(a)] whereas the *A1*-Cosp-H-13, 14 and 15 exons, on the other hand, appeared to be more related to alleles from chinook salmon *Oncorhynchus tshawytscha* (Walbaum) (Onts-H4 and Onts-A*1) and coho salmon *O. kisutch* (Walbaum) (Onki-A*7 and Onki-A*1).

The 11 sequences of the *A2* exon were 168 bp in length. Thirty-nine of the 56 amino acids (70%) were variable with 16 in the peptide binding region. However, two conserved residues that point into the cleft of the PBR out of three detected were retained throughout all whitefish *A2* sequences (T at position 143 and Y at 160; Fig. 1). Neighbour joining analysis of the *A2* amino acid sequences from whitefish with alleles from closely and more distantly related teleosts showed that three of them (*A2*-Cosp-H-1, 2 and 3) clustered with sequences from the guppy *Poecilia reticulata* (Peters) (PRUAA3) and salmonid alleles of the

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A1                10                  30                  50                  70
Consensus FYTASS~EVP NFPEFVAVGM VDGVQMVHYD SN~~SQRAVP KQDWMNKQTD ~~TQYWERNT GNLLGSQQTF KIDINILKQ
A1-Cosp-H-1 ---T------ ---------- ---------- ---------S D---I----- --------EK --------I- -----T-M-
A1-Cosp-H-2 ---T------ ---------- ---------- -S-------S D---I----- --------EK --------I- -----T-M-
A1-Cosp-H-3 ---------- ---------- ---------- ---------S D---I----- --------EK --------I- -----T-L-
A1-Cosp-H-4 ---------- ---------- ---------- ---------S D---I----- --------EK --------I- -----T-M-
A1-Cosp-H-5 -F-------- ---------- ---------- ---------S D---I----- --------EK --------I- -----T-L-
A1-Cosp-H-6 ---------- ---------- -----ID--- ---------- ------N--- --------EK --------I- -----T---
A1-Cosp-H-7 ---------- ------V--- ---------- ---------- -------R-- --------E- --F------- -VS-DNV-P
A1-Cosp-H-8 ---------- ---------- -----ID--- ---------- ---------- ------D--- -I--V----- -----T---
A1-Cosp-H-9 ---------- ---------- -----ID--- ---------- -------R-- ------D--- -I--V----- -----T---
A1-Cosp-H-10 -F-------- ------I--- ------FY-- ---------- ---------- --P---KTQ- -IA--E--N- -VN-DN--P
A1-Cosp-H-11 ---------- ---------- -----I---- ------K--S ---------- ------QIV- QRF----RI- -V--D----
A1-Cosp-H-12 ---------- ------G--- ---------- ---------- ----V--AA- --P------- --FMA----- -V--DNI-P
A1-Cosp-H-13 ---------- ------T--- -----ID--- ---------- ----V--AA- --P------- -IYM-A---- -AN-E-A--
A1-Cosp-H-14 ---------- ------G--- ------D--- ---------- ------RA-E TLP-----V- -SYM-A--A- -AN-D-A--
A1-Cosp-H-15 ---------- ---------- --D---D--- ---------- ------RA-E TLP-----V- -SYM-A--A- -AN-D-A--
Onki-A*7 ---------- ------V--- ---------- ---------- ----V--AA- ---------- ---K------ -AN-D-V--
Onki-A*1α1 ---------- ------VA-- ---------- ---------- ----V--AA- ---------- ELGK-A---- -AN-D-A--
Onts-A*1 ---------- ------V--- ---------- ---------- -------AAE TL-----S-- -IDK-A---- -AN-D-A--
Onts-H4 ---------- ------V--- ---------- ---------- ----V--AA- ---------- -IFK-A---- -AN---A--
Sasa-A*1 -------G-- -----MV--- ---------- -------M-- --------T- --AE------ -IAFD---V- -D-V------
Sasa-A*2 -------G-- -----MV--- -----ID--- -------M-- ----V---A- --AE------ -IAFD---V- -D-V------
Sasa-B*1α1 -------K-T ------V--- -------L-- ----I-KV-- EHV-----A- ---E-----R EKFFD--H-- -AYPH----
Onmy-A*1α1 -------G-T ------V--- ---------- ---------S --------T- --T----Q-- -I-----R-- -AGVD----
Onmy-A*4 -------G-- ------V--- ---------- ---------S ----V---A- --T----Q-- -I-~~~R-- -AGVD----
PRUAA3 -------Q-- ------V-A- --D------- ----TGN--- -----R-NM- --Q---DGQ- -IFQAT---- -AN-E-A--
PRUAA30 ---G---Q-- ------V-A- -GD------- -----GK--- ------AAA- ---------- --F--A---- -AN-E-A--
Dare137534 I---TK-GL- D------AT- ---M-VNYF- -E--IKEVI- R-E-VRGAV- --E-F-Q--- QIRSNMH-L- -NN---AME
Auha-705 -C-ETP-G-Q SI------AF --E--IGDFN NV--RGAEPK K---IKFFA- -H-EHL-WYS SISKQ-H-V- -AN-E-FR-
Auha-706 -------Q-- ------T--- --D--VDY-- -D--TEK-E- ----IARNT- --Q------- DIYR----S- -AN-E-V--
Cyca-UAw1 ----TT-GIS ---RL-D--- LN-EPISM-- -T----KK-- --K--AENL- --YE—NSAT- EMRKARD-V- FSN-QV-MD
A1-HLA-G -SA-V-RPGR GE-R-I-M-Y --DS-F-RF- -DSACP-ME- RAP-V~E-EG --PE---EE- R-TKAHA--D RMNLQT-RG
PBR  *           * * *                                 . t* .t*.t** .t*.t**.t*   **t
Polymorphic  p                                                    p  ppp   pp     p   pp
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A2                110                 130                 150
Consensus VTEGFDQYGY DGEDFLAFDL KTLTWIAPTP QAVITKHKWD SDKANNEQWK NYLTQT
A2-Cosp-H-1 -----R-F-- ------VY-M -R------KQ ---F-Q---N --Q-GLVF-N --IS--
A2-Cosp-H-2 EAD--R-L-- ------GY-M -RF-----KQ ---KIQY-LN --QSGLIF-- --IS--
A2-Cosp-H-3 EAD--R-L-- ------GY-M -RF-----KQ ----SQQ-LN --QSGLIL-- --IS--
A2-Cosp-H-4 -----E-F-- -----I-L-- N--------- --L------- ----F---E- H-Y--E
A2-Cosp-H-5 -------F-- -----I---- -------TM- --LV------ ----F---N- H--M--
A2-Cosp-H-6 -----M---- -----I---- N------TM- --LV------ ----F---N- H--M--
A2-Cosp-H-7 -----Y-F-- -----V---- --K------- ---------- RNI-----R- H----E
A2-Cosp-H-8 A----N-F-- -----V-L-- --E------- ---------- NVRE-TMH-- --H---
A2-Cosp-H-9 ---------- -----VV--- --K------- ----S---L- RNI----RN- H-----
A2-Cosp-H-10 ---------- -----V---- --K------- ---------- RNM-L--NMN ------
A2-Cosp-H-11 F-D--HHI-- --I-L----- --E----AV- --FNS-L--E -NPS-I-RE- L----E
SasaL24943 ---------- ---------- ---------- -SL---L--- --T-Y---E- -----I
Sasa-B*1α2 -G--T-H-- ------GY-M --F-----KQ --E--RL--N H-Q-GLAFRH ------
Sasa-a*1 --------- ---------- ---K------ ------L--- NNM-QIQ-D- H-----
Sasa-A*6 --------- ---------- ---K------ ------L--- --T-Q--YR- --Y---
OnmyARO-f2 A----Y---- ---------- --T------- ------Q--- -NT--E-RR- H----E
Onmy-UA*1 I-REDF-L-- --A---SL-K S----T-ANQ K-----L--- ATG-EANFQ- ---EN-
Onmy-A*1α2 F----Y---- -----I-L-- --TK------ ------L--- -NT--T-Y-N -----S
Onki-A*1α2 ----E-H-- -----I---- --TK---M- ---------- --T-A--YK- --Y---
Onki-B*1 Q----G-L-- N--N--EY-M -----KSRKQ --NFMQDE-N --ISRLGF-- --FS--
Onts-UA*6 I-R-DV-F-- --A---SL-K S----T-ANQ K-----L--- ATG-VANFD- ---EN-
Onts-B*1 Q----G-L-- N--N--EY-M -----KSRKQ --NFMQDE-N --ISRLGF-- --FS--
PRUAA3 QVT-YR-F-- ------TWNT E-N-----QQ --E-S-NT-N N---SL-FY- ---N-I
PRUAA30 EIK-YT-F-- --D--SV--- --ES-T--VT E--V-T---- N--GL-AG-V -----N
Dare137534 T-R-HW---- -----IS--K N---NVVAN- ------N--- AN--Q--YR- Q--ENQ
Auha-705 EIN--N-F-- ------VL-- ------T-K- ------LR-- AY--RL-GNR -F-GHR
Auha-706 EVK--E-D-- ------S--M --E-F-T-V- --IV----LE -NRGFIA-KA --Y--I
Cyca-UAw1 TKR-YM---- --Q--ISL-K N---FT-AN- --M---N--E ANR-EA---- G--ENM
HLA-A*0231 FLR-YH--A- --K-YI-LKE DLRS-T-ADM A-QT-----E ~AAHVA--LR A--EG-
PBR        * *                          * tb*   ttt* tb*. t* .tb
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FIG. 1. (a).

B-locus described in Miller & Withler (1998) [Fig. 2(b)]. The other sequences (*A2*-Cosp-H-4, 5, 6 ,7, 9, 10, respectively) clustered among several sequences from other fish species and in particular among the alleles of the salmonid A-locus (Miller & Withler, 1998) indicating that the intraspecific differentiation of sequences exceeds interspecific variation (trans-species polymorphism).

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B1                  30                  50                  70                  90
Consensus  DIEYIDSYVF NKVEYIRFNS TVGKYVGYTE HGVKNAEAWN KGQE~LAQEL GELERYCKHN AAIDYSAILD KTGEQGSLAP LTVL
B1-Cosp-H-1 ---L----Y- -----V---N ---------- Y--------- T--------- --------Y- -D-Y------ ---------- ----
B1-Cosp-H-2 ---L----Y- -----V---N ---------- Y--------- T--V------ ---------- DP-Y------ ---------- ----
B1-Cosp-H-3 ---L----Y- -----V---N ---------- ---------- ---------- -----F--Y- ---Y------ --------T- ----
B1-Cosp-H-4 ---L----Y- -Q---V---- ---------- Y--------- ---------- -----V---- ---------- --------T- --I-
B1-Cosp-H-5 ---F----Y- ----H----- ---------- ---------- T--D------ ---------- DP-Y------ ---------- ----
B1-Cosp-H-6 G-------Y- ---------- ---------- ---------- ---------- -T------N- DP-Y------ ---------- ----
B1-Cosp-H-7 ---------- --A------- ---------- ---------- ---------- -T------P- ---------- E--------- ----
B1-Cosp-H-8 ---F--A--- ---------- ---------- ----T--T-- -------G-- -----F---- --NH-R---- ---------- ----
B1-Cosp-H-9 G--------- --A------- ---------- ---------- ---------- -Q--S----- D--Y------ --------T- ----
B1-Cosp-H-10 G--------- ----H----- ---------- F--------- ---------- ----G----- ---------- ---------- ----
B1-Cosp-H-11 G--------- -Q--H----- ---------- L--------- ---------- -A--G----S ---------- ---------- ----
B1-Cosp-H-12 G--L------ ----N----- ---------- L-L------- ---------- --------PS ---------- ---------- ----
B1-Cosp-H-13 G--L-N---- -Q--N----- ---------- L-L------- -------R-- -L--G---P- ---------- ---------- ----
B1-Cosp-H-14 G--------- -Q---V---- ---------- ---------- ---------- --------Y- ---------- ---------- ----
B1-Cosp-H-15 ---------- ----H----- ---------- ---------- T--V------ ---------- DP-Y------ ---------- I---
B1-Cosp-H-16 ---------- ----H----- ---------- L--------- ---------- -A--G----- ---------- ---------- ----
B1-Cosp-H-17 ---------- -----V---N ---------- Y--------- T--------- --------Y- -D-Y------ ---------- ----
B1-Cosp-H-18 ---------- ---------- ---------- L-L------- SDASF----Q -N---F---- ---Y------ ---------- ----
B1-Cosp-H-19 ---------- ----D----- ----F----- ---------- SDASL----Q -Q--S---N- ---------- ---------- I---
B1-Cosp-H-20 ---------- ---------- ----F----- ---------- SDASL----Q -Q--S---N- ---------- ---------- ----
Sana561G    --------- --A------- ---------- -------T-- ---SE----- ---------- --NH---VV- --
Onmy.race1  --------- ---------- ---RF----- ---------- SDAGI-G--Q AQ-S----P- -D-------- --
Onts-Wr2    G--F-H---- ----H----- ---R------ L-L------- --PQ--G--Q A----V--P- --LH-R
B1-OntsH-1  G--F------ --A------- ---R------ L--------- --PQ--G--Q A----F--P- --LH-R---- --
Sasa-c144   G--L-T---- -QA-N----- ----F----- ---------- --P----G-- -V------F- -P-------- --
Onmy-DAB    G--F---FF- ----D----- ---R------ ---------- SDAGI-G--Q A----V---- -DLH------ --
Brre-DEB    D-GVH-N-I- --DV------ -L-YF----- ---Y--QL-K QRYQL-E--R AHED-F--Y- -E---NN--G --
Cyca-Dme2B  DMVFV-N-I- --DV-VQ--- ---E------ L--RS--S-- -DPNI-Q--R A-V--F---- -E-RQ
Meau-236C   D----L--YY --I--A--S- S------F-K ---Y--DR-- NDPAE-NNRR AQK----V-- IG--
Mosa-S-2    N----F--YY --L-FL--S- SE-EF----- L------RF- -DPTY---RR A-K----LT- IN---QNA-- -S
HLA-DRB1 O4S RVRFL-R-FY HQE-YV--D- D--E-RAV-- L-RPS--Y-- SQKDL-E-RR A-VDT--R-- YGVG
PBR             * * *     **          *         *   **    *  * **   *   *  **   ** **
Polymorphic      p p         pp                   p              p   p   p        p  pp
```

FIG. 1. (b).

FIG. 1. Amino acid sequences of *Coregonus sp. Mhc* class I *A1*, *A2* and class II *B1* alleles. –, Identity with the consensus sequence; ∼, gaps introduced to improve the alignment. Codon numbers for each exon start with numbers corresponding to human sequences. Codons associated with the human peptide binding region (PBR) are shown. Notations for the location of amino acids within the PBR are from Miller *et al.* (1997): *, points into the cleft; *t*, points up and potentially interacts with the T-cell receptor; . , points away from the cleft; b, is intermediate in position; p, residues polymorphic in human genes. For abbreviations, Genbank accession numbers and references, see Table II.

POLYMORPHISM OF *Mhc* CLASS II*B* GENES

For the whitefish class II *B1* alleles, a 249–252 bp PCR product could be obtained. The primers used for the amplification of those sequences (Table I) yielded a fragment of only 213 bp in other salmonids including chinook salmon, indicating an insertion of 12 amino acids at the 3′ end into the whitefish genes (Fig. 1; Miller *et al.*, 1997). When compared with the human genes, 32 of the 84 amino acids were polymorphic and 14 were variable within the PBR (Fig. 1). Two residues that are conserved on the mammalian beta chain (W61 and N82) were also conserved in the *Coregonus B1* sequences (Fig. 1). There was an insertion of one codon at position 67 in three sequences of the *B1* exon (*B1*-Cosp-18, 19 and 20). The high level of amino acid similarity within the *B1* exon resulted in the tight clustering in the neighbour joining tree [Fig. 2(c)]. However, clustering of two alleles from other salmonid species (*Salvelinus namaycush* (Walbaum), Sana561G and *Salmo salar*, Sasa-c144) within the clade was observed.

OCCURRENCE OF MULTIPLE LOCI

Most individuals displayed two different sequences from two clones examined, indicating either a high degree of heterozygosity or the presence of multiple loci. Eleven individuals from a total of 14 displayed two different sequences for the *A1*

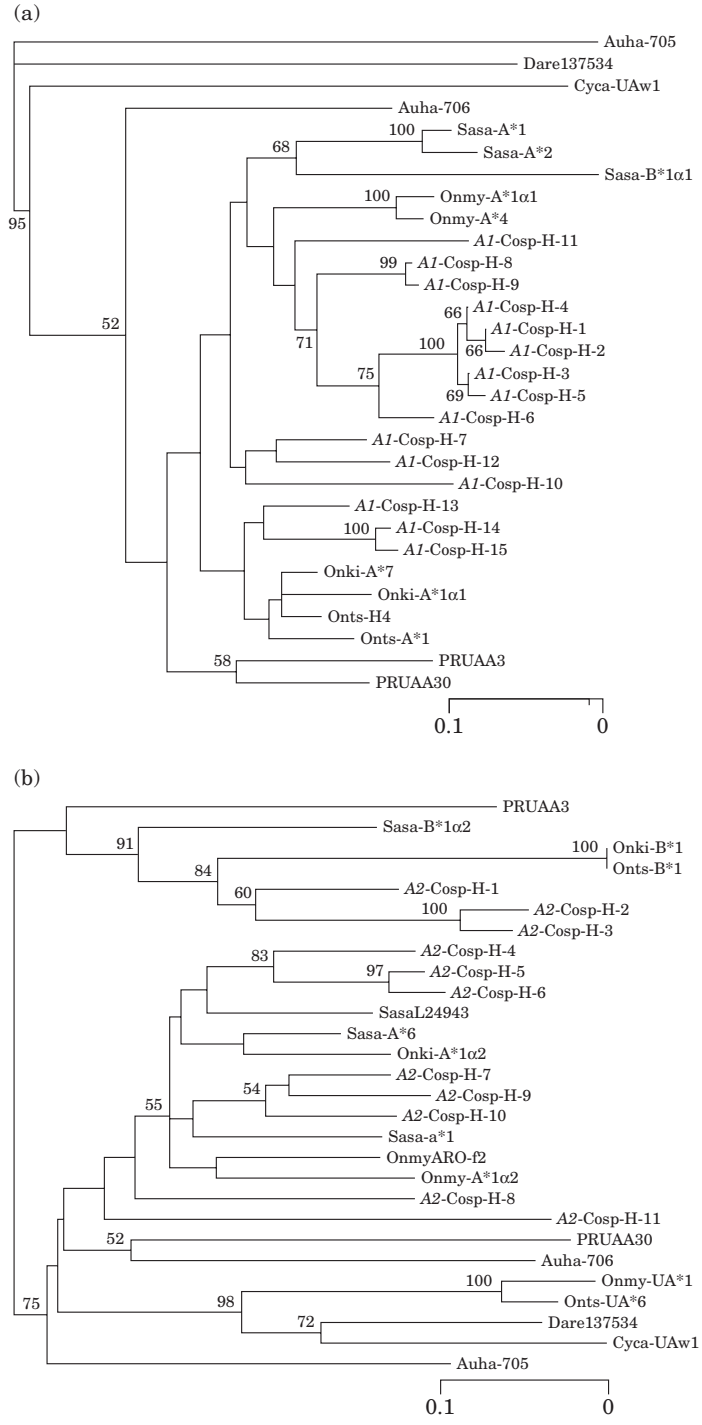
(a)
Auha-705
Dare137534
Cyca-UAw1
Auha-706
Sasa-A*1
Sasa-A*2
Sasa-B*1α1
Onmy-A*1α1
Onmy-A*4
A1-Cosp-H-11
A1-Cosp-H-8
A1-Cosp-H-9
A1-Cosp-H-4
A1-Cosp-H-1
A1-Cosp-H-2
A1-Cosp-H-3
A1-Cosp-H-5
A1-Cosp-H-6
A1-Cosp-H-7
A1-Cosp-H-12
A1-Cosp-H-10
A1-Cosp-H-13
A1-Cosp-H-14
A1-Cosp-H-15
Onki-A*7
Onki-A*1α1
Onts-H4
Onts-A*1
PRUAA3
PRUAA30
100
68
95
100
99
66
52
71
100
66
75
69
100
58
0.1
0
(b)
PRUAA3
Sasa-B*1α2
91
100
Onki-B*1
Onts-B*1
84
60
A2-Cosp-H-1
100
A2-Cosp-H-2
A2-Cosp-H-3
83
A2-Cosp-H-4
97
A2-Cosp-H-5
A2-Cosp-H-6
SasaL24943
Sasa-A*6
Onki-A*1α2
A2-Cosp-H-7
54
A2-Cosp-H-9
55
A2-Cosp-H-10
Sasa-a*1
OnmyARO-f2
Onmy-A*1α2
A2-Cosp-H-8
A2-Cosp-H-11
52
PRUAA30
Auha-706
100
Onmy-UA*1
75
98
Onts-UA*6
72
Dare137534
Cyca-UAw1
Auha-705
0.1
0

FIG. 2. (a) and (b).

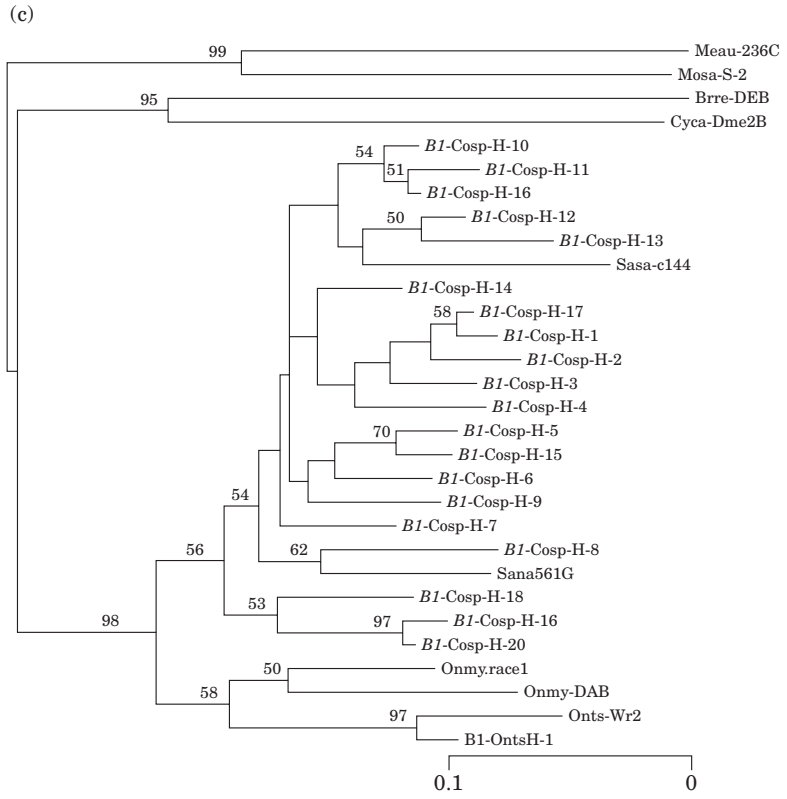

FIG. 2. (c).

FIG. 2. Phylogenetic tree showing relationships of the whitefish *Mhc* class I and II genes to representative *Mhc* genes of other fish species. The dendrograms were constructed by the neighbour-joining method (Saitou & Nei, 1987) using the *Mhc* class I and II amino acid sequences shown in Fig. 1. Sequence names are defined in the legend of Fig. 2. Numbers indicate bootstrap values (1000 replicates). (a) *Mhc* class I *A1* exons; (b) *Mhc* class I *A2* exons; (c) *Mhc* class II *B1* exons. Note that, for the *Mhc* class I genes derived from Miller & Withler (1998; see Table II), the letters A, B and UA in the sequence designations refer to distinct loci.

exon (79%). For the *A2* and the *B1* exons, 7 and 12 individuals were found to contain two variable sequences, respectively. In addition, sequencing analysis of 30 additional clones (10 per exon) from one individual revealed four different sequences for the two class I exons and five for the class II *B1* exon. Therefore, at least two class I and three class II loci are present in the type of whitefish analysed here.

NUCLEOTIDE SUBSTITUTION RATES

For the *Mhc* class I *A1* and class II *B1* sequences, nonsynonymous substitution (*dn*) at peptide binding regions (PBR) occurred at significantly higher frequencies than synonymous substitution (*ds*) (ratio of 1·96, $P<0·001$ and 2·38, $P<0·001$, respectively, Table IV). Conversely, an excess of *ds* versus *dn* was determined for the *A2* exon even within the PBR (0·561 *v.* 0·464, $P<0·001$, Table IV). However, the nonsynonymous substitution rate was significantly higher in the peptide binding region than within the rest of the gene ($P<0·001$; Table IV).

TABLE IV. Numbers of synonymous (*ds*) and nonsynonymous (*dn*) substitutions per nucleotide in whitefish *Mhc* class I *A1*, *A2* and class II *B1* sequences. Values of *ds* and *dn* over the entire sequence (Total), and for nucleotides with the peptide binding region (PBR) and outside the PBR (Non-PBR) are given

Exon	Region	Codons	*ds* (S.E.)	*dn* (S.E.)	*dn* /*ds*
Class I					
A1	Non-PBR	48	0·091 (0·038)	0·063 (0·018)	0·69
	PBR	26	0·113 (0·055)	0·221 (0·038)	1·96
	Total	74	0·097 (0·030)	0·117 (0·019)	1·20
A2	Non-PBR	37	0·764 (0·251)	0·170 (0·137)	0·22
	PBR	19	0·561 (0·506)	0·464 (0·211)	0·83
	Total	56	0·691 (0·289)	0·258 (0·123)	0·37
Class II					
B1	Non-PBR	62	0·050 (0·017)	0·074 (0·016)	1·46
	PBR	21	0·048 (0·05)	0·115 (0·027)	2·38
	Total	83	0·044 (0·016)	0·079 (0·014)	1·8

S.E., Standard error.

DISCUSSION

The class I *A1* and class II *B1* exons of the whitefish *Mhc* examined in this study are similar to the sequences from Atlantic salmon and other salmonids. These gene regions display high levels of polymorphism and retention of conserved residues when compared with mammalian genes (Miller *et al.*, 1997). Therefore, these two exons are probably derived from classical salmonid *Mhc* loci (Grimholt *et al.*, 1993; Miller *et al.*, 1997). The high rates of nonsynonymous substitution observed in the PBR of *Coregonus Mhc* genes, which exceed the synonymous substitution, indicate the presence of balancing or frequency-dependent selection at those loci (Nei & Hughes, 1991). The same kind of result was found in a variety of salmonid fish including rainbow trout, Atlantic salmon and chinook salmon (Miller & Withler, 1996; Miller *et al.*, 1997). The class I *A2* exon exhibited much higher variation (only 39% of amino acid sequence similarity between the most divergent whitefish sequences) but still resembled the other salmonid sequences. In this exon, the synonymous substitution rate was higher than nonsynonymous substitution within the PBR, suggesting that balancing selection at these loci is absent or occurs to a much lesser extent.

The finding of four distinct *A1* and *A2* nucleotide sequences in one individual demonstrates the occurrence of at least two class I loci in *Coregonus*. For the class II genes, a minimum of three loci are present since five different sequences could be obtained from the same individual. The observations for the class I loci are in accordance with the results of previous work showing the presence of multiple loci in salmonids (Grimholt *et al.*, 1994; Miller & Withler, 1998). However, more than one class II locus has been reported so far only for sockeye salmon *Oncorhynchus nerka* (Walbaum) (Miller & Withler, 1996).

Trans-species polymorphism is the sharing of similar allelic lineages by different species. For *Mhc* genes, this kind of polymorphism has been reported

for a wide variety of species including mammals and bony fishes (Klein *et al.*, 1998). In the Salmonidae, trans-species polymorphism has been described at class I loci in a number of Pacific salmonids. In this study, since we cannot attribute the sequences to specific loci, the interpretation of the phylogenetic trees regarding trans-species polymorphism may be biased. For example, Miller & Withler (1998) showed that salmonid exon 3 sequences exhibit a very distinctive clustering by locus first (rather than by species first), suggesting that the differentiation of these loci predate the formation of the salmoniformes.

In the neighbour joining analysis, the *Coregonus A2* sequences formed two distinct groups which were more related to alleles of two distinct salmonid loci than between themselves. However, within one of these apparently locus-specific clusters, some *Coregonus A2* sequences were more related to alleles from other salmonid species which indicates trans-species polymorphism. For the *A1* sequences, inter- and intra-locus variability are less well defined than for the *A2* exon (Miller & Withler, 1998). Therefore, it is not possible to determine whether trans-species polymorphism or locus-specific clustering is more likely to account for the finding that some *A1* whitefish sequences were more related to other salmonid rather than to other whitefish sequences.

Indications for trans-species polymorphism for salmonid *Mhc* class II genes, particularly for pink chum salmon *Oncorhynchus keta* (Walbaum) and sockeye salmon, have been reported (Miller & Withler, 1996). The present investigation shows that sequences from whitefish are more related to two alleles from salmonids (Sana561G and Sasa-c144) than to other coregonid sequences, perhaps indicating trans-species polymorphism for the whitefish *Mhc* class II *B1* exon. However, considering the aforementioned observation of multiple loci occurrence this could also reflect a clustering according to distinctive loci already present in a common ancestor. With the exception of those two alleles from lake trout and Atlantic salmon, phylogenetic analysis groups all the whitefish class II *B1* sequences into a single clade. Thus, it is likely that most of them are derived from a common ancestor which was distinct from ancestors of the other taxonomic groups. This result is consistent with the evolution pattern of *Mhc* class II *B* genes observed in other bony fishes (Klein & Horejsi, 1997).

In conclusion, several genes from the immune system of whitefish, an economically and ecologically important fish species have been described. The cloning and sequence analysis of *Mhc* genes provides an excellent additional tool for the study of population genetics, in particular for the analysis of the mode of evolution of species flocks within the Central Alpine region. They also represent molecular markers for the study of host-pathogen interactions, e.g. the host response to bacterial furunculosis.

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