

Temperature and length-dependent modulation of the MH class II β gene expression in brook charr (*Salvelinus fontinalis*) by a cis-acting minisatellite

Sébastien Croisetière^{a,*}, Louis Bernatchez^b, Pierre Belhumeur^a

^a Département de Microbiologie et Immunologie, Université de Montréal, Montréal, Québec H3C 3J7, Canada

^b Institut de Biologie Intégrative et des Systèmes (IBIS), Université Laval, Québec, Québec G1 V 0A6, Canada

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ABSTRACT

It is widely recognized that the variation in gene regulation is an important factor from which evolutionary changes in diverse aspects of phenotype can be observed in all organisms. Distinctive elements with functional roles on gene regulation have been identified within the non-coding part of the genome, including repeated elements. Major histocompatibility complex (MHC) genes have been the subject of an abundant literature which made them unique candidates for studies of adaptation in natural populations. Yet, the vast majority of studies on MHC genes have dealt with patterns of polymorphism in sequence variation while very few paid attention to the possible implication of differential expression in adaptive responses. In this paper, we report the identification of a polymorphic minisatellite formed of a 32 nucleotides motif (38% G+C) involved in regulation of the major histocompatibility class II β gene (MHII β) of brook charr (*Salvelinus fontinalis*). Our main objectives were: to analyze the variability of this minisatellite found in the second intron of the MHII β gene and to document its effect to the variation of expression level of this gene under different environmental conditions. Distinctive number of the minisatellite repeats were associated with each different MHII β alleles identified from exon 2 sequences. Relative expression levels of specific alleles in heterozygous individuals were determined from fish lymphocytes in different genotypes. We found that alleles carrying the longest minisatellite showed a significant 1.67–2.56-fold reduction in the transcript expression relatively to the shortest one. Results obtained in three different genotypes also indicated that the repressive activity associated to the longest minisatellite was more effective at 18 °C compared to 6 °C. In contrast, no significant difference was observed in transcript levels between alleles with comparable minisatellite length at both temperatures. We also depicted a significant up-regulation of the total MHII β transcript at 6 °C relative to 18 °C. These results reveal for the first time that a temperature-sensitive minisatellite could potentially play an important role in the gene regulation of the adaptive immune response in fishes.

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1. Introduction

More than three decades ago, King and Wilson (1975) proposed that the phenotypic divergence between human and chimpanzees was mostly attributable to changes in gene regulation rather than changes in the protein-coding sequences of the gene. It is now recognized that the variation in gene regulation is an important factor from which evolutionary changes in diverse aspects of phenotype can be observed in all organisms (Wray et al., 2003; Bird et al., 2007). Indeed, numerous studies demonstrated the correlation between variation in cis-regulatory sequences and significant modification in morphology, physiology or behaviour (Wray, 2007). In the last few years, several groups used the approach of genome-wide asso-

ciation analysis to identify the genetic basis of the variation in gene expression (Brem and Kruglyak, 2005; Cheung et al., 2005; Stranger et al., 2005). Being advantageous at multiple levels, these studies have the inconvenient of solely identifying the single nucleotide polymorphisms (SNPs) or marker in linkage disequilibrium with a determinant responsible for the variation of the expression level of the gene. Moreover, the correlation between the marker and the gene expression level requires a direct allele-specific expression measurement to confirm a cis-acting effect and further investigations to identify the mechanism responsible of the effect (Pastinen and Hudson, 2004).

Non-coding DNA other than the one found in the transcriptional regulatory region (i.e. promoter, enhancer and suppressor), considered for a long time as “junk” DNA, recently received increasing interest (Dermitzakis et al., 2002; Mouse Genome Sequencing Consortium et al., 2002; Thomas et al., 2003; Siepel et al., 2005). Distinctive elements with functional roles in the regulation of gene

* Corresponding author. Tel.: +1 514 343 6111x1432; fax: +1 514 343 5701.

E-mail address: sebastien.croisetiere@umontreal.ca (S. Croisetière).

expression have been identified within the non-coding part of the genome. These non-coding functional elements include introns, satellite DNAs and transposons, as well as pseudogenes and non-coding RNA genes (see Eddy, 2001; Balakirev and Ayala, 2003; Beauregard et al., 2008; Usdin, 2008 for reviews).

The different roles associated to introns include those in alternative splicing, transcriptional regulation, nuclear export, translation and chromatin structure. Moreover, the evolution of intron size in eukaryotes can be imputable to different models. The “selection for economy” model argues that natural selection favours short introns in highly expressed genes to minimize the cost of transcription and other molecular processes (Castillo-Davis et al., 2002; Eisenberg and Levanon, 2003). On the other hand, the “genomic design” model hypothesizes that the greater length of introns found in tissue-specific genes, possibly resulting from the accumulation of multispecies conserved sequences (MCSs) (Pozzoli et al., 2007), is an indication of their functional and regulatory complexity (Vinogradov, 2004, 2006). Furthermore, repeated sequences are frequently found in introns of higher eukaryotes. These repeated sequences are classified in two large families, called “tandem repeats” and “dispersed repeats” (Richard et al., 2008). The first family includes satellite DNAs, where the subfamilies of microsatellites and minisatellites are respectively tandemly repeated sequences of short (<10 bp) or longer motives (>10 bp and <100 bp) (Charlesworth et al., 1994). The second family contains transposons from which three subdivisions of retrotransposons can be identified; long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs) and long terminal repeats (LTRs) (Wicker et al., 2007). Both these families of repeated sequences have been shown to be implicated in regulation of gene expression (Ugarkovic, 2005; Feschotte, 2008; Tomilin, 2008).

Located in coding or non-coding regions of genomes, microsatellites (simple sequence repeats, SSRs) expansion or contraction can produce various effects on chromatin organization, regulation of gene activity, recombination, DNA replication, cell cycle, mismatch repair system, etc. (Li et al., 2002, 2004). SSRs variation is also well known for being responsible of human diseases such as neurological disorders and cancers (Atkin, 2001; Orr and Zoghbi, 2007), and also for contributing to normal variation in brain and behavioral traits (Fondon et al., 2008). These expanded repeats have the potential to adopt altered DNA secondary structures that confer genetic instability, leading eventually to phenotypic changes (Wells et al., 2005; Wang and Vasquez, 2006). On the other hand, functions associated to minisatellites (variable number of tandem repeats, VNTRs) are far less well characterized with the exception of their implication in recombination events and gene expression modulation (Bois and Jeffreys, 1999; Richard and Pâques, 2000). Probably the best characterized minisatellite regulating gene expression is the one found at the insulin (*INS*) gene locus (Bennett et al., 1995), for which the implication in susceptibility to human type 1 diabetes has been known for more than two decades (Bell et al., 1984). Other regulatory VNTRs were identified in additional genes as well: cystatin B (*CSTB*) (Laloti et al., 1997), serotonin transporter (*5-HTT*) (MacKenzie and Quinn, 1999), dopamine transporter (*DAT1*) (Mill et al., 2002) and type 2 bradykinin receptor (*B₂*) (Zamorano et al., 2006).

Classical major histocompatibility complex class II (MHCII) molecules are cell-surface glycoproteins specialized in the presentation of peptides, mainly derived from extracellular proteins, to the antigen receptor of CD4 T cells in the adaptive immune system. With the MHC class I molecules, they both represent the most polymorphic genes known to date, generally with multiple loci and high allelic diversity at each of these loci (Trowsdale and Parham, 2004). Classical major histocompatibility genes have been the subject of an abundant literature over the last 20 years or so. In particular, these genes offer several assets that made them unique candi-

dates for studies of adaptation in natural populations (Bernatchez and Landry, 2003). Yet, the vast majority of studies on MHC genes deal with patterns of polymorphism in sequence variation while very few paid attention to the possible implication of differential expression in adaptive responses. This is surprising since it has been known for many years that the modulation of the immune response is not only determined by the polymorphism of MHCII genes, but also by the cell surface expression correlation with the transcriptional level of the gene (Glimcher and Kara, 1992).

In a previous study, we identified a 32 bp motif minisatellite located in the second intron of the MH class II β (MHII β) gene in brook charr (*Salvelinus fontinalis*) (Croisetière et al., 2008). Here, our main objective is to investigate the possible implication of this minisatellite in the MHII β gene expression. More specifically, we (i) analyzed the variability of this minisatellite found in the second intron of the MHII β gene in brook charr and (ii) documented its effect to the variation of the expression level of this gene under different environmental conditions. Precise expression levels were determined directly from fish lymphocytes. In addition to the identification of TNF- α and LPS as regulator of the MHII expression in rainbow trout macrophages (Knight et al., 1998), the promoter characterisation of the MHII genes in Atlantic salmon (Syed et al., 2003) and the modulation of the transcriptional activity of immune system genes by low temperature (Rodrigues et al., 1998; Nath et al., 2006), results presented here improve our understanding of the factors contributing to the modulation of the expression of the MHII genes in teleosts.

2. Materials and methods

2.1. Fish

Individuals of approximately 50 g from a domestic strain of brook charr from a local hatchery (Chesterville, Québec, Canada) were used to evaluate the total expression level (cumulative of both alleles) of the MHII β gene in a natural environment. Our previous work confirmed the existence of a single copy of this gene in the brook charr (Croisetière et al., 2008). A sample of 96 individuals from this population was previously genotyped at MHII β gene which revealed eight different alleles (exon 2 coding sequence) with a distinct minisatellite size associated to each one (data not shown). Directly at the hatchery, head kidney tissue samples were taken for total RNA isolation from 23 random individuals in August when average water temperature was 18 °C and from 24 other individuals in November when average temperature was 6 °C.

A population of 93 brook charr originating from the Rupert River (Rupert strain) in northwestern Québec, Canada, and associated waterways were maintained in a controlled environment at the LARSA (Laboratoire de Recherches des Sciences Aquatiques; Université Laval). These fish of approximately 1 kg were tagged and genotyped at the MHII β locus (*DAB*) by SSCP (Single Strand Conformational Polymorphism) as previously described (Croisetière et al., 2008). Knowing their respective genotype, fish could be specifically selected for the length of the minisatellites they were carrying at the MHII β gene and used in real-time RT-PCR with specific primers to determine allelic expression. Before proceeding to blood sampling, fish were acclimatized 5–7 days at specified temperature (6 °C and 18 °C) to attenuate the effect of stress and assure a steady state expression of the MHII β gene.

2.2. RNA isolation from peripheral blood leukocytes (PBL) and head kidneys

Head kidneys from domestic fish (Chesterville) of each group (6 °C and 18 °C) were isolated to evaluate the total expression of

MHII β genes. Fish were sacrificed with an overdose of tricaine methanesulfonate (MS-222) (Sigma) before removing the head kidney that was mashed into a disposable dish using a syringe. An equivalent volume of Trizol (Invitrogen) was added before transferring the tissue sample to an Eppendorf for total RNA extraction according to the manufacturer's protocol. For each fish, a fin sample was taken for genomic DNA isolation.

RNA from PBL of brook charr (Rupert strain) was also extracted to evaluate the relative expression levels of MHII β alleles at 6 °C and 18 °C. Since it is non-lethal, RNA extraction from PBL compared to head kidneys allowed us to test gene expression of the same fish population at both experimental temperatures. Furthermore, this allowed the evaluation of the expression level of the MHII β gene for different alleles in a second strain and type of tissue. Fish were first anesthetised with 0.1% of tricaine methanesulfonate (MS-222) (Sigma). The blood was collected from the caudal vein using a heparinised 3 ml syringe with a 21G1 needle (Becton Dickinson) and added to 1 volume of ice-cold PBS containing 10 U/ml heparin sodium salt (Sigma). A fraction of this cellular suspension (1 ml) was then spun at 500 \times g for 5 min at 4 °C and the buffy coat and plasma transferred to a new Eppendorf. The PBL were then pelleted and resuspended in Trizol (Invitrogen) for total RNA extraction according to the manufacturer's protocol. Dimethyl sulfoxide (DMSO; 7% final concentration) was added to the remaining blood sample in PBS and frozen for subsequent genomic DNA isolation.

2.3. Polymerase chain reaction (PCR), cloning and sequencing

PCR was performed on genomic DNA from domestic strain fish to evaluate the size(s) of the minisatellite present in the second intron of the MHII β alleles. The PCR fragment containing the minisatellite was obtained with primers located in conserved regions of the second intron, after analysis of thirteen alleles in brook charr (data not shown). The sequence of the sense primer P399, located 69–91 base pair (bp) before the first minisatellite motif, was 5'-TTCTTGAATRGTTTTATAGCTGCAAC-3' and the antisense primer P400, located 37 or 81 bp after the last motif, was 5'-CATCGCTGTASTTTATAAATTACTTC-3'. PCR was carried out in a total volume of 50 μ l containing 20–40 ng of genomic DNA, 0.2 mM dNTPs, 7 μ l of 10 \times PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 2 mM MgCl₂, 5 pmol each of P399 and P400 primers, 1 unit of *Taq* DNA polymerase. The following PCR conditions were used: denaturation for 3 min at 94 °C followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 45 °C and extension for 2 min at 72 °C. The final extension was for 10 min at 72 °C.

For each allele of the Rupert strain previously identified (Croisetière et al., 2008), a MHII β gene fragment containing the complete exon coding for the β 1 domain (second exon) and the subsequent intron (second intron) was obtained by PCR on genomic DNA using primers in conserved regions for cloning and sequencing. The sequence of the sense primer SP4503, located at the end of the first intron of the gene, was 5'-CCTGTATTTATGTTCTCCTTTTCAGATGG-3' and the antisense primer P232, located at the N-terminal region of the β 2 domain (exon 3), was 5'-CTGAGCTCAGTCTGACATGG-3'. The reactions were carried out in a total volume of 50 μ l containing 20–40 ng of genomic DNA, 0.2 mM dNTPs, 7 μ l of 10 \times Expand high fidelity buffer (Roche), 2 mM MgCl₂, 5 pmol each of SP4503 and P232 primers, 2.6 units of Expand high fidelity enzyme mix (Roche) in a thermocycler (Biometra). The following PCR conditions were used: denaturation for 3 min at 94 °C followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C and extension for 3 min at 72 °C. The final extension was for 10 min at 72 °C.

PCR products, from 1350 to 2700 bp depending on MHII β Rupert alleles, were subsequently purified and extracted from agarose gel using the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare) and subcloned in both orientations in pT7Blue-2 vector (Novagen) in accordance with the recommended protocol from the manufacturer.

Sequencing of both strands was performed on a 3730 DNA analyser (Applied Biosystems) using plasmid primers P235-Blue2S-Tag (5'-ACCGCTGCTGCTAAATTCGAA-3') and P247 (5'-CGGAATTCTATAGTGTACC-3') as well as intron 2 internal sense primer P399 (5'-TTCTTGAATRGTTTTATAGCTGCAAC-3') and antisense primer P400 (5'-CATCGCTGTASTTTATAAATTACTTC-3'). Since the length of one particular allele (*Safo-DAB*0101*) was too long to reach the nucleotide sequences from both sides (see Section 3), an Exonuclease III (New England Biolabs) digestion of the vector containing the allele was realized to allow the sequencing through the minisatellite.

2.4. Reverse transcriptase (RT)

Quantification of extracted RNA from fish of both the domestic and Rupert strain was done on a ND-1000 Spectrophotometer (NanoDrop) after treating with the DNA-free kit (Ambion) and before cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen). Briefly, 500 ng of total RNA was mixed with oligo(dT)_{12–18} primer (Invitrogen), dNTPs (GE Healthcare) in a volume of 12 μ l and denatured at 65 °C for 5 min. This was followed by addition of 5 \times FS buffer, DTT, RNaseOUT (Invitrogen) and 200 units of SuperScript RT in a 20 μ l final reaction volume for incubation at 42 °C for 50 min, as recommended by the manufacturer.

2.5. Quantitative PCR

Quantitative PCR was performed using the FastStart SYBR Green Master (Roche Diagnostics) on a Rotor-Gene 3000 (Corbet Research). The primers used in these experiments are detailed in Table 1. All new primers were designed to flank a region containing an intron, to assure that amplification of contaminating DNA was controlled, which has not been detected in melting curve analysis. Amplification mixture (25 μ l) contained template cDNA (2 μ l of 1/20 dilution), 2 \times FastStart SYBR Green Master (12.5 μ l) and 300 nM forward and reverse primers. The cycling conditions comprised 15 min polymerase activation and denaturation step at 95 °C, followed by 40 cycles at 95 °C for 15 s, 60 °C for 45 s and 72 °C for 30 s. All samples were run in triplicate with non-template controls (NTC). Relative quantification of gene expression levels was done by comparing difference in C_T values by the $\Delta\Delta$ C_T method (Critical factors for successful real-time PCR, Qiagen), since the PCR efficiency was nearly equivalent for pairs of primers. For the domestic strain, EFIA_B, β 2m, GAPDH and TCR α were evaluated as reference genes to normalize the results before calculating the relative expression levels. The stability of the reference genes was evaluated with the Bestkeeper tool (Pfaffl et al., 2004). We kept the elongation factor I alpha, paralog B (EFIA_B) as reference gene, as it showed the lowest variability in our samples and also because it has been found suitable for gene expression assay in different studies (Olsvik et al., 2005; Jorgensen et al., 2006; Ingerslev et al., 2006). Within the population of 93 brook charr (Rupert strain) kept at the LARSA, we identified four genotypes from which the allelic expression levels could be analyzed at temperatures of 18 °C and 6 °C: *Safo-DAB*0201*0301*, *Safo-DAB*0401*0301*, *Safo-DAB*0101*0301* and *Safo-DAB*0101*0201*. The sizes of the minisatellites present in the MHII β gene from these individuals were respectively: 27 and 20, 40 and 20, 69 and 20, 69 and 27 repetitions of the minisatellite motif (Table 2). The relative allelic

Table 1
Primer sequences for quantitative RT-PCR in brook charr.

Gene	Allele	Name	Sequence (5'→3')
MHIIβ ^a	*0101	P451-Fwd GGAAGTATGTTGGATACACTGAGTAT
	*0201 and *0301	P452-Fwd GTATGTTGGATACACTGAGCTG
	*0101 to *0401 (generic)	P456-Fwd	GTGGGGAAGTATGTTGGATACACTGAG
	*0401	P478-Fwd GGAAGTATGTTGGATACACTGAGCAT
	*0101, *0201 and *0401	P537-Fwd	. . . CTAGGGGAGCTGGAGCGTGT
	*0301	P538-Fwd	GAGCTAGGGGAGCTGGAGAGTTA
	*0101 and *0201	P539-Fwd	. . . GCATGGTGCAGAGTTTATAGACTCTTACTAC
	*0301 and *0401	P540-Fwd	CCTGCATGGTATAGAGTTTATAGACTCTTATGTT
	*0101 to *0401	P232-Rev	CTGAGCTCAGTCTGACATGG
*0101 to *0401 (generic)	P457-Rev	ATGGCAGGGTGTCTGCCACTA	
β2m	N/A	P454-Fwd	AGAGTGTGGATTACACCAGCCA ^b
	N/A	P455-Rev	TCCTTACATATCTGCCTCCAGGT ^b
EFIA _B	N/A	P460-Fwd	TGCCCTCCAGGATGTCTAC ^c
	N/A	P461-Rev	CACGGCCACAGGTA ^c
GAPDH	N/A	P462-Fwd	AGATGTGGCCATCAACGACCC ^d
	N/A	P463-Rev	AAACGTCCGTGGGTGGAGTCATA ^d
TCRα	N/A	P464-Fwd	GACAGCTACTACAGCCAGGTT ^e
	N/A	P465-Rev	CAGAATGGTCAGGGATAGGAAGTT ^e

Abbreviations: MHIIβ, Major histocompatibility class II gene beta; β2m, beta-2 microglobulin; EFIA_B, elongation factor I alpha, paralogue B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TCRα, T cell receptor alpha; Fwd, forward; Rev, reverse; N/A, not applicable. Dots (.) are introduced to aligned primers. ^aPrimer sequences are from Croisetière et al. (2008). ^bFrom conserved region of *Oncorhynchus mykiss* gene sequences. ^cReference: Olsvik et al. (2005). ^d*Salmo salar* gene sequence. GeneBank accession no. AM230811. ^eReference: Moore et al. (2005).

expression levels were calculated by comparing differences in C_T values in the same sample with allele-specific primers. Samples were included in different qPCR runs to control the reproductivity of the results. Differences for MHIIβ expression levels as a function of minisatellite length or temperature were compared using the *t*-test with bilateral distribution for samples with homogeneous variance.

2.6. Sequence analysis

Nucleotide sequences from the MHIIβ second intron alleles of the Rupert strain were submitted to GenBank (accession nos. GQ253476, GQ253477, GQ253478, GQ253479, GQ253480

and GQ253481). The coding sequences of the six MHIIβ alleles can also be found (GenBank accession nos. EU478851, EU478852, EU478853, EU478854, EU478855 and EU478856). Alignment of the nucleotide sequences was performed using ClustalX (Thompson et al., 1994) and MEGA 4 (Tamura et al., 2007). Identity of the intronic sequences was determined by pairwise-comparison of nucleotide sequences with the Syn-SCAN program (Gonzales et al., 2002). GeneBank published sequences from Atlantic salmon, *Salmo salar* (*Sasa-DAB*0201*; AJ439067 and *Sasa-DAB*0301*; AJ439069) and *Oncorhynchus* species (*Onmy-Su2a*; U34715, *Onne-Wk136e*; U34713, *Onne-Wk136a*; U34712 and *Onke-An13c*; U34702) (Miller and Withler, 1996) were included in the alignment.

Table 2
Characteristics of the second intron in MHIIβ alleles from brook charr (*Safo-DAB**) and other salmonid fishes.

Allele	Intron length (nt.)	Minisatellite			<i>Hpa</i> I SINE	
		Motif ^a	No. repeats	Purity (%)	Partial	Complete
<i>Safo-DAB*0101</i>	2410	I	69 ^b	88.1	No	No
<i>Safo-DAB*0201</i>	1382	I	27	99.7	No	No
<i>Safo-DAB*0301</i>	1095	I	20	99.4	No	No
<i>Safo-DAB*0401</i>	1696	I	40	99.1	No	No
<i>Safo-DAB*0501</i>	1023	I	19	95.6	No	No
<i>Safo-DAB*0601</i>	1255	I	25	99.6	No	No
	1334	I	25 ^c	95.4	No	No
<i>Sasa-DAB*0201</i>		II	6 ^c	98.7		
<i>Sasa-DAB*0301</i> ^d	478	I	≥7	92.0	No	N/A
<i>Onmy-Su2a</i>	563	I	1	88	Yes	No
<i>Onne-Wk136e</i>	547	I	1	88	Yes	No
<i>Onne-Wk136a</i>	731	I	1	88	Yes	Yes
<i>Onke-An13c</i>	773	I	1	78	Yes	Yes

N/A, Not available.

^a Consensus sequence, type I (32 nt.): ACATTACAGTATGACTAGTCAGCTATGTAGTG; consensus sequence, type II (40 nt.): TATGATAACATCCAATAATATATGGTGAACATG-GAATA.

^b 10 partial motifs.

^c 1 partial motif.

^d Incomplete intronic sequence.

3. Results

3.1. Nucleotides sequences and minisatellite from the second intron of MHII  gene

The first objective of this study was to document the variability of a minisatellite found in the second intron of the MHII  gene in brook charr. As a first step, we sequenced the second intron of the six brook charr MHII  alleles of the Rupert strain identified in a previous study (Croiseti re et al., 2008). Characteristics of the second intron of these alleles and of other salmonids MHII  gene are given in Table 2. The intron length differed among all six exon alleles iden-

tified in brook charr of the Rupert strain and ranged from 1023 to 2410 nucleotides. The alignments of the second intron nucleotide sequences flanking a minisatellite in 5' (Fig. 1) and 3' (Fig. 2) are presented for selected salmonid species to illustrate the diversity found at this locus. A schematic representation of this diversity in the second intron of the MHII  gene of salmonids is also shown in Fig. 3.

Four regions of the intron indicated as Box I to Box IV showed variable level of sequence divergence in pairwise comparisons (Figs. 1–3). The first 261 nucleotides of the second intron (Box I) showed pairwise divergence values ranging from 1.20% to 7.02% (mean of 5.52%) among brook charr alleles to a maximum of 12.9%

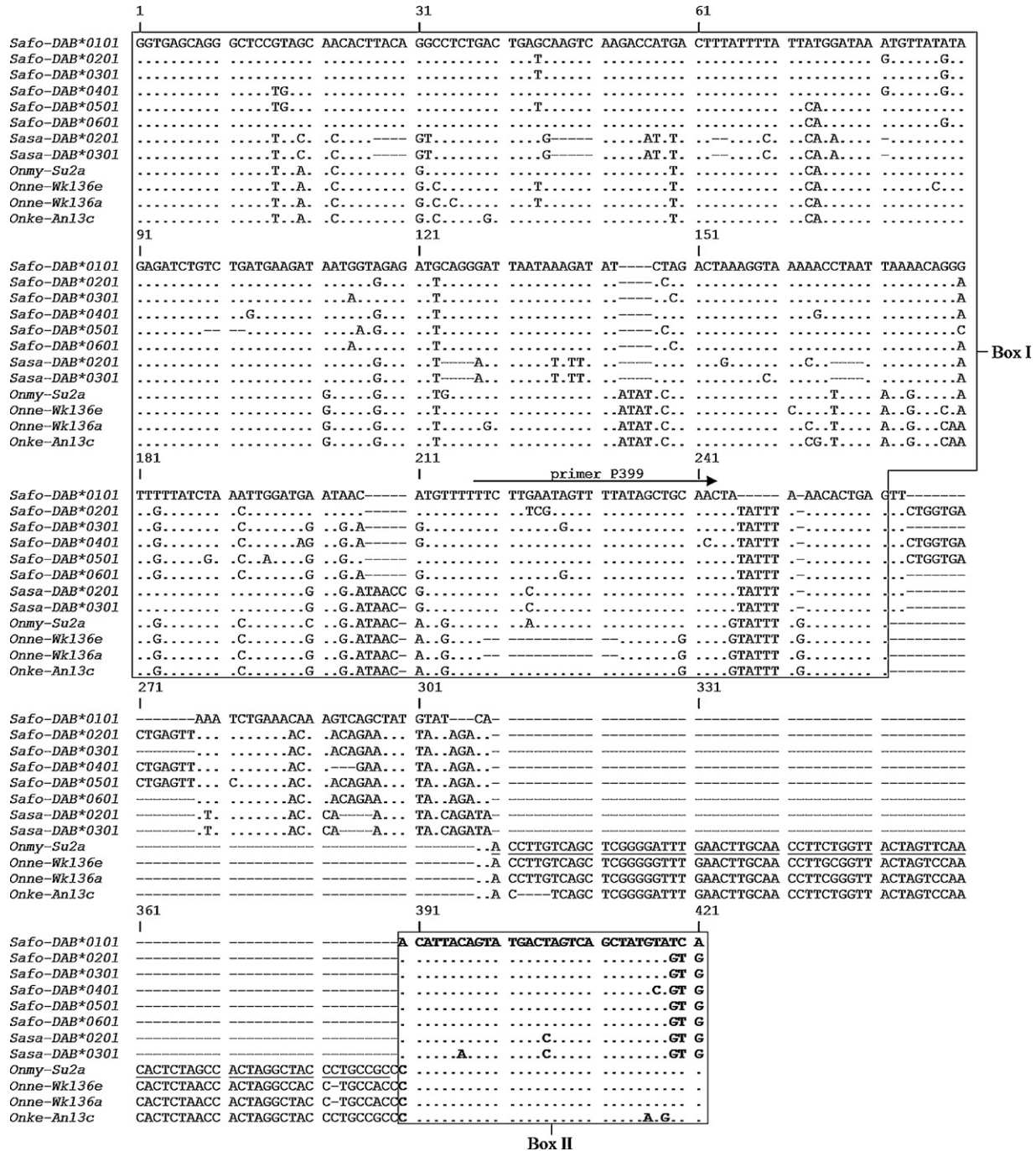


Fig. 1. Alignment of the second intron nucleotide sequences preceding the minisatellite found in the MHII  gene from brook charr and other salmonids. The first minisatellite motif sequence of each allele is in bold. Dots (.) indicate identity with *Safo-DAB*0101*; dashes (-), gaps introduced to maximize the alignment. Portions of the intronic sequences showing high conservation are identified with boxes (I–II). The partial *Hpa* I SINE sequence is underlined. GeneBank accession numbers for Atlantic salmon (*Salmo salar*; Sasa) and Pacific salmon (*Oncorhynchus* species; Onmy, Onne and Onke) sequences are AJ439067, AJ439069 and U34715, U34713, U34712, U34702 respectively.

(mean of 8.46%) when compared to other salmonid species. Box II corresponds to the first minisatellite motif from brook charr and Atlantic salmon, and the sole motif reported in Pacific salmon species. This 32 nucleotide sequence diverged by 0 to 4 nucleotides (mean of 1.33 nt.; 4.17%) within brook charr while divergence for up to 8 nucleotides were found (mean of 3.05 nt.; 9.52%) between species. Box III comprises 36 nucleotides with divergence ranging from 0 to 5 nucleotides (mean of 2.27; 9.45%) among brook charr alleles and up to 9 substitutions (mean of 4.22 nt.; 13.4%) among species. Box IV is composed of the last 58 nucleotides of the intron with divergence values ranging from 0.00% to 5.00% (mean of 2.33%) within brook charr alleles and up to 17.9% (mean of 7.07%) among species.

The minisatellite identified within the second intron has a consensus motif of 32 nucleotides (type I in Table 2): ACAT-TACAGTATGACTAGTCAGCTATG TAGTG; which is 38% G+C rich.

Search for homologue sequences did not reveal its occurrence in salmonid species other than Atlantic salmon. PCR amplification of a fragment containing this minisatellite with the primer pairs P399/P400 revealed that each allele contains a different number of the minisatellite repeats with 69, 27, 20, 40, 19 and 25 repetitions found in the *Safo-DAB*0101* to *Safo-DAB*0601* allele, respectively (Table 2). An example of the minisatellite found in allele *Safo-DAB*0301* is shown (Fig. 4). With the exception of the *Safo-DAB*0101* allele, sequencing also revealed that the purity of the repeated motif was very high, ranging from 95.6% to 99.7%. In contrast, allele *Safo-DAB*0101* showed a high level of mutation over the whole minisatellite length; with 86% (59 out of 69) of the motifs being mutated in comparison with the consensus sequence. Moreover, these mutated motifs included ten additional ones that showed a deletion of about 50% of the nucleotides relative to the consensus motif (Fig. 3).

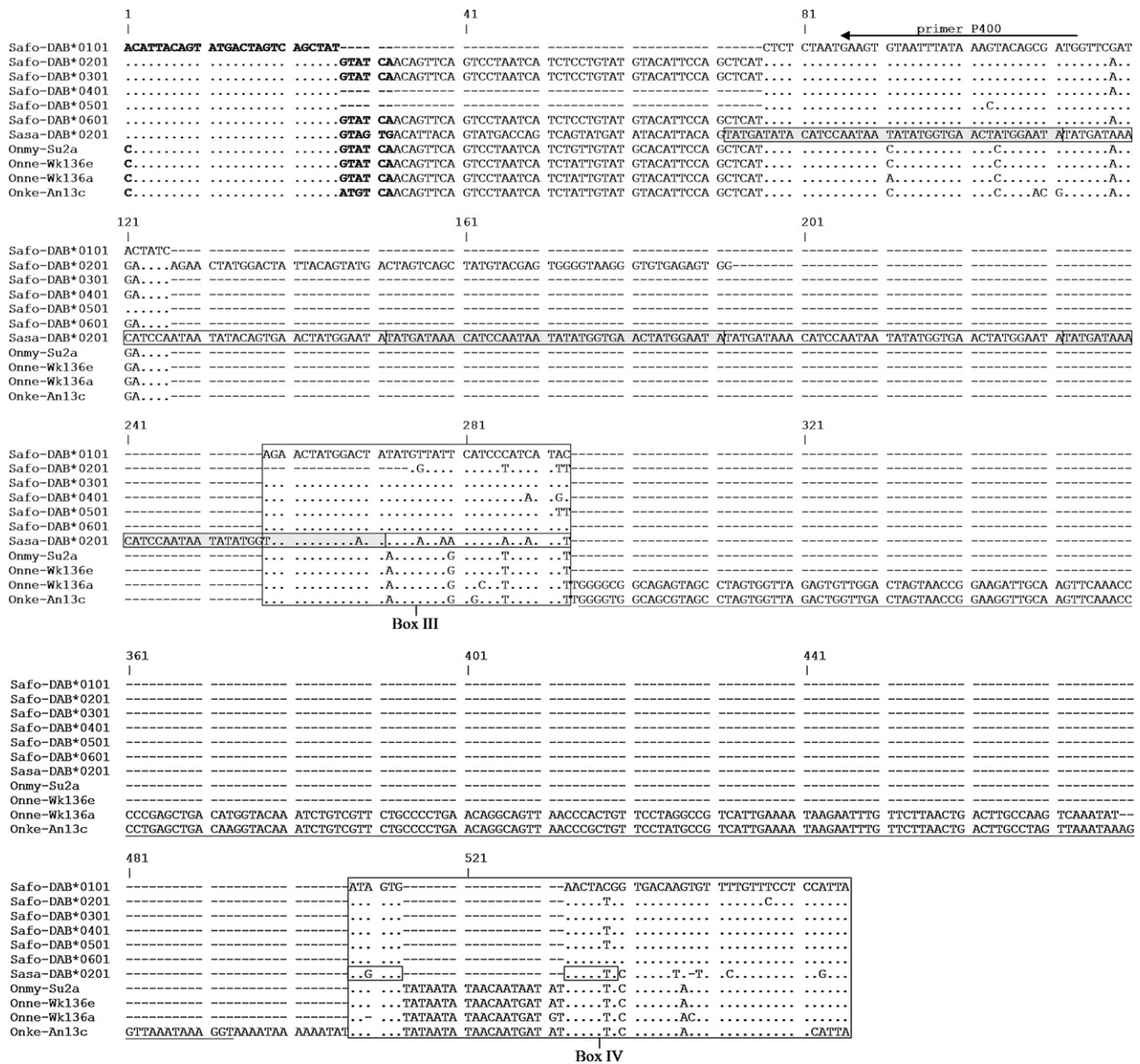


Fig. 2. Alignment of the second intron nucleotide sequences following the minisatellite found in the MHIIβ gene from brook charr and other salmonids. The last minisatellite motif sequence of each allele is in bold. Dots (.) indicate identity with *Safo-DAB*0101*; dashes (-), gaps introduced to maximize the alignment. Portions of the intronic sequences showing high conservation are identified with boxes (III–IV). The motifs (type II) forming the second minisatellite in Atlantic salmon are alternatively indicated by a grey and white rectangle. The complete *Hpa* I SINE sequence is underlined. GeneBank accession numbers for Atlantic salmon (*Salmo salar*; Sasa) and Pacific salmon (*Oncorhynchus* species; Onmy, Onne and Onke) sequences are AJ439067, AJ439069 and U34715, U34713, U34712, U34702 respectively.

3.2. *MHIIβ* gene expression level is inversely correlated to minisatellite length

A quantitative real-time RT-PCR experiment was realized on head kidney RNA from the domestic strain to evaluate the possible effect of minisatellite length on gene expression level of *MHIIβ* at both temperatures of 18 °C and 6 °C. This strain was selected because the fish were exposed to natural fish farm environment and also because of availability of specimens. However, since the genotypes of the individuals at the fish farm are variable in function of new incorporated stocks, the coding sequence of the *MHIIβ* gene was not considered and the focus was oriented on the minisatellite length found in the second intron.

To estimate the size of the minisatellite present in both *MHIIβ* genes, a PCR amplification with the P399/P400 conserved primer pair was realized on genomic DNA from 23 individuals for which the head kidney was sampled at 18 °C and 24 individuals at sampled at 6 °C directly at the fish farm. Depending on *MHIIβ* allele, PCR products of approximately 750, 925, 975, 1600 and 2000 bp, corresponding to approximately 19, 25, 27, 46 and 59 motif repetitions respectively, were generated (Fig. 5A). We identified 23 homozygote and 24 heterozygote individuals in the 47 fish sam-

pled at both temperatures, with the former ones clearly showing increased intensity of the PCR product (e.g. #4, #8 at 18 °C). Fish carrying both short minisatellites, heterozygote (#3 and #15; 19 and 25 repeats) or homozygote (#4 and #14; 25 repeats), and long minisatellites, homozygote (#1 and #7; 46 repeats), were selected at 18 °C, while at 6 °C fish were also selected for short minisatellites, heterozygote (#10 and #22; 19 and 25 repeats) or homozygote (#8 and #15; 25 repeats), and long minisatellites (#12; 46 and 59 repeats) for the quantitative RT-PCR experiment.

Fig. 5B shows the relative total *MHIIβ* gene expression values (generic P456/P457 primer pair) in head kidney for short or long minisatellites present in the gene, at temperatures of 18 °C and 6 °C with *EFlA_B* as reference genes. The results are the cumulative of two RT-PCR experiments where each sample is tested each time in triplicate. At 18 °C, the *MHIIβ* genes having a short minisatellite (19 and 26 repeats) showed a relative expression of 1.12 ± 0.12 while it was 0.75 ± 0.17 for the genes with a long minisatellite (46 repeats). This real-time PCR results demonstrated a significant 1.49-fold down-regulation (*t*-test, *p* < 0.01, *n* = 8 and 4) of the *MHIIβ* expression for gene carrying a long minisatellite. At 6 °C, the relative expression was 2.31 ± 0.27 for the genes with a short minisatellite and

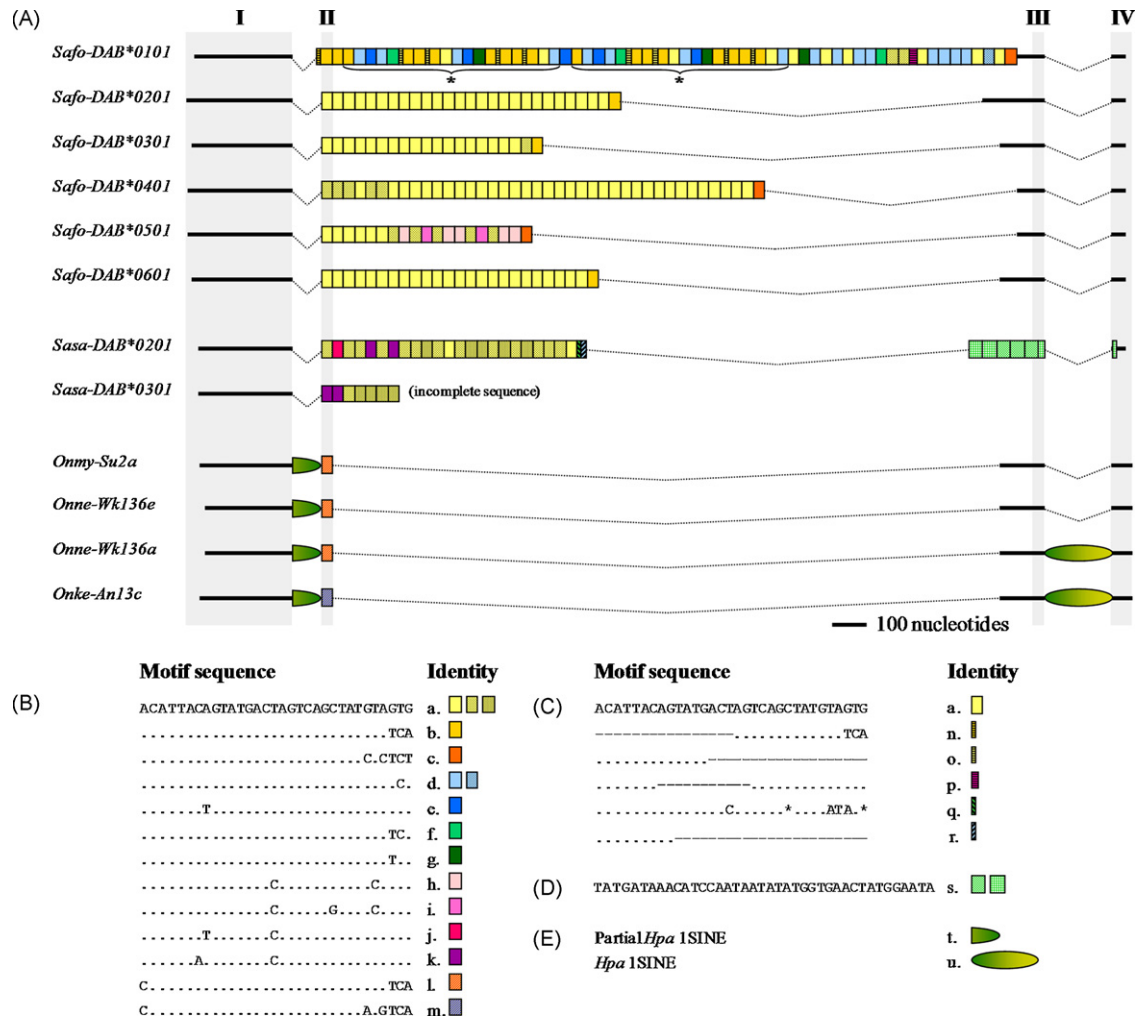


Fig. 3. Properties of the second intron of the *MHIIβ* gene in salmonids. (A) Schematic representation of the allelic diversity found in the *MHIIβ* gene of various salmonid species. Identity of the motifs and elements correspond to the letters and color shapes indicated in (B), (C), (D) and (E). The sequence of 22 motifs repeated twice in *Safo-DAB*0101* is marked by an asterisks (*). (B) Complete motif sequences with corresponding identity indicated below by a letter and color boxes. Motifs with one or two mutations relative to the original are represented by boxes with few or many shading respectively. Dots (.) indicate identity with the consensus motif. (C) Partial motif sequences with the element identity indicated below. Asterisks (*) indicate gaps introduced to maximize the alignment; dashes (-), sequence not related to the motif. (D) Motif sequence of the second minisatellite (type II) present in the second intron of *MHIIβ* gene from Atlantic salmon. (E) Identity of the partial and complete *Hpa* I SINES.

Safo-DAB*0301 intron 2

EXON 2 ◀ GGTGAGCAGGGCTCCGTAGCAACACTTACAGGCCTC
 TGACTGATCAAGTCAAGACCATGACATTTATTTATTATGGATAAA
 TGTTATGTAGAGATCTGTCTGATGAAGATAAATAGTAGAGATTCAG
 GGATTAATAAAGATATCTCGACTAAAGGTAACCTAATTAATAAA
 CAGGATGTTATCTAAGTGGATGGATGAAGTGTTTTCTTGAA
 TGGTTTTATAGCTGCAACTATATTTAAACACTGAGTTAAATCTGA
 AAACAAACAGAAATATTAATAGACA
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 1
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 2
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 3
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 4
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 5
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 6
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 7
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 8
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 9
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 10
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 11
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 12
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 13
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 14
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 15
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 16
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 17
 ACATTACAGTATGACTAGTCAGCTATGTAAGT >Repeat 18
 ACATTACAGTAGGACTAGTCAGCTATGTAAGT >Repeat 19
 ACATTACAGTATGACTAGTCAGCTATGTAATCA >Repeat 20
 ACAGTTCAGTCCATAATCATCTCCTGTATGTACATTCAGCTCATC
 TCTCTAATGAAGTGTAATTTATAAAGTACAGCGATGGTTCAATGA
 TATCAGAAGTATGGACTATATGTTATTCATCCCATCATACATAGT
 GAACACTACGGTGACAAGTGTTTTGTTTTCTCCATTA ▶ **EXON 3**

Fig. 4. Example of a minisatellite sequence within in the second intron of brook charr MHIIB gene, allele *Safo-DAB*0301*. The intron of this allele contains 20 perfect repeats of the 32 nucleotide motif. The purity and numbers of motifs found in brook charr and salmonids alleles are presented in Table 2.

1.56 ± 0.05 for the genes with a long one, representing a significant 1.48-fold down-regulation (*t*-test, *p* < 0.001, *n* = 8 and *n* = 2) for the MHIIB gene carrying a long minisatellite. These results further indicated that the lower amount of expression associated to the long minisatellite is observed at both temperatures. It has been previously demonstrated that the expression of MHIIB genes was influenced by temperature in rainbow trout (*Oncorhynchus mykiss*) (Nath et al., 2006). Then, we finally compared the total expression level of MHIIB gene from fish having a short minisatellite at both temperatures and observed a significant 2.08-fold up-regulation of the MHIIB transcript at 6 °C relative to 18 °C (*t*-test, *p* < 0.001, *n* = 8).

3.3. The minisatellite from MHIIB gene represents a functional cis-acting polymorphism

Since the variation in MHIIB expression level seems to be modulated by the size of the minisatellite present in the gene, we developed a real-time RT-PCR assay to quantify the relative expression level of both alleles in peripheral blood leukocytes (PBL) from the same heterozygous fish. Values indicate the relative allelic expression levels of MHIIB for the target allele in function of the second one used as reference. The results from eight individuals of *Safo-DAB*0201/*0301* genotype (27 and 20 repeats) indicated an average MHIIB relative allelic expression of 0.97 ± 0.06 at 18 °C, and 1.00 ± 0.02 for seven individuals at 6 °C (primers P537–P538/P457) (Fig. 6). This observation is not primer specific given that it was observed with a second pair of primers: P539–P540/P232 (data not shown). In fish of this genotype, the analysis of the relative allelic

expression revealed no significant difference in the transcript levels between these alleles at both temperatures.

We also analyzed individuals with the *Safo-DAB*0401/*0301* genotype (40 and 20 repeats), for which the difference in minisatellite length is more important. In those fish, we detected an allelic imbalance in the expression profile between both alleles. The relative expression level of the *Safo-DAB*0401* allele in function of *Safo-DAB*0301* allele, as studied in five individuals, was 0.51 ± 0.06 and 0.59 ± 0.06 at 18 °C and 6 °C, respectively (primers P452–P478/P457) (Fig. 6). This represents a 1.96-fold and 1.69-fold reduction of the expression for the allele carrying the longer minisatellite. Moreover, these values indicate a significant difference in the expression levels between 18 °C and 6 °C (*t*-test: *p* < 0.05, *n* = 9).

The last two genotypes for which we analyzed the relative expression levels were *Safo-DAB*0101/*0301* (69 and 20 repeats) and *Safo-DAB*0101/*0201* (69 and 27 repeats), with the *Safo-DAB*0101* allele carrying the longest minisatellite found in this population present in both ones. In the former genotype, found in three individuals (2 analyzed at 6 °C because of one mortality between sampling), the relative allelic expression was 0.45 ± 0.05 at 18 °C and 0.60 ± 0.07 at 6 °C (primers P451–P452/P457). This corresponded to a significant 2.22-fold and 1.67-fold reduction in the expression of the *Safo-DAB*0101* allele compared to *Safo-DAB*0301* at both temperatures (*t*-test: *p* < 0.001, *n* = 9 (18 °C), *n* = 6 (6 °C)) (Fig. 6). This result was confirmed with the pair of primer P539–P540/P232 (data not shown). Found in 11 individuals, the second genotype in which *Safo-DAB*0101* was present also showed a reduction in the transcript expression of the allele carrying the longest minisatellite and a significant difference in the relative expression levels between temperatures (*t*-test: *p* < 0.05, *n* = 10). A relative allelic expression of 0.39 ± 0.06 (2.56-fold reduction) and 0.51 ± 0.03 (1.96-fold reduction) at 18 °C and 6 °C respectively was found for the *Safo-DAB*0101/*0201* genotype (Fig. 6). The results presented for the last three genotypes (*Safo-DAB*0401/*0301*, *Safo-DAB*0101/*0301* and *Safo-DAB*0101/*0201*) further indicates that the repressive activity associated to the longest minisatellite was more effective at 18 °C compared to 6 °C and therefore highlighted a functional cis-acting polymorphism for the minisatellite present in the second intron of the MHIIB gene.

4. Discussion

4.1. Instability of the minisatellite found in the second intron of MHIIB gene

In this study, a minisatellite composed of a 32 nucleotides motif was identified in the second intron of the MHIIB gene in brook charr. This minisatellite has already been revealed in the same gene in Atlantic salmon (Stet et al., 2002) and as a single 32 nucleotides motif in Pacific salmon (*Oncorhynchus*) species (Miller and Withler, 1996). Search for homologue sequences of the minisatellite did not reveal its occurrence in other species. The length of the intron found in brook charr is comparable to that found in Atlantic salmon, but longer than for Pacific salmon. The presence of the motif as a single or as a minisatellite sequence in all salmonids species, combined with the low sequence divergence of Box I and Box IV support the hypothesis of a single and same ancestral expressed MHIIB locus in salmonids (Glamann, 1995; Langefors et al., 2000).

Atlantic salmon minisatellites (type I) are showing an increase accumulation of mutations with purity of 92.0% and 95.4% over the entire length. For microsatellites, it has been demonstrated that expansion and contraction mutations on the tandem repeat tend to remove the imperfections found in the motifs by the mechanism of polymerase slippage, while in the absence of this purifying activ-

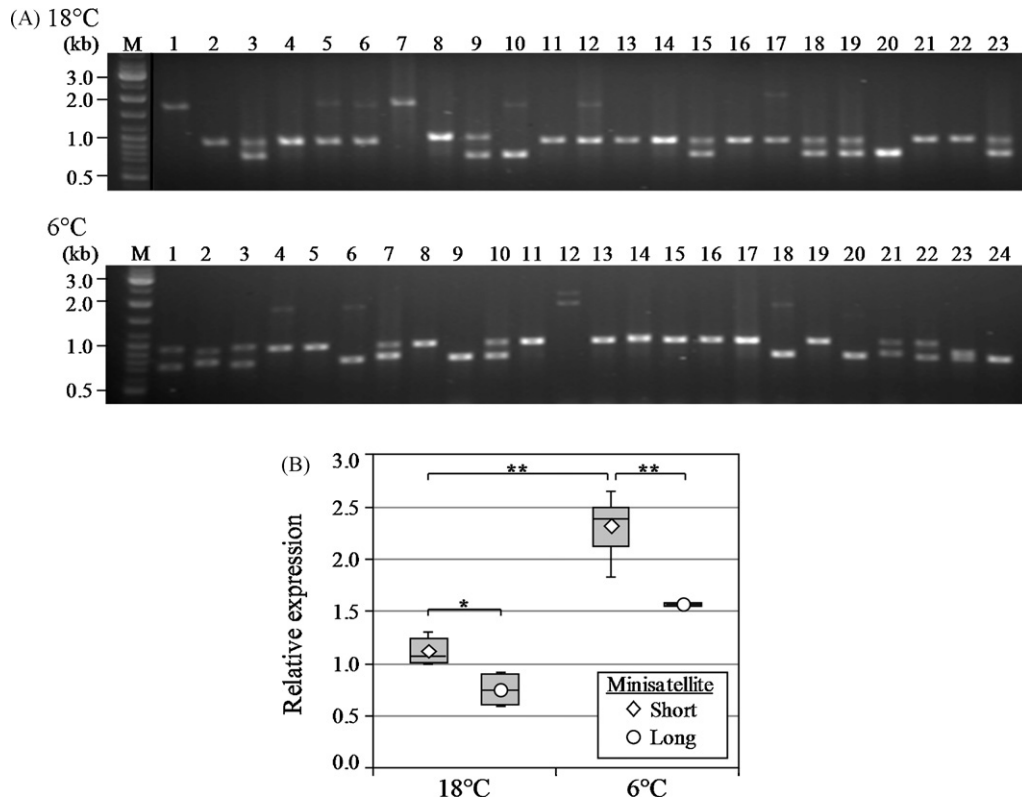


Fig. 5. MHIIB expression level in brook charr head kidney as a function of minisatellite length and temperature. (A) Genomic PCR showing the length of the minisatellite present in the second intron of MHIIB alleles (domestic strain) amplified with the P399/P400 primers. A total of 23 fish and 24 fish were sampled at 18 °C and 6 °C, respectively. Short and long minisatellites have 19 or 25 and 46 or 59 repeats of the 32 nucleotide motif. (B) Box plots giving the relative expression distributions, evaluated by real-time RT-PCR, of total MHIIB mRNA from fish with variable minisatellite length. Symbol legend: diamond, short minisatellite; circle, long minisatellite. Results from samples at 18 °C and 6 °C are normalized for EFlA_β. The top and bottom of each rectangle represent the 75th and 25th percentiles, respectively; the bar and symbol in the rectangle indicate the median and average expression value respectively; the top and bottom whiskers show the maximal and minimal allelic expression value. Results are the cumulative data of two independent real-time RT-PCR experiments where samples are analyzed in triplicate. Comparisons of the distributions using the *t*-test returned significant results: **p* < 0.01 and ***p* < 0.001.

ity, the motifs accumulate punctual mutations (Ellegren, 2000). Thus, if some extrapolation can be done for minisatellite, this would suggest that the high minisatellite purity found in the brook charr MHIIB gene, with the exception of the *Safo-DAB*0101* and Atlantic salmon alleles, are indicative of a purifying activity, presumably a

consequence of a selection force preserving further possibility of expansion or contraction of the minisatellite.

Analysis of the motif sequences found in *Safo-DAB*0101* allele revealed the perfect repetition of a DNA fragment within the minisatellite composed of 22 motifs representing 704 nucleotides

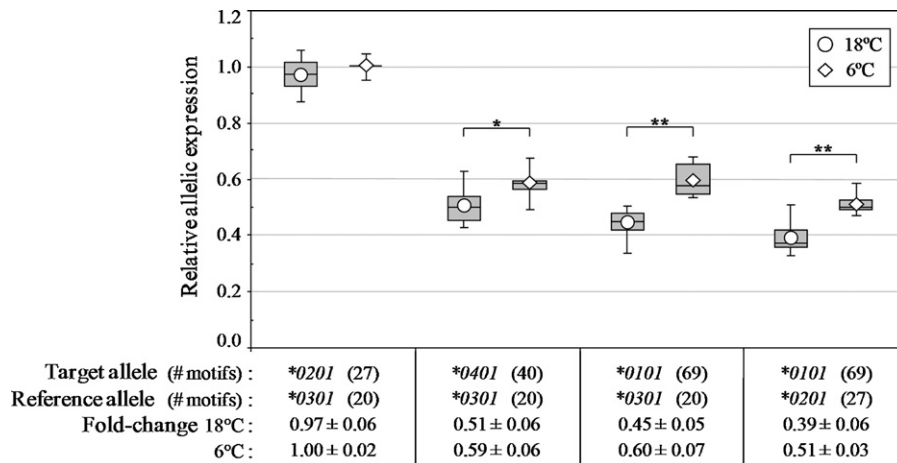


Fig. 6. Expression level of MHIIB alleles as a function of both minisatellite number of repeats and temperature. Box plots showing the relative allelic expression distributions of MHIIB mRNA in brook charrs' lymphocytes (Rupert strain) at 18 °C and 6 °C. The allelic expression levels are expressed in function of the other one in parenthesis. The top and bottom of each rectangle represent the 75th and 25th percentiles, respectively; the bar and symbol in the rectangle indicate the median and average allelic expression value respectively; the top and bottom whiskers show the maximal and minimal allelic expression value. Symbol legend: circle, value at 18 °C; diamond, value at 6 °C. Values presented are from a single (*Safo-DAB*0201*/**0301* and *Safo-DAB*0101*/**0201*), two (*Safo-DAB*0401*/**0301*) or three (*Safo-DAB*0101*/**0301*) independent real time RT-PCR experiment, where all samples are analyzed in triplicate. Comparisons of the distributions using the *t*-test returned significant results: **p* < 0.05 and ***p* < 0.001.

identified by asterisks in Fig. 3. Since the minisatellite found in this allele is composed of motifs showing a complex and high level of polymorphism, this repetition seems to reflect a recombination event that occurred previously and support this mechanism to explain the expansion of this minisatellite sequence as previously suggested (Debrauwere et al., 1997).

4.2. Minisatellite modulation of the MHII β gene expression level

We also described a functional effect associated to the presence of the polymorphic minisatellite on the transcriptional activity of the MHII β gene. Regulation of the transcriptional activity by minisatellites has already been demonstrated in few studies (e.g. Pugliese et al., 1997; MacKenzie and Quinn, 1999; Zamorano et al., 2006). This genetic element is added to a different minisatellite (type II 40 nucleotides motif, Figs. 2, 3 and Table 2), a partial and a complete *Hpa* I SINE that were identified previously at the same location in salmonids (Figs. 1–3 and Table 2) (Miller and Withler, 1996; Stet et al., 2002). The possibility of MHII β gene expression modulation offered by these types of genetic elements could constitute a fine tuning mechanism to ensure an appropriate immune response to pathogens, while limiting deleterious effects to host tissues.

In fact, two strains of brook charr demonstrated a reduction in MHII β gene expression for different alleles possessing the longest minisatellite. The effect was also seen at the total RNA expression level in head kidney tissue (Fig. 5) or by comparison of the allele's relative expression level in lymphocytes of heterozygous fish (Fig. 6). This analysis of the transcriptional allelic variation allows the detection of small differences observed between alleles and also eliminates the inter-individual variation that could come from different genetic background as well as environmental or physiological conditions rather than direct genetic factors. Also, the real-time RT-PCR results on heterozygous fish (Fig. 6) were subsequently confirmed with different probes for the same allele (Table 1). With the exception of the few last 3' nucleotides that were allele specific, the primers for the quantification between these alleles were localized at the same position at the end of the second exon and produced same size amplicons with minimal variation in nucleotide sequence. The selection of housekeeping gene is also known to be a critical factor in gene expression experiments. We used EF1A β that was reported with the most stable transcription level in a selection of genes in Atlantic salmon (Olsvik et al., 2005). This reference gene also showed a stable level of expression at both temperatures in the tissues tested for the quantification experiments (Croisetière et al., in preparation). Moreover, we observed this effect in multiple heterozygous individuals presenting three different genotypes where the MHII β alleles carrying the longest minisatellite (*Safo-DAB*0101* and *Safo-DAB*0401*) showed reduce transcript expression when compared to the allele with the shortest one (Fig. 6). It is noteworthy that the difference between the expression level of the allele *Safo-DAB*0101* relatively to *Safo-DAB*0301* was not greater than *Safo-DAB*0401* relatively to *Safo-DAB*0301*, even if the number of motif found in *Safo-DAB*0101* is superior (69 versus 40). One possible explanation for this could be the presence of multiple mutations in this allele that decrease its impact.

Finally, our results also confirmed that reduced expression level associated to alleles carrying a long minisatellite is modulated by the temperature. The temperature effect on teleost MH gene expression has been addressed for more than a decade now (Rodrigues et al., 1998). Here, results clearly showed a more pronounced difference of MHII β gene expression level at 18 °C between alleles of different minisatellite length (Fig. 6). This could possibly reflect a greater thermodynamical energy for the adoption of a DNA secondary structure as functional mechanism.

4.3. Reduced immune gene expression at high temperature in brook charr?

When we compared at both temperatures the total expression level of the MHII β genes carrying short minisatellites, we found that the expression was 2.08-fold higher at 6 °C than at 18 °C (Fig. 5). The difference was approximately of the same order for long minisatellites, however with fewer samples tested. This increased expression at low temperature is in accordance with the data of Raida and Buchmann (2007) for constitutive expression of MHII β gene in rainbow trouts' head kidney. In their paper, Table 4 indicates a difference of 0.8 raw C $_T$ between MHCII expression at 5 °C and 15 °C, while the expression of the reference gene EF1- α was stable. Since MHCII and EF1- α show almost the same PCR efficiency (101.1% vs 100.0%), this represents a 1.74-fold higher expression of MHII β at 5 °C, a result in accordance with ours. On the other hand, Nath et al. (2006) observed a downregulation of MHII β gene expression at mRNA and protein levels in rainbow trout at 2 °C. However, this conflicting result seems to be the consequence of the exposition to temperature below the immunologically nonpermissive temperature of ~4 °C (Fryer et al., 1976; Bly and Clem, 1991) and near the lower lethal temperature of -0.7 °C for salmonids (Saunders, 1995). Furthermore, since the gene coding for the ribosomal protein S11 also shows a decrease in transcript at 2 °C, the effect reported by Nath et al. (2006) seems to be the result of a more general and severe repression of the transcription.

From the evaluation of the four candidate housekeeping genes that was realized, the β 2m gene was surprisingly ranked as the worst gene in the samples tested. A close examination of the C $_T$ obtained with the β 2m gene revealed an interesting fact. The mean of the C $_T$ at 18 °C for the β 2m expression was 18.18 \pm S.E. 0.44 while it was of 16.89 \pm S.E. 0.40 at 6 °C. This difference was significant (*t*-test: *p* < 0.001, *n* = 6) and represented a 2.78-fold higher expression of β 2m at 6 °C once corrected with the reference gene EF1A β (data not shown). This result concord with studies performed in Atlantic salmon and rainbow trout that show no decrease of the β 2m transcript after 10 days at temperature of 2 °C (Kales et al., 2006), unlike to a reduction of the expression at 6 °C reported in common carp (Rodrigues et al., 1998). The reasons why Kales et al. (2006) did not detect a significant variation in the β 2m gene expression at low temperature could be explained by the low sensitivity of their technique or the differences in the temperatures to which the fish have been exposed. The conflictual data described in this report regarding the expression level of the MHII β and the β 2m as a function of temperature clearly highlights the need of further investigations on immune gene expression level at boundary or extreme habitat temperatures.

One can question what could be the advantages for fish to reduce their MHII β expression level at higher temperature? By reducing the MHII β gene expression level at higher temperature (and possibly other immune relevant genes), the advantage could also be a diminution of the energy cost to mount an efficient immune response. This hypothesis is based on the fact that temperature is important in governing the rate at which body processes are carried out. This might be the consequences of an increased cellular metabolism and/or functional activity. It has been suggested that membrane fluidity could be involved in the activation of T cells in function of temperature (Vallejo et al., 1992). As previously mentioned, other studies also demonstrated that some fish MH genes have a temperature-dependent expression level (Rodrigues et al., 1998; Kales et al., 2006; Nath et al., 2006). Another possible advantage on the field of energy economy implicates a fine tuning by the minisatellite of the MHII β gene expression level. The closely linkage of the minisatellite with the exon 2 coding for the protein β 1 domain and responsible of antigen presentation suggests a co-evolution of both elements. Since it is known

that specific alleles of MHII β gene correlates with increase resistance to pathogen (Langefors et al., 2001; Grimholt et al., 2003; Croisetière et al., 2008), the selection for an allele with a particular minisatellite length from another one coding for an identical protein, but with a different minisatellite length, is clearly possible. The basis for this selection would be an optimal expression level of the allele, involving a certain energy cost, in function of the efficiency to present antigens and induce an appropriate immune response. Here, it would be very interesting to develop transgenic fish with the same MHII β -coding sequence, but differing at minisatellite length, and to test them in challenge experiment against bacteria to evaluate the contribution of gene expression level to the resistance and susceptibility to pathogens.

4.4. Other elements contributing to the polymorphism of the MHII β gene in salmonids

Two additional elements could be distinguished from the analysis of the second intron nucleotide sequences of the MHII β gene in salmonids: a second minisatellite composed of a 40 nucleotides motif (type II) found exclusively in Atlantic salmon (Figs. 2, 3 and Table 2, small white and grey rectangles) and a *Hpa* I SINE (Figs. 1–3 and Table 2) (Kido et al., 1991; Miller and Withler, 1996). The accumulation of genetic element in the second intron of the salmonids MHII β gene with its expression restricted to specialized tissues (Ting and Trowsdale, 2002) support the hypothesis of 'genomic design' model for the evolution of this intron (Pozzoli et al., 2007).

SINEs contain an internal RNA polymerase III promoter that consists of an A box downstream from the transcription start site, and a B box located approximately 50 bp downstream from the B box (Roy et al., 2000). Moreover, it contains several potential transcription factor binding sites. Wang et al. (2004) reported that SINE retroposition does not appear randomly in the direction of insertion, with all SINEs within known host gene in rainbow trout inserted in the opposite orientation to the host gene transcription. As they suggested, insertion in both orientations could interfere with gene expression, the insertion in the same orientation showing the greatest possibility of effects. The fact that the complete *Hpa* I SINE found in brook charr MHII β gene is in the same orientation and shows almost intact A box and B box thus suggests that this element might present a regulatory role modulating the expression of the gene during stress period or pathogen infection. Furthermore, the closely insertion of two *Hpa* I SINEs in the same gene is unexpected since SINEs insertion is believed to be random (Kido et al., 1995). The dual insertion events detected in brook charr MHII β gene is puzzling and calls for further studies on the mechanisms of SINEs retroposition and regulatory functions.

The presence of multiple elements for which the implication in recombination events has been demonstrated previously probably highlights the importance of this type of mechanism in generating polymorphism of the MHII β gene. The high concentration and variability of elements found in this intron, just downstream of the exon 2 coding for the domain implicated in peptide binding and presentation could reflect the selective pressure to assure polymorphism of the MHII β gene. A higher incidence of these elements in the MHII β gene of salmonids may be the consequence of the fact that it is the only MHII locus identified thus far (Glamann, 1995; Langefors et al., 2000). Allele and gene conversion were identified as a mechanism to generate polymorphism of MHC in multiple organisms (Pease et al., 1983; Zangenberg et al., 1995). Also, it was demonstrated that the *Eb* gene of the mouse contains a recombinational hotspot located within the second intron. Interestingly, a segment of the intron showed strong similarity to retroposon long terminal repeat (LTR), *env*, and *pol* genes indicating that this segment of

the second intron may have evolved through retroposon insertion (Zimmerer and Passmore, 1991).

4.5. Identification of a new regulatory element submitted to natural selection?

MHII β gene exhibit polymorphism that is undoubtedly relevant for resistance or susceptibility toward infectious diseases (Trowsdale and Parham, 2004; Dionne et al., 2009), where different mechanisms are responsible for the evolution and maintenance of this polymorphism (Bernatchez and Landry, 2003; Aguilar and Garza, 2007). Since teleosts express a single "minimal, essential" locus of both α and β chain forming the MHII molecule (Glamann, 1995; Langefors et al., 2000) and also MHII α and MHII β loci are genetically linked in Atlantic salmon (Stet et al., 2002), this animal model was used in association studies to identify resistance and susceptibility alleles, with the focus on the coding sequences (Langefors et al., 2001; Grimholt et al., 2003; Croisetière et al., 2008). Other studies demonstrated the implication of non-coding region polymorphism in the modulation of the MHCII β transcriptional activity. The expression level of MHCII molecules was shown to be affected by mutations in the promoter, some of them revealing binding sites for transcription factors (Shewey et al., 1992; Baumgart et al., 1998). The results presented in this paper clearly demonstrate the implication of a new mechanism to fine tune the expression of the MHII β gene. Some reports are emerging to implicate microsatellite in morphological and behavioral evolution in vertebrates (Fondon and Garner, 2004; Hammock and Young, 2005) indicating a more important contribution of VNTR regulating phenotypic expression than previously presumed. Given the amply documented implication of the MHCII β gene in the immune response and fitness, this report is the first, to our knowledge, to present a minisatellite that could potentially be submitted to selective forces and therefore play an important role in the immune response of fish populations in the wild.

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References

- Aguilar, A., Garza, J.C., 2007. Patterns of historical balancing selection on the salmonid major histocompatibility complex class II beta gene. *J. Mol. Evol.* 65, 34–43.
- Atkin, N.B., 2001. Microsatellite instability. *Cytogenet. Cell. Genet.* 92, 177–181.
- Balakirev, E.S., Ayala, F.J., 2003. Pseudogenes: are they "junk" or functional DNA? *Annu. Rev. Genet.* 37, 123–151.
- Baumgart, M., Moos, V., Schuhbauer, D., Müller, B., 1998. Differential expression of major histocompatibility complex class II genes on murine macrophages associated with T cell cytokine profile and protective/suppressive effects. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6936–6940.
- Beauregard, A., Curcio, M.J., Belfort, M., 2008. The take and give between retrotransposable elements and their hosts. *Annu. Rev. Genet.* 42, 587–617.
- Bell, G.I., Horita, S., Karam, J.H., 1984. A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. *Diabetes* 33, 176–183.
- Bennett, S.T., Lucassen, A.M., Gough, S.C., et al. (17 co-authors), 1995. Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. *Nat. Genet.* 9, 284–292.
- Bernatchez, L., Landry, C., 2003. MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? *J. Evol. Biol.* 16, 363–377.

- Bird, C.P., Stranger, B.E., Liu, M., Thomas, D.J., Ingle, C.E., Beazley, C., Miller, W., Hurler, M.E., Dermitzakis, E.T., 2007. Fast-evolving noncoding sequences in the human genome. *Genome Biol.* 8, R118.
- Bly, J.E., Clem, L.W., 1991. Temperature-mediated processes in teleost immunity: in vitro immunosuppression induced by in vivo low temperature in channel catfish. *Vet. Immunol. Immunopathol.* 28, 365–377.
- Bois, P., Jeffreys, A.J., 1999. Minisatellite instability and germline mutation. *Cell. Mol. Life Sci.* 55, 1636–1648.
- Brem, R.B., Kruglyak, L., 2005. The landscape of genetic complexity across 5700 gene expression traits in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 102, 1572–1577.
- Castillo-Davis, C.I., Mekhedov, S.L., Hartl, D.L., Koonin, E.V., Kondrashov, F.A., 2002. Selection for short introns in highly expressed genes. *Nat. Genet.* 31, 415–418.
- Charlesworth, B., Sniegowski, P., Stephan, W., 1994. The evolutionary dynamics of repetitive DNA in eukaryotes. *Nature* 371, 215–220.
- Cheung, V.G., Spielman, R.S., Ewens, K.G., Weber, T.M., Morley, M., Burdick, J.T., 2005. Mapping determinants of human gene expression by regional and genome-wide association. *Nature* 437, 1365–1369.
- Croisètière, S., Tarte, P.D., Bernatchez, L., Belhumeur, P., 2008. Identification of MHC class II beta resistance/susceptibility alleles to *Aeromonas salmonicida* in brook charr (*Salvelinus fontinalis*). *Mol. Immunol.* 45, 3107–3116.
- Debrauwere, H., Gendrel, C.G., Lecham, S., Dutreix, M., 1997. Differences and similarities between various tandem repeat sequences: minisatellites and microsatellites. *Biochimie* 79, 577–586.
- Dermitzakis, E.T., Raymond, A., Lyle, R., Scamuffa, N., Ucla, C., Deutsch, S., Stevenson, B.J., Flegel, V., Bucher, P., Jongeneel, C.V., Antonarakis, S.E., 2002. Numerous potentially functional but non-genic conserved sequences on human chromosome 21. *Nature* 420, 578–582.
- Dionne, M., Miller, K.M., Dodson, J.J., Bernatchez, L., 2009. MHC standing genetic variation and pathogen resistance in wild Atlantic salmon. *Philos. Trans. R. Soc. Lond.* 364, 1555–1565.
- Eddy, S.R., 2001. Non-coding RNA genes and the modern RNA world. *Nat. Rev. Genet.* 2, 919–929.
- Eisenberg, E., Levanon, E.Y., 2003. Human housekeeping genes are compact. *Trends Genet.* 19, 362–365.
- Ellegren, H., 2000. Microsatellite mutations in the germline: implications for evolutionary inference. *Trends Genet.* 16, 551–558.
- Feschotte, C., 2008. Transposable elements and the evolution of regulatory networks. *Nat. Rev. Genet.* 9, 397–405.
- Fondon 3rd, J.W., Garner, H.R., 2004. Molecular origins of rapid and continuous morphological evolution. *Proc. Natl. Acad. Sci. U.S.A.* 101, 18058–18063.
- Fondon 3rd, J.W., Hammock, E.A., Hannan, A.J., King, D.G., 2008. Simple sequence repeats: genetic modulators of brain function and behavior. *Trends Neurosci.* 31, 328–334.
- Fryer, J.L., Pilcher, K.S., Sanders, J.E., Rohovec, J.S., Zinn, J.L., Groberg, W.J., McCoy, R.H., 1976. Temperature, infectious disease, and the immune response in salmonid fish. *NTIS* 191, 72.
- Glamann, J., 1995. Complete coding sequence of rainbow trout Mhc II beta chain. *Scand. J. Immunol.* 41, 365–372.
- Glimcher, L.H., Kara, C.J., 1992. Sequences and factors: a guide to MHC class-II transcription. *Annu. Rev. Immunol.* 10, 13–49.
- Gonzales, M.J., Dugan, J.M., Shafer, R.W., 2002. Synonymous-non-synonymous mutation rates between sequences containing ambiguous nucleotides (SynSCAN). *Bioinformatics* 18, 886–887.
- Grimholt, U., Larsen, S., Nordmo, R., Midtlyng, P., Kjoeglum, S., Storset, A., Saebø, S., Stet, R.J., 2003. MHC polymorphism and disease resistance in Atlantic salmon (*Salmo salar*): facing pathogens with single expressed major histocompatibility class I and class II loci. *Immunogenetics* 55, 210–219.
- Hammock, E.A., Young, L.J., 2005. Microsatellite instability generates diversity in brain and sociobehavioral traits. *Science* 308, 1630–1634.
- Ingerslev, H.C., Pettersen, E.F., Jakobsen, R.A., Petersen, C.B., Wergeland, H.I., 2006. Expression profiling and validation of reference gene candidates in immune relevant tissues and cells from Atlantic salmon (*Salmo salar* L.). *Mol. Immunol.* 43, 1194–1201.
- Jorgensen, S.M., Kleveland, E.J., Grimholt, U., Gjoen, T., 2006. Validation of reference genes for real-time polymerase chain reaction studies in Atlantic salmon. *Mar. Biotechnol. (NY)* 8, 398–408.
- Kales, S., Parks-Dely, J., Schulte, P., Dixon, B., 2006. Beta-2-microglobulin gene expression is maintained in rainbow trout and Atlantic salmon kept at low temperatures. *Fish Shellfish Immunol.* 21, 176–186.
- Kido, Y., Aono, M., Yamaki, T., Matsumoto, K., Murata, S., Saneyoshi, M., Okada, N., 1991. Shaping and reshaping of salmonid genomes by amplification of tRNA-derived retroposons during evolution. *Proc. Natl. Acad. Sci. U.S.A.* 88, 2326–2330.
- Kido, Y., Saitoh, M., Murata, S., Okada, N., 1995. Evolution of the active sequences of the HpaI short interspersed elements. *J. Mol. Evol.* 41, 986–995.
- King, M.C., Wilson, A.C., 1975. Evolution at two levels in humans and chimpanzees. *Science* 188, 107–116.
- Knight, J., Stet, R.J.M., Secombes, C.J., 1998. Modulation of MHC class II expression in rainbow trout *Oncorhynchus mykiss* macrophages by TNF α and LPS. *Fish Shellfish Immunol.* 8, 545–553.
- Lalio, M.D., Scott, H.S., Buresi, C., Rossier, C., Bottani, A., Morris, M.A., Malafosse, A., Antonarakis, S.E., 1997. Dodecamer repeat expansion in cystatin B gene in progressive myoclonus epilepsy. *Nature* 386, 847–851.
- Langefors, A., Lohm, J., Von Schantz, T., Grahn, M., 2000. Screening of Mhc variation in Atlantic salmon (*Salmo salar*): a comparison of restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE) and sequencing. *Mol. Ecol.* 9, 215–219.
- Langefors, A., Lohm, J., Grahn, M., Andersen, O., von Schantz, T., 2001. Association between major histocompatibility complex class II B alleles and resistance to *Aeromonas salmonicida* in Atlantic salmon. *Proc. Biol. Sci.* 268, 479–485.
- Li, Y.C., Korol, A.B., Fahima, T., Beiles, A., Nevo, E., 2002. Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. *Mol. Ecol.* 11, 2453–2465.
- Li, Y.C., Korol, A.B., Fahima, T., Nevo, E., 2004. Microsatellites within genes: structure, function, and evolution. *Mol. Biol. Evol.* 21, 991–1007.
- MacKenzie, A., Quinn, J., 1999. A serotonin transporter gene intron 2 polymorphic region, correlated with affective disorders, has allele-dependent differential enhancer-like properties in the mouse embryo. *Proc. Natl. Acad. Sci. U.S.A.* 96, 15251–15255.
- Mill, J., Asherson, P., Browes, C., D'Souza, U., Craig, I., 2002. Expression of the dopamine transporter gene is regulated by the 3' UTR VNTR: evidence from brain and lymphocytes using quantitative RT-PCR. *Am. J. Med. Genet.* 114, 975–979.
- Miller, K.M., Withler, R.E., 1996. Sequence analysis of a polymorphic Mhc class II gene in Pacific salmon. *Immunogenetics* 43, 337–351.
- Moore, L.J., Somamoto, T., Lie, K.K., Dijkstra, J.M., Hordvik, I., 2005. Characterisation of salmon and trout CD8alpha and CD8beta. *Mol. Immunol.* 42, 1225–1234.
- Mouse Genome Sequencing Consortium, Waterston, R.H., Lindblad-Toh, K., et al. (223 co-authors), 2002. Initial sequencing and comparative analysis of the mouse genome. *Nature* 420, 520–562.
- Nath, S., Kales, S., Fujiki, K., Dixon, B., 2006. Major histocompatibility class II genes in rainbow trout (*Oncorhynchus mykiss*) exhibit temperature dependent down-regulation. *Immunogenetics* 58, 443–453.
- Olsvik, P.A., Lie, K.K., Jordal, A.E., Nilsen, T.O., Hordvik, I., 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Mol. Biol.* 6, 21.
- Orr, H.T., Zoghbi, H.Y., 2007. Trinucleotide repeat disorders. *Annu. Rev. Neurosci.* 30, 575–621.
- Pastinen, T., Hudson, T.J., 2004. *cis*-Acting regulatory variation in the human genome. *Science* 306, 647–650.
- Pease, L.R., Schulze, D.H., Pfaffenbach, G.M., Nathenson, S.G., 1983. Spontaneous H-2 mutants provide evidence that a copy mechanism analogous to gene conversion generates polymorphism in the major histocompatibility complex. *Proc. Natl. Acad. Sci. U.S.A.* 80, 242–246.
- Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* 26, 509–515.
- Pozzoli, U., Menozzi, G., Comi, G.P., Cagliani, R., Bresolin, N., Sironi, M., 2007. Intron size in mammals: complexity comes to terms with economy. *Trends Genet.* 23, 20–24.
- Pugliese, A., Zeller, M., Fernandez Jr, A., Zalcborg, L.J., Bartlett, R.J., Ricordi, C., Pietropaolo, M., Eisenbarth, G.S., Bennett, S.T., Patel, D.D., 1997. The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. *Nat. Genet.* 15, 293–297.
- Raida, M.K., Buchmann, K., 2007. Temperature-dependent expression of immune-relevant genes in rainbow trout following *Yersinia ruckeri* vaccination. *Dis. Aquat. Organ.* 77, 41–52.
- Richard, G.F., Pâques, F., 2000. Mini- and microsatellite expansions: the recombination connection. *EMBO Rep.* 1, 122–126.
- Richard, G.F., Kerrest, A., Dujon, B., 2008. Comparative genomics and molecular dynamics of DNA repeats in eukaryotes. *Microbiol. Mol. Biol. Rev.* 72, 686–727.
- Rodrigues, P.N., Dixon, B., Roelofs, J., Rombout, J.H., Egberts, E., Pohajdak, B., Stet, R.J., 1998. Expression and temperature-dependent regulation of the beta2-microglobulin (Cyca-B2m) gene in a cold-blooded vertebrate, the common carp (*Cyprinus carpio* L.). *Dev. Immunol.* 5, 263–275.
- Roy, A.M., West, N.C., Rao, A., Adhikari, P., Alemán, C., Barnes, A.P., Deininger, P.L., 2000. Upstream flanking sequences and transcription of SINES. *J. Mol. Biol.* 302, 17–25.
- Saunders, R., 1995. Salmon aquaculture: present status and prospectus for the future. In: Boghen, A. (Ed.), *Cold-water Aquaculture in Atlantic Canada*. The Canadian Institute for Research On Regional Development, Moncton, New Brunswick, Canada, pp. 35–82.
- Shewey, L.M., Beaty, J.S., Andersen, L.C., Nepom, G.T., 1992. Differential expression of related HLA class II DQ beta genes caused by nucleotide variation in transcriptional regulatory elements. *J. Immunol.* 148, 1265–1273.
- Siepel, A., Bejerano, G., Pedersen, J.S., et al. (16 co-authors), 2005. Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. *Genome Res.* 15, 1034–1050.
- Stet, R.J., de Vries, B., Mudde, K., Hermesen, T., van Heerwaarden, J., Shum, B.P., Grimholt, U., 2002. Unique haplotypes of co-segregating major histocompatibility class II A and class II B alleles in Atlantic salmon (*Salmo salar*) give rise to diverse class II genotypes. *Immunogenetics* 54, 320–331.
- Stranger, B.E., Forrest, M.S., Clark, A.G., et al. (12 co-authors), 2005. Genome-wide associations of gene expression variation in humans. *PLoS Genet.* 1, e78.
- Syed, M., Vestreheim, O., Mikkelsen, B., Lundin, M., 2003. Isolation of the promoters of Atlantic salmon MHCII genes. *Mar. Biotechnol.* 5, 253–260.
- Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.
- Thomas, J.W., Touchman, J.W., Blakesley, R.W., et al. (71 co-authors), 2003. Comparative analyses of multi-species sequences from targeted genomic regions. *Nature* 424, 788–793.

- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Ting, J.P., Trowsdale, J., 2002. Genetic control of MHC class II expression. *Cell* 109 (Suppl.), S21–S33.
- Tomilin, N.V., 2008. Regulation of mammalian gene expression by retroelements and non-coding tandem repeats. *Bioessays* 30, 338–348.
- Trowsdale, J., Parham, P., 2004. Mini-review: defense strategies and immunity-related genes. *Eur. J. Immunol.* 34, 7–17.
- Ugarkovic, D., 2005. Functional elements residing within satellite DNAs. *EMBO Rep.* 6, 1035–1039.
- Usdin, K., 2008. The biological effects of simple tandem repeats: lessons from the repeat expansion diseases. *Genome Res.* 18, 1011–1019.
- Vallejo, A.N., Miller, N.W., Clem, L.W., 1992. Cellular pathway(s) of antigen processing in fish APC: effect of varying in vitro temperatures on antigen catabolism. *Dev. Comp. Immunol.* 16, 367–381.
- Vinogradov, A.E., 2004. Compactness of human housekeeping genes: selection for economy or genomic design? *Trends Genet.* 20, 248–253.
- Vinogradov, A.E., 2006. “Genome design” model: evidence from conserved intronic sequence in human-mouse comparison. *Genome Res.* 16, 347–354.
- Wang, G., Vasquez, K.M., 2006. Non-B DNA structure-induced genetic instability. *Mutat. Res.* 598, 103–119.
- Wang, T., Johnson, N., Zou, J., Bols, N., Secombes, C.J., 2004. Sequencing and expression of the second allele of the interleukin-1beta1 gene in rainbow trout (*Oncorhynchus mykiss*): identification of a novel SINE in the third intron. *Fish Shellfish Immunol.* 16, 335–358.
- Wells, R.D., Dere, R., Hebert, M.L., Napierala, M., Son, L.S., 2005. Advances in mechanisms of genetic instability related to hereditary neurological diseases. *Nucleic Acids Res.* 33, 3785–3798.
- Wicker, T., Sabot, F., Hua-Van, A., et al. (13 co-authors), 2007. A unified classification system for eukaryotic transposable elements. *Nat. Rev. Genet.* 8, 973–982.
- Wray, G.A., 2007. The evolutionary significance of cis-regulatory mutations. *Nat. Rev. Genet.* 8, 206–216.
- Wray, G.A., Hahn, M.W., Abouheif, E., Balhoff, J.P., Pizer, M., Rockman, M.V., Romano, L.A., 2003. The evolution of transcriptional regulation in eukaryotes. *Mol. Biol. Evol.* 20, 1377–1419.
- Zamorano, R., Suchindran, S., Gainer, J.V., 2006. 3'-Untranslated region of the type 2 bradykinin receptor is a potent regulator of gene expression. *Am. J. Physiol. Renal Physiol.* 290, F456–F464.
- Zangenberg, G., Huang, M.M., Arnheim, N., Erlich, H., 1995. New HLA-DPB1 alleles generated by interallelic gene conversion detected by analysis of sperm. *Nat. Genet.* 10, 407–414.
- Zimmerer, E.J., Passmore, H.C., 1991. Structural and genetic properties of the Eb recombinational hotspot in the mouse. *Immunogenetics* 33, 132–140.