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Evidence for two *transferrin* loci in the *Salmo trutta* genome

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Summary

To determine the organization of *transferrin* (*TF*) locus in the *Salmo trutta* genome, partial DNA and cDNA sequencing, fluorescent *in situ* hybridization (FISH) and *Salmo salar* BAC analysis were performed. *TF* expression levels and copy number prediction were assessed using real-time PCR. In addition to two previously reported DNA *TF* variant sequences of *S. trutta* and *Salmo marmoratus* (*TF1*), two novel variant sequences (*TF2*) were revealed in both species. Variant-specific sequence tags, characterizing two variants for each *TF* type (*TF1* and *TF2*), were identified in genomic clones from each of the F1 hybrids between *S. trutta* and *S. marmoratus*. These clearly documented double heterozygote status at the *TF* loci. The real-time PCR data showed that each of the two *TF* types (*TF1* and *TF2*) existed in one copy only and that the transcription of *TF2* was considerably lower compared with *TF1*. Using FISH, hybridization signals were observed on two medium-sized acrocentric chromosomes of *S. trutta* karyotype. A *TF* type-specific PCR followed by a restriction analysis revealed the presence of two *TF* loci in the majority of analysed BAC clones. It was concluded that the *TF* gene is duplicated in the genome of *S. trutta*, and that the two *TF* loci are located adjacent to one another on the same chromosome. The differing transcription levels of *TF1* and *TF2* appear to depend on the corresponding promoter activity, which at least for *TF2* seems to vary between different *Salmo* congeners.

Keywords chromosomes, fluorescent *in situ* hybridization, real-time PCR, *Salmo marmoratus*, *Salmo trutta*, *transferrin*.

Introduction

Molecular markers have been applied to answer many biological questions, ranging from gene mapping to population genetics, phylogenetic reconstruction, paternity testing and forensic applications. Allozymes have been one of the most widely used types of biochemical markers in fish biology (Utter *et al.* 1987), and represented the initial tool for brown trout (*Salmo trutta*) genetic studies in the 1980s and 1990s (e.g. Ryman 1983; Ferguson 1989; Hindar *et al.* 1991; García-Marín *et al.* 1999). Because of several drawbacks of allozyme markers (see Schlötterer 2004 for review), the introduction of recombinant DNA technology promoted a shift from enzyme- to DNA-based markers (Schlötterer 2004). Although most DNA studies have been based on mitochondrial DNA and highly variable nuclear DNA markers like microsatellites, there may still be some advantages in the use of polymorphisms involving protein-coding loci. For instance, diagnostic markers for specific populations are more likely to be found at loci under weak selection than at those that are neutral (Ferguson 1994). In order to increase the information content of the biochemical markers, some of them have been converted to DNA format (e.g. Powell *et al.* 1992). An example of a protein that has been important in the population genetics and phylogenetic studies of brown trout is the one encoded by the *lactate dehydrogenase-C1** (*LDH-C1**) gene. This is also one of the few markers in salmonids for which a DNA-based method has been developed, providing a means for routine screening of highly informative *LDH-C1** polymorphisms in brown trout population studies (McMeel *et al.* 2001).

Another biochemical marker that has been frequently used for differentiation of salmonid populations and that has emerged as highly informative in trout typing is

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transferrin (TF). Several electrophoretic variants have been described in trout (*Salmo* sp.), enabling differentiation among evolutionarily distinct lineages and hybrid swarms. For example, the electromorph *TF*75* was found to be private and fixed in marble trout (*Salmo marmoratus*; Giuffra *et al.* 1996) and was used as a diagnostic tool for discriminating marble trout in its hybrids with brown trout (Berrebi *et al.* 2000).

In addition to its exploitation in population genetics and phylogenetic studies, the *TF* locus has significant physiological relevance. In fish, it is found in blood serum and interstitial spaces. In terms of tertiary chemical structure, *TF* consists of two homologous lobes, each containing a single highly conserved iron-binding domain. It has high binding affinity for iron and keeps free iron at low concentrations in blood (Aisen & Listowsky 1980). Because iron is often a limiting nutrient for bacterial growth, iron binding by *TF* provides resistance to bacterial infection (Suzumoto *et al.* 1977). The rate of evolution at non-synonymous sites is significantly higher than the rate at synonymous sites; so positive selection for new alleles has played an important role in the evolution of *TF* in some salmon species (Ford *et al.* 1999; Ford 2000, 2001) including brown trout (Antunes *et al.* 2002).

Based on mRNA isolated from liver tissue of Atlantic salmon (*Salmo salar*), two types of *TF* genes, convincingly transcribed from two different loci, were previously described (Kvingedal *et al.* 1993; Kvingedal 1994). The genes produced the same amount of RNA transcripts and had only slight differences in promoter sequences (Kvingedal 1994). In subsequent *TF* investigations, which included mRNA studies on *S. trutta* and various species of Pacific salmons (*Oncorhynchus nerka*, *Oncorhynchus masou*, *Oncorhynchus kisutch*, *Oncorhynchus mykiss*, *Oncorhynchus tshawytscha* and *Oncorhynchus kisutch*) and chars (*Salvelinus pluvius*, *Salvelinus fontinalis* and *Salvelinus namaycush*), not more than one type of *TF* gene has been observed (Tange *et al.* 1997; Lee *et al.* 1998; Ford *et al.* 1999). The same conclusion could be inferred from later, DNA-level studies performed on *O. tshawytscha*, *O. kisutch* and *S. trutta* (Ford *et al.* 1999; Antunes *et al.* 2002). On the contrary, as inferred from DNA-level RFLP analysis of *O. mykiss*, the presence of two *TF* gene copies was proposed (Tange *et al.* 1997). The existence of two copies of the *TF* gene has been recently supported by an analysis of microsatellites adjacent to the *TF* gene in *S. trutta* and *S. salar*; analysis of backcross families showed that duplicated *TF*-linked microsatellites had no recombination indicating tandem duplication of the *TF* gene in both species (Antunes *et al.* 2005; Gharbi *et al.* 2006).

Presumed duplication of the *TF* gene and its non-neutral evolution call into the question its suitability to be used as a population genetic and phylogenetic marker. The main goal of the study was therefore to reveal the genomic organization of the *TF* gene in order to (i) determine the number of *TF* gene copies represented in the genome of *S. trutta*; (ii) define the location of existing copies in the genome; and (iii) understand the contradictory reports concerning the arrangement of the *TF* gene in the Salmonidae family.

Materials and methods

Samples

Total DNA was isolated from fin tissue of *S. trutta*, *S. marmoratus*, *S. salar* and F_1 hybrids between *S. trutta* and *S. marmoratus* with known hybridization history (hereafter referred to as hybrids) using standard high-salt extraction methods (Sambrook *et al.* 1989). RNA was isolated from *S. trutta* and *S. marmoratus* livers, which were dissected and frozen immediately in liquid nitrogen. RNA was purified directly from frozen tissue using TRIZOL Reagent (Gibco BRL) according to the manufacturer's instructions. Isolated RNA was treated by RQ RNase-Free DNase (Promega) and reverse-transcribed by M-MLV Reverse Transciptase (Promega) and oligo(dT) primers.

DNA sequencing

Nucleotide sequences between exons 5 and 7 of the *TF* gene in *S. trutta*, *S. marmoratus*, *S. salar* and hybrids were amplified by primers TFex5/7-F (5′-GCCAGCTGTGTAAGGG AGAC-3′) and TFex5/7-R (5′-CTTGACGGCCACCAGTTT-3′), which were designed on the basis of a previously published *S. trutta* cDNA *TF* sequence (Lee *et al.* 1998). PCR amplification was carried out in a 20-μl reaction volume containing 50 ng DNA, 0.5 μM both primers, 0.2 mM dNTPs, 1.25 mM $MgCl_2$, 10 mM Tris–HCl, 50 mM KCl and 0.5 U *Taq* DNA polymerase (Fermentas). Thermal cycling was performed with an initial denaturation at 94 °C for 5 min followed by 35 cycles of 45 s at 94 °C, 45 s at 58 °C and 1 min at 72 °C.

Cloning

PCR products representing variant-specific fragments of two *TF* gene types were ligated into pGEM-T Easy Vector (Promega). Transformation of competent bacterial cells DH5α (Invitrogen) was performed according to the manufacturer's instructions. Cloned DNA inserts were sequenced using the TFex5/7-F primer pair.

cDNA sequencing

Specific primers for the amplification of *TF* from *S. trutta* and *S. marmoratus* cDNA samples were designed either on the basis of previously published cDNA for *S. trutta* (Lee *et al.* 1998) or on the basis of sequences (EU436165–EU436168) obtained from cloned fragments between exons 5 and 7. These primers were TFStr1-F (5′-CATGAAACTGCTTCTCC TCTCA-3′), TFStr1-R (5′-GGCCACCAGTTTGTTGTAGA-3′),

(a)

TFStr1-F TFStr1-R

TFStr2-F TFStr2-R

TFStr3-F TFStr3-R

(b)

TFStr1C-F TFStr1Cpg-R

TFcDNApg-F TFStr2-R

TFcDNApg-F TFStr3A-R

Figure 1 Strategy for PCR amplification of two gene types, *TF1* (a) and *TF2* (b).

TFStr2-F (5′-GAGCTCCCATCGACAGCTAC-3′), TFStr2-R (5′-CCTGGCAGAGGAGTTCAAAG-3′), TFStr3-F (5′-GGAGC CTTCAGATGTCTGGT-3′), TFStr3-R (5′-TTGGTTGGACTGGT TTGGTT-3′), TFStr1C-F (5′-GCGTAAAGTCGGAGCAAGAG-3′), TFStr1Cpg-R (5′-CTGTCCTCATCTCCTCCCCT-3′), TFcDNA pg-F (5′-CAAGGAGCCCTACTATGACCACGC-3′), TFStr2-R (5′-CCTGGCAGAGGAGTTCAAAG-3′) and TFStr3A-R (5′-GGACCTCAGTGTTTGGTTTGG-3′) (Fig. 1a,b). PCR amplification was carried out in 30-μl reaction volumes containing 50 ng cDNA, 0.5 μM each primer, 0.2 mM dNTPs, 0.85 mM $MgCl_2$, 10 mM Tris–HCl, 50 mM KCl and 0.5 U *Taq* DNA polymerase. In PCR using the TFStr1C-F and TFStr1Cpg-R primers, 1 mM $MgCl_2$ was used. Thermal cycling was performed in a PTC-100^{TM} Programmable Thermal Controller (MJ Research) with an initial denaturation at 94 °C for 5 min followed by 35 cycles of 45 s at 94 °C, 45 s at 65 °C and 1 min 30 s at 72 °C. Sequencing of PCR fragments was performed by Macrogen.

Data analysis

Relationships between the sequences of *TF1* and *TF2* (exons 5–7) from *S. trutta*, *S. marmoratus* and *S. salar* were represented by a neighbour-joining phylogenetic tree constructed in MEGA version 4 (Tamura *et al.* 2007). Protein-coding nucleotide sequences (cDNA of *TF1* and *TF2* for *S. trutta* and *S. marmoratus* respectively) were tested for positive selection (H_A: $dN > dS$) by the Nei–Gojobori method and *p*-distance model (Nei & Gojobori 1986) using MEGA version 4 (Tamura *et al.* 2007).

Fluorescent *in situ* hybridization

Metaphase chromosomes were prepared from brown trout leucocytes by following a modified protocol from Fujiwara *et al.* (2001). BACs containing the *S. salar TF* gene (Thorsen *et al.* 2005) were purchased from BACPAC Resources (Children's Hospital Oakland Research Institute) and used as a probe for fluorescent *in situ* hybridization (FISH). BACs were isolated from bacterial colonies using the Very Low-Copy Plasmid/Cosmid Purification kit (Qiagen).

Nucleotide sequences between *TF* exons 5 and 7 were amplified by primers TFex5/7-F and TFex5/7-R. PCR products were digested with the restriction enzyme PsuI. Restriction was carried out in a 20-μl reaction volume containing 8–10 μg BAC DNA, 1× Buffer B and 25 U PsuI (Fermentas). The digestion mixture was incubated at 37 °C overnight. Restriction profiles were analysed on a 1.5% agarose gel. Single-colour FISH was performed as previously described (Pinkel *et al.* 1986) on cultured lymphocytes from salmonids. Slides for FISH were prepared by following standard cytogenetic protocols, and pre-treated to optimize hybridization. After pepsin treatment (0.01% pepsin in 0.01 N HCl for 15 min), slides were incubated three times in 2× SSC for 3 min, fixed in a 4% formaldehyde/50 mM $MgCl_2$/2× SSC solution for 10 min, incubated three times in 2× SSC for 3 min, dehydrated in ethanol series and air-dried. Denaturation of slides was carried out in 70% formamide/2× SSC for 3 min at 72 °C, followed by rehydration in an ethanol series at −20 °C and air-drying.

A BAC clone was used for the detection of the *TF* gene in cells. A total of 1 μg of DNA probe was labelled with digoxigenin (Boehringer) by nick translation using the appropriate amount of DNase. Nick-translated DNA probe (250–500 ng/μl) was co-precipitated with 5 μg of human Cot-1 DNA. The probe mix was re-suspended in a hybridization buffer, denatured at 72 °C for 10 min, applied to the denatured slide and incubated overnight in a moist chamber at 37 °C. Post-hybridization washes were carried out in 0.4× SSC at 72 °C for 2 min and in 2× SSC/0.05% Tween at room temperature for 1 min. Immunodetection was carried out using monoclonal fluorescein-labelled digoxigenin–rhodamin antibodies. Finally, slides were counterstained with 4,6-diamino-2-phenyl-indole (DAPI). FISH images were captured with a monochrome CCD camera (Carl Zeiss) on an Axioplan 2 Fluorescence Microscope (Carl Zeiss) with appropriate filters. Image analysis was performed using CytoVision (Applied Imaging).

Real-time PCR

Four *S. trutta* and two *S. marmoratus* samples were analysed using real-time PCR to determine the number of copies of the two *TF* gene types and the levels of expression of the two genes. Exon 13, where the most pronounced differences between both *TF* gene types were found, was selected to design primers and probes using ASSAYS-BY-DESIGN FILE BUILDER software (Applied Biosystems). The nucleotide sequences of primers and TaqMan MGB probes are shown in Table 1. To determine the number of *TF* gene types and their transcription levels, a species-specific DNA fragment containing microsatellite locus *BFRO002* (Sušnik *et al.* 1997) and a standard control DNA fragment encoding 18S rRNA (Applied Biosystems) respectively were used as internal controls.

Real-time PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Each

Table 1 TaqMan assays used for *TF1* and *TF2* and microsatellite *BFRO002*, an internal control.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	TaqMan MGB probe (5′–3′)
TF1	CTACCTTCTGTGCCCAGTGTAAG	GGCTTTGCACATGTCCTCATCT	CCCACGGGTTTCCCA
TF2	CCCTTCTGTGCCCAGTGTAAG	GCTTTGCACCTGTCCTCATCT	CCCCTGGCCATCCCA
BFRO002	TGCCATCTAGTAGACCCTCACATC	CAGGCTAGTTCAAACAGTTGGAGAT	ATGCCTCGTTGACTCTT

20-μl reaction contained 10 μl 2× TaqMan Universal PCR Master Mix, 18 μM each primer, 5 μM probe and 2 μl diluted DNA (0.01–100 ng). PCR was achieved with a 10-min activation and denaturation step at 95 °C, followed by 45 cycles of 15 s at 95 °C and 60 s at 60 °C. Baseline and threshold cycle (C_t) calculations were set automatically with ABI Prism 7900 SDS software version 2.3. Twofold serial dilutions of DNA or cDNA were made for efficiency calculations. Five serial dilutions were analysed in separate sample wells. Negative controls were run for each master mix.

Results

DNA sequences

Sequence analysis of cloned DNA inserts of *S. trutta*, *S. marmoratus* and hybrids revealed four different DNA sequence variants ranging from 569 to 579 bp. Two of them originating from *S. trutta* (EU436166) and *S. marmoratus* (EU436167) respectively corresponded to the ones previously described for *S. trutta* and *S. marmoratus* in Antunes *et al.* (2002) and were designated as *TF1*. The other two, one represented by *S. trutta* (EU436165) and the other by *S. marmoratus* (EU436168), have not been described previously and were designated as *TF2*. Intra-specific differences between *TF1* and *TF2* were found to be greater than inter-specific ones between *TF1* and *TF2* (Table 2). All four variants were identified in genomic clones from each of the F_1 hybrids between *S. trutta* and *S. marmoratus*, clearly documenting double heterozygote status at both *TF* loci.

Table 2 Inter- and intra-specific nucleotide and aa differences (in percentages) between *TF1* and *TF2* at the DNA level, cDNA level and aa level, and number of synonymous and non-synonymous mutations.

	Genetic differences (%)			Mutations	
Compared sequences	DNA	cDNA	aa	Syn	Non-syn
Salmo marmoratus: *TF1/TF2*	3.87	2.52	4.95	8	34
Salmo trutta: *TF1/TF2*	3.53	2.01	4.17	5	27
TF1: *Salmo marmoratus/Salmo trutta*	2.19	0.93	1.85	3	13
TF2: *Salmo marmoratus/Salmo trutta*	1.18	0.62	1.23	2	9

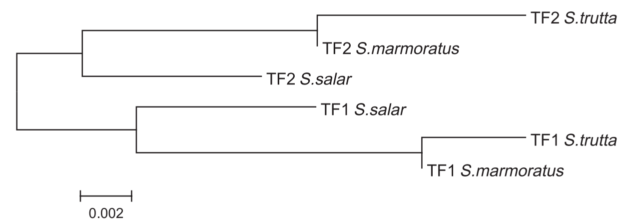

Figure 2 Relationships between *TF1* and *TF2* sequences from exon 5 to 7 originating from *Salmo trutta*, *Salmo marmoratus* and *Salmo salar* as inferred from the neighbour-joining tree.

Sequence analysis of cloned *TF* inserts of *S. salar* (EU436169, EU436170) pointed to the same gene organization revealing *TF1* and *TF2* variants. The relationships between *TF1* and *TF2* originating from all the three species are presented in Fig. 2.

cDNA and amino acid sequences

A total of 2354 bp of *TF1* cDNA (EU436173) and 2272 bp of *TF2* cDNA (EU436174) were resolved by sequence analysis in *S. trutta*, and 2317 bp of *TF1* cDNA (EU436171) and 2241 bp of *TF2* cDNA (EU436172) were resolved in *S. marmoratus*. The 5′ ends of the sequences were missing, while the sequences at the 3′ end were complete and of full length.

Tests of positive selection for *TF1* cDNA in *S. trutta* and *S. marmoratus* and *TF2* cDNA in *S. trutta* and *S. marmoratus* revealed a highly significant prevalence ($P = 0.001$) of non-synonymous sites over synonymous sites in both variants (from almost four- to sixfold; Table 2). After nucleotide sequence translation into a peptide, 679 and 652 amino acids (aa) were derived in *TF1* and *TF2* in *S. trutta*, and 673 and 647 in *TF1* and *TF2* in *S. marmoratus* respectively. Comparing the 647-aa-long region of strong sequence homology between *TF1* and *TF2*, 16 mutation sites in common for both variants were detected within each species. The remaining mutations, either inter- or intra-specific, were found to be apomorphic (five for *TF1 S. trutta*, 10 for *TF1 S. marmoratus*, three for *TF2 S. trutta* and *TF2 S. marmoratus*).

With respect to *S. trutta* and *S. marmoratus*, all iron-binding sites were found to be conserved in *TF1* including homologous iron-binding sites originating from each of the two *TF* homologous lobes (i.e. 1-5, 2-6, 3-7 and 4-8; Fig. 3). Considering *TF2* in both *S. trutta* and *S. marmoratus*, histidine (H) was found at the third iron-binding site instead of

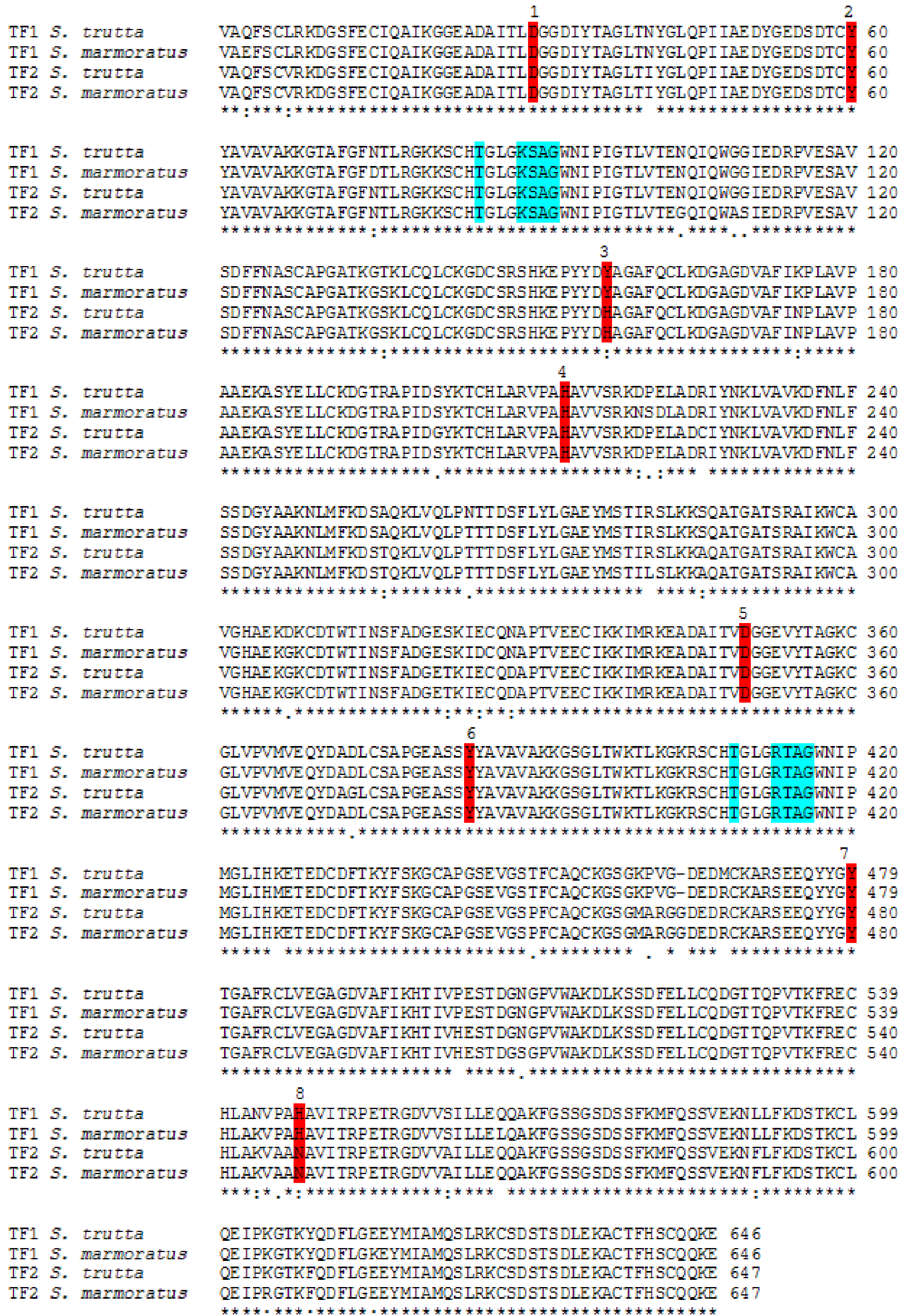

Figure 3 Amino-acid sequence alignment of *TF1* and *TF2* (from aa at position 40 to the stop codon) for *Salmo trutta* and *Salmo marmoratus*. Iron-binding sites and CO_3^{2-}-binding sites are designated with dark (red) and light grey (blue) respectively. The delimitation between the N- and C-terminal domains of transferrin is at aa position 285.

tyrosine (Y) in *TF1* and asparagine (N) at the eighth site instead of H in *TF1* (Fig. 3). All three amino acids have polar character, but H is charged while Y and N are uncharged. There were no differences within CO_3^{2-}-binding sites between *TF1* and *TF2*; however, the two binding sites, each located at in different *TF* lobe, differed to certain extent (Fig. 3).

BAC analysis and FISH

For *S. salar TF*, an RFLP protocol was developed (using PsuI) to cut a single site in *TF2*, which yielded two fragments (258 and 392 bp); *TF1* lacked this restriction site. Using this RFLP protocol, 13 BACs were tested (from exons 5 to 7). Four BACs were found to contain the *TF1* fragment, two BACs contained the *TF2* fragment and the remaining seven BACs contained both the *TF1* and *TF2* fragments (Fig. 4).

On metaphase chromosomes, fluorescent signals were observed on two medium-sized acrocentric *S. trutta* chromosomes (Fig. 5). In addition, two fluorescent signals were detected on an inter-phase nucleus (not shown).

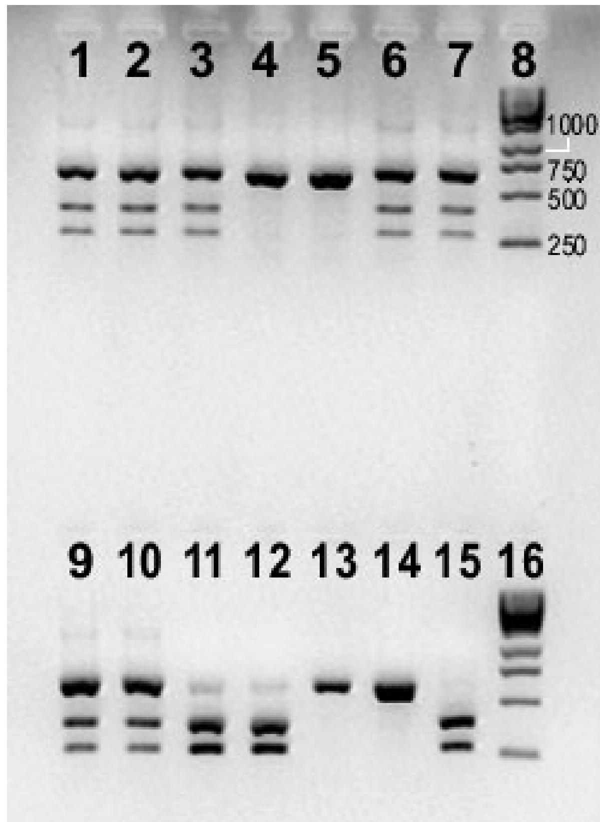

Figure 4 PCR-RFLP of 13 BAC clones digested with PsuI. Lanes 1–7 and 9–14: RFLP patterns of 13 BAC clones; lane 15: PCR of *Salmo trutta* genomic DNA, restricted with PsuI. Lanes 8 and 16: 1-kb M.W. marker (Fermentas).

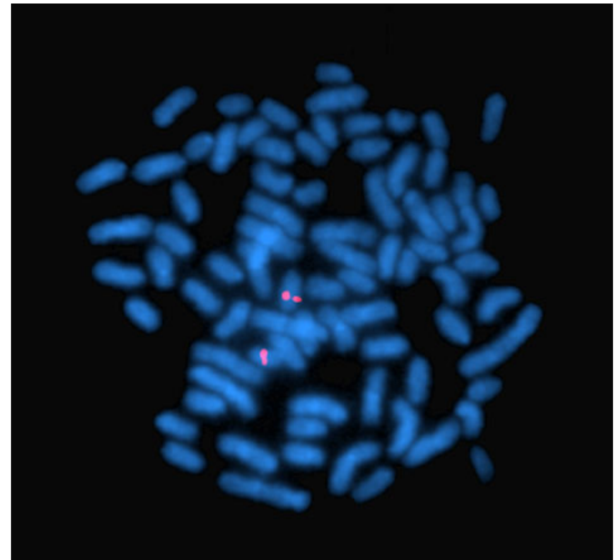

Figure 5 FISH of *Salmo trutta* metaphase chromosomes hybridized with *Salmo salar* BAC DNA.

Real-time PCR

Based on genomic DNA derived from six brown trout samples, the signal of fluorescence (at dilutions of 1:100 and 1:1000) passed the line of fluorescent threshold on average at the 27.01 and 27.20 cycles (SD = 1.76 and 1.56 respectively) for *TF1* and *TF2* respectively. The calculated ratio between the initial number of *TF1* and *TF2* DNA molecules was on average 1.2 (SD = 0.21) in favour of *TF1* (Fig. 6). Using an internal control (microsatellite *BFRO002*), the fluorescence signal passed the line of fluorescent threshold at the 27.93 cycle on average (SD = 0.55).

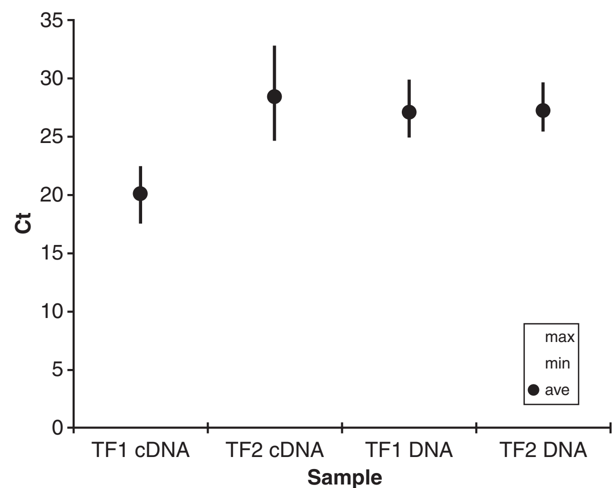

Figure 6 Mean threshold cycles during real-time PCR of *TF1* and *TF2* cDNA and of *TF1* and *TF2* DNA, for inferring RNA expression and for comparing the number of copies respectively between the *TF1* and *TF2* genes.

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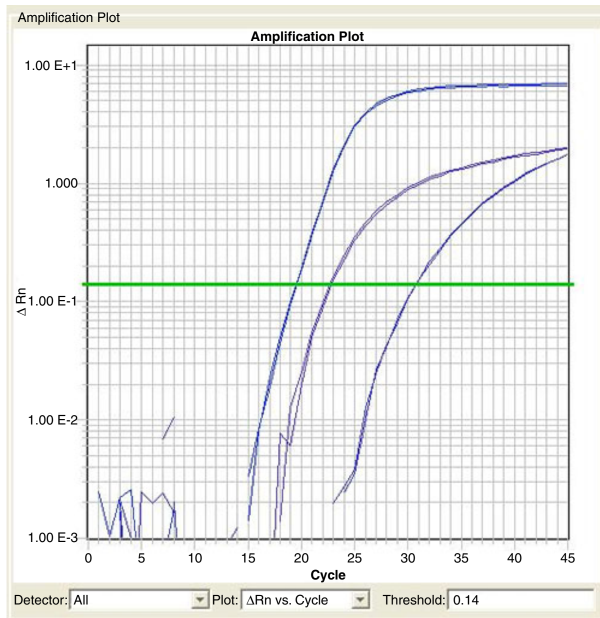

Figure 7 Transcription of *TF1* and *TF2* in the liver of *Salmo trutta*, assayed by real-time PCR. The threshold cycle (C_t) for *TF1* is 20th and for *TF2* is 31st. The threshold cycle for internal control is between them (ΔRn, normalized reporter signal; Cycle, number of cycles; mid-horizontal line, threshold line).

As inferred from six samples of cDNA derived from the trout liver, the signal of fluorescence (at dilutions of 1:10 and 1:100) passed the fluorescent threshold line at the 20.00 cycle on average (SD = 1.97) for *TF1* and at the 28.27th cycle on average (SD = 3.69) for *TF2* (Figs 6 & 7). The calculated ratios between the initial number of *TF1* and *TF2* cDNA molecules were on average 685.7 (SD = 773.34) in favour of *TF1*.

Discussion

This study unequivocally shows that the *TF* gene is duplicated in the genomes of *S. trutta* and *S. marmoratus* and by analogy, probably also in the genomes of other *Salmo* congeners. This duplication has been demonstrated in two ways: (i) based on both DNA and cDNA sequence analysis of *TF* and (ii) based on cloned genomic DNA sequences of F_1 progeny obtained by controlled reciprocal hybridization of *S. marmoratus* and *S. trutta*. In these hybrids, four different *TF* sequences, two of them characteristic of *S. marmoratus* and two of *S. trutta*, were revealed after PCR and cloning, indicating that these sequences originate from two gene loci rather than from a single locus. This observation confirms the initial, partial or indirect observations of previous investigators pointing to the *TF* duplication in *S. salar* and *S. trutta* (Kvingedal *et al.* 1993; Antunes *et al.* 2005; Gharbi *et al.* 2006), and disagrees with the results of cDNA analysis where researchers were not able to prove *TF* duplication in any of the salmonid genera (Tange *et al.* 1997; Lee *et al.* 1998; Ford *et al.* 1999).

We demonstrated that in *S. trutta* and *S. marmoratus*, *TF1* was transcribed at a higher level than *TF2*. The observed ratios between *TF1* and *TF2* transcripts were found to vary considerably; however, the trend (i.e. that the *TF1* transcripts outnumber the *TF2* transcripts) remained the same across all the samples analysed. We did not observe any species-specific transcription differences. The ratios between *TF1* and *TF2* transcripts may be a consequence of genetic interactions, epigenetic effects and environmental factors because of different environments from which the tested animals originated; e.g. marble trout came from one hatchery, while the brown trout came from another. In addition, the samples were taken during different periods of the year. Therefore, it seems very likely that the analysed animals were not directly comparable in terms of *TF* transcription, which may explain the different *TF1*/*TF2* ratios.

Considering our results showing that *TF2* was poorly transcribed in comparison to *TF1*, it is not surprising that cDNA analyses performed in some previous studies (Tange *et al.* 1997; Lee *et al.* 1998; Ford *et al.* 1999) concealed *TF* duplication. An exception seems to be *S. salar*, whose *TF1* and *TF2* genes appear to be, according to Kvingedal *et al.* (1993) and Kvingedal (1994), equally transcribed, both having a functional promotor. Taking into account this observation, it can be assumed that different transcription levels of *TF1* and *TF2* could depend on corresponding promoter activity, which at least for *TF2* seems to vary considerably between different *Salmo* congeners.

TF is a protein of significant physiological importance and, accordingly, would be expected to be highly conserved. The fact that the sequence comparison of *TF1* and *TF2* coding regions between and within individuals showed considerably higher rate of evolution at non-synonymous than at synonymous sites may, at least from this point of view, appear to be a surprising result. Nevertheless, this phenomenon, as previously described in *S. salar*, *S. trutta* and some *Oncorhynchus* species (Ford *et al.* 1999; Antunes *et al.* 2002), was explained as positive selection because of selective pressure governed by interactions/competition between host *TF* and the iron-scavenging proteins of pathogenic bacteria (Ford 2000, 2001). The amino acids on the surface of the protein (exposed for the contact by bacterial proteins) were subjected to more efficient positive selection than the amino acids in the interior of the protein (see Ford *et al.* 1999 for detailed explanation). Most interestingly, higher rates of evolution at non-synonymous sites compared with synonymous sites were found also in *TF2*, which indicates the same pattern of selection and consequently points to its active role. Assuming this notion and considering the fact that *TF2* contains a sequence of bases that could potentially encode a protein, a question arises as to why the transcription of *TF2* is relatively low. One possible explanation might be that the DNA sequence of the *TF2*

promoter has been affected by some mutation(s), which in turn affect the transcription efficiency of *TF2*. Because the pattern of positive selection was still conserved in *TF2*, this event must have happened relatively recently.

Using real-time PCR, we showed that *TF1* and *TF2* were represented by the same number of genomic copies. In addition, the detection of only one genetic variant in *TF1* and *TF2* within a species indicated that each of the two gene types existed in one copy only. This notion is in congruence with Antunes *et al.* (2005), who found no more than four alleles at a microsatellite locus located within the *S. trutta TF* gene, indicating that each *TF* type appeared in a single copy. Real-time PCR of *TF1* and *TF2* showed almost double amplification efficiency compared with an internal control. Because the internal control was a microsatellite locus, the apparent discrepancy may be explained by the assumption that PCR amplification of the microsatellite locus was less effective because of specific tandem repeat structure.

Considering the *TF* duplication in the *S. trutta* genome and the FISH results exhibiting a single fluorescent signal on each homologous chromosome, it could be deduced that the duplicated genes are adjacently located and that the fluorescent signals of both are overlapping. On the basis of BAC fragment lengths, which are about 188 kb (Thorsen *et al.* 2005), it could be inferred that this is the maximum distance separating the genes. The brown trout karyotype is composed of several distinct chromosome groups including seven pairs of metacentric, three pairs of submetacentric, one pair of large submetacentric and 29 pairs of subtelocentric to acrocentric chromosomes of decreasing size (Woznick *et al.* 2000). FISH signals were observed at a pericentromeric region of a medium-sized acrocentric pair. According to Antunes *et al.* (2005) and Gharbi *et al.* (2006), who indirectly determined the position of the *TF* gene via the linkage map of *S. trutta*, *TF* genes were classified within the linkage group BT-16.

At a glance, *TF* duplication in the *S. trutta* genome could be explained by a well-known theory of ancestral salmonid tetraploidization, which is responsible for most gene duplications in salmonid genomes (Allendorf & Thorgaard 1984). However, keeping in mind the tandem organization of *TF* genes, it seems highly unlikely that in the process of genome re-diploidization, which followed the tetraploidization event, the duplicated genes emerged side by side on the same chromosome. Tandem organization of the *TF* gene and a *TF* pseudogene was reported also for *Homo sapiens* (Schaeffer *et al.* 1987), a species that was never subjected to chromosome tetraploidization. With respect to the ancestral *TF* locus, which should have emerged in the *S. trutta* genome through a tetraploidization event, one can hypothesize that it has been lost by mutations or that 'diploidization does not necessarily happen simultaneously for all chromosomes or even for all loci on a particular chromosome' (Wolfe 2001).

The obtained results represent a new perspective of the role of *TF* as a phylogenetic marker, and shed new light on the interpretation of already existing data on the *TF* locus/loci in terms of its molecular organization; however, in order to better understand the molecular basis of quantitative differences in *TF* expression between the two loci, further promoter studies are needed.

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