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Divergence of Paralogous Growth-Hormone-Encoding Genes and Their Promoters in Salmonidae

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Abstract—In many fish species, including salmonids, the growth-hormone is encoded by two duplicated paralogous genes, *gh1* and *gh2*. Both genes were already in place at the time of divergence of species in this group. A comparison of the entire sequence of these genes of salmonids has shown that their conserved regions are associated with exons, while their most variable regions correspond to introns. Introns *C* and *D* include putative regulatory elements (sites Pit-1, CRE, and ERE), that are also conserved. In chars, the degree of polymorphism of *gh2* gene is 2–3 times as large as that in *gh1* gene. However, a comparison across all Salmonidae species would not extent this observation to other species. In both these chars' genes, the promoters are conserved mainly because they correspond to putative regulatory sequences (TATA box, binding sites for the pituitary transcription factor Pit-1 (F1–F4), CRE, GRE and RAR/RXR elements). The promoter of *gh2* gene has a greater degree of polymorphism compared with *gh1* gene promoter in all investigated species of salmonids. The observed differences in the rates of accumulation of changes in growth hormone encoding paralogs could be explained by differences in the intensity of selection.

Keywords: growth-hormone genes, nucleotide diversity, gene duplication, *Salvelinus*, Salmonidae, *cis*-regulatory regions

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The duplication of DNA sequences and genes, in particular, are one of the major factors of the evolution. These genes may arise in different ways: the duplication of the entire genome or of the individual chromosomes and by unequal crossing-over [1, 2]. The fate of duplicated genes may also be different. Most frequently they lose their function as a result of accumulation of mutations in one of the paralogs, so one of the copies become pseudogene. In other cases, both genes have the same function, but they were transcribed in different tissues. In rare cases, the duplicated gene acquires a new, substantially different function; a so-called function separation or subfunctionalization occurs [2–4]. It is also assumed that the duplicated sequences may play an important role in the eukaryotic genome, which protects it from harmful mutations and contributes it to the genetic stability of the organism [5].

Abbreviations: *gh1* and *gh2* are paralogous genes of the growth hormone; Pit-1 is pituitary-specific transcription factor; CRE the response element on cAMP; GRE the response element on glucocorticoids; RAR/RXR is the retinoic acid receptor; ERE is the response element on estrogenes.

It is believed that the whole genome duplication occurred [1, 2] in the history of all organisms. It was accompanied by diploidization of chromosomes, in which a significant portion of multiple genes was lost. A part of the remaining duplications arises in the genome (which is especially true for fish) in the form of multiple gene families. The traces of four stages of duplication (4R) revealed in the order Salmonidae. The last one has occurred in more recent times, as in some species diploidization had not been completed, and quadrivalent chromosomes combinations are detected periodically during mitosis [6]. Therefore, salmonids are a good model system for studying the processes of the origin, evolution, and functionalization of duplicated genes.

Growth-hormone gene in the genome of vertebrates is usually represented by one copy, although there are exceptions [7–9]. In some fish species, including salmonids, growth hormone is encoded by two unlinked paralogous genes, *gh1* and *gh2* [10–16]. Furthermore, another nonfunctional pseudogene, *gh*-ψ, was detected in *Y*-chromosome of chinook salmon and coho salmon [17, 18]. Apparently, both genes exist throughout the time of species divergence in this

group (25–100 million years) [6], and are probably both functionally active [17, 19].

The structure of the growth-hormone gene in fish is typical for vertebrates; it comprises six exons and five introns [10, 20–23]; genes with five exons and four introns are rare [24–26]. One of the approaches to understanding the functional significance of the genes and their various sites is a comparative DNA analysis of different species within the taxa. Conserved sequences in representatives of the same taxa gives grounds to consider these sites to be important for gene functioning. The high rate of nucleotide substitutions accumulation suggests of either a low functional significance of the site, or of the impact of the positive selection [1, 27].

In the present study, we compared the complete nucleotide sequences of genes *gh1* and *gh2*, including the promoter regions, in the representatives of the three genera of the family Salmonidae, i.e., *Salvelinus*, *Salmo*, and *Oncorhynchus*.

EXPERIMENTAL PART

Materials

During the investigation, one individual for each of the four species of the northwestern part of the Pacific Ocean chars, northern Dolly Varden trout (*Salvelinus malma*), southern Dolly Varden (*S. curilus*), Levanidov char (*S. levanidovi*), and Tarants char (*S. taranetzi*) were used. The individuals were obtained from the collection of laboratory of genetics of National Scientific Center of Marine Biology, Russian Academy of Sciences. The growth-hormone gene nucleotide sequences of the chars of the genus *Salvelinus* with a structural part (introns and exons) and 5'-adjacent region were deposited in Genbank (GeneBank NCBI) under the following numbers (in order of mention): (*gh1*—KF772972.2, *gh2*—KF772976.2); (*gh1*—KF772971.2, *gh2*—KF772975.2);(*gh1*—KF772974.2, *gh2*—KF772978.2); (*gh1*—KF772973.2, *gh2*—KF772977.2). The nucleotide sequences of the growth-hormone gene of Atlantic salmon (*Salmo salar*, *gh1*—EU621898.1, *gh2*—EU621899.1); chum salmon (*Oncorhynchus keta*, *gh2*—L04688.1); rainbow trout (*Oncorhynchus mykiss*, *gh2*—J03797.1); sockeye salmon (*Oncorhynchus nerka*, *gh1*—U14551.2, *gh2*—U14535.2); chinook salmon (*Oncorhynchus tshawytscha*, *gh1*—EU621900.1, *gh2*—EU621901.1), and *Coregonus lavaretus* (*gh*—AB001865.1) were taken from the GeneBank/NCBI.

The methods used to obtain growth-hormone gene nucleotide sequences (amplification, sequencing, and molecular cloning) have been previously described [19].

Nucleotide sequence analysis and multiple alignment were performed using MEGA-6.0 software package [28]. To align the sequences MUSCLE algorithm was used [29]. Phylogenetic reconstruction was performed by Bayesian Inference (BI), using the software MrBayes v. 3.2.6 [30], and also by the neighbor-joining method (NJ) of MEGA-6.0 software [28]. An optimal model of nucleotide substitutions (T92 + G) was selected using the jModelTest 2.1.1 software [31] according to the Akaike information criterion. Nucleotide diversity (π) was evaluated in the DnaSP-5.10.01 software [32] using the sliding window method.

RESULTS

Phylogeny of the Growth-Hormone Gene

Phylogenetic scheme of divergence of the transcribed part of paralogous genes based on Bayesian Inference is shown in Fig. 1. The tree constructed based on the NJ has a similar topology. Each of the paralogous genes of all studied species forms a separate cluster. As expected, within each cluster, the species of the genera *Salvelinus*, *Oncorhynchus*, and *Salmo* are divided into separate subclusters. The exception is gene *gh2* of *S. salar*, which is found in the subcluster of the chars of the genus *Salvelinus*.

Variability in the Structural Sequences of the Gene

The genes of all compared species include six exons (I–VI) and five introns (*A*, *B*, *C*, *D*, and *E*). In the case of chars, the length of the transcribed sequences have the following values. In *S. malma*, *gh1* is 3673 bp, and *gh2*, 3305 bp; in *S. surilus*, *gh1* is 3666 bp and *gh2*, 3305 bp; in *S. levanidovi*, *gh1* is 3672 bp and *gh2* is 3299 bp; in *S. taranetzi*, *gh1* is 3675 bp and *gh2*, 3300 bp. The size of each exon of the two genes is identical in all of the compared species, and the gene length varies due to the different length of introns.

The results of the sliding window analysis of the transcribed sequence of growth hormone paralogous genes in chars of the genus *Salvelinus* and a comparison of this sequence in different salmonids (*S. salar*, *O. nerka*, and *O. tshawytscha*) are shown in Figs. 2a–2d. It can be seen that the conserved regions are generally associated with exons. The peaks of variable regions inhered to the intron sequences. It can be seen (Figs. 2a, 2b) that the *gh1* gene of char is more conserved compared with the *gh2* gene, as the peak height variability of the *gh2* gene two to three times greater than the *gh1* gene. However, there no large differences in a comparison with the corresponding genes of other species of the Salmonidae family (Figs. 2c, 2d). The highest peaks, which correspond to the nucleotide substitutions in the *gh1* gene, occur in intron sequences *A*, *C*, *D*, and *E*, and intron *B* is conserved. The *gh2* gene introns *A*, *B*, and *D* are more variable than in the *gh1* gene. Intron *C* is also relatively conserved.

Intron sequences are variable not through the whole length, there are also conserved regions. It is seen that the variability peaks fall mainly on the central part of the intron and the areas adjacent to the exons, are more conserved. As was shown previously,

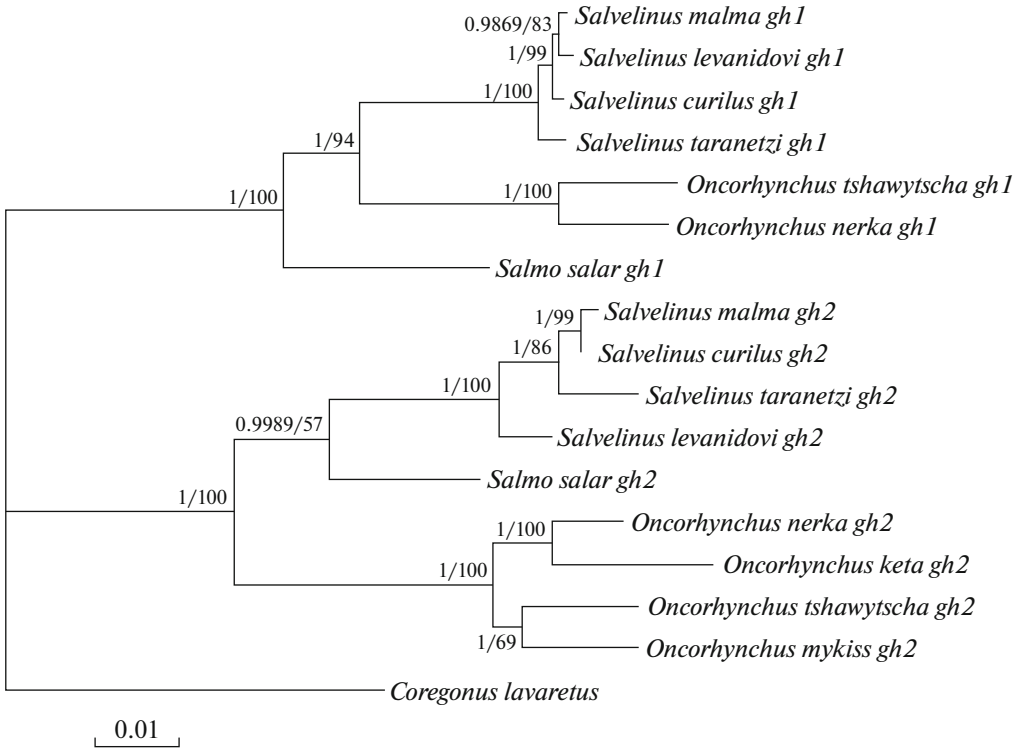

Fig. 1. Phylogenetic reconstruction by Bayesian method. Nodes of branches listed posterior probability/bootstrap support for BI/NJ trees. *gh1* of *Coregonus lavaretus* was used as an external group.

the conserved regions of introns may also be due to the presence of potential regulatory elements in them [19, 33–35]. So, in intron *C*, the regulatory elements of the ERE that are responsible for interactions with the estrogen receptor [34] were detected, as well as consensus sequences of binding sites of pituitary-specific transcription factor Pit-1 (A/T_3NCAT), a part of the two AT-rich regions [33]. The cAMP response element (CRE) is located in intron *D* [19, 35].

CRE sequences within each paralogous gene are identical in all compared species. The CRE-element of *gh1* gene is a palindromic sequence (ACTGCAGT), element CRE of the *gh2* gene differs from it by a single nucleotide substitution (ACTGTAGT). The ERE sequence of the *gh2* gene is identical in all of the examined species (GGGCAAGCAGATC) and differs by one nucleotide from the ERE located in the *gh1* gene (GGGCAAGCAGACC). ERE sequences of the *gh1* gene contain one nucleotide replacement in three species (*S. salar*, *S. curilus*, *S. malma*).

Sequences of the Pit-1 binding sites found earlier in *gh2* gene of *O. mykiss* [34] are less conserved in the comparison of the two genes. The sequence of the site Pit-1 of the first AT-rich region (region 1) is relatively conserved. Pit-1 sites located in the second region (region 2) are more variable (table).

Variability in the 5'-Adjacent Promoter Regions

The lengths of determined 5'-flanking end sites of the growth-hormone genes *gh1* and *gh2* in chars of the genus *Salvelinus* is on average 1200 bp. The results of analyzing the sites compared to some previously obtained [19, 36] indicate that, apparently, the length of the functional regulatory region is only about 300 bp. Therefore, the distribution of variable sites was evaluated in 5'-*cis*-adjacent sections with a length of 300 bp in chars of the genus *Salvelinus*, as well as in other species of salmon (Figs. 3a–3d). In this region, in addition to the TATA box, several possible regulatory elements were found: Pit-1 binding sites (F1–F4) responsible for the interaction with the Pit-1 transcription factor; CRE responsible for binding to cAMP, GRE responsible for binding to glucocorticoid receptor, and a sequence that binds to the retinoic acid receptor (RAR/RXR) [19, 35, 36].

It has been shown that the promoter sites of both genes of the representatives of the genus *Salvelinus* are conserved over almost the entire length (Figs. 3a, 3b). Thus, in the *gh1* gene, there are two variable sites; one is directly adjacent to the TATA box, while the other is located between the F1 and F2 sites (Fig. 3a). Two variable sites were also detected in the *gh2* gene, but they differ in localization. One is in close proximity to the F3 site, while the second is located between the F3 and F4 sites (Fig. 3b).

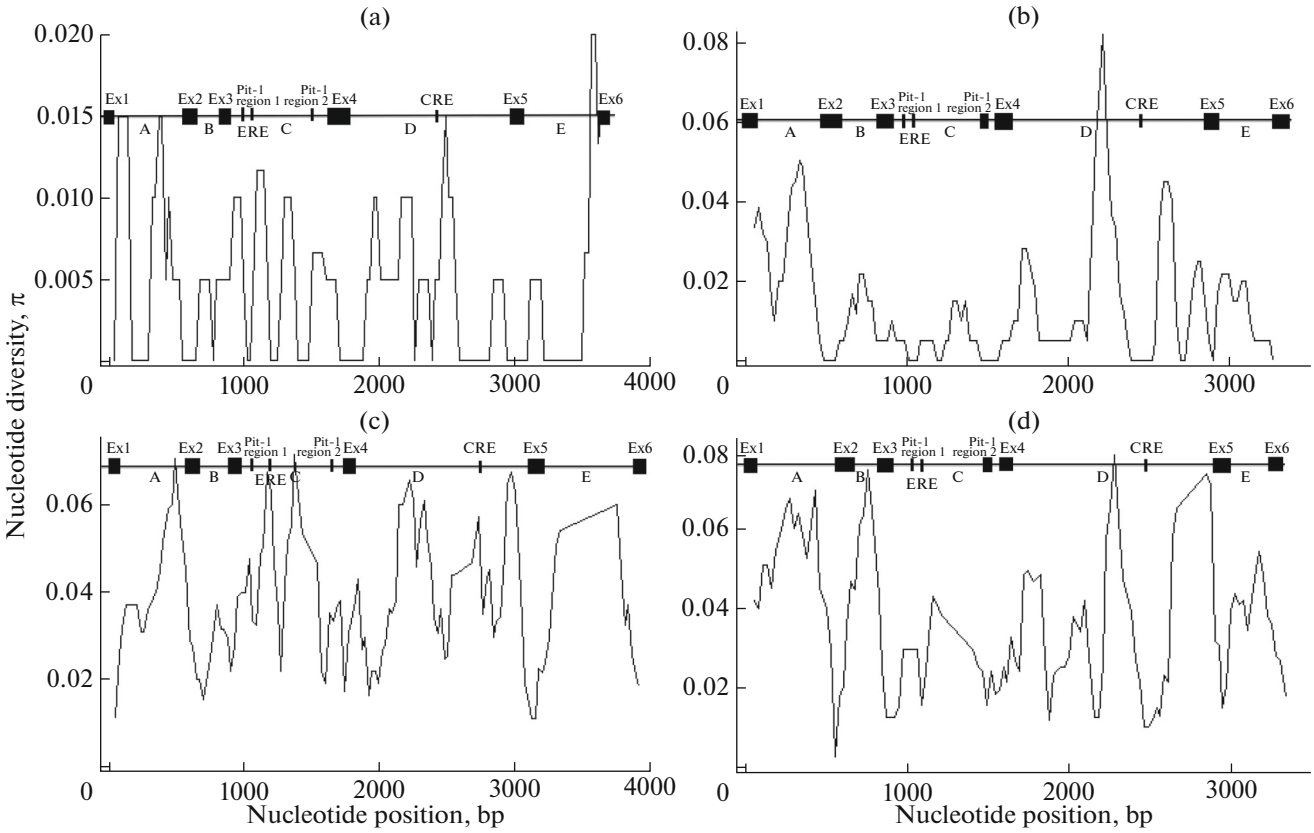

Fig. 2. Analysis of nucleotide diversity (π) of *gh1* and *gh2* genes of Salmonidae family representatives by the sliding window method. Frame size is 100 bp; interval, 25 bp. Nucleotide position is layed off as *X* axis; nucleotide diversity, as *Y* axis. (a) Comparision of chars of the genus *Salvelinus gh1* gene; (b) comparison of chars of the genus *Salvelinus gh2* gene; (c) comparision of *gh1* gene of the representatives of the three genera of the family Salmonidae: *Oncorhynchus*, *Salmo*, and *Salvelinus*; (d) comparison of *gh2* gene of representatives of three genera of the Salmonidae family, i.e., *Oncorhynchus*, *Salmo*, and *Salvelinus*. Exons and possible regulatory elements are marked with black rectangles. Thin lines marked introns.

A comparative analysis of the promoter regions of growth-hormone *gh1* gene of chars with other salmon species (*S. salar*, *O. nerka*, and *O. tshawytscha*) revealed a conserved TATA box, F1 site, and CRE sequences. The F2 and F3 sites are less conserved, and the highest level of variability is typical of the F4 site. It should be noted that the F2 site overlaps on one end with a GRE response element and on the other end with the element of RAR/RXR [19, 35]. This element is conserved, and only one nucleotide substitution was found in the gene of *O. nerka*. Two nucleotide substitutions were found in the GRE element. Both are in the representatives of *Oncorhynchus* genus (Fig. 3c). Interestingly, the nonoverlap of the sequence of these elements for the F2 site of *O. tshawytscha* contains four nucleotides and has substitutions in three positions, and all three are transversions (Fig. 3c). The mainly variable sequences are located near the F1 and F2 sites. There is also a highly conserved site (approximately 50 bp) adjacent to F4. In all salmonids species, this sequence is in the 5'-promoter regions of the growth-hormone genes. However, during a search in the databases, we were unable to find any other homologues in other vertebrates.

The variability in the promoter sequence of the *gh2* gene for all studied salmonids species is more pronounced than in the *gh1* gene (Fig. 3d). The TATA box, F1 section, RAR/RXR, and CRE elements are also conserved. The moderate level of conservation is specific to the F4 site and some part of the F3 site. Sequences between F1 and F2 sites are mainly variable, as well as between F4 and F3 sites with the binding transcription factor Pit-1. Three nucleotide changes (Fig. 3d), which are also transversions, are revealed in the region of the F2-site sequence, consisted of the four nucleotides, and does not overlap with RAR/RXR and GRE elements, as is the case with the *gh1* gene.

DISCUSSION

The divergence pattern of gene-transcribed regions, including the adjacent *cis*-regulatory region, is consistent with previously published patterns obtained based on intronic sequences [37]. This sup-

Sequences of AT-rich regions of intron *C* for the *gh* genes

Gen	Species	Region 1 Site 1	Region 2 Site 2	Region 2 Site 3
gh2	*O. mykiss*	CGATTCC-AAC ATGAATAA TAGGG	TTCTCCAGCC ATGTATCATGTAAA T	GATATGGCATCTCAAGCTGTAC AATTACAT CTCAAC
	O. nerka	CAATTCC-AAC ATGAATAA TAGGG	TTCTCCAGCA ATGTGTCATGTAAA T	GATATGGCATCTCAAGCTGTAC AATTACAA CTCAAC
	O. tshawytscha	CAATTCC-AAC ATGAATAA TAGGG	TTCTCCAGCA ATGTATCATGTAAA T	GATATGGCATCTCAAGCTGTAC AATTACAA CTCAAC
	S. curilus	TGATTCC-AAA ATAAATAA TAGGG	TTCTCCAGCC ATGTATCATGTAAA T	GATATGGCATCTCAAGCTGTAC AA−TACAA CTCAAC
	S. malma	TGATTCC-AAA ATAAATAA TAGGG	TTCTCCAGCC ATGTATCATGTAAA T	GATATGGCATCTCAAGCTGTAC AA−TACAA CTCAAC
	S. taranetzi	TGATTCC-AAA ATAAATAA TAGGG	TTCTCCAGCC ATGTATCATGTAAA T	GATATGGCATCTCAAGCTGTAC AA−TACAA CTCAAC
	S. levanidovi	TGATTCC-AAA ATAAATAA TAGGG	TTCTCCAGCC ATGTATCATGTAAA T	GATATGGCATCTCAAGCTGTAC AA−TACAA CTCAAC
	S. salar	AGATTCC-AAA ATAAATAA TAGGG	TTCTCCAGCC ATGTATCATGTAAA T	GATATGGCATCTCAAGCTGTAC AA−TACAA CTCAAC
gh1	*O. nerka*	AGATTCCAAAA ATAAATAA TAGGG	----------ATCATGTAAA T	-----AGGGCATCTCAAGCTGTAC AA−TACAA CTCAAC
	O. tshawytscha	AGATTCCAAAA ATAAATAA TAGGG	----------ATCATGTAAA T	-----AGGGCATCTCAAGCTGTA− AA−TACAA CTCAAC
	S. curilus	TGATTCC−AAA ATAAATAA CAGGG	----------ATCATGTAAA T	-----AGGGCATCTCAAGCTGTAC AA−TACAA CTTCAT
	S. malma	TGATTCC−AAA ATAAATAA CAGGG	----------ATCATGTAAA T	-----AGGGCATCTCAAGCTGTAC AA−TACAA CTTCAT
	S. taranetzi	TGATTCC−AAA ATAAATAA CAGGG	----------ATCATGTAAA T	-----AGGGCATCTCAAGCTGTAC AA−TACAA CTTCAT
	S. levanidovi	TGATTCC−AAA ATAAATAA CAGGG	----------ATCATGTAAA T	-----AGGGCATCTCAAGCTGTAC AA−TACAA CTTCAT
	S. salar	TGATTAC−AAA ATAAATAA TATGG	----------ATCATGTAAA T	-----AGGGAATCTCAAGCTGTAC AA−TACAA CGCAAC

Pit-1-binding sites are shown in the frames.

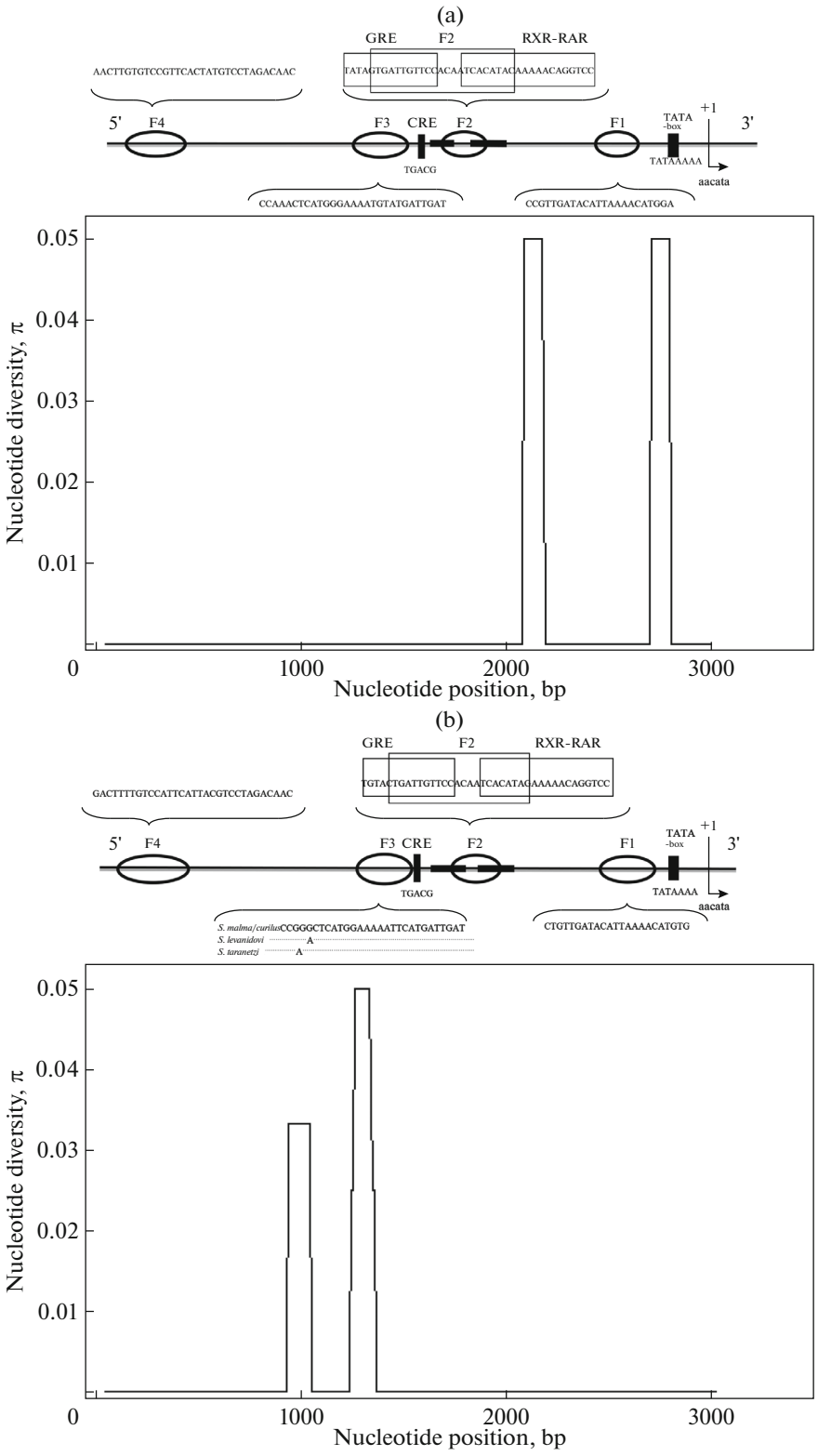

Fig. 3. Analysis of nucleotide diversity of *gh1* and *gh2* genes promoter regions of the representatives of the family Salmonidae by a sliding window method. Frame size is 25 bp; interval, 1 bp. Nucleotide position is layed off as *X* axis; nucleotide diversity (π), as *Y* axis. (a) Comparison of promoter regions of *gh1* gene of chars of the genus *Salvelinus*; (b) comparison of *gh2* gene promoter regions of chars of the genus *Salvelinus*; (c) comparison of *gh1* gene promoter regions of the representatives of three genera of the family Salmonidae: *Oncorhynchus*, *Salmo*, and *Salvelinus*; (d) comparison of *gh2* gene promoter regions of the representatives of the three genera of the Salmonidae family: *Oncorhynchus*, *Salmo*, and *Salvelinus*. Black rectangle marked TATA box, CRE-, GRE- and RXR-RAR-sequences; ovals marked Pit1-binding sites, F1–F4.

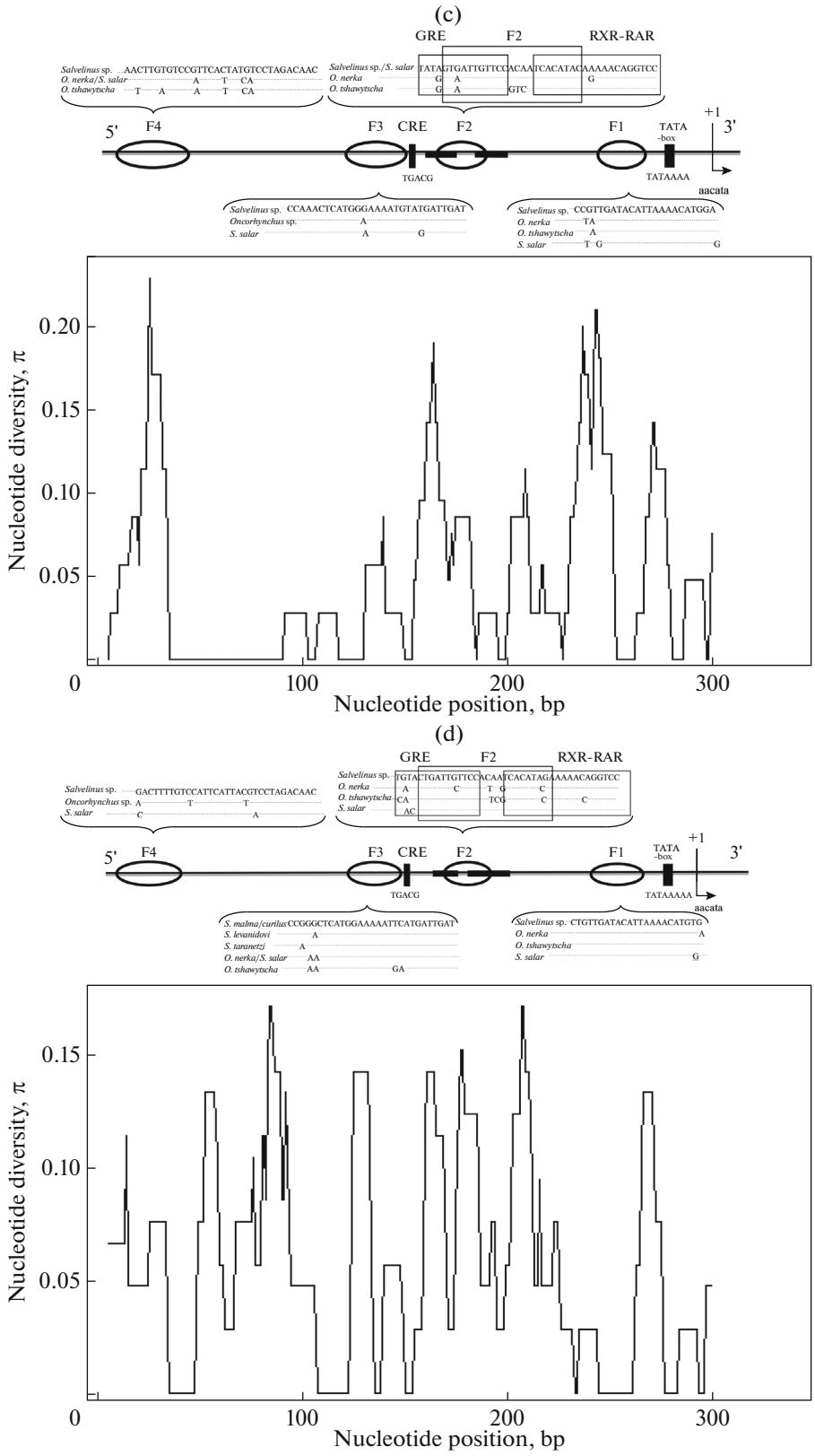
(c)
GRE
F2
RXR-RAR
Salvelinus sp. AACTTGTGTCCGTTCACTATGTCCTAGACAAC
O. nerka/S. salar A T CA
O. tshawytscha T A A T CA
Salvelinus sp./S. salar TATAGTGATTGTTCCACAATCACATACAAAAACAGGTCC
O. nerka G A G
O. tshawytscha G A GTC
5' F4 F3 CRE F2 F1 TATA -box +1 3'
TGACG
TATAAAA
aacata
Salvelinus sp. CCAAACTCATGGGAAAATGTATGATTGAT
Oncorhynchus sp. A
S. salar A G
Salvelinus sp. CCGTTGATACATTAAAACATGGA
O. nerka TA
O. tshawytscha A
S. salar T G G
Nucleotide diversity, π
0.20
0.15
0.10
0.05
0
100
200
300
Nucleotide position, bp
(d)
GRE
F2
RXR-RAR
Salvelinus sp. GACTTTTGTCCATTCATTACGTCCTAGACAAC
Oncorhynchus sp. A T T
S. salar C A
Salvelinus sp. TGTACTGATTGTTCCACAATCACATAGAAAACAGGTCC
O. nerka A C T G C
O. tshawytscha CA TCG C C
S. salar AC
5' F4 F3 CRE F2 F1 TATA -box +1 3'
TGACG
TATAAAA
aacata
S. malma/curilus CCGGGCTCATGGAAAAATTCATGATTGAT
S. levanidovi A
S. taranetzi A
O. nerka/S. salar AA
O. tshawytscha AA GA
Salvelinus sp. CTGTTGATACATTAAAACATGTG
O. nerka A
O. tshawytscha
S. salar G
Nucleotide diversity, π
0.15
0.10
0.05
0
100
200
300
Nucleotide position, bp

Fig. 3. (Contd.).

ports the notion of a long-standing and independent evolution of paralogous genes of growth hormones in salmon fish. It is clearly seen (Fig. 1) that the accumulation of nucleotide changes (mutations) in each of the paralogous genes is defined by the time of independent divergence. The high conservation of coding sequences (exons) of paralogous genes of the growth hormone in salmonid fish is determined by the probable functionality of the both genes [17]; but their functions are different (genes are subfuncionalized [39]) and, thus, are exposed to the cleaning (negative) selection. According to our data, mutations in both genes of the growth hormone accumulate predominantly at the intron sites of the genes. In many cases, mainly variable peaks fall to the middle part of the intron sequences. This may be due to the presence of the splice sites, which are under the influence of the selection [40], on the intron ends, and also to the influence of interfering selection (hitchhiking), wherein the sequences adjacent to the functionally important sequences (exons) are also under the influence of the selection [27, 38]. In addition, the regulatory sequences located in the introns can affect the rate of nucleotide changes accumulation within introns. In our case, ERE-element sequence and binding sites of pituitary-specific transcription factors Pit-1 were detected in intron *C*, while the CRE-element sequence was detected in intron *D*. In both genes of all studied species, these sites are conserved and, hence, under the influence of the selection. Interestingly, the Pit-1-binding sites and CRE-element are located in the promoter region, as well as within introns [34]. However, the consensus nucleotide sequences in the promoter and introns are different. It can be assumed that, if these regulatory regions work cooperatively (or, conversely, on the antagonism principle when bound to ligand), these differences determine the subfunctionalization of paralogous genes.

In general, when comparing the growth-hormone gene sequences of chars (Figs. 2a, 2b) it becomes obvious that the *gh1* gene is more conservative than the *gh2* gene. To a lesser extent, this pattern can be traced when comparing the corresponding genes of all salmonids species (Figs. 2c, 2d).

The same trend can be seen during the analysis of promoter sequences. The high conservation inside *gh1* gene corresponds to the high conservation of the promoter region. This fact is consistent with the assumption that one of the duplicated copies of the gene must be under a strong selection pressure, while the other must be under a weaker selection pressure [27].

The four-base sequence localized at the F2-site Pit-1-binding region, which is rich in transversions in both genes, is of special interest. The nature of this hot spot (mutatios harbor) remains unknown.

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