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Comparison of phylogenetic analysis in the natural *salmonids* by using growth hormone (GH) gene

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ABSTRACT

To days, The Growth hormone (GH) gene is more important in the regulator of metabolism, osmoregulation, reproduction and skeletal growth in Livestock. GH almost in all of animals has been same function that mentioned. This hormone also is exciting skeletal cellular for more growth and replication. In *Salmonids* for specially, furthermore, GH gene in population of *salmonids* can be used as the studies of phylogenetics and finding ancient and pedigree of *salmonids* that some researchers used from GH gene in *salmo salar* and *salmo trutta* for studies of phylogenetics. In this study we had done sequence of fragments of GH gene in *salmo trutta caspius* full length of almost 2048 bp. and deposited in GeneBank (accession number, JN24163) For sequencing of GH gene in the *Salmo trutta caspius*, first was extracted DNA genomics from bloods and the muscles of *salmons*, in related to, we designed three pairs of primers from first to end of the GH gene in same sequences from *salmo salar* and *salmo trutta* that reported in GeneBank. After sequencing of fragments we analyzed fragments and compared with other sequences in *salmonid* fishes. In this research our aims, study of amount variation in the between *salmo trutta caspius* species with *Atlantic salmon* and also, study amount of phylogenetic variation between *Salmo trutta caspius* with other *salmons* regarding to the GH gene.

Keywords: *Salmo trutta caspius*, sequencing, Phylogenetic analysis, growth hormone gene

INTRODUCTION

Growth hormone (GH) gene has been very benefits in natural *salmonids*. To increase of body composition, health, milk production, aging and other same functions is important in GH gene (Lincoln *et al*., 1995; Cook *et al*., 2000; Devlin *et al*., 2004). The growth hormone receptor on target cells by transducting the myogenic stimulating signal across the cell membrane and inducing the transcription of many genes, including IGF-I (Rotwein *et al*., 1994).The GH gene however effective on the cell growth rate, but also, it has been other function for polymorphism of populations in animals. GH gene in bovine changed a single nucleotide polymorphism in fifth exon (Lucy *et al*., 1991; Zhang *et al*., 1992;Yao *et al*., 1996). The many genes were used for polymorphism of *salmonids*, mitochondrial DNA has been studied extensively in numerous fish species by restriction site analysis of the entire mitochondrial genome or by 'micro restriction' mapping and direct sequencing of the selectively amplified genes (Beckenbach, 1991; Bernatchez *et al*., 1992; Whitmore *et al*., 1992; Ovenden *et al*., 1993), that used some genes for this means, including, cytochrome b gene in *salmo treutta fario* (Rezaei and Akhshabi, 2012), cytochrome b in *salmo trutta caspius* (Jamshidi and Kalbasi, 2009), and other genes in mitochondrial DNA. The mitochondrial DNA more inherited

maternal traits than paternal traits,but in GH gene more inherited paternal trait on the nuclear DNA genomics. GH gene in *salmonids* have is duplicated because tetraploids origin and as a consequence, two forms of GH are produced. (Agellon *et al*., 1988; Rentier-Delrue *et al*., 1989). The GH sequence in teleost species have recently cloned, such as *goldfish* (*Carassius auratus*) (Lee *et al*., 2001), *turbot* (*Scophthalmus maximusi*) (Calduch-Giner *et al*., 2001), *Japanese eel* (*Anguilla japonica*) (Ozaki *et al*., 2002), *black seabream* (*Acanthopagrus schlegeli*) (Tse *et al*., 2003), *rainbow trout* (*Oncorhynchus mykiss*) (Very *et al*., 2005) and *gilthead seabream* (*Sparusaurata*) (Calduch-Giner *et al*., 2003; Saera-Vila *et al*., 2005). In natural *salmonids*, including, *salmo salar*, *salmo trutta* and *salmo trutta caspius*, GH gene investigated for comparison of phylogenetics and the relationship between ancient of natural *salmonids*. In this study we analysed the among of variation and relationship between natural *salmons* by using GH gene that other researchers used this gene for polymorphism of populations *salmonids*.

MATERIALS AND METHODS

Samples Fishes: The adult fishes including male and female *salmon* was caught from muscles and bloods for DNA extraction. These *salmons* had 2-3 years old age that originated from the Rivers of Sardabrood and Dohezar of Tonekabon-Iran.

DNA extraction: DNA extracted by kit (produced by Chromous Company kit, Bangalore-India) was used from 2-3 grams muscles and also 1 cc bloods. The *salmons* were caught after anesthetic of that, samples were muscle fins and also blood. These samples immediately had cold on the temperature of 20°c for next experiments.

Primers: Three pairs of primers were designed according to method of designing primers by DNAMAN program computer and also BLAST network system. In this regards, we used some GH gene that reported in GeneBank including, *salmo salar* and *salmo trutta*, because we assumed these sequences had high homology with *salmo trutta caspius*. The fragment of primers including:

Product Size 1495 bp.

Primer_Set_I_For.
AATCATCCTTGGCAATTAAGAG
Primer_Set_I_Rev.
CCTTAGTTGAAGGCACTGAGGT

Product Size 1500 bp.

Primer_Set_II_For.
GCATGTTATGCCCTTTAAAACC
Primer_Set_II_Rev.
CAGTCCTGTGGCCTTCAAGT

Product Size 1493 bp.

Primer_Set_III_For.
TGAACTCAAAGTCAATGAAAAGTCA
Primer_Set_III_Rev.
AACCCTGGAGACAGGCTCTT

PCR Amplification: Different set of parameters (Gradient Cycle, Primer Concentration variation, Magnesium Chloride variation, PCR Cycle variation) have been set to standardize the PCR amplification of DNA with above primer sets. PCR was performed using primers (PCR cycle conditions are mentioned below), including:

Template DNA: 1.0 µl
Forward primer (100ng/ ml) 2.0µl
PCR Cycle condition:

94°C	94°C	55°C	72°C	94°C
5 min	30 sec	30 sec	1 min	5 min
	35 cycles			

Reverse primer (100ng/ ml) 2.0 µl
dNTP mix (2.5mM each) 2.0 µl
10X ChromTaq Assay buffer 5.0 µl
ChromTaq enzyme (3U/ ml) 0.5 µl
Water: 37.5 µl
Total Reaction volume: 50.0 µl

Electrophoresis: Amplified GH gene full length was separated by one percent agarose gel electrophoresis. Gels were loaded at approximately 100 V until the Bromophenol blue dye front reached the

end of the gel. After electrophoresis, the DNA full length was visualized ethidium bromide and then was taken photos by gel DOC Bio RAD Company.

GEL Extraction and PCR purification by the kit SPIN-50 (RKT33): The kit is designed for rapid purification of plasmid DNA from standard or low-melt agarose in TAE or TBE solution. Features of the kit: High quality DNA and no phenol chloroform required. PCR products were gel eluted and sequenced using gene specific forward and reverse primer. Finally, the PCR products were sent to the Choromous Geni Company-India for doing sequencing.

Sequencing of fragments: Fragments of GH gene were amplified, these fragments the was loaded on the gel electrophoresis, first, purified by extraction of gel electrophoresis kit by method of mentioned: **A.** Cut the DNA fragment from the Agarose gel with a clean, sharp cutter. **B.** Weigh the gel slice in a 2 ml micro centrifuge tube **C.** Add 3 volumes of Gel extraction buffer to 1 volume of gel (100mg) C. Incubate the tube at 55°C for 5-10 min (or until the gel piece has completely dissolved). Mix the sample by inverting the tube every 2-3 min during the incubation to solublize agarose completely. For >2%agarose gels, increase the incubation time. **D.** After the gel piece has dissolved completely, add 1 gel volume of isopropanol to the tube and mix (If the Agarose gel piece is 100mg, add 100µl isopropanol). **E.** Place the spin column in a 2ml collection tube provided. **F.** Load the gel extracted solution mixed with isopropanol on to the spin column (6000g for each time). **G.** Spin at 13,000g for 1 min at Room Temperature. Discard the contents of the collection tube. Place the spin column back in the same collection tube. **H.** Add 500 µl of wash buffer to the column. Spin at 13,000g for 1 min at room temperature. Discard the contents of the collection tube. Place the spin column back in the same collection tube. **K.** Repeat step 9. **L.** Spin the empty column with the collection tube at 13000g for 3 min at RT. **M.** Place the spin column in a fresh 1.5ml micro centrifuge tube. **N.** Add 15 µl of Elution Buffer on to the spin column. **O.** Keep the vial along with the spin column at Room temperature for 2min**. Z.** Spin at 13,000g for 1 min at room temperature. **P.** Again add 15 µl of Elution Buffer on to the spin column. **Q.** Keep the vial along with the spin column at Room temperature for 2min. Spin at 13000g for 1 min at room temperature. **R.** Purified DNA is collected in the tube. DNA purified after were dried and ready for sequencing by primer walking.

Designing of primer: A fragment of DNA were designed from end of DNA for amplify of DNA sequences. Including, ATCTGGTAGAGCCTGACTCCA

RESULTS

Study variations at DNA level contribute to the genetic characterization of *Salmons* we used GH of gene. According to the annotation GH genes, these are genes linked to economic traits and polymorphism genetics which are governed by many genes, following to the sequences of the *salmon* GH gene were published in the BLASTn on the National Centre for Biotechnology Information (NCBI) Network Service, was designed a fragment of almost 3kb. Hence, genomic DNA was extracted from blood samples *salmo trutta caspius* (Fig. 1).

PCR amplification gel photo: According to reported sequences about GH genes at NCBI network, we have expected full length almost 3.5 kb from PCR products but are shown approximately 3 kb from full length *salmo trutta caspius* (Fig. 2).

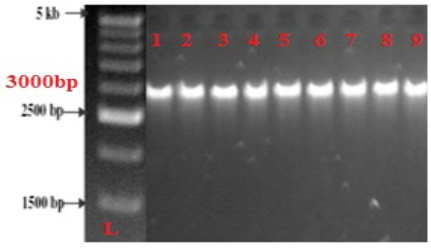

Fig. 2: PCR amplification of Growth hormone gene (~ 3 kb) *Salmo trutta caspius* from samples 1 to 9. PCR Products were loaded on 1% agarose gel. **L**: 500bp DNA ladder.

Fig. 1: Genomic DNA was extracted from blood sample *Salmo trutta caspius* was loaded on 1% agarose gel.

Exons highlighted in the reference sequence:

GATCTAATGTGTTATATTCGCCTACATTACTTTCACATTTCCACAAACTCCAAAGTATTTCCTTTCAAATGGTATCA
ATAATATGCATATCCTTGCTTCAGGTCCTGAGCTACAGGCAGTTAGATTTGGTTATGTCATTTCAGGTGAAAATTGG
GAAAAAAAGGGTCCGATCCTTAAGAGGTTTTAATGCCATAGGACATTCAATTTGACAATAAACAATAAAATATTG
GTGCTGATAAAGAAGCAATATAATACATTTGTCAAATACTGCATGTTATCTACAGTACCACAGGTGGAATGGCAG
AATAACCGGTGTTGTGTGTGTATGTGTGTGTGTAACTTGTGTCCATTCATTACATCCTAGACAACAGAGGTTTGTGT
TGTGTTTTGACCCTAATTCGTTCAGTCATCAAGTAAGTTGTTTTTTTAGGACACGTCCCCTCTTCCAAACTCATGGAA
AAATGTAGGATTGATTTGACGCATTATAGTGATTGTTCCACAATCACATACAAAAACAGGTCCTATTAATGAAAGG
TGGTAAATGGATGAAAATCTCATGTTTCCTCCTGGTGATACATTAAAACATGGGTTCCCCATCTATAAAAACAGTG
GTCCCAAACAAACAGCAACATACTCAACCGACCACCGCACTTTCAAGTTAAGTAATCATCCTTGGCAATTAAGAGT
AAAAATGGGACAAGGTAAGCCTGCTTTTTCTGTCTATTTCTTTTTTCAGTGGGAAGTCAGTGTACCATTTAGTACAA
TTTAACTTACACATTTAATCACTGAGGCAGGGGCCAACACGGCAGAGAAAAGTGAACAAGTATTCTACTACTATG
AGGTTATAAATCTATTGACACAGAACCACCTGCTTTAACAACCTAACTATGTGATCTATAACATTTACATTTGAGTC
GTTTAGCAGACGCTCTTATCCAGAGCGACTTACAGGAGCAATTAGGGTTAAGTGCCTTGCTCAAGGGCACGTCGAC
AGATTTCTCACCTAGTCAGCTCAGGGATTGAAACCAGTAACCTTTCAATTACTTACCCAACGCTCTTAACCGCTAG
GCTATTGGTGTTCGATGGCTGAGAATATCTAACTAATGTATCTCACCATAATTCGACTTACTCGTTTTATACATTTG
TTATTTTCTCTTTCTTTTAGTGTTTCTGCTGATGCCAGTCTTACTGGTCAGTTGTTTTCTGAGCCAAGGGGCAGCGAT
GGAAAACCAACGGCTCTTCAACATCGCGGTCAACCGGGTGCAACATCTCCACCTAATGGCTCAGAAGATGTTCAA
TGACTTTGTAAGACAGCTTTTGAATCTTCTTTTGACATATCAAATAGTGTATCAATGATGTTCTTCTTCTTGTAGAC
AGTGTCCTCTTTACACAACCCTCGTGGCAACAACAAAAAAATCTCTCTCCCTTCTTTGTGATTTTGTGCAGGAAGGT
ACCCTGTTGCCTGATGAACGCAGACAGCTGAACAAGATATTCCTGCTGGACTTCTGTAACTCTGACTCCATCGTGA
GCCCAATCGACAAGCTTGAGACTCAGAAGAGTTCAGTAAGTAACCTGGTTGAGACAATTATGCATGTTATGCCCTT
TAAAACCATATAAAAGTGTAAAATTGTGACAGGTCCACTCTGCTATTCACCTTAAATATGAATTCCTCCATGATGC
ATGATTCCAAAATAAATAATATGGCATCTCAATTTGAACAATCGATAGAACTTAGTCATTAGTTATTGGGAAAGCA
GACCACCAATTATCTAAACTCCAATTTATAAATGTTTTAATTTGAATTTTTTTACCATTATTTAACTAGGCAAGTCA
ATTAAGAACAAATTCTCATTTACAATGACAAGCAGAGGCTGCATCATGCATGGCTGTCGAGTGGCGCACGAGTCT
AAGGCACTGCATCTCAGTGTTAGAGGTGTCACTACAGACCCTGGTTCGATTCCAGACTGTATTACAAATGGCTGTG
ATTGGGAGTCCCATAGGCGACACGCAATTGGCCCACCGTCGTTAGGGTTTGGCCGGGGTTGGCGGTCAAATAAAA
AAAAAATGGTGGAAATGAAATCTAGCCATGACAGAGAGTTTAACTGTACATGTAAAATTGGCATTAACACATTGC
TATACCTCAGTGCCTTCAACTAAGGTAGGTAAAACAACCACATATCAAAGTCATTGCAAGTAAAACCATCACTCTC
TAAATCGGTGGTTTCTCTACGTCTACATTCTCCGTTTTGTGCTTTTCTGTCCAGGAAACCAGCCCCAAAGGTTTTTA
ACTCAATCATGTAAATAGGGAATCTCAAGCTGTACAATACAACGCAACTTCATTTTCCAATAATCTGTGGTTTCTCT
ACATCTACACACACCACAGGTCCTGAAGCTGCTCCATATCTCTTTCCGTCTGATTGAATCCTGGGAGTACCCTAGCC
AGACCCTGACCATCTCCAACAGCCTAATGGTCAGAAACTCCAACCAGATCTCTGAGAAGCTCAGCGACCTCAAAG
TGGGCATCAACCTGCTCATCAAGGTAAAGAAAGGAGGGAGAACAATGACCATTTGTGGTGCCACACTTTGTGCAC
TGTAAACCCCAAGGCATTTTTAACTCAAATACTTCTAGTAAGTTGAACTCAAAGTCAATGAAAAGTCATTATTACT
TAAAATGTTTATGTGGTACTGGCTCAAAACTAAATGAGAAGTGACATCAACACAATTTTTTAAAGTTATAACAAAT
TAACTTTTTATCCAGCATGCTCTACTGCAGGTAGATTTTTTGGAATTGTTTTTAACTATCTGTGTTTTGCATGTACAG
GACATTGAGTGATTGATTCATCGTATGCTACACAAAGATATATAACATACATTTTTCAACATTTTCACAAAGATGA
ATAAGTTACCAGAATTTTGCAAACCCGACTTGCAGGCCTGATGTGGCCTTAAACTATGAGTTTCAGGCCACTGTAT
TAGGGTACACGTACGCCTCAAAATACGGTCTTATGAGATATGTAATGTATTGTTATAAAGAGTTGAATTACAATGA
TAATATTTGCCTAGGAATTAACTTGAAGGCCACAGGACTGAAAATGAATGACAACCATGTCTCTGTTACTAACAAA
TACAGTCATGGGTGATAACTACAATTCACTCAAAAAGGCCAGGCACACTGGGAAATGATATTGGGGACGTGGCTT
AGTGAGGGCATTACTAAAAAATGTCAAGCTGATACAACTCAAATCTGGACCCTTCACAGGGTGACTAGAGTAATG
ACTAACTGCAGTCAGATTCTATATATTAAGTGCAACGGGTTTCCTAAAACGTTTTGAGTAATGACAGCACATTGGG
TTTTACAGTGACATGAAAGTGAAATACCTCTATGCTTTCCTAGTTAGAAAGCATAGTGTAGGACCACGTTTGCCTC
TTCTCAGCAGATCTTTCAGTGCTTTACATTGTGATGGGGTAAATAACCTCATCTATCATCACTAATATTGACTATAT
CAGTAACACCCCATTCAATGACTGAATATCAGCCCATTCAAGGATATTTATGCATGCGTCTTTTGCTGTGTGTGCTT
TCAGAAAGGCCCAATAAACAAATATTGATATGCACACATCCACCCCACCATGCATCTCTCTCTGTCTCCCACAGGG
GAGCCAGGATGGCGTACTGAGCCTGGATGACAATGACTCTCAGCAGCTGCCCCCCTACGGGAACTACTACCAGAA
CCTGGGGGGGCGACGGCAACGTCAGGAGGAACTATGAGTTGTTGGCCTGCTTCAAGAAGGACATGCACAAGGTGCA
AAACCATGTTGCCTTCTATTTCATGTGCCTTCCTATATTTTCTACAGTGCGTTTCTTGTGCTCTCTATTGCAAAGTAT
CTTTGGGTCTTTAACCCATATATTATTACTATTATTGTTCATTGATCAAGACTGTTCTCGAGAAAGGTCTAGTGACC
TAGAACACTCACATTAAAATGTGTCAACTATAACCCATTCTTCTATTTTTCCCCCAAGGTCGAGACCTACCTGACCG
TCGCCAAGTGCAGGAAGTCACTGGAGGCCAACTGCACTCTGTAGACGTGGGCTGGAGAGGCAGCCAGCAAGAGCC
TGTCTCCAGGGTTCGGTTTCCCAGATACAGATTAGGCCTTGCCCTGCACTGAAGAGCATTTTCAATTGAGATTCTCC
ATTAAACGTGCTTTTTAGTCTAGAGTAGATTTAATTTGGATCTGGTAGAGCCTGACTCCAGGGGTTTTCAGGAATTT
GCATTTTGTTCTCTGAAATCAACAACAGCACTTTCTATATTGACT

Fig. 3: Yellow: Exons, Green: Primers designed for amplification of GH gene in *salmo trutta caspius* that originated from *Salmo salar* sequence of GH gene had reported in GeneBank, Blue: primer designed for sequencing and amplification

DISCUSSION

The family *Salmonidae* comprises eleven genera and includes *salmon*, *trout*, *charr*, *freshwater whitefishes*, *ciscos* and graylings (Nelson, 2006). Many *salmonid* species are of considerable genetics polymorphism, economic, social and environmental importance. Many genes engaged on the growth cells, metabolism and anabolism in the natural *salmonids*,

specially GH gene. This gene has two types, type I and type II. (Agellon *et al*., 1988 and 1989; Rentier-Delrue *et al*., 1989). The growth hormone gene in *teleost* (*Siluriforms* and *Cypriniforms*) which consist of the five exon type and four intron type, while in *salmoniforms*, *Perciforms* and *Tetradontiforms*, which consist of six exons and five introns (six exon type) (Moriyama *et al*., 2006). There are some genes that finding ancestral of *salmonids*, these genes including, mitochondrial genomics that more inherited of maternal traits (Allendorf *et al*., 1984; Bernatchez *et al*., 1992; Bernatchez, 1995), and GH gene that inherited of paternal traits (Gross and Nilsson, 1995). The aim of this study was to annotate the coding sequence of the GH gene and perform a GH-based phylogenetic analysis within the *salmonids* family. The GH gene as a natural marker for studies of evolutionary genetics of various fishes because of its sequence conservation, in *salmonids* (Marins *et al*., 2003; Chen *et al*., 2004; Pinheiro *et al*., 2008). According those results, The *salmo trutta caspius* possible has been ancestral with *salmo salar*,*salmo trutta* and homology with *onchorhyncus mykiss* and *chun salmon*. The analysis and sequenced provided valuable information about the mode of evaluation of these DNA sequences. In this project we analyzed the rate of homology between population *salmonids* by GH genes, there were some documents that they are alleles at a common ancient, from 12000-14000 years ago. Berg *et al*., (1962) proposed that *salmo trutta* had originated from Atlantic Ocean that had been migrated to White Sea and then left to Russia in Caspian Sea. Also they proposed that these *salmons* is related to deep, and the rivers of around Caspian Sea is very good for passing period of smolt and egg laid by adult *salmons* those will select the rivers of connected to Caspian Sea, in fact these results introduced and denoted that *salmo trutta caspius* can be originated from *Atlantic salmons*.

The phylogenetic tree: The evolutionary hypothesis of a phylogeny can be graphically represented by a phylogenetic tree. The neighbor joining algorithm, (Saitou and Nei, 1987), on the other hand, builds a tree where the evolutionary rates are free to differ in different lineages.

The same results phylogenetic tree in figure 4 and figure 5, are shown that denoted and annotated, *salmo trutta caspius*, *salmo salar*, *salmo trutta*, *onchorhyncus mykiss*, and other *salmonids* had common ancient according hypothesis by Berg, 1962. In figure 4 distance of between *salmo trutta caspius* with *salmo salar* is longer (74 to 89%) than *rainbow trout* (94%), however for generally distance genetics between sequences of GH gene is not very long and among of homology is very high.

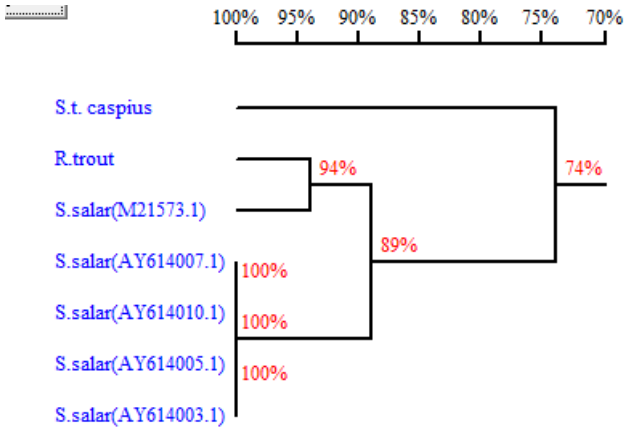

Fig. 4: Results of phylogenetic analysis sequences of the GH gene in *salmo trutta caspius* and *Rainbow trout*, *Salmo salar*(different sequences that reported in GeneBank). The consensus tree is shown

obtained high homology between sequences, however in *salmo trutta caspius* the rate of homology less than other *salmons*. These results obtained from DNAMAN computer program.

Fig. 5: Results of phylogenetic analysis sequences of the GH gene in *salmo trutta caspius* and *Salmo salar*(different sequences that reported in GeneBank). The consensus tree is shown obtained high homology between sequences, however in *salmo trutta caspius* the rate of homology less than other *salmons*. These results obtained from BLAST NCBI Network program.

CONCLUSION

In this study we shows in *salmo trutta caspius* GH gene can be marker genetics for analysis of polymorphism populations in *salmons*. However the among of homology in GH gene denote for finding of pedigree is good but also these gene more inherited paternal traits to offspring, so we proposed that use other gene from mitochondrial genomics that inherited maternal traits and also microsatellites fragments in length of DNA genomics, with compare these information finally we can surely analyse phylogenetic populations of *salmonids*, however information of GH gene are very benefit for evolutionary and also finding ancestral of population *salmons.*

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