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Genome-Wide Determination, Characterization and Bioinformatics Analysis of Cold Shock Protein (*CSP*) Genes In Atlantic Salmon (*Salmo salar*), Carp (*Cyprinus carpio*) and Rainbow Trout (*Oncahorynchus mykiss*)

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ABSTRACT

Atlantic salmon (*Salmo salar*) is the symbol of the underwater world; carp (*Cyprinus carpio*) is a freshwater fish that gives its name to the Cyprinidae family and rainbow trout (*Oncorhynchus mykiss*) is a species of Salmonidae family, like as salmon. These three types of fish are important in the world because they are made of fish farming. The cold shock proteins (CSP) were first described in *Escherichia coli* and are the conserved proteins that have the activity to protect the organism against cold temperatures. They can be characterized by general stress response in fish. There is no comprehensive study related to the identification of CSPs in fish genomes. In this study, determination, characterization and bioinformatics analysis of CSPs were carried out in the genomes of salmon, carp and rainbow trout. The study results represent preliminary knowledge about understanding of the effects of these proteins in cold tolerance in economically important fish species.

Anahtar Kelimeler:
Somon Balığı
Sazan Balığı
Gökkuşağı Alabalığı
Biyoinformatik
Soğuk Şoku Proteini

ÖZ

Atlantik somonu *(Salmo salar*) sualtı dünyasının simgesidir; sazan balığı (*Cyprinus carpio*), sazangiller (Cyprinidae) familyasına adını veren tatlı su balığıdır ve gökkuşağı alabalığı (*Oncorhynchus mykiss*) somon gibi Salmonidae ailesine ait bir balık türüdür. Bu üç balık türü dünyada en çok çiftçiliği yapılan balık türlerinden olduğu için önemlidir. Soğuk şoku proteinleri (CSP) ise ilk kez *Escherichia coli*'de tanımlanmış olup organizmayı soğuğa karşı koruma aktivitesine sahip olan korunmuş proteinlerdir. Bunlar, balıklardaki genel stres tepkisi ile karakterize edilebilir. Balık genomlarında CSP proteinlerinin tanımlanması ile ilgili kapsamlı bir çalışma yoktur. Bu çalışmada, Atlantik somonu, sazan balığı ve gökkuşağı alabalığı genomlarında CSP proteinlerinin belirlenmesi, karakterizasyonu ve biyoinformatik analizleri gerçekleştirilmiştir. Çalışma sonuçları, ekonomik öneme sahip balık türlerinde bu proteinlerin soğuk toleransındaki etkilerinin anlaşılması için ön bilgi sunmaktadır.

1. Introduction

Atlantic salmon (*Salmo salar*) is a fish species of Salmonidae family, composed of 11 genera and 70 species, living in the North Atlantic Ocean, exhibiting a wide range of ecological adaptations and using a variety of marine and freshwater habitats [1]. Atlantic salmon is an icon and king in the underwater world. However, today, like many types of water, its survival has been threatened [2]. Therefore, it is one of the most important species for fish farming worldwide.

The carp (*Cyprinus carpio*) is a freshwater fish that gives its name to the cyprinids (Cyprinidae) family [3]. Common carps are native to Europe and Asia and have been introduced to all parts of the world except the poles. These are the third most common fish species worldwide [4] and the dates they spend as farm fishes are based on the Roman period. Carp is used as food in many areas, but is also considered harmful in several regions due to its ability to compete with local fish stocks [5]. Carp is an important food fish in most parts of the world, except in Australia and North America, where the fish are thought to be tasteless [6, 7].

The rainbow trout (*Oncorhynchus mykiss*) is native to North America, along the eastern North Pacific region, through the Aleutic Islands from Mexico and the Kamchatka Peninsula [8]. The rainbow trout is classified as *Oncorhynchus mykiss*, and Salmonidae, which is of the same breed as the Pacific salmon and which contains various trout (Salvelinus sp.), such as Atlantic salmon (*Salmo salar*), Arctic char (*Salvelinus alpinus*), Arctic grayling (*Thymallus arcticus*) and whitefish (Coregonus sp.) belongs to the family. The rainbow trout may tolerate a wide range of water temperatures and other environmental variables, such as water quality, but require high levels of oxygenated water and develop at water

temperatures of 13-18° C. They are very valuable nutrients and can be grown with a pigmented (red) or non-pigmented (white) meat depending on their nutrition. Rainbow trout is the most common farm trout in the world, which has been cultured for hundreds of years [9].

All living cells are capable of responding to changes in the environment, such as temperature, pressure, osmotic stress, and oxygen availability. For example; at the cellular level, acclimation or adaptation in response to the temperature flow occurs with changes in metabolic rate, intracellular pH, ion concentration, membrane composition, and gene expression. An increase in temperature results in a specific heat shock response shared by all organisms, from bacteria to mammals. The heat shock response is featured by the synthesis of a number of highly conserved heat shock proteins. Cellular responses to a decrease in temperature have not been well studied and no conserved cold inducible protein set has been identified among all organisms. There are adaptive mechanisms used by many organisms in response to cold temperature. These include changes in membrane fluidity and changes in the protein translation mechanism of the cell [10]. Membrane fluidity decreases on temperature drop affecting membrane-related cellular functions. Organisms overcome this by reducing the degree of saturation in membrane phospholipids to achieve greater flexibility. This condition is described in *Escherichia coli*. As in Bacillus and Synechococcus, the synthesis and stability of membrane-bound desaturase is followed by a cold shock. Cold shock allows secondary structures to stabilize in RNA and DNA, leading to a decrease in the efficiency of translation, transcription and DNA replication. The harmful effects of cold shock are overcome by stimulation of cold shock proteins (CSP). CSPA, a major cold shock protein, was first identified from *E. coli* and has since been identified in various Gram-positive and Gram-negative bacteria, including homologs, psychrotrophic, psychophilic, mesophilic and thermophilic strains [11].

CSPs are among the most conserved proteins. The characteristic feature is that it comprises one or more cold shock domains (CSD) having nucleic acid binding properties. This condition equips these proteins with pleiotropic functions, such as the regulation of transcription, translation and splicing [12]. CSPs eliminate some of the harmful effects of temperature drop and thus help to adapt the cells. After the emergency cold shock response, the synthesis of CSPs is reduced and the synthesis of other proteins is increasing. This allows the cells to grow at low temperature, although at a lower rate. CSPs are known to be important during the cold shock response, but recent studies have shown that CSPs may play a wider role in the stress tolerance of bacteria [13].

A cold shock that reduces body temperature to the lower limit of the thermal range of an organism can cause severe inferior disorders and mortality. The magnitude of the cold shock response depends on both the temperature drop rate and the magnitude of the change according to the thermal tolerance limits [14, 15]. While the cold shock stress response is generally thought to be an adaptive response to maintain homeostasis, prolonged or severe temperature changes other than a certain tolerance change may ultimately result in mortality [16] or otherwise affect the health and condition of the organism [17].

CSPs are generally better defined in bacteria. Although there is no comprehensive study related to the identification of CSPs in fish genomes, they have been characterized by general stress response in fish [17]. When these three fish species are considered, *Salmo salar* is at the risk of extinction and *Salmo salar*, *Cyprinus carpio* and *Oncahorynchus mykiss* are the most consumed fishes in the world and this indicates the importance of these fishes. In this study, it was aimed to identify genes encoding for CSP in *Salmo salar*, *Cyprinus carpio and Oncahorynchus myciss* genomes and their settlements, phylogenetic relations, estimated biological roles, molecular functions, cellular settlements, target miRNAs and secondary structures and locations on chromosomes.

2. Material and Method

The NCBI (https://www.ncbi.nlm.nih.gov/) database was used to identify CSPs and BLASTP analysis (Protein Blast-Sequence Comparison) was performed from the NCBI database. The presence of protein sequences in the CSP family was checked by Pfam domain analysis and various physical and chemical parameters were calculated using the Expasy Prot Param Tool (https://web.expasy.org/protparam/). pH range, molecular weights, variability states and isoelectric effect (pI) values were reached with this analysis.

The certain amino acid sequences were identified by the MEGA7 program [18] and were used for sequence alignment. Multiple sequence alignments were performed with ClustalW program. The exon-intron regions were determined by using the GSDS2.0 (http://gsds.cbi.pku.edu.cn/) database [19], using the genomic sequences and the CDS sequences of the CSP sequences. A phylogenetic tree was created by the Neighbor Joining method that gave the closest tree to the evolutionary process. For the phylogenetic tree, the Bootstrap 1000 value, which is a 1000 repetitive boot tab, was selected and the phylogenetic tree was generated with the desired analysis [20]. The resulting phylogenetic tree was visualized in ITOL and the relationship between evolutionary processes has been made more prominent. The database used as DNA motif screening tool (MEME) [21] was used to identify protected motifs. In the analysis, the maximum number of motifs and the widths of the motifs were ≥ 2 and ≤ 300, respectively. Following the identification of the MEME motifs, the InterPro database and InterProScan were also scanned [22].

Functional analyzes of CSP protein sequences were performed using the Blast2GO program [23]. These functional analyzes were carried out in three steps. In the first step, maps were loaded with the sequences loaded in the program (BLASTp), and in the second step maps related to the BLAST results were prepared (MAPPING) and in the third step, the

information file was prepared for the sequences (ANNOTATION). With this program, three categories were created and biological functions, cellular content and molecular functions were determined by GO classification.

miRNAs were determined in order to identify miRNA-controlled gene targets in the *CSP* genes in Atlantic salmon, carp and rainbow trout and to understand the miRNA functions. For this, miRBase v20.0 (http://www.mirbase.org/) was used and previously known animal miRNA precursors were detected. The plant miRNA database was used to identify miRNAs targeting the *CSP* genes found in salmon, carp and rainbow trout. In addition, all known plant miRNAs were identified using the psRNA Target Server (https://plantgrn.noble.org/psRNATarget/) database to align the *CSP* gene transcripts in Atlantic salmon, carp and rainbow trout and defined miRNAs in these fishes. All known animal miRNAs and their target or targets were identified by the parameters described. The miRNA targets detected by computer screening were confirmed. BLASTX screening was also performed for the analysis of gene homologs. Using the protein sequences, the 3D structure of the CSPs was estimated by Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The reliability of the model was confirmed by other numerical data [24].

3. Results and Discussion

CSP genes from *Salmo salar*, *Cyprinus carpio* and *Oncorhynchus mykiss* were named as *SsaCSP*, *CcaCSP* and *OmyCSP*, respectively. According to the results, 86 genes for *SsaCSP*, 15 genes for *CcaCSP* and 60 genes for *OmyCSP* were identified. Carp, Atlantic salmon and rainbow trout have 2n = 58, 2n = 100 and 2n = 58 chromosomes, respectively. Based on the analysis of *Salmo salar* genome by using CSP sequences, chromosomal location of *SsaCSP* genes on 13th and 15th carp chromosomes are noteworthy and most of the *SsaCSP* genes located on these two chromosomes. This finding shows that the 13th and 15th chromosomes have an important role for the SsaCSP family members (**Figure 1**). *CcaCSP* gene family members were only on 8 chromosomes of carp. In this family, the maximum gene placement was located in the scaffold. Since the localizations of the genes in this family are largely in the scaffold, it is unclear which chromosome plays a role for CSPs (**Figure 1**). Gene placement of *OmyCSP* gene family on 9th and 16th on chromosomes was quite remarkable. On the 9th and 16th chromosome pairs, there were 23 and 21 *CSP* genes, respectively. The other chromosomes had an average of 1-2 *CSP* genes. This table clearly shows that the 9th and 16th chromosomes are directly related to CSPs. The presence of only 1 gene on scaffold provides stronger interpretations when compared to other gene families (**Figure 1**).

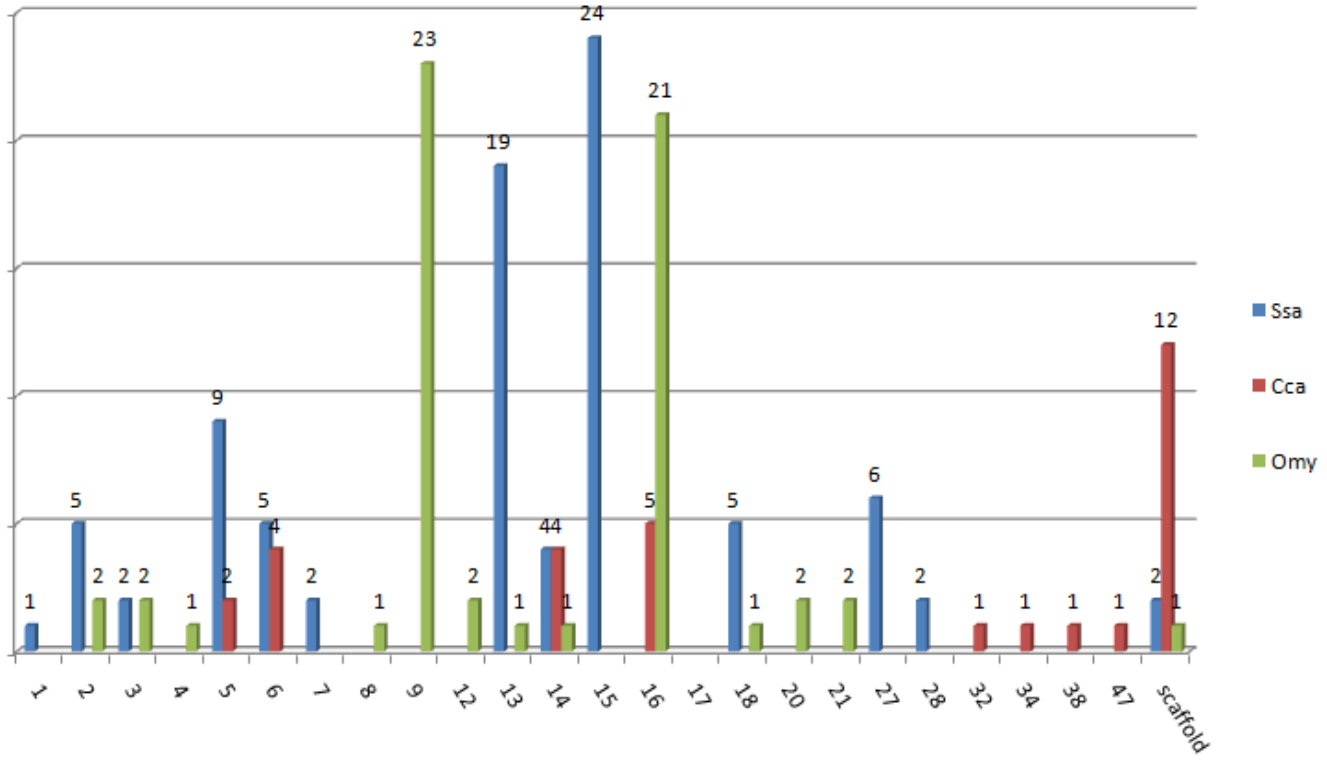

Figure 1. Chromosomal distribution of *SsaCSP*, *CcaCSP* and *OmyCSP* genes in Atlantic salmon, carp and rainbow genomes, respectively

The protein lengths determined for the SsaCSP family members were between 108-939 aa. The average molecular weight of this family members was 75 kDa. Thirty-eight SsaCSPs were acidic and 48 SsaCSPs were found to be basic. Although the number of proteins with basic character was greater than the number of proteins with acidic character, the average of pI values was 7.90 (**Table S1 in appendix**).

CcaCSPs had 101-315 aa in length and the average length of these proteins were 228 aa. The average molecular weight of this protein family in common carp was 25.5 kDa (Since a large amount of scaffold was detected in the *CcaCSP* gene family, it was not taken into account in the protein length and molecular weight average). Two of CcaCSP proteins were found to be acidic and 29 of CcaCSPs were basic. The number of proteins with basic character was more than the number of proteins with acidic character among CcaCSPs and the average of pI values was 8.97 (**Table S2 in appendix**).

The protein length determined for the OmyCSP family was between 130-1862 aa and the average length was 667 aa. The average molecular weight of this protein family was 73.9 kDa. Thirty-four of the OmyCSPs were acidic and 26 OmyCSPs were basic. However, the average of pI values was 7.48. This was due to the strong basic character of basic proteins among OmyCSPs (**Table S3 in appendix**). It can be concluded that CSPs from Atlantic salmon, carp and rainbow trout were mainly basic proteins.

Phylogenetic tree analysis was performed by using MEGA7 (Molecular Evolutionary Genetics Analysis) program to determine the evolutionary relationships among SsaCSP, CcaCSP and OmyCSPs. In this analysis, the Neighbor Joining Method was used. According to the phylogenetic tree analysis, SsaCSPs, CcaCSPs and OmyCSPs were categorized in 9, 8 and 5 main classes, respectively (**Figure 2**). Based on the phylogenetic distribution of the SsaCSP family, the highest number of proteins was found in the 7th class. The most of the CcaCSPs were determined in the 8th class. Besides, the highest number of proteins were defined in the 5th class when phylogenetic distribution of the OmyCSP family was analyzed.

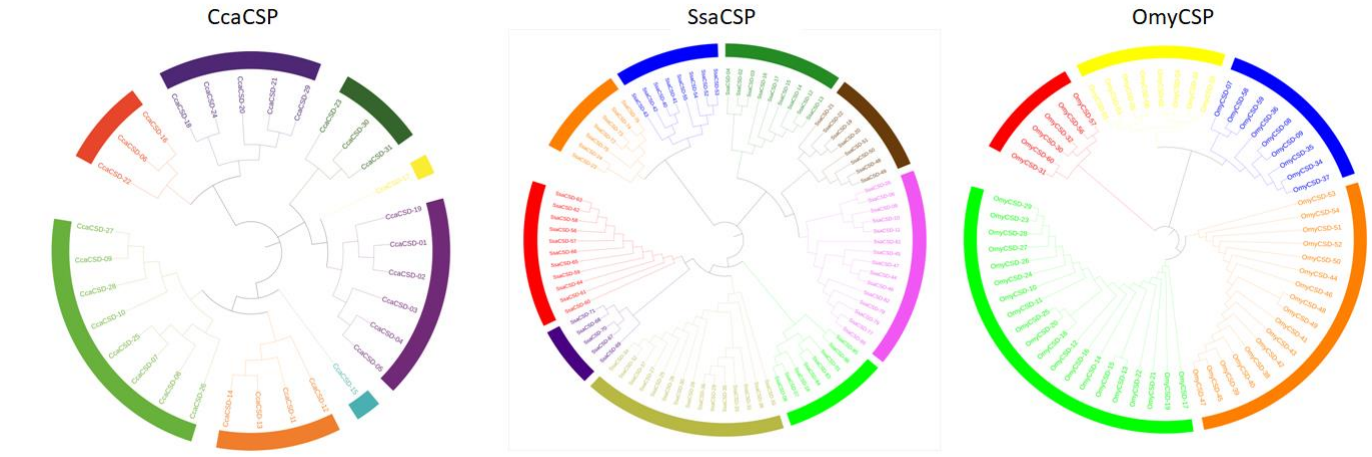

Figure 2. Phylogenetic distribution of Atlantic salmon, carp and rainbow trout CSPs drawn by the Mega7 program

In order to test the reliability of the phylogenetic tree, the motif compositions of the SsaCSP, CcaCSP and OmyCSPs were examined. Eighty-six for SsaCSP, 31 for CcaCSP and 60 for OmyCSP amino acid sequences of protein were loaded into the MEME database. A total of 20 different conserved motifs were identified for SsaCSP (**Table 1A**), CcaCSP (**Table 1B**), OmyCSP (**Table 1C**) proteins. Protected motifs, sequences and motif lengths of proteins were determined. According to those analysis, proteins containing similar motif patterns were found to be in the same cluster in the phylogenetic tree. In addition, motif patterns were similar in each of CSPs in s Atlantic salmon, carp and rainbow trout as species level, which can be attributed to the characteristic structure of the CSPs.

Table 1 Amino acid composition of the (A) *Salmo salar*, (B) *Common carp* and (C) *Oncorhynchus mykiss* CSP motifs

Motif no.	Sites	E-value	Amino acid sequence composition of motif	Width (aa)
(A)				
Motif 1	66	6.5e-1985	EVFYLTYGPDDIEKECHLPPQPKKSFYMZTNKHTGAVSAHN	41
Motif 2	81	4.9e-1196	NVRDGFGFIKCVDRDARMFFHFSD	24
Motif 3	63	6.9e-2193	FVSYSKLDMEGFRSLQEGEKVEFTFNESKRGLZQSAVTGPIGNRCVGTER	50
Motif 4	31	1.3e-1514	LLGYIATLKDNFGFIETANHDQEIFFHYSELCGDLENLELGDTVEYTLSK	50
Motif 5	31	1.2e-1498	NNGHTAFANGTAAGIRETGVVEKLLTSYGFIQCSERQARLFFHCSQYNGN	50
Motif 6	31	7.9e-1487	KCQNYSFGIVGMANKADCLQKGEMVKFQLCTVAQTGQKMACNVVPQRKAL	50
Motif 7	31	5.5e-1475	MRCQGVVCATKEAFGFIERADVVKEIFFHYSEFKGDLEALQAGDDVEFTI	50
Motif 8	31	1.3e-1206	LLEGDHVQFNISTDRRDKLERATNIDILPDTFHFTKESREM	41
Motif 9	31	1.2e-1446	RLLAQGTVIFEDISIEQFEGTVIKVIPKVPTKNQNDPLPGRICARISFTD	50
Motif 10	31	1.3e-1435	FSVILNQRTGKCSACNVRRVSEGPKPVATPRPDRLVNRLKSITLDDASAP	50
Motif 11	31	7.2e-1431	DMLSAQRNHAVRIKKLPKGTVSFHTQSEQRFVGVVEKEATAAITNNKSAS	50
Motif 12	31	1.5e-1341	NKVSAEKVTKVVAVNGVGQDVGETVMLGKVVRPLRSVDPSQTEYQGLIEL	50

Motif 13	31	2.1e-1336	LQELKIGDDVEFEVSSDRRTGKPIAVKLLKIKPEVLPEERISGQVGPDSH	50
Motif 14	24	1.1e-1075	MERVHSEPPLARNTASATSVVAIPRSFSVSHKKHKRTPLYQRSMSFDPGM	50
Motif 15	51	2.5e-842	LVIVRQPRGPDNSKGFNVERKTRQPGVID	29
Motif 16	31	5.1e-1127	VECVKDQFGFITYEVGESKKLFFHVKEVHDGLELQTGDEVE	41
Motif 17	24	5.4e-881	ASPFTVLHGYIHPVVSAIPTHLDGKSAPGQVPTGSVCYERN	41
Motif 18	29	1.9e-679	WKGFVEFTLPASPPAAFVSADLSSTSPVGLSLSPYGRSCDP	41
Motif 19	52	1.8e-529	KKKDKEAEEGVISYEDCGVKL	21
Motif 20	59	1.1e-357	EGQLHISDEVEFTVV	15
(B)				
Motif 1	26	5.8e-385	VIATKVLGTVKWFNVRNGYGFINRN	25
Motif 2	20	2.0e-478	RKYLRSVGDGETVEFDVVEGEKGAEAANVTGPGGVPVQGSK	41
Motif 3	12	5.4e-437	YPPYFVQRRYGRRPPYTNAPQRGEMTEGGEGDENQGGPDQGNKPMRQNYY	50
Motif 4	12	1.6e-417	GQNQEPRQRRYRRNFNYRRRRPQTTKPQDGKDSKAADASAEKSAAPEAEQ	50
Motif 5	12	1.1e-419	YAADRNRYRRFPRRRGPPRDYQENYQSDGEAREKREEEENVPEGEMQQQQ	50
Motif 6	17	1.0e-223	AAETQQPPQPAADAESPSSPAAAATAGDK	29
Motif 7	21	6.4e-169	KEDVFVHQTAIKKNN	15
Motif 8	12	1.6e-126	RGPPRPRPVREGEEDKENQDE	21
Motif 9	10	2.0e-126	VPVEGDEVTYKVCSIPPKHKKIQAVEVVITHLAPGTKHETW	41
Motif 10	9	1.5e-053	GLLPSPLPTKRTRTYSATVRA	21
Motif 11	11	2.9e-036	SAEPEESTSPDLSPLSPESASQPSSFPFP	29
Motif 12	12	1.4e-032	PTYPGRRR	8
Motif 13	7	6.1e-027	VQKRRKKGDRCYNCGGLDHH	20
Motif 14	5	3.2e-026	SREGVPLDPPVDVFVHQSKLH	21
Motif 15	3	6.7e-022	KCVDRDARMFFHFSEVLEESQLHISDEVEFTVVPDMLSAQRNHAVRIKKL	50
Motif 16	2	7.3e-022	NKGDCLQKGEMVKFQLCTVAQTGQKMACNIVPQRRALVECVKDQFGFITY	50
Motif 17	2	2.1e-020	NFGFIETANHDQEIFFHYSEVCGDVDNMDLGDTVEYTLSKGKGNKISAEK	50
Motif 18	2	6.8e-020	MGIRETGVVEKLLASYGFIQCSERQARLFFHCSQYNGNLQELKIGDDVEF	50
Motif 19	2	3.4e-019	TLDTGDKVNFYMETNKHTGAVSAHNIVLVKKKQSRCQGVVCATKEAFGFI	50
Motif 20	3	1.3e-017	KVPTKNQNDPLPGRISARINFTDKELLFGEKDTKSKVTLLEGDHVQFNI	49
(C)				
Motif 1	37	1.0e-1899	YLTYTPDDIEGNMHLDTGDKVSFYMETNKHTGAVSAHNIVLVKKKQMRCQ	50
Motif 2	37	2.9e-1817	DGTKCQNYSFGIVGMANKADCLQKGEMVKFQLCTVAQTGQKMACNVVPQR	50
Motif 3	37	5.6e-1859	TIKILNRTVNTKRLLGYIATLKDNFGFIETANHDQEIFFHYSELCGDLEN	50
Motif 4	52	3.3e-1577	AMRDGFGFIKCVDRDARMFFHFSEVLEEGQLHISDEVEFTVV	42
Motif 5	37	2.1e-1837	NNGHTAFANGTAAGIRETGVVEKLLTSYGFIQCSERQARLFFHCSQYNGN	50
Motif 6	37	7.6e-1781	NQNDPLPGRICARISFTDKELLFGEKDTKSKVTLLEGDHVQFNISTDRRD	50
Motif 7	37	2.6e-1769	MDMLSAQRNHAVRIKKLPKGTVSFHTQSEQRFVGVVEKEATAAITNNKSA	50
Motif 8	37	1.1e-1760	FGFITYEVGESKKLFFHVKEVHDGLELQTGDEVEFSVILNQRTGKCSACN	50
Motif 9	37	3.5e-1754	CATKEAFGFIERADVVKEIFFHYSEFKGDLEALQAGDDVEFTIKERNGKE	50
Motif 10	37	8.3e-1733	AEEGVISYEDCGVKLTVSYHVKDLEGATQPQAGDKVEFSINEVKRTGQQS	50
Motif 11	37	1.1e-1721	ATPRPDRLVNRLKSITLDDASAPRLVIVRQPRGPDNSKGFNVERKTRQPG	50
Motif 12	37	3.1e-1636	LQELKIGDDVEFEVSSDRRTGKPIAVKLLKIKPEVLPEERISGQVGPDSH	50
Motif 13	37	1.6e-1643	KGNKVSAEKVTKVVAVNGVGQDVGETVMLGKVVRPLRSVDPSQTEYQGLI	50
Motif 14	29	2.0e-1285	MGSPWKGFVEFTLPTSPPAAFISADLSSTSPIGLSLSPYGRSCFPVPTPL	50
Motif 15	27	6.8e-1225	MERVHSEPPLARNTAPSTSAVAIPRSFSVSHKKHKRTPLYQRSMSFDPGM	50
Motif 16	26	4.3e-966	SPFTVLHGYIHPVVSAIPTHLDGKSAPGQVPTGSVCYERNG	41
Motif 17	37	1.4e-931	DVRLLAQGTVIFEDISIEQFEGTVVKVIP	29
Motif 18	37	6.4e-710	RATNIDILPDTFHFTKESREM	21
Motif 19	45	1.5e-292	LELGTVEYTL	11
Motif 20	41	1.2e-257	VRRVSEGPKPV	11

Micro RNAs (miRNA) are products of a family of small non-coding RNAs. miRNAs break down the mRNA of the target gene to inhibit the expression of them [25]. miRBase v20.0 (http://www.mirbase.org/) was used and previously known animal miRNA precursors were downloaded. miRNAs targeting SsaCSP, CcaCSP and OmyCSP transcripts were evaluated in the miRNA database and then animal miRNAs were chosen. Two important parameters were used to identify these genes. The maximum expectation threshold was set to 3.0. The second parameter was the UPE maximum energy value. This value represents the energy required to open the secondary structure of the target region of the mRNA. According to the results, SsaCSP transcripts were targeted by 116 different miRNAs. Thirty-five different SsaCSP transcripts were targeted by these miRNAs. CcaCSP-31 was the most targeted transcript among CcaCSPs by the 13 miRNAs. miRNA targets of 14 different CcaCSP transcripts were identified. Besides, 25 of OmyCSP transcripts were targeted by 90 different miRNAs. Identification of miRNAs targeting CSP transcripts may be informative for functional genomics studies (**Figure 3**).

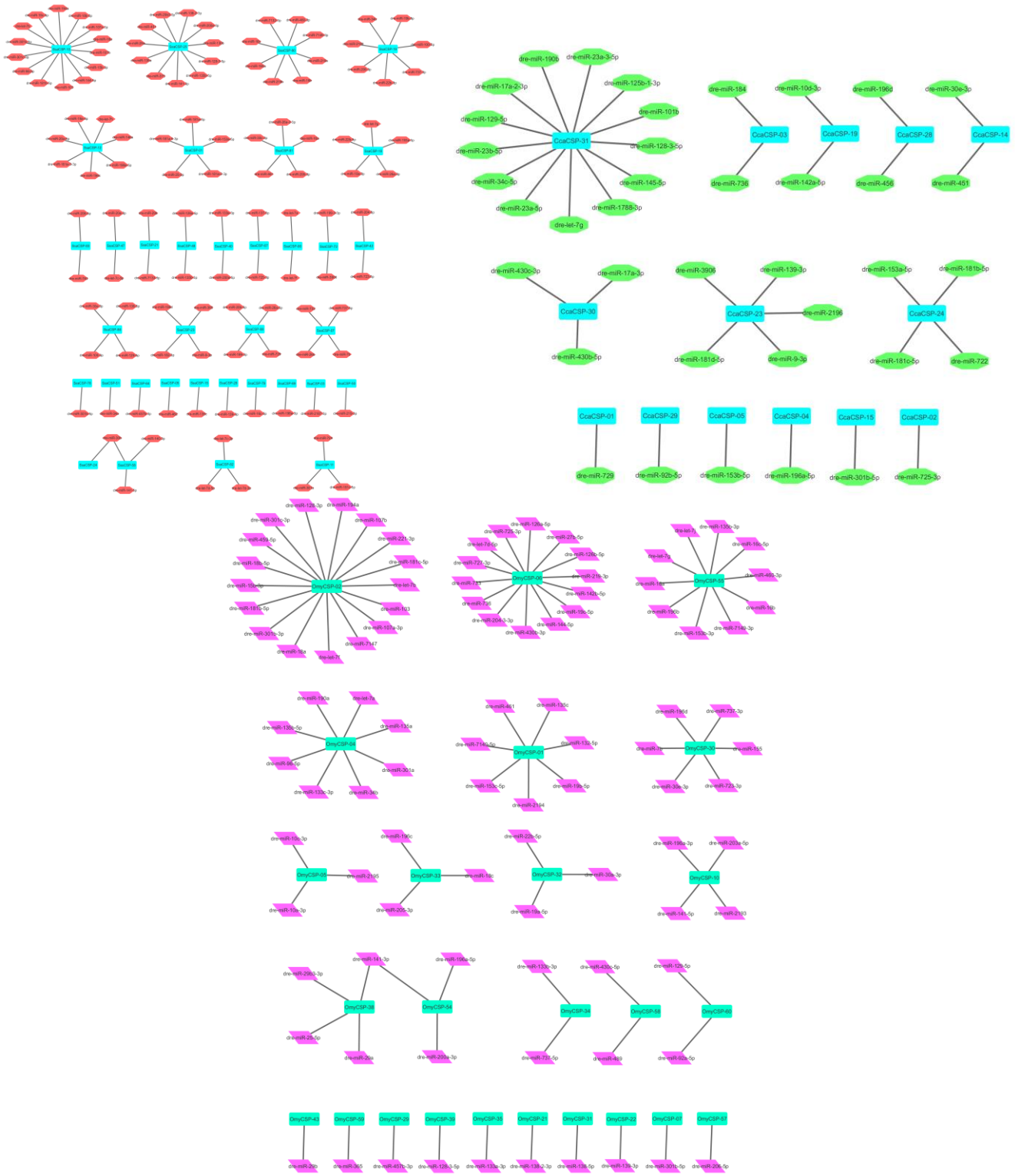

Figure 3. miRNA targets of SsaCSP, CcaCSP and OmyCSP transcripts

Using the Phyre2 database, BLASTP screening was performed for the CSPs found in the Protein Data Bank (PDB) and three-dimensional homology modeling of the SsaCSP, CcaCSP and OmyCSPs was performed. This database was used to estimate the structure, function, and analysis of mutations. There were 86 proteins for SsaCSP, 31 proteins for CcaCSP and 60 proteins for OmyCSP. A three-dimensional structure of 86 SsaCSP proteins was found in the similarity ratio of approximately 2 proteins with 90% reliability (**Figure 4**). A three-dimensional structure was determined from a total of 31 CcaCSPs in the similarity ratio of approximately 3 proteins with 90% reliability (**Fig. 4).** A three-dimensional structure was determined in the similarity ratio of 60 OmyCSP proteins with approximately 90% confidence in 1 protein (**Figure 4**). These protein models, which were estimated to have three-dimensional structures, might be useful in advanced studies for understanding of the function of CSPs in Atlantic salmon, carp and rainbow trout species. According to the 3-dimensional structure, OB (oligonucleotide binding) folding sites were found in CcaCSP protein. α-helix structures were generally

observed to be dominant in SsaCSPs. Rarely, β-layered structure was observed. There were also determined α-helix structures in OmyCSPs.

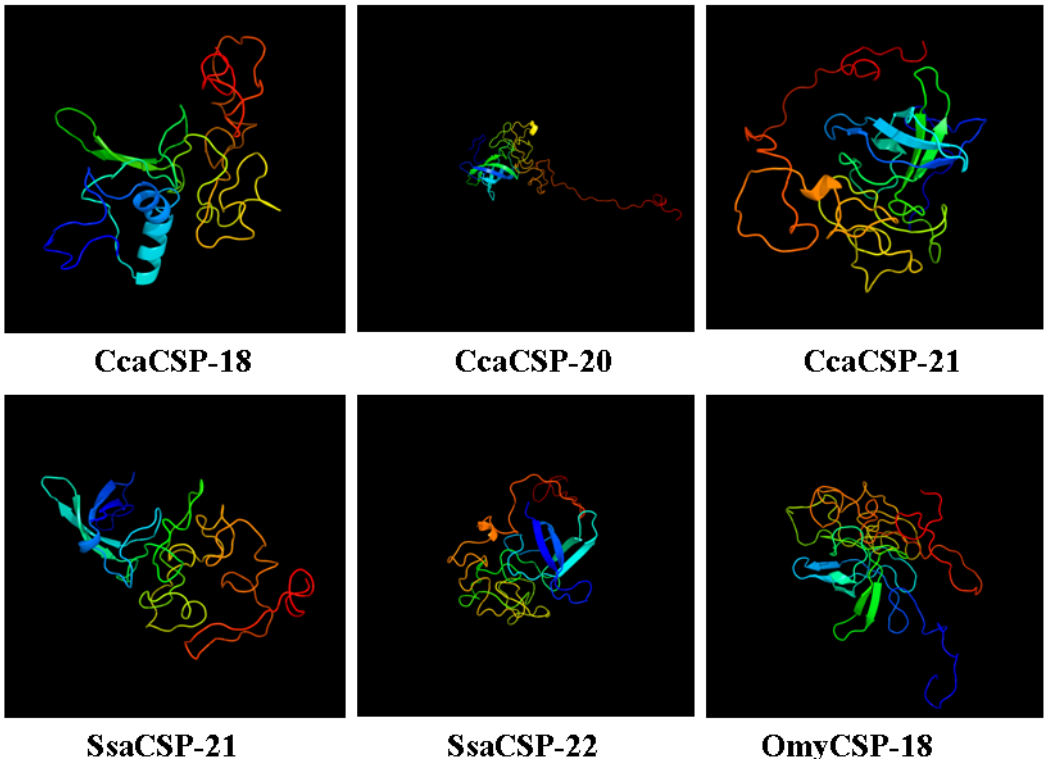

Figure 4. Predicted three-dimensional structure of SsaCSPs, CcaCSPs, OmyCSPs

Blast2Go program was used to understand the cellular localization, biological roles and molecular functions of SsaCSP, CcaCSP, OmyCSPs. In the light of the data obtained, the binding activity for SsaCSP, CcaCSP, OmyCSPs was determined as the molecular function. Binding to ATP, metal ions and cations were determined as the most binding activity for the SsaCSP, CcaCSP, OmyCSPs. SsaCSPs had function in biological regulation, metabolic and cellular processes. CcaCSPs were mainly located in the cell part and organelles and played important roles in metabolic and cellular processes and biological regulation. OmyCSPs had roles in metabolic and cellular processes and regulation of biological processes. (**Figure 5**).

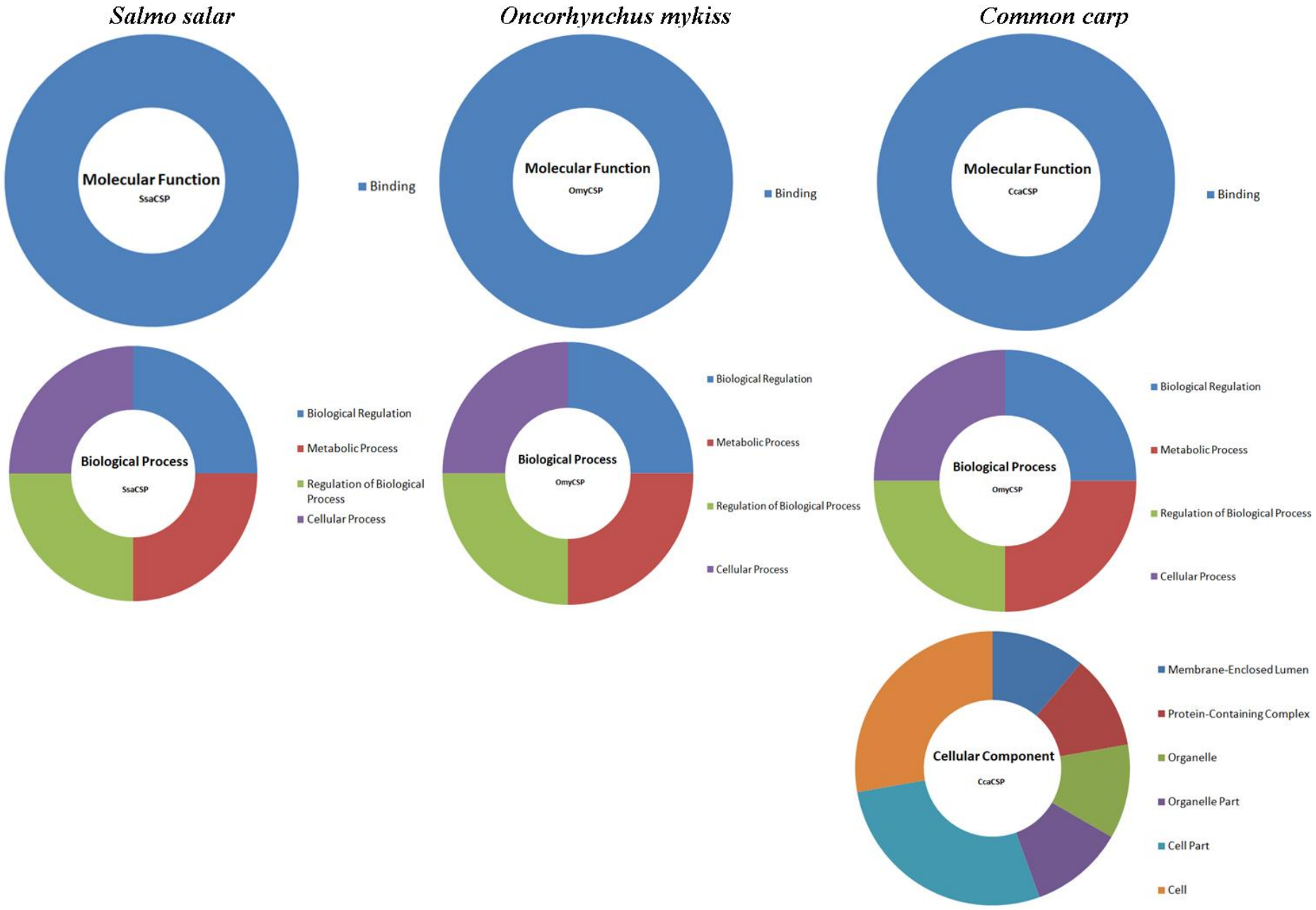

Figure 5. Functional analysis of SsaCSP, CcaCSP and OmyCSPs

Gene structure display server (http://gsds.cbi.pku.edu.cn/) was utilized to determine exon and intron structures of *SsaCSP*, *CcaCSP*, *OmyCSP* genes (**Figures 6**). All of the *SsaCSP*, *CcaCSP* and *OmyCSP* genes had introns. When the exon-intron structures of *SsaCSP*, *CcaCSP*, *OmyCSP* genes were compared in the phylogenetic trees, genes had similar exon-intron regions were found to be in the same phylogenic clusters in the trees.

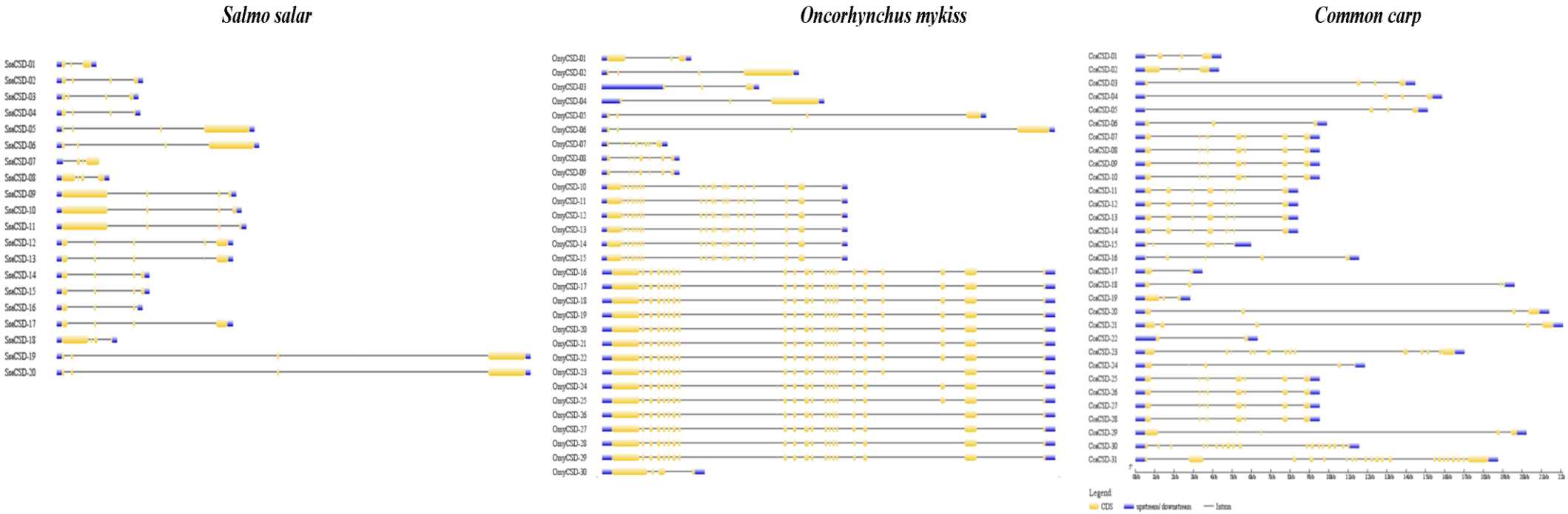

Figure 6. Exon-intron analysis of *SsaCSP*, *CcaCSP*, *OmyCSP* genes

As a result, there are not many studies on CSPs in fish. This protein region has been found in the zebrafish (*Danio rerio*), which is the model organism among fishes, but there is no similar study in the literature. The CSP family was found in a single-cell organism such as *Bacillus*, *Escherichia coli*. This includes CSPA, CSPC and CSPD family members in unicellular cells and is classified as CSPB, CSPC, and CSPD according to their function rather than CSP [11]. However, there is no such distinction in fish. It was found that these fish species were found in the *L. migrateria, ruets* and EST libraries. In the *L. migrateria* library, there were 43.481 motifs belonging to CSP. *Ruets* had 75 motifs. CSP was found in EST gene library with the number of 45.481. This shows the limited working area of CSPs in the fish. Current study represents the determination and characterization of CSP family members in these valuable fish species. This kind of gene identification studies open new perspectives to analyze functions of CSP family members in fishes. In addition, study results represent preliminary knowledge about understanding of the effects of these proteins in cold tolerance in these economically important fish species.

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Appendix

Table S1. Supplementary table for SsaCSPs

ID	NCBI Accession No.	Physical position on *Salmo Salar* genome			Protein length (aa)	Molecular weight (Da)	pI	Instability index	Stable or unstable
		Chromosome	Start position (bp)	End Position (bp)					
SsaCSP-01	XP_014063589.1	1	74,309,848	74,312,833	158	17480.85	8.82	53.35	unstable
SsaCSP-02	XP_014013043.1	2	14,826,588	14,834,258	262	28502.73	9.35	72.92	unstable
SsaCSP-03	XP_014013050.1	2	14,826,588	14,834,258	236	25961.05	9.62	74.30	unstable
SsaCSP-04	XP_014013059.1	2	14,826,588	14,834,258	145	15852.85	6.20	52.76	unstable
SsaCSP-05	XP_014022707.1	2	30,471,155	30,490,497	202	22117.74	6.86	59.61	unstable
SsaCSP-06	XP_014022716.1	2	30,471,155	30,490,497	195	21371.95	6.86	60.44	unstable
SsaCSP-07	NP_001133414.1	3	65.477.156	65.481.446	159	17459,82	9,26	55,65	unstable
SsaCSP-08	XP_014048617.1	3	65,477,156	65,481,446	159	17459.82	9.26	55.65	unstable
SsaCSP-09	XP_014055906.1	5	50,224,632	50,242,696	202	22170.89	8.35	64.51	unstable
SsaCSP-10	XP_014055908.1	5	50,224,632	50,242,696	195	21425.10	8.35	65.51	unstable
SsaCSP-11	XP_014055909.1	5	50,224,632	50,242,696	195	21425.10	8.35	65.51	unstable
SsaCSP-12	XP_014057001.1	5	65,451,183	65,467,908	285	31180.01	9.95	72.59	unstable
SsaCSP-13	XP_014057003.1	5	65,451,183	65,467,908	257	28094.38	9.92	75.81	unstable
SsaCSP-14	XP_014057004.1	5	65,451,183	65,467,908	252	27532.78	9.75	77.35	unstable
SsaCSP-15	XP_014057005.1	5	65,451,183	65,467,908	251	27475.73	9.75	77.13	unstable
SsaCSP-16	XP_014057007.1	5	65,451,183	65,467,908	226	24975.01	9.96	81.05	unstable

SsaCSP-17	XP_014057008.1	5	65,451,183	65,467,908	226	24975.01	9.96	81.05	unstable
SsaCSP-18	XP_014059274.1	6	33,682,575	33,687,644	159	17530.85	9.00	57.39	unstable
SsaCSP-19	XP_014060071.1	6	45,455,711	45,502,322	271	29388.34	8.64	66.63	unstable
SsaCSP-20	XP_014060072.1	6	45,455,711	45,502,322	267	29003.91	8.64	64.82	unstable
SsaCSP-21	XP_014060073.1	6	45,455,711	45,502,322	190	20665.36	8.35	79.73	unstable
SsaCSP-22	XP_014060074.1	6	45,455,711	45,502,322	190	20665.36	8.35	79.73	unstable
SsaCSP-23	XP_014062241.1	7	12,010,561	12,016,017	335	36643.33	10.19	80.82	unstable
SsaCSP-24	XP_014062242.1	7	12,010,561	12,016,017	331	36261.86	10.16	82.42	unstable
SsaCSP-25	XP_013990799.1	13	26.009.382	26.044.770	939	104041,31	6,95	36,08	stable
SsaCSP-26	XP_013990800.1	13	26.009.382	26.044.770	935	103541,7	6,71	36,28	stable
SsaCSP-27	XP_013990801.1	13	26.009.382	26.044.770	932	103234,39	6,95	36,05	stable
SsaCSP-28	XP_013990802.1	13	26.009.382	26.044.770	931	103226,31	6,71	35,64	stable
SsaCSP-29	XP_013990803.1	13	26.009.382	26.044.770	931	103041,08	a	35,01	stable
SsaCSP-30	XP_013990804.1	13	26.009.382	26.044.770	927	102726,7	6,52	35,84	stable
SsaCSP-31	XP_013990805.1	13	26.009.382	26.044.770	923	102226,08	6,45	34,55	stable
SsaCSP-32	XP_013990807.1	13	26.009.382	26.044.770	920	102028,06	7,08	35,79	stable
SsaCSP-33	XP_013990808.1	13	26.009.382	26.044.770	919	101726,47	6,31	34,75	stable
SsaCSP-34	XP_013990809.1	13	26.009.382	26.044.770	913	101221,15	7,08	35,75	stable
SsaCSP-35	XP_013990810.1	13	26.009.382	26.044.770	912	100919,55	6,31	34,71	stable

SsaCSP-36	XP_013990811.1	13	26.009.382	26.044.770	893	98906,31	6,31	34,38	stable
SsaCSP-37	XP_013990812.1	13	26.009.382	26.044.770	887	98718,27	7,33	33,44	stable
SsaCSP-38	XP_013990813.1	13	26.009.382	26.044.770	886	98159,58	6,46	34,37	stable
SsaCSP-39	XP_013990814.1	13	26.009.382	26.044.770	844	93877,73	6,46	32,04	stable
SsaCSP-40	XP_013991015.1	13	31.436.802	31.445.267	326	36365,72	9,71	60,63	stable.
SsaCSP-41	XP_013991016.1	13	31.436.802	31.445.267	322	35809,05	9,58	60,9	stable
SsaCSP-42	XP_013991017.1	13	31.436.802	31.445.267	305	33820,84	9,47	58,12	stable
SsaCSP-43	NP_001133216.1	13	31.436.802	31.445.267	301	33264,17	9,29	58,37	unstable
SsaCSP-44	XP_013997885.1	14	68.017.031	68.027.537	204	22532,07	6,66	65,79	stable
SsaCSP-45	XP_013997886.1	14	68.017.031	68.027.537	202	22084,52	6,18	64,7	stable
SsaCSP-46	XP_013997887.1	14	68.017.031	68.027.537	195	21284,65	6,18	64,47	stable
SsaCSP-47	XP_013997888.1	14	68.017.031	68.027.537	195	21298,72	6,39	63,69	stable
SsaCSP-48	XP_013999364.1	15	22.395.252	22.438.885	237	25670,05	8,88	68,03	stable
SsaCSP-49	XP_013999365.1	15	22.395.252	22.438.885	233	25285,62	8,88	65,98	stable
SsaCSP-50	XP_013999366.1	15	22.395.252	22.438.885	231	25069,28	8,75	68,36	stable
SsaCSP-51	XP_013999367.1	15	22.395.252	22.438.885	190	20669,44	8,97	73,19	stable
SsaCSP-52	XP_014001654.1	15	66,117,700	66,124,504	379	42113.05	9.57	70.43	unstable
SsaCSP-53	XP_014001655.1	15	66,117,700	66,124,504	378	42025.97	9.57	69.37	unstable
SsaCSP-54	XP_014001656.1	15	66,117,700	66,124,504	358	39568.17	9.34	68.87	unstable

SsaCSP-55	XP_014001657.1	15	66,117,700	66,124,504	357	39481.09	9.34	67.74	unstable
SsaCSP-56	XP_014001115.1	15	75.504.333	75.523.784	932	103181,39	6,52	34,55	stable
SsaCSP-57	XP_014001116.1	15	75.504.333	75.523.784	876	97304,57	6,45	32,3	stable
SsaCSP-58	XP_014001117.1	15	75.504.333	75.523.784	925	102374,48	6,52	34,5	stable
SsaCSP-59	XP_014001118.1	15	75.504.333	75.523.784	924	102283,35	6,52	33,89	stable
SsaCSP-60	XP_014001119.1	15	75.504.333	75.523.784	868	96406,53	6,44	31,58	stable
SsaCSP-61	XP_014001120.1	15	75.504.333	75.523.784	861	95599,61	6,44	31,51	stable
SsaCSP-62	XP_014001121.1	15	75.504.333	75.523.784	913	101168,15	6,55	34,23	stable
SsaCSP-63	XP_014001122.1	15	75.504.333	75.523.784	906	100361,24	6,55	34,17	stable
SsaCSP-64	XP_014001123.1	15	75.504.333	75.523.784	842	93586,37	6,46	31,09	stable
SsaCSP-65	XP_014001124.1	15	75.504.333	75.523.784	880	97804,18	6,61	32,11	stable
SsaCSP-66	XP_014001126.1	15	75.504.333	75.523.784	880	97804,18	6,61	32,11	stable
SsaCSP-67	NP_001167093.1	15	75.504.333	75.523.784	854	94950,98	6,13	30,69	stable
SsaCSP-68	XP_014001127.1	15	75,504,333	75,522,784	879	97291.55	6.12	32.38	stable
SsaCSP-69	XP_014001128.1	15	75,504,333	75,522,784	837	92965.65	6.12	30.16	stable
SsaCSP-70	XP_014001129.1	15	75,504,333	75,522,784	837	92965.65	6.12	30.16	stable
SsaCSP-71	XP_014001130.1	15	75,504,333	75,522,784	818	90952.41	6.10	29.70	stable
SsaCSP-72	NP_001133543.1	18	47.970.066	47.975.989	328	35950,69	9,94	81,7	unstable
SsaCSP-73	XP_014010961.1	18	47,970,066	47,974,989	339	37095.02	9.98	79.22	unstable

SsaCSP-74	XP_014010962.1	18	47,970,066	47,974,989	335	36713.55	9.94	80.78	unstable
SsaCSP-75	XP_014010963.1	18	47,970,066	47,974,989	332	36332.16	9.98	80.10	unstable
SsaCSP-76	XP_014010964.1	18	47,970,066	47,974,989	328	35950.69	9.94	81.70	unstable
SsaCSP-77	XP_014033333.1	27	19,676,671	19,691,042	204	22228.81	7.57	51.49	unstable
SsaCSP-78	XP_014033334.1	27	19,676,671	19,691,042	202	21919.48	6.65	53.78	unstable
SsaCSP-79	XP_014033335.1	27	19,676,671	19,691,042	195	21065.55	6.65	53.78	unstable
SsaCSP-80	XP_014033336.1	27	19,676,671	19,691,042	195	21079.62	7.02	52.99	unstable
SsaCSP-81	XP_014033337.1	27	19,676,671	19,691,042	175	19070.75	8.24	59.07	unstable
SsaCSP-82	XP_014033338.1	27	19,676,671	19,691,042	159	17347.53	8.12	53.21	unstable
SsaCSP-83	XP_014035109.1	28	13,878,289	13,888,222	158	17493.88	8.93	51.57	unstable
SsaCSP-84	XP_014035110.1	28	13,878,289	13,888,222	158	17493.88	8.93	51.57	unstable
SsaCSP-85	XP_014042228.1	scaffold	2,089	4,413	108	11519.15	6.05	25.02	stable
SsaCSP-86	XP_014039437.1	scaffold	13,179	15,319	167	17742.03	8.57	65.89	unstable

Table S2. Supplementary table for CcaCSPs

ID	NCBI Accession No.	Physical position on *Cyprinus carpio* genome			Protein length (aa)	Molecular weight (Da)	pI	Instability index	Stable or unstable
		Chromosome	Start position (bp)	End Position (bp)					
CcaCSP-01	XP_018925526.1	5	18,459,016	18,462,463	154	16856.21	9.30	60.69	unstable
CcaCSP-02	XP_018925532.1	5	18,459,016	18,462,463	154	16856.21	9.30	60.69	unstable
CcaCSP-03	XP_018931080.1	6	7,067,314	7,082,199	158	16923.21	8.91	71.16	unstable
CcaCSP-04	XP_018931086.1	6	7,067,314	7,082,199	158	16923.21	8.91	71.16	unstable
CcaCSP-05	XP_018931094.1	6	7,067,314	7,082,199	158	16923.21	8.91	71.16	unstable
CcaCSP-06	XP_018927839.1	6	11,490,177	11,499,086	201	22000.64	8.36	62.56	unstable
CcaCSP-07	XP_018963132.1	14	1,145,141	1,153,681	313	35663.85	9.58	66.21	unstable
CcaCSP-08	XP_018963133.1	14	1,145,141	1,153,681	312	35576.77	9.58	64.36	unstable
CcaCSP-09	XP_018963134.1	14	1,145,141	1,153,681	309	35076.16	9.44	65.76	unstable
CcaCSP-10	XP_018963135.1	14	1,145,141	1,153,681	308	34989.09	9.44	64.44	unstable
CcaCSP-11	XP_018964282.1	16	2,464,171	2,471,589	315	35914.01	9.56	67.45	unstable
CcaCSP-12	XP_018964283.1	16	2,464,171	2,471,589	314	35826.93	9.56	67.02	unstable
CcaCSP-13	XP_018964284.1	16	2,464,171	2,471,589	311	35383.38	9.42	69.05	unstable
CcaCSP-14	XP_018964285.1	16	2,464,171	2,471,589	310	35296.30	9.42	67.76	unstable
CcaCSP-15	XP_018964078.1	16	6,798,175	6,803,164	214	23815.09	9.78	68.96	unstable

Table S3. Supplementary table for OmyCSPs

ID	NCBI Accession No.	Physical position on *Oncorhynchus mykiss* genome			Protein length (aa)	Molecular weight (Da)	pI	Instability index	Stable or unstable
		Chromosome	Start *position* (bp)	End Position (bp)					
OmyCSP-01	XP_021481619.1	2	9,987,049	9,994,437	130	14105.98	9.44	61.44	unstable
OmyCSP-02	XP_021417535.1	2	23,615,665	23633142	202	22166.81	7.15	62.48	unstable
OmyCSP-03	XP_021437991.1	3	22,181,984	22195726	251	27291.40	9.66	82.57	unstable
OmyCSP-04	XP_021438459.1	3	33,644,075	33663929	195	21393.01	7.15	59.06	unstable
OmyCSP-05	XP_021454089.1	4	49,774,027	49809035	227	24494.67	8.53	70.08	unstable
OmyCSP-06	XP_021467181.1	8	8,446,940	8488379	227	24619.77	8.59	66.55	unstable
OmyCSP-07	XP_021470938.1	9	18,761,008	18766173	331	36214.33	10.10	83.29	unstable
OmyCSP-08	XP_021471492.1	9	36,175,698	36181996	379	42027.88	9.56	69.78	unstable
OmyCSP-09	XP_021471493.1	9	36,175,698	36181996	358	39483.00	9.31	68.18	unstable
OmyCSP-10	XP_021471853.1	9	45,229,012	45251043	940	104261.69	6.82	35.49	stable
OmyCSP-11	XP_021471854.1	9	45,229,012	45251043	936	103762.07	6.61	35.69	stable
OmyCSP-12	XP_021471856.1	9	45,229,012	45251043	933	103454.77	6.82	35.45	stable
OmyCSP-13	XP_021471857.1	9	45,229,012	45251043	932	103243.42	6.52	34.70	stable
OmyCSP-14	XP_021471858.1	9	45,229,012	45251043	932	103363.64	6.82	34.84	stable
OmyCSP-15	XP_021471859.1	9	45,229,012	45251043	928	102743.81	6.38	34.90	stable
OmyCSP-16	XP_021471860.1	9	45,229,012	45251043	928	102864.03	6.61	35.04	stable
OmyCSP-17	XP_021471861.1	9	45,229,012	45251043	924	102345.38	6.52	34.04	stable
OmyCSP-18	XP_021471862.1	9	45,229,012	45,251,043	921	102248.44	6.91	35.19	stable
OmyCSP-19	XP_021471863.1	9	45,229,012	45,251,043	920	101845.77	6.37	34.24	stable

OmyCSP-20	XP_021471864.1	9	45,229,012	45,251,043	914	101441.53	6.91	35.14	stable
OmyCSP-21	XP_021471865.1	9	45,229,012	45,251,043	913	101038.85	6.37	34.19	stable
OmyCSP-22	XP_021471866.1	9	45,229,012	45,251,043	894	99025.61	6.38	33.85	stable
OmyCSP-23	XP_021471867.1	9	45,229,012	45,251,043	888	98838.45	6.95	32.94	stable
OmyCSP-24	XP_021471868.1	9	45,229,012	45,251,043	887	98339.84	6.31	33.09	stable
OmyCSP-25	XP_021471869.1	9	45,229,012	45,251,043	868	96326.60	6.31	32.71	stable
OmyCSP-26	XP_021471870.1	9	45,229,012	45,251,043	845	93999.91	6.31	31.06	stable
OmyCSP-27	XP_021471871.1	9	45,229,012	45,251,043	838	93193.00	6.31	30.98	stable
OmyCSP-28	XP_021471872.1	9	45,229,012	45,251,043	826	91986.67	6.31	30.62	stable
OmyCSP-29	XP_021471873.1	9	45,229,012	45,251,043	819	91179.76	6.31	30.53	stable
OmyCSP-30	XP_021480819.1	12	72,079,696	72,083,911	159	17383.72	9.00	57.72	unstable
OmyCSP-31	XP_021481521.1	12	88,577,019	88,644,765	170	17851.11	8.24	57.48	unstable
OmyCSP-32	XP_021413690.1	13	39,520,408	39524430	159	17420.73	9.00	55.70	unstable
OmyCSP-33	XP_021417799.1	14	20,554,543	20563643	195	21253.67	6.00	66.08	unstable
OmyCSP-34	NP_001158512.1	16	34,115,146	34124611	301	33120.04	9.47	57.89	unstable
OmyCSP-35	XP_021420068.1	16	34,115,146	34124611	326	36221.59	9.82	60.19	unstable
OmyCSP-36	XP_021420069.1	16	34,115,146	34124611	322	35664.92	9.71	60.45	unstable
OmyCSP-37	XP_021420070.1	16	34,115,146	34124611	305	33676.71	9.62	57.65	unstable
OmyCSP-38	XP_021422074.1	16	49,176,543	49214631	939	104132.52	7.12	38.22	stable
OmyCSP-39	XP_021422075.1	16	49,176,543	49214631	935	103632.91	6.82	38.43	stable
OmyCSP-40	XP_021422076.1	16	49,176,543	49214631	932	103325.00	7.12	38.20	stable
OmyCSP-41	XP_021422078.1	16	49,176,543	49214631	931	103317.52	6.82	37.79	stable
OmyCSP-42	XP_021422079.1	16	49,176,543	49214631	1862	206431.79	6.78	37.49	stable

OmyCSP-43	XP_021422080.1	16	49,176,543	49214631	927	102817.91	6.61	38.01	stable
OmyCSP-44	XP_021422081.1	16	49,176,543	49214631	923	102317.29	6.52	36.73	stable
OmyCSP-45	XP_021422082.1	16	49,176,543	49214631	920	102119.27	7.29	37.97	stable
OmyCSP-46	XP_021422083.1	16	49,176,543	49214631	919	101817.68	6.38	36.94	stable
OmyCSP-47	XP_021422084.1	16	49,176,543	49214631	913	101312.36	7.29	37.95	stable
OmyCSP-48	XP_021422085.1	16	49,176,543	49214631	912	101010.76	6.38	36.91	stable
OmyCSP-49	XP_021422086.1	16	49,176,543	49214631	893	98997.52	6.39	36.63	stable
OmyCSP-50	XP_021422087.1	16	49,176,543	49214631	887	98837.54	7.58	35.71	stable
OmyCSP-51	XP_021422089.1	16	49,176,543	49214631	886	98237.78	6.46	36.08	stable
OmyCSP-52	XP_021422090.1	16	49,176,543	49214631	867	96224.54	6.49	35.77	stable
OmyCSP-53	XP_021422091.1	16	49,176,543	49214631	844	93955.93	6.46	33.84	stable
OmyCSP-54	XP_021422092.1	16	49,176,543	49214631	825	91942.69	6.49	33.47	stable
OmyCSP-55	XP_021428532.1	18	41,939,025	41955903	195	21093.57	6.39	57.79	unstable
OmyCSP-56	XP_021431664.1	20	16,564,828	16572932	158	17493.88	8.93	51.57	unstable
OmyCSP-57	XP_021431665.1	20	16,564,828	16572932	316	34969.74	9.06	51.60	unstable
OmyCSP-58	XP_021433847.1	21	42,643,840	42648716	333	36429.95	10.10	79.46	unstable
OmyCSP-59	XP_021433848.1	21	42,643,840	42648716	329	36048.48	10.07	81.05	unstable
OmyCSP-60	XP_021448749.1	scaffold	0,502	832	170	17851.11	8.24	57.48	unstable