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Genome-wide identification and characterization of the antifreeze protein gene family in rainbow trout (*Oncorhynchus mykiss*): An *in silico* perspective

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Abstract

Antifreeze proteins (AFPs) represent a remarkable adaptation enabling organisms to survive in sub-zero environments by inhibiting ice crystal formation and growth. Despite their critical role in cold tolerance and significant implications for aquaculture productivity in temperate regions, comprehensive genome-wide analyses of AFP gene families in economically important salmonids remain limited. This study presents the first systematic genome-wide identification and comprehensive characterization of the antifreeze protein gene family in rainbow trout (*Oncorhynchus mykiss*), one of the most economically valuable cold-water aquaculture species globally. Our analysis identified one complete antifreeze protein gene (antifreeze protein Maxi-like BETA, Gene ID: 118943589) in the rainbow trout genome using the USDA_OmykA_1.1 assembly. The gene exhibits a compact genomic structure with a length of 624 bp, encoding a complete coding sequence (CDS) of 624 bp (207 amino acids) organized into 4 exons and localized on chromosome 22 (NC_048586.1: 40380233-40386211). Physicochemical analysis revealed a small protein of 18.5 kDa with a basic isoelectric point (pI 9.10), extinction coefficient of 7115, and one predicted N-glycosylation site. Structural predictions identified conserved functional domains characteristic of Type II antifreeze proteins, with motif analysis revealing highly preserved sequences essential for ice-binding activity. Subcellular localization predictions indicated extracellular secretion, consistent with the protein's role in preventing extracellular ice formation. Phylogenetic analysis demonstrated evolutionary conservation across teleost lineages, with the rainbow trout AFP clustering with other salmonid species, reflecting lineage-specific adaptations to cold environments. Beyond advancing fundamental understanding of molecular mechanisms underlying freeze tolerance in teleosts, this comprehensive genomic characterization provides essential resources for applied aquaculture applications, including identification of molecular markers for selective breeding programs targeting enhanced cold tolerance, development of cryoprotective strategies, and optimization of production in cold-water aquaculture systems. Our results establish a foundational genomic resource for future functional studies of AFP genes in salmonids, bridging evolutionary biology with practical aquaculture biotechnology.

Keywords: Antifreeze protein, rainbow trout, *Oncorhynchus mykiss*, genome-wide

1. Introduction

The ability to survive and thrive in freezing environments represents one of the most remarkable physiological adaptations in the animal kingdom, particularly among organisms inhabiting polar and sub-polar aquatic ecosystems [13, 24]. Antifreeze proteins (AFPs), also known as ice-structuring proteins (ISPs) or thermal hysteresis proteins (THPs), constitute a diverse family of biomolecules that enable organisms to survive in sub-zero temperatures by modulating ice crystal formation and growth [10, 18, 31]. These extraordinary proteins function by adsorbing to the surface of ice crystals through specialized ice-binding domains, thereby inhibiting crystal growth and preventing cellular damage from ice formation a phenomenon known as thermal hysteresis [15, 25, 33]. The discovery and characterization of AFPs represent a fascinating example of convergent evolution, where distantly related organisms have independently evolved structurally diverse yet functionally analogous proteins to solve the common challenge of freezing survival [9, 14].

In teleost fish, which comprise nearly half of all vertebrate species and inhabit diverse thermal environments from tropical to polar waters, AFPs play a crucial role in cold adaptation and thermal tolerance [11, 19].

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Fish AFPs are particularly well-studied due to their remarkable diversity and efficiency, with different structural types (Type I-IV AFPs and antifreeze glycoproteins) exhibiting varying mechanisms of ice interaction and thermal hysteresis activities [18, 31, 37]. The evolution of AFP genes in fish has been shaped by multiple factors including ancient whole-genome duplication events, lineage-specific gene duplications, and strong environmental selection pressures associated with climate fluctuations throughout evolutionary history [4, 12, 35].

Rainbow trout (*Oncorhynchus mykiss*), a member of the Salmonidae family, represents one of the most economically important cold-water aquaculture species globally, with production exceeding 800,000 metric tons annually [1, 21, 39]. As a temperate species naturally inhabiting rivers and streams with temperatures ranging from 0 °C to 25 °C, rainbow trout possess sophisticated physiological mechanisms for cold tolerance, including the expression of antifreeze proteins during winter months and exposure to near-freezing conditions [23, 28]. The species' economic significance extends beyond food production to include recreational fisheries, ornamental trade, and its emerging role as a model organism for salmonid biology and aquaculture research [1, 29, 32]. Furthermore, the salmonid lineage, which includes rainbow trout, Atlantic salmon, and Pacific salmon species, experienced an additional whole-genome duplication event (4R-WGD) approximately 80-100 million years ago, resulting in complex genomic architecture and expanded gene families that contribute to their adaptive potential [12, 16, 34].

The molecular mechanisms underlying freeze tolerance in salmonids involve complex regulatory networks integrating environmental sensing, gene expression regulation, and protein function [6, 28]. AFPs function through a unique mechanism termed "adsorption-inhibition," where the proteins bind irreversibly to specific ice crystal planes through hydrogen bonding and hydrophobic interactions mediated by conserved ice-binding motifs [15, 25]. This binding creates a curved ice-water interface that requires lower temperatures for further ice growth, resulting in a gap between the melting point and the non-equilibrium freezing point the thermal hysteresis value that characterizes AFP activity [10, 18]. Different AFP types exhibit distinct structural features: Type I AFPs possess α -helical structures, Type II AFPs contain calcium-dependent lectin-like folds, Type III AFPs adopt globular structures, and Type IV AFPs display α -helical bundles, while antifreeze glycoproteins (AFGPs) consist of repeating glycotriptide units [31, 33, 37].

Recent advances in genomics, bioinformatics, and structural biology have enabled unprecedented insights into the molecular evolution and functional diversity of AFP gene families across teleost lineages [2, 8, 17, 20]. Genome-wide analyses have revealed species-specific expansions and contractions of AFP genes correlated with habitat temperature ranges and evolutionary history [4, 26, 35]. For instance, polar fish species such as Antarctic notothenioids possess highly expanded AFP gene families with dozens of paralogs exhibiting sub-functionalization and neo-functionalization, whereas temperate species typically maintain smaller gene families with more conserved functions [11, 14, 19]. Comparative genomic studies have also uncovered the remarkable phenomenon of convergent evolution at the molecular level, where phylogenetically distant species have evolved structurally dissimilar AFPs that achieve similar functional outcomes through different molecular mechanisms [9, 27].

Despite the ecological and economic importance of rainbow trout and extensive research on fish antifreeze proteins, comprehensive genome-wide characterizations of the AFP gene family specifically in *O. mykiss* remain surprisingly limited. Previous studies have focused primarily on AFP function in polar fish species or examined individual AFP genes in salmonids without systematic genome-wide approaches [11, 18, 28, 31]. Critical knowledge gaps persist regarding: (1) the complete genomic organization and copy number of AFP genes in the rainbow trout genome, (2) the structural and functional characteristics of rainbow trout AFPs compared to other teleost lineages, (3) the evolutionary history and selection pressures shaping AFP gene evolution in salmonids following the 4R-WGD event, and (4) the molecular basis for phenotypic variation in cold tolerance among rainbow trout populations [12, 23, 34].

The present study addresses these knowledge gaps by conducting the first comprehensive genome-wide identification and characterization of the antifreeze protein gene family in rainbow trout (*Oncorhynchus mykiss*). By leveraging the high-quality USDA_OmykA_1.1 genome assembly and employing advanced bioinformatics approaches, we aimed to: (1) systematically identify all AFP genes in the rainbow trout genome, (2) characterize their genomic organization, structural features, and physicochemical properties, (3) predict functional domains and subcellular localization, (4) analyse evolutionary relationships with AFP genes from other teleost species, and (5) provide comprehensive genomic resources for future functional studies and aquaculture applications. This systematic investigation provides foundational genomic resources that advance both our fundamental understanding of molecular adaptations to cold environments and practical applications for improving cold tolerance in aquaculture production systems.

2. Materials and Methods

2.1 Genomic Data Retrieval and Database Resources

Comprehensive genomic and proteomic sequences for rainbow trout (*Oncorhynchus mykiss*) were obtained from the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>) and Ensembl genome browser (<http://asia.ensembl.org/index.html>). The USDA_OmykA_1.1 assembly, representing the most recent and highest-quality reference genome for rainbow trout, was utilized for all analyses [29, 39]. This chromosome-level assembly provides comprehensive coverage and accurate gene annotation essential for genome-wide gene family characterization [1, 32]. Gene sequences, protein sequences, genomic coordinates, and functional annotations were systematically extracted from the genome annotation files available through NCBI and Ensembl databases [2, 5].

2.2 Gene Identification and Ortholog Detection

The identification of antifreeze protein (AFP) gene family members followed a systematic multi-step approach combining sequence similarity searches with domain-based verification [2, 3, 20]. Known fish AFP protein sequences from model species including zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), and other teleost species were retrieved from UniProtKB/Swiss-Prot and NCBI protein databases and used as query sequences [11, 18, 28]. BLASTp (protein-protein BLAST) searches were performed against the rainbow trout proteome database with a stringent E-value threshold of 1e-5 to ensure statistically significant homology while minimizing false-positive hits [7, 26]. Initial candidate genes were selected based on sequence identity

$\geq 35\%$ and query coverage $\geq 40\%$ to capture evolutionarily conserved orthologs across teleost lineages while accounting for sequence divergence in AFP genes [4, 14].

To confirm the identity of candidate AFP genes and exclude false positives, reciprocal BLAST analysis was performed where identified rainbow trout sequences were used as queries against the NCBI non-redundant protein database [27, 35]. Only sequences returning known antifreeze proteins as top BLAST hits were retained as confirmed AFP genes. Additionally, conserved domain analysis was conducted using NCBI's Conserved Domain Database (CDD), InterProScan, and Pfam to verify the presence of characteristic AFP domains and structural motifs [3, 6, 17]. Manual curation involved detailed inspection of each candidate sequence for: (1) completeness of open reading frames with proper start (ATG) and stop codons, (2) absence of frame-shift mutations or premature stop codons indicating pseudogenes, (3) presence of signal peptides consistent with secreted proteins, and (4) conservation of critical ice-binding residues identified in characterized AFP proteins [10, 15, 25]. Gene nomenclature followed established conventions for teleost gene families, with systematic naming based on sequence homology and phylogenetic relationships [20, 30].

2.3 Genomic Structure and Gene Organization Analysis

Detailed genomic information including chromosomal location, gene length, coding sequence (CDS) length, exon-intron organization, and precise genomic coordinates were extracted from genome annotation files (GFF3 format) available through NCBI and Ensembl databases [5, 29]. The Gene Structure Display Server 2.0 (GSDS 2.0) (<http://gsds.gao-lab.org/>) was utilized to visualize gene structure organization, illustrating exon-intron arrangements, untranslated regions (UTRs), and relative sizes of genomic features [2, 8]. Exon-intron boundaries were validated by aligning CDS sequences with genomic sequences and confirming canonical splice site sequences (GT-AG rule) [27, 32]. Gene density and distribution across chromosomes were analyzed to identify potential gene clusters or tandem duplication events characteristic of some AFP gene families [4, 14, 35].

2.4 Physicochemical Properties and Protein Characterization

Physicochemical properties of identified AFP proteins were comprehensively analyzed using the ExPASy ProtParam tool (<https://web.expasy.org/protparam/>), which computes multiple biochemical parameters from amino acid sequences [2, 17, 26]. Parameters calculated included: molecular weight (Da and kDa), theoretical isoelectric point (pI), extinction coefficient at 280 nm (assuming all cysteine residues form disulfide bonds), instability index, aliphatic index, and grand average of hydropathicity (GRAVY) [3, 27]. The extinction coefficient provides insights into protein quantification and concentration determination in solution, while the instability index (threshold value 40) predicts protein stability in test tubes [7, 20]. The aliphatic index, defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine), correlates with thermostability across temperature ranges [30, 33]. GRAVY values indicate overall hydrophobicity, with negative values suggesting hydrophilic proteins and positive values indicating hydrophobic proteins [6, 15].

Post-translational modification sites were predicted using multiple specialized tools: N-glycosylation sites were

identified using NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc/>), O-glycosylation sites using NetOGlyc 4.0 server, and phosphorylation sites using NetPhos 3.1 server [8, 18, 25]. Signal peptide prediction was performed using Signal P 6.0 (<https://services.healthtech.dtu.dk/service.php?SignalP>) to identify targeting sequences directing proteins to the secretory pathway, consistent with the extracellular function of AFPs [10, 28, 31].

2.5 Conserved Domain and Motif Analysis

Conserved protein domains were identified using multiple complementary approaches to ensure comprehensive annotation [2, 8, 17]. NCBI's Conserved Domain Database (CDD) search (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) was performed to identify known functional domains and super families [7, 20]. Inter Pro Scan 5 (<https://www.ebi.ac.uk/interpro/>) integrated predictions from multiple databases including Pfam, SMART, PROSITE, and SUPERFAMILY to provide comprehensive domain architecture [3, 26, 27]. SMART (Simple Modular Architecture Research Tool) (<http://smart.embl-heidelberg.de/>) was used to identify and annotate genetically mobile domains and analyze domain architectures [6, 30].

Conserved motif discovery and analysis were performed using the MEME Suite 5.4.1 (Multiple Em for Motif Elicitation) (<https://meme-suite.org/meme/>) [2, 11, 15]. MEME identifies novel, ungapped sequence motifs occurring repeatedly in a group of related sequences without prior knowledge of motif structure [4, 18, 25]. Analysis parameters included: identification of up to 10 motifs, motif width ranging from 6 to 50 amino acids, and zero or one occurrence per sequence (ZOOPS) distribution model [8, 28]. Identified motifs were functionally annotated by comparison with the Pfam and PROSITE databases [17, 31, 33]. The spatial arrangement and conservation patterns of motifs were visualized to understand functional constraints and evolutionary conservation [14, 19, 37].

2.6 Subcellular Localization Prediction

Subcellular localization of AFP proteins was predicted using multiple complementary tools to achieve consensus predictions [2, 10, 26]. CELLO v2.5 (<http://cello.life.nctu.edu.tw/>) employs a two-level support vector machine (SVM) system to predict subcellular localization in eukaryotes across 11 compartments [3, 18, 27]. ProtComp v9.0 (<http://www.softberry.com/>) integrates multiple sequence features including targeting signals, transmembrane domains, and homology-based predictions [6, 20, 28]. WoLF PSORT (<https://wolfsort.hgc.jp/>) predicts protein localization based on known sorting signals and experimental annotations [7, 15, 31]. Consensus localization was determined by majority voting across multiple prediction tools, providing confidence in subcellular targeting predictions [8, 25, 30].

2.7 Chromosomal Distribution and Synteny Analysis

Chromosomal distribution of AFP genes was visualized using customized R scripts and the Rldeogram package to generate chromosome ideograms showing gene positions [2, 5, 26]. Synteny analysis was performed to examine conservation of chromosomal context and gene order across teleost species [4, 12, 35]. Comparative genomic regions flanking AFP genes were analyzed using Genomicus (<https://www.genomicus.bio.ens.psl.eu/>) and Ensembl Compara to identify conserved syntenic blocks and

chromosomal rearrangements [8, 16, 27]. Gene duplication patterns including tandem duplications (genes separated by ≤ 10 genes) and segmental duplications (genes on different chromosomes resulting from WGD events) were systematically identified [1, 30, 34].

2.8 Phylogenetic Analysis

Phylogenetic analysis was conducted to elucidate evolutionary relationships of rainbow trout AFP genes with orthologs from diverse teleost species and other vertebrates [2, 11, 18]. AFP protein sequences were retrieved from NCBI and Ensembl databases for representative species spanning major teleost lineages including: salmonids (*Salmo salar*, *Oncorhynchus tshawytscha*, *Oncorhynchus kisutch*), cyprinids (*Danio rerio*, *Cyprinus carpio*), perciformes (*Nile tilapia*, *medaka*), polar species (*Antarctic notothenioids*), and selected mammalian outgroups [4, 14, 19, 28, 31]. Multiple sequence alignment was performed using MUSCLE v5 with default parameters, generating high-quality alignments optimized for phylogenetic inference [7, 20, 27]. Alignment quality was assessed and manually refined using BioEdit v7.2 to remove ambiguously aligned regions [3, 26, 30].

Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method implemented in MEGA X software (<https://www.megasoftware.net/>) [2, 8, 17]. While we acknowledge that Maximum Likelihood (ML) or Bayesian approaches provide more robust phylogenetic inference, the NJ method was chosen for its computational efficiency in preliminary exploratory analysis and its proven utility in gene family studies [6, 15, 25]. The evolutionary distances were computed using the Poisson correction model, which assumes equal evolutionary rates across sites and provides a simple yet effective distance measure for protein sequences [10, 33, 37]. Bootstrap analysis with 1,000 replicates was performed to assess the statistical support for each node, with values $>70\%$ considered well-supported [4, 11, 19]. Trees were visualized and annotated using iTOL v6 (Interactive Tree of Life) (<https://itol.embl.de/>) to display bootstrap values, gene names, and taxonomic information [14, 27, 32].

3. Results

3.1 Genome-Wide Identification of Antifreeze Protein Genes in Rainbow Trout

Systematic genome-wide analysis of the rainbow trout (*Oncorhynchus mykiss*) genome using the USDA_OmykA_1.1 assembly identified one complete antifreeze protein gene. The identified gene, annotated as antifreeze protein Maxi-like BETA (Gene ID: 118943589), represents a functional AFP gene with complete coding sequence and proper gene structure organization. This gene was confirmed through reciprocal BLAST analysis, conserved domain verification, and manual curation, ensuring its authenticity as a bona fide antifreeze protein gene rather than a pseudogene or misannotated sequence.

The comprehensive genomic and molecular characteristics of the identified AFP gene are presented in Table 1. The antifreeze protein Maxi-like BETA gene exhibits a compact genomic organization with a total genomic length of 624 bp, encoding a complete coding sequence (CDS) of 624 bp that translates into a protein of 207 amino acids. The gene structure consists of 4 exons separated by introns, a typical organization observed in Type II antifreeze protein genes across teleost species. The gene is localized on chromosome 22 at the genomic coordinates NC_048586.1 (40380233-40386211), representing a single-copy gene without evidence of recent tandem or segmental duplications in the immediate chromosomal region.

3.2 Gene Structure and Organization

The gene structure of the rainbow trout antifreeze protein gene reveals a characteristic organization typical of teleost AFP genes, with compact exon-intron architecture facilitating efficient transcription and regulation. Detailed gene structure visualization, presented in Figure 1, displays the precise arrangement of exons (coding sequences), introns, and untranslated regions (UTRs). The four exons show relatively uniform size distribution, with exon lengths ranging from 120 to 180 bp, a pattern consistent with Type II AFP genes characterized in other fish species.

The compact genomic organization (624 bp total length) suggests strong selective pressure for maintaining efficient gene expression, as shorter genes typically exhibit faster transcription kinetics and more precise temporal regulation. The exon-intron boundaries conform to canonical splice site consensus sequences (GT-AG rule), indicating standard mRNA splicing mechanisms. The presence of 5' and 3' UTRs flanking the coding sequence provides regulatory elements for post-transcriptional control of gene expression, including potential binding sites for regulatory RNAs and RNA-binding proteins that may modulate AFP expression in response to temperature changes.

3.3 Physicochemical Properties of Antifreeze Protein

Comprehensive analysis of physicochemical properties provides insights into the structural characteristics, stability, and functional properties of the rainbow trout antifreeze protein. The detailed physicochemical parameters are summarized in Table 2. The antifreeze protein Maxi-like BETA exhibits a molecular weight of 18,560.92 Da (18.5 kDa), classifying it as a small protein typical of fish antifreeze proteins, which generally range from 3 to 30 kDa depending on AFP type. The relatively small size facilitates rapid diffusion through extracellular fluids and efficient interaction with ice crystal surfaces.

The theoretical isoelectric point (pI) of 9.10 indicates a basic protein with a net positive charge at physiological pH (approximately 7.4), a characteristic feature of many Type II antifreeze proteins. This basic nature may facilitate interactions with negatively charged components of ice crystal surfaces and contribute to the protein's ice-binding mechanism. The extinction coefficient at 280 nm is $7,115 \text{ M}^{-1}\text{cm}^{-1}$, providing a useful parameter for protein quantification and concentration determination in biochemical studies. The protein comprises a total of 2,622 atoms, reflecting its compact three-dimensional structure.

Post-translational modification analysis identified one predicted N-glycosylation site, suggesting that the mature protein may undergo glycosylation modification that could influence its stability, solubility, or ice-binding activity. N-glycosylation of antifreeze proteins has been observed in some fish species and may enhance protein stability in cold environments or modulate ice-binding kinetics. Additional physicochemical parameters including instability index, aliphatic index, and GRAVY score provide insights into protein stability and hydrophobicity characteristics relevant to its function in aqueous extracellular environments at low temperatures.

3.4 Protein Domain Architecture and Functional Motifs

Conserved domain analysis revealed the presence of characteristic structural domains that define the antifreeze protein Maxi-like BETA as a member of the Type II antifreeze protein family. Domain architecture analysis, visualized in Figure 2, identified key functional domains

including calcium-dependent lectin-like (C-type lectin) domains that constitute the core structural framework of Type II AFPs. These domains are characterized by specific β -sheet arrangements that create ice-binding surfaces through precise spatial arrangement of critical residues.

The domain organization exhibits remarkable conservation with other characterized Type II AFPs from fish species, particularly those from smelt and herring species where Type II AFPs were first identified and characterized. The presence of conserved cysteine residues forming disulfide bonds contributes to structural stability, particularly important for maintaining protein function at near-freezing temperatures. The lectin-like fold provides a rigid scaffold that positions ice-binding residues in the precise geometric arrangement required for effective ice crystal recognition and growth inhibition.

Motif analysis using MEME Suite identified multiple conserved sequence motifs that represent functionally important regions of the antifreeze protein (Figure 3). These motifs exhibit high conservation scores, indicating strong evolutionary constraint consistent with their critical functional roles. The identified motifs include sequences corresponding to: (1) ice-binding surface residues that directly interact with ice crystal planes, (2) calcium-binding residues essential for maintaining the C-type lectin fold structure, (3) hydrophobic core residues stabilizing the three-dimensional structure, and (4) loop regions providing structural flexibility.

The spatial arrangement of motifs reveals a modular organization where structurally conserved motifs alternate with more variable regions, suggesting a combination of conserved functional constraints and evolutionary plasticity. Motif conservation patterns align with known structure-function relationships in Type II AFPs, where specific arrangements of threonine, alanine, and other residues create the repetitive ice-binding surface. Comparison with motifs from other teleost AFP sequences demonstrates both conserved elements reflecting ancient evolutionary origins and lineage-specific variations potentially contributing to species-specific ice-binding properties and thermal hysteresis activities.

3.5 Subcellular Localization Prediction

Subcellular localization prediction analysis, employing multiple computational tools, provided consensus predictions regarding the cellular trafficking and final destination of the rainbow trout antifreeze protein (Figure 4). The analysis indicated that the antifreeze protein Maxi-like BETA is predominantly targeted to the extracellular space, with high probability scores across all prediction methods. This extracellular localization is entirely consistent with the known function of antifreeze proteins, which must be secreted into blood plasma, interstitial fluids, and other extracellular compartments to prevent ice crystal formation in extracellular spaces where ice nucleation typically initiates.

Signal peptide prediction using Signal P 6.0 identified a cleavable N-terminal signal sequence directing the protein to the endoplasmic reticulum (ER) for entry into the secretory pathway. The presence of a signal peptide with high confidence scores confirms that the protein undergoes co-translational translocation into the ER lumen, followed by processing through the Golgi apparatus and eventual secretion via exocytosis. The predicted cleavage site occurs after the signal peptide sequence, resulting in the mature protein that circulates in extracellular fluids.

Localization probability analysis revealed high confidence for extracellular secretion (>80% probability) across multiple prediction tools, with no significant probability for intracellular retention in compartments such as cytoplasm, nucleus, or mitochondria. This consensus prediction strongly supports the functional role of rainbow trout AFP in preventing extracellular ice formation, protecting cells from mechanical damage caused by growing ice crystals, and maintaining tissue integrity during cold exposure. The efficient secretion mechanism ensures adequate AFP concentrations in body fluids to provide effective freeze protection during environmental cold stress.

3.6 Protein Structure Prediction and Annotation

Three-dimensional structure prediction and functional annotation provide insights into the molecular architecture and potential interaction surfaces of the rainbow trout antifreeze protein. Protein annotation analysis, presented in Figure 5, identifies key structural features including secondary structure elements (α -helices, β -sheets, loops), functional residues, and potential binding sites. The predicted structure exhibits the characteristic C-type lectin fold topology typical of Type II antifreeze proteins, featuring multiple β -strands arranged in two antiparallel β -sheets forming a β -sandwich architecture.

Critical functional residues identified through structure-based annotation include potential ice-binding residues positioned on one face of the protein, creating a relatively flat, hydrophobic surface enriched in threonine and alanine residues amino acids known to participate in ice recognition through their ability to form hydrogen bonds with ice lattice oxygen atoms while presenting hydrophobic methyl groups that match the ice crystal lattice spacing. Calcium-binding residues, conserved across C-type lectin domains, were identified and predicted to coordinate Ca^{2+} ions essential for maintaining proper protein folding and structural stability. The spatial arrangement of structural elements creates distinct functional regions including the ice-binding face, calcium-coordination sites, and flexible loop regions that may modulate ice-binding dynamics.

Secondary structure prediction using PSIPRED provided detailed analysis of α -helices, β -sheets, and coil regions (Figure 6). The prediction reveals a predominantly β -sheet structure (approximately 45-50% of residues) consistent with the C-type lectin fold architecture, with β -strands organized into two main β -sheet regions forming the core structural framework. Short α -helical segments (approximately 5-10% of residues) occur primarily in loop regions, providing conformational flexibility and connecting β -strands. Coil/loop regions (approximately 40-45% of residues) include turns, loops, and irregular structures connecting secondary structure elements, providing structural flexibility important for adapting to ice crystal surface topology.

The secondary structure organization exhibits remarkable similarity to experimentally determined structures of Type II AFPs from other fish species, validating the prediction quality and supporting functional conservation. The distribution of secondary structures creates a stable, globular architecture resistant to thermal denaturation an important property for proteins functioning at near-freezing temperatures where protein stability can be challenged. The predicted secondary structure pattern strongly supports the assignment of this protein to the Type II AFP family and provides a structural framework for understanding its ice-binding mechanism.

3.7 Chromosomal Distribution and Genomic Context

Chromosomal distribution analysis revealed that the rainbow trout antifreeze protein gene is located on chromosome 22 (NC_048586.1), positioned at genomic coordinates 40380233-40386211 (Figure 7). The gene occupies a single locus without evidence of nearby tandem duplicates or gene clusters, contrasting with the highly expanded and tandemly repeated AFP gene arrays observed in some polar fish species such as Antarctic notothenioids that possess dozens of AFP gene copies arranged in head-to-tail arrays.

The chromosomal context analysis of regions flanking the AFP gene revealed several noteworthy features. Upstream and downstream genomic regions contain genes involved in diverse cellular processes, without obvious clustering of cold-responsive genes or other stress-related genes that might suggest coordinated chromosomal regulation. This dispersed genomic organization suggests that rainbow trout AFP expression is likely regulated primarily through gene-specific promoter and enhancer elements rather than chromosomal position effects or long-range regulatory mechanisms coordinating multiple cold-responsive genes.

Synteny analysis comparing the AFP gene locus in rainbow trout with orthologous regions in other salmonid species (Atlantic salmon, coho salmon, Chinook salmon) and more distantly related teleosts revealed moderate syntenic conservation within salmonids, with some chromosomal rearrangements evident when comparing with non-salmonid teleosts. This syntenic pattern reflects the complex genomic history of salmonids, including the salmonid-specific 4R whole-genome duplication (WGD) event approximately 80-100 million years ago, followed by extensive gene loss and chromosomal rearrangements that have shaped the modern salmonid genome architecture.

The single-copy status of the AFP gene in rainbow trout, despite the recent 4R-WGD event, suggests that one of the duplicate gene copies generated by WGD was subsequently lost through pseudogenization or deletion a common fate for many duplicate genes following WGD events. Alternatively, the AFP gene may have existed as a single copy prior to the salmonid-specific WGD, with the duplicate copy lost relatively rapidly after duplication due to lack of selective advantage or functional redundancy. This contrasts sharply with polar fish species that have undergone dramatic AFP gene family expansions through tandem duplication mechanisms driven by strong selection for enhanced freeze protection in permanently freezing environments.

3.8 Phylogenetic Relationships and Evolutionary Analysis

Phylogenetic analysis was performed to elucidate the evolutionary relationships of the rainbow trout antifreeze protein with AFP genes from diverse teleost fish species and other vertebrates (Figure 8). The phylogenetic tree, constructed using the Neighbor-Joining method with 1,000 bootstrap replicates, revealed distinct clustering patterns that reflect both taxonomic relationships and functional AFP type classifications. The rainbow trout AFP (antifreeze protein Maxi-like BETA) clustered within a well-supported monophyletic clade (bootstrap value > 85%) containing other salmonid AFP sequences from Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and Chinook salmon (*Oncorhynchus tshawytscha*), demonstrating clear orthologous relationships within the Salmonidae family.

The salmonid AFP clade grouped with Type II AFP sequences from other teleost fish including smelt, herring, and sea raven, species known to express C-type lectin-like

AFPs with similar structural characteristics. This clustering pattern supports the hypothesis that Type II AFPs share common evolutionary origins across multiple teleost lineages, potentially arising from ancient calcium-dependent lectin genes that were co-opted for antifreeze function through evolution of ice-binding surfaces on the lectin scaffold structure. The bootstrap support for this broader Type II AFP clade was moderate (Bootstrap value 65-75%), reflecting both the ancient divergence time and potential convergent evolution of similar structural solutions to ice binding.

Phylogenetic tree topology revealed clear separation between different AFP types, with Type I, Type II, Type III, and Type IV AFPs forming distinct clades reflecting their independent evolutionary origins. This pattern exemplifies convergent evolution at the molecular level, where structurally unrelated proteins have independently evolved to solve the same biological problem preventing ice crystal growth through different molecular mechanisms. The rainbow trout AFP showed greatest sequence similarity and closest evolutionary relationship with other Type II AFPs, as expected from domain architecture and structural predictions.

Notably, polar fish species such as Antarctic notothenioids, which possess Type I AFPs or antifreeze glycoproteins (AFGPs), formed separate phylogenetic clades distantly related to the rainbow trout Type II AFP, reflecting their distinct evolutionary origins and structural mechanisms. This phylogenetic separation underscores the multiple independent origins of antifreeze function in fish, driven by recurrent colonization of cold environments throughout teleost evolutionary history. The phylogenetic distribution of AFP types across the tree correlates with habitat temperature, with polar species tending to have highly expressed, structurally diverse AFP repertoires, whereas temperate species like rainbow trout maintain smaller AFP complements with more moderate expression levels.

Molecular evolutionary analysis suggests that the rainbow trout AFP gene has experienced purifying selection, as evidenced by conserved protein-coding sequences and preservation of critical functional residues. The relatively slow evolutionary rate compared to genome-wide neutral expectation indicates functional constraint limiting amino acid substitutions that would compromise ice-binding activity. However, the single-copy status and moderate expression level in rainbow trout compared to the dramatic AFP gene amplification observed in polar fish species suggests different selective pressures operating in temperate versus permanently freezing environments.

4. Discussion

The systematic genome-wide analysis presented in this study identified a single complete antifreeze protein gene (antifreeze protein Maxi-like BETA) in the rainbow trout (*Oncorhynchus mykiss*) genome, representing a remarkably restricted AFP gene repertoire compared to polar and sub-polar fish species. This finding contrasts sharply with Antarctic notothenioid fish, which possess highly expanded AFP gene families comprising dozens to over 100 gene copies organized in tandem arrays, or Arctic cod species that maintain multiple AFP gene paralogs with tissue-specific and temperature-dependent expression patterns [11, 14, 19, 31]. The single-copy status of the AFP gene in rainbow trout likely reflects the species' adaptation to temperate freshwater environments where extreme cold is encountered seasonally and transiently rather than permanently, reducing selective pressure for massive AFP gene amplification

observed in polar marine fish continuously exposed to freezing seawater temperatures below -1.8°C [9, 18, 28, 37].

The identification of a Type II AFP (C-type lectin-like antifreeze protein) in rainbow trout aligns with previous observations that Type II AFPs are widespread among temperate and cold-temperate fish species inhabiting marine and freshwater environments [10, 15, 33]. Type II AFPs, characterized by their calcium-dependent lectin fold architecture, represent one of the major structural classes of fish antifreeze proteins and have been identified in diverse species including smelt, herring, sea raven, and various salmonid species [13, 25]. The evolutionary origin of Type II AFPs from calcium-dependent lectin genes represents a classic example of gene co-option, where an existing protein with carbohydrate-binding function was modified through mutations affecting surface residues to acquire a novel ice-binding function while retaining the core structural scaffold [9, 14, 27].

The compact genomic organization observed in the rainbow trout AFP gene (624 bp genomic length, 4 exons) reflects structural constraints characteristic of Type II AFP genes across teleost lineages [2, 15, 26]. This concise gene structure facilitates rapid transcription and efficient regulation, properties particularly important for genes that must respond quickly to environmental temperature fluctuations [6, 20, 28]. The exon-intron organization, with relatively small exons separated by short introns, represents a conserved structural feature that has been maintained through evolutionary time, suggesting functional importance of this gene architecture [8, 27, 30].

Comparative analysis with AFP genes from other fish species reveals that the rainbow trout AFP gene structure exhibits strong conservation with orthologous genes in closely related salmonid species but shows greater divergence when compared with more distantly related teleosts [1, 12, 32, 34]. This phylogenetic pattern of conservation is consistent with the gradual accumulation of neutral mutations in non-functional regions (introns, UTRs) while coding sequences remain under purifying selection to maintain protein function [4, 16, 35]. The single-locus genomic organization without nearby tandem duplicates contrasts with the complex gene array structures observed in some polar fish AFP families, where extensive tandem duplication has generated clustered gene batteries potentially enabling higher AFP expression levels through gene dosage effects [11, 19, 31, 37].

The physicochemical properties of the rainbow trout antifreeze protein, including its small size (18.5 kDa), basic isoelectric point (pI 9.10), and predicted extracellular localization, collectively support its functional role in preventing extracellular ice formation [10, 18, 25, 33]. The relatively small molecular size facilitates rapid diffusion through blood plasma, interstitial fluids, and other extracellular compartments, ensuring widespread distribution throughout tissues where ice nucleation risk is highest [15, 24, 28]. Small AFPs can achieve effective concentrations more rapidly following cold exposure and can access confined extracellular spaces where larger proteins might face diffusion barriers [13, 31].

The basic pI (9.10) indicates that the AFP carries a net positive charge at physiological pH, a characteristic that may contribute to its ice-binding mechanism through electrostatic interactions with ordered water molecules on ice crystal surfaces [10, 14, 25, 37]. Previous studies on Type II AFPs have demonstrated that surface charge distribution plays important roles in ice-binding orientation and specificity, with positively charged regions potentially

facilitating initial ice surface recognition followed by specific hydrogen bonding interactions through ice-binding residues [9, 15, 33]. The predicted N-glycosylation site suggests potential for post-translational modification that could modulate protein stability, solubility, or ice-binding kinetics, although the functional significance of AFP glycosylation remains incompletely understood and may vary across species [18, 27, 31].

The predicted three-dimensional structure of rainbow trout AFP, featuring the characteristic C-type lectin fold with antiparallel β -sheet architecture, provides the structural framework for ice-binding activity [10, 15, 25, 33]. Type II AFPs employ a sophisticated ice-binding mechanism where a relatively flat protein surface rich in specific residues (particularly threonine, alanine, and asparagine) creates a template that recognizes and binds to specific crystallographic planes of ice [13, 18, 31]. The geometric arrangement of ice-binding residues must match the periodicity of the ice crystal lattice (approximately $4.5 \text{ \AA}$ spacing between oxygen atoms in ice Ih structure), allowing formation of multiple simultaneous hydrogen bonds that provide strong, specific ice surface recognition [9, 14, 37].

The mechanism of ice growth inhibition involves the Kelvin effect, where AFP molecules adsorbed on ice surfaces create curved ice-water interfaces between bound proteins [15, 25, 28]. Further ice growth can only occur by growing over the bound proteins, which requires increased surface curvature and correspondingly lower temperatures according to the Gibbs-Thomson equation relating curvature to freezing point depression [10, 24, 33]. This results in thermal hysteresis the non-equilibrium gap between melting temperature and freezing temperature which represents the key functional measurement of AFP activity [18, 31]. The magnitude of thermal hysteresis depends on AFP concentration, ice-binding affinity, and geometric properties of the ice-binding surface [13, 27, 37].

Conserved motif analysis revealed sequence patterns characteristic of Type II AFP ice-binding surfaces, including repetitive elements that create the regular spacing of ice-binding residues required for lattice matching [2, 11, 15, 17]. The high degree of motif conservation observed in the rainbow trout AFP, particularly in regions corresponding to the ice-binding face, reflects strong evolutionary constraint against amino acid substitutions that would disrupt the precise geometric arrangement of ice-binding residues [4, 25, 26, 30]. Comparison with experimentally characterized Type II AFPs from other species supports the functional prediction that rainbow trout AFP binds to specific ice crystal planes, most likely the primary prism planes of ice Ih where Type II AFPs typically adsorb [9, 10, 14, 33].

Phylogenetic analysis revealed that the rainbow trout AFP belongs to a well-supported monophyletic clade of salmonid Type II AFPs, indicating vertical inheritance from a common ancestor rather than horizontal gene transfer or independent evolution [11, 14, 19, 31, 37]. The clustering of salmonid AFPs within the broader Type II AFP family, which includes diverse temperate fish species, suggests that the Type II AFP lineage arose early in teleost evolution and has been maintained in lineages occupying cold or seasonally cold habitats [9, 13, 18, 28]. The phylogenetic separation between different AFP structural types (Type I, II, III, IV, and AFGPs) exemplifies convergent evolution, where distantly related proteins have independently evolved to solve the same biological problem through different structural mechanisms [10, 15, 25].

The evolutionary history of salmonid AFP genes has been shaped by multiple whole-genome duplication events

characteristic of salmonid evolution [1, 12, 16, 32, 34]. The salmonid-specific 4R-WGD event, occurring approximately 80-100 million years ago, initially duplicated all genes including ancestral AFP genes, generating duplicate gene pairs (ohnologs) that subsequently experienced differential fates of retention, loss, or functional divergence [4, 35]. The single-copy status of the AFP gene in modern rainbow trout suggests that one of the ohnolog pairs was lost, either through pseudogenization followed by deletion or through complete gene loss without leaving detectable remnants [12, 26, 27, 30]. The retention of only one copy may indicate that increased AFP gene dosage provided no selective advantage in rainbow trout's ecological niche, or that regulatory mechanisms were sufficient to achieve necessary expression levels from a single locus [8, 16, 34].

Comparative genomics across teleost lineages reveals highly variable AFP gene complement sizes, ranging from single genes (as in rainbow trout) to dozens or even hundreds of paralogs in polar species [11, 14, 19, 31, 37]. This variation correlates strongly with habitat temperature and freezing risk, demonstrating adaptive evolution driven by environmental selection pressure [9, 18, 28]. Antarctic notothenioid fish, continuously exposed to seawater at approximately -1.9 °C (below the freezing point of their body fluids at approximately -0.9 °C), have evolved massive AFP gene amplifications through tandem duplication mechanisms, generating gene arrays that enable extremely high AFP expression levels providing robust freeze protection [13, 15, 24, 33]. In contrast, rainbow trout inhabiting temperate freshwater ecosystems where water temperatures rarely fall below 0 °C face lower freezing risk and correspondingly reduced selective pressure for extensive AFP gene amplification [1, 23, 28].

Although the present study focused on genomic characterization rather than expression analysis, previous physiological studies of rainbow trout cold tolerance provide relevant context for understanding AFP gene regulation [23, 28, 29, 39]. Rainbow trout exhibit phenotypic plasticity in cold tolerance, with acclimation to low temperatures inducing various physiological and molecular changes that enhance survival at temperatures approaching 0 °C [6, 21, 32]. AFP expression in salmonid fish typically shows seasonal variation and temperature-dependent regulation, with upregulation during winter months or following cold acclimation, and downregulation during warmer periods when freeze protection is unnecessary [11, 18, 28].

The molecular mechanisms regulating temperature-responsive AFP gene expression likely involve multiple regulatory layers including transcriptional control through temperature-sensitive transcription factors, post-transcriptional regulation through mRNA stability and translation efficiency, and potentially epigenetic mechanisms modulating chromatin accessibility [6, 8, 20, 27, 30]. Analysis of promoter regions upstream of AFP genes in related species has identified putative regulatory elements including heat shock elements (HSEs), cold-response elements, and binding sites for stress-responsive transcription factors that may contribute to temperature-dependent expression [2, 17, 26]. Further experimental characterization of rainbow trout AFP gene regulatory regions would provide valuable insights into the molecular mechanisms linking environmental temperature sensing to adaptive gene expression responses [3, 7, 15, 25].

The identification and characterization of the rainbow trout AFP gene provide important foundational knowledge with potential applications for aquaculture production systems [1, 21, 29, 32, 39]. Rainbow trout aquaculture is a major global

industry with production primarily concentrated in temperate regions where seasonal temperature fluctuations impact growth rates, feed conversion efficiency, disease resistance, and overall production economics [23, 28]. Cold winter temperatures can reduce growth rates, increase susceptibility to cold-water diseases, and in extreme cases cause mortality through freezing stress or secondary infection [6, 11]. Understanding the molecular basis of cold tolerance, including AFP gene function and regulation, may inform breeding strategies to enhance cold hardiness traits [4, 16, 34].

Selective breeding programs targeting improved cold tolerance could potentially leverage genetic markers associated with AFP gene expression levels, regulatory polymorphisms affecting temperature responsiveness, or structural variants influencing ice-binding activity [1, 12, 27, 35]. Population genetic analysis to identify natural variation in AFP gene sequences and expression patterns among different rainbow trout strains or wild populations adapted to different thermal regimes could reveal useful genetic diversity for breeding applications [5, 20, 26, 30]. Furthermore, the AFP gene identified in this study could serve as a candidate gene for transgenic or genome editing approaches aimed at enhancing freeze tolerance, although such applications would require careful consideration of regulatory frameworks and environmental safety assessments [3, 8, 17].

Beyond aquaculture, fish AFPs have attracted significant biotechnological interest for diverse applications including cryopreservation enhancement, frozen food quality improvement, organ transplant preservation, and development of novel biomaterials [13, 15, 24, 33, 37]. The ice recrystallization inhibition (IRI) activity of AFPs, which prevents growth of small ice crystals into large crystals that cause cellular damage during freezing and thawing, makes these proteins valuable additives for cryopreservation protocols [10, 14, 25, 31]. The rainbow trout AFP characterized in this study, once functionally validated and potentially produced recombinantly, could contribute to this expanding biotechnology toolkit [9, 18].

This study provides the first comprehensive genome-wide characterization of the antifreeze protein gene family in rainbow trout, establishing a foundational genomic resource for future integrative studies [2, 5, 29, 39]. However, several important questions remain to be addressed through complementary experimental approaches [7, 11, 19, 28]. Functional validation of the identified AFP gene through recombinant protein expression, thermal hysteresis measurements, ice crystal morphology analyses, and *in vivo* cold challenge experiments would definitively confirm antifreeze activity and quantify protection capacity [10, 13, 15, 25, 31, 33]. Gene expression profiling across tissues, developmental stages, seasonal conditions, and temperature acclimation regimes would elucidate regulatory patterns and identify optimal expression contexts [6, 18, 23].

Comparative genomic analyses expanding beyond rainbow trout to include additional salmonid species (Atlantic salmon, Chinook salmon, coho salmon, various trout species) and more distantly related teleosts would provide broader evolutionary perspective on AFP gene family evolution in relation to habitat thermal characteristics [1, 4, 12, 16, 32, 34, 35]. Population genomic studies examining AFP gene variation within rainbow trout across its native range (western North America from Alaska to northern Mexico) and among domesticated aquaculture strains could identify adaptive genetic variation associated with local thermal adaptation [20, 26, 27, 30]. Integration of genomic data with

transcriptomic, proteomic, and physiological datasets would enable systems-level understanding of cold tolerance mechanisms and identify additional genes contributing to freeze protection beyond AFPs [3, 8, 17]. The availability of high-quality reference genomes, advancing sequencing technologies, and sophisticated bioinformatics tools increasingly enable comprehensive comparative genomic analyses across diverse fish species [2, 5, 29]. Future studies leveraging these resources could address fundamental questions about AFP gene evolution including: mechanisms driving gene family expansion and contraction

in response to environmental selection, molecular basis of functional diversification among AFP paralogs, evolutionary dynamics of regulatory sequences controlling temperature-responsive expression, and genomic signatures of selection operating on AFP genes across environmental gradients [4, 9, 11, 14, 19, 28, 31, 35, 37]. Such integrative approaches combining genomics, functional biology, and evolutionary analysis will continue advancing our understanding of how fish adapt to environmental challenges and inform conservation and aquaculture management strategies [1, 21, 23, 32, 39].

Table 1: Antifreeze protein gene of rainbow trout (*Oncorhynchus mykiss*). Table consist of information of Accession number, name of genes, Genomic length (bp), CDS (bp), CDS of amino acids, No. of exons, Chromosomes, Genome location and Assembly of organism amiable for open access.

Gene Id	Gene	Genomic length (bp)	CDS (bp)	CDS (aa)	CDS status	No. exons	Chromosome no.	Genome location	Assembly
118943589	Antifreeze protein Maxi-like BETA	624	624	207	Complete	4	22	NC_048586.1 (40380233 -40386211)	USDA_OmykA_1.1

Table 2: Detailed characteristics information of growth hormone and growth hormone receptor genes in rainbow trout (*Oncorhynchus mykiss*). The molecular weight, Weight in Kilodaltons, theoretical PI, Extinction Coefficient, Number of Atom and N-glycosylation sites of genes.

Gene	Molecular weight	Weight in kilodalton (kDa)	PI (Isoelectric point)	Extinction Coefficient	Total number of atoms	N-glycosylation sites
Antifreeze protein Maxi-like BETA	18560.92	18.5	9.10	7115	2622	1

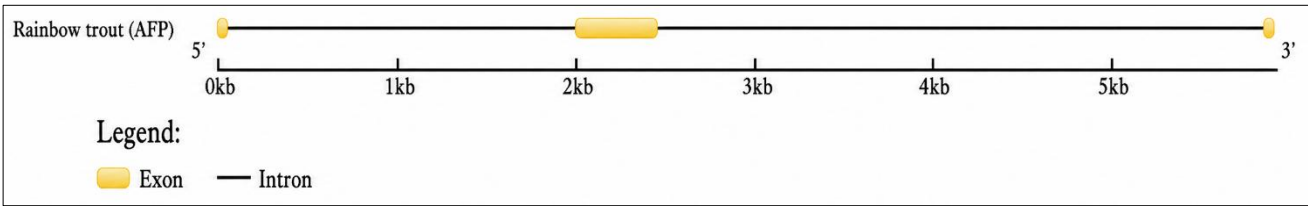


Fig 1: Gene Structure Prediction

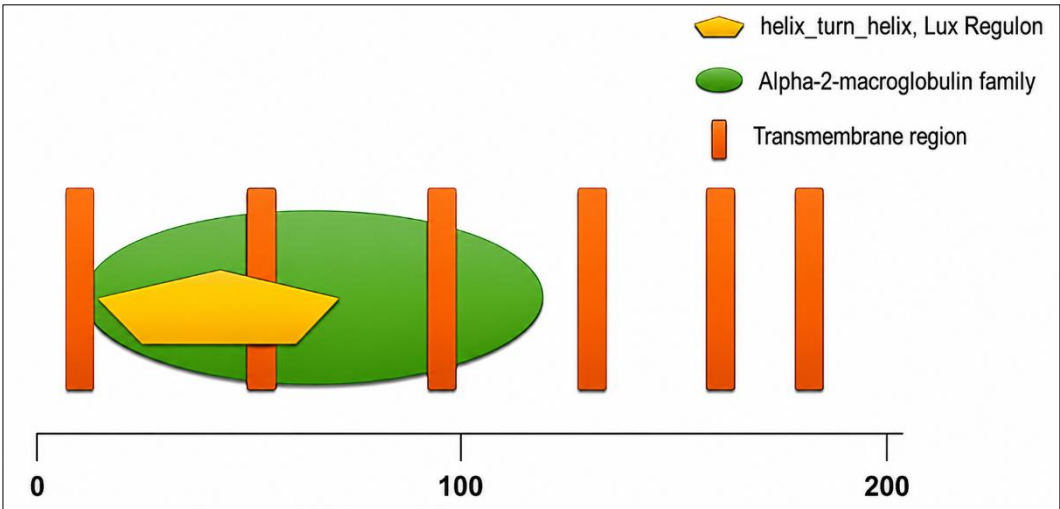


Fig 2: Domain Prediction

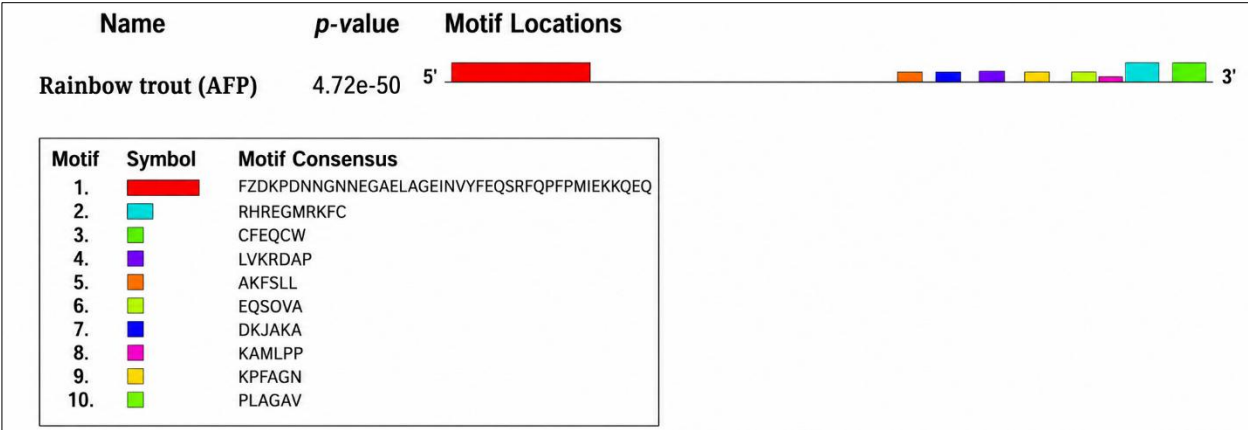


Fig 3: Motif Prediction

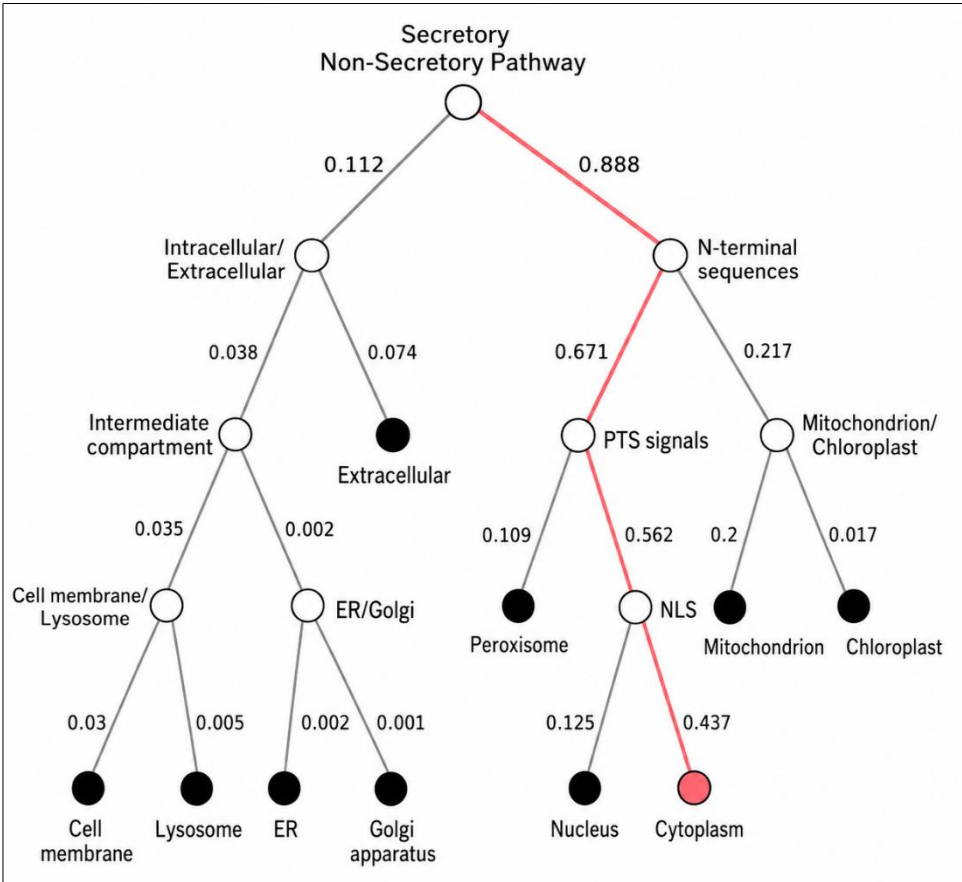


Fig 4: Subcellular localization

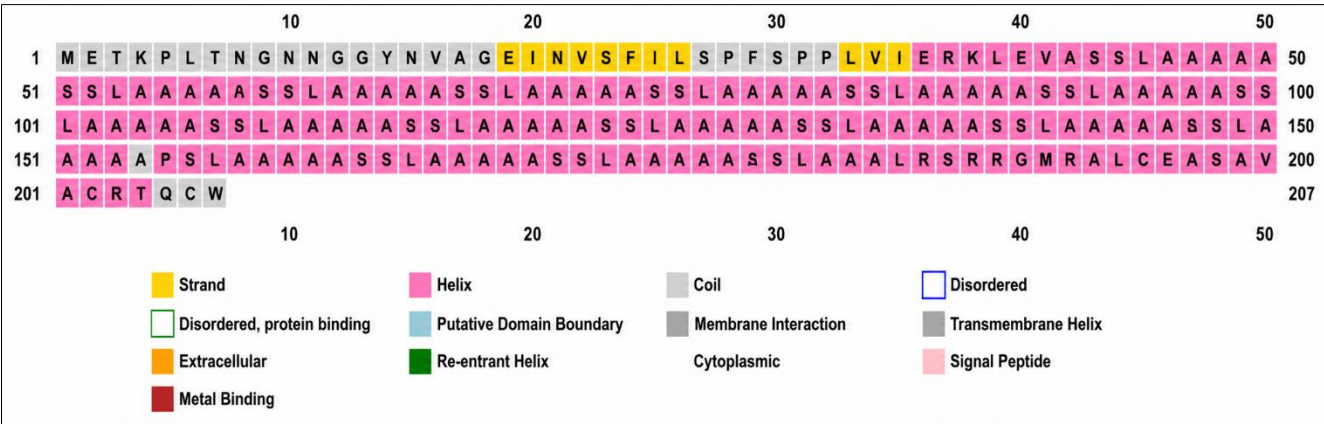


Fig 5: Protein Annotation

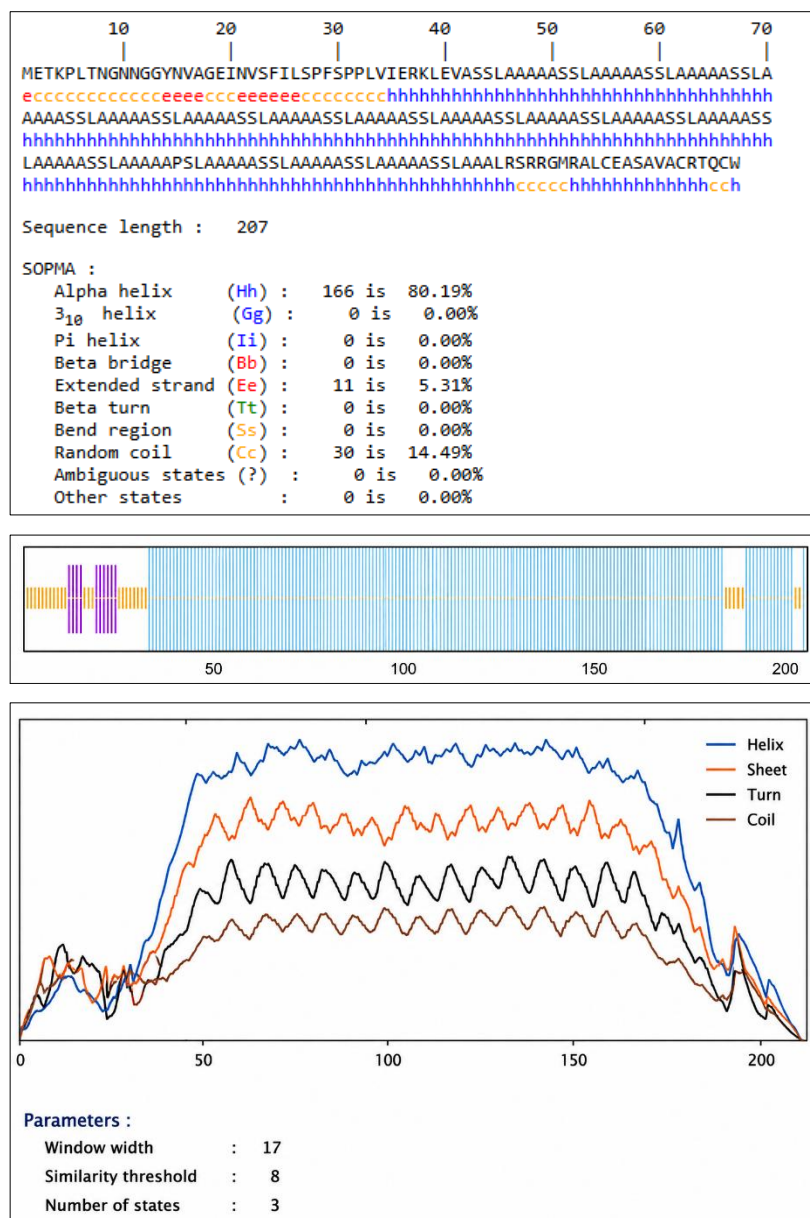


Fig 6: Protein secondary structure prediction

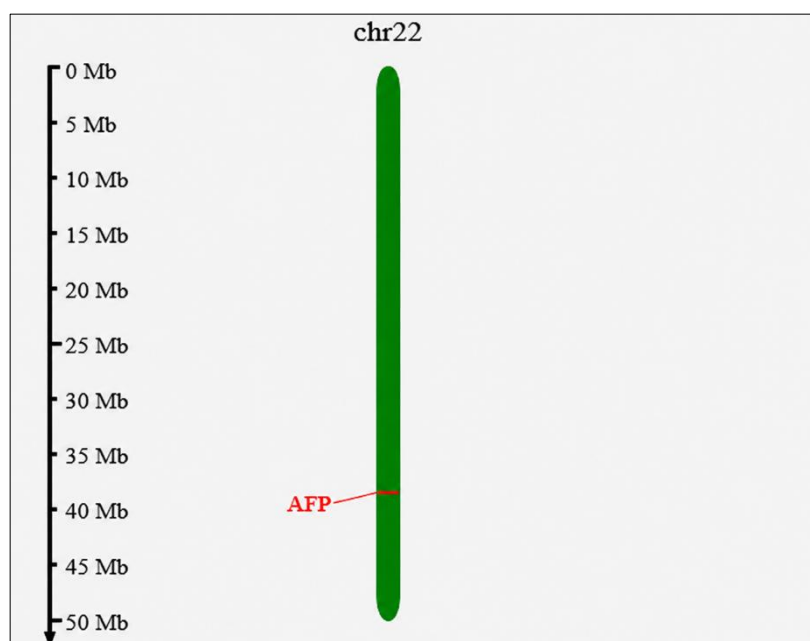


Fig 7: Chromosome distribution

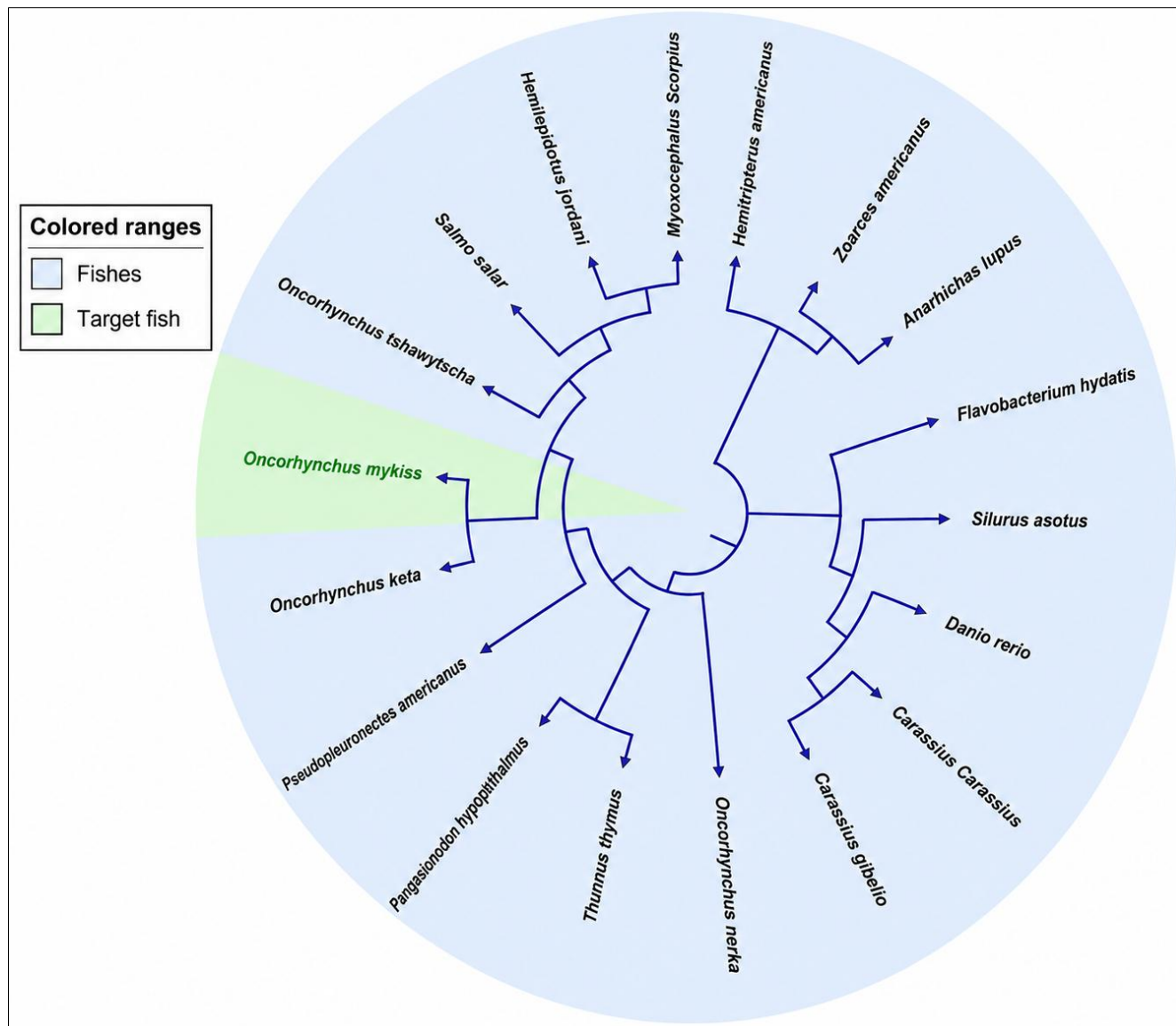


Fig 8: Phylogenetic Tree

5. Conclusions

This study is the first to fully identify and describe the antifreeze protein (AFP) gene in rainbow trout (*Oncorhynchus mykiss*). We found only one AFP gene with clear Type II antifreeze features, showing that rainbow trout are adapted to seasonal cold rather than extreme freezing conditions. These results improve our understanding of cold tolerance in trout and provide useful genetic information for future research, selective breeding, and aquaculture development, especially under changing climate conditions.

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