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Identification of Conserved Repetitive Amino Acid Motifs in Antifreeze Proteins of Selected Helopini Species (Coleoptera: Tenebrionidae)

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ABSTRACT

Antifreeze proteins (AFP) protect the organisms against freezing by increasing freezing tolerance. Insects inhabiting cold regions have been reported to utilize AFPs as part of their cold adaptation mechanisms. The tribe Helopini, a member of the Tenebrionidae family, which is distributed across cold environments ranging from lowlands to high-altitude areas. *Cylindrinotus charlesi* Nabozhenko, 2015, *Odocnemis anatolica* Pic, 1899, *Nalassus clavicornis* Allard, 1876, and *Xanthomus ovulus* Seidlitz, 1895 - four species belonging to the tribe Helopini (Coleoptera, Tenebrionidae) - are active during cold periods of the year and are known to inhabit cold environments such as under stones near snow or buried in cold dune areas in winter or early spring. Due to their cold habitat preferences, the presence of AFPs in these Helopini species was investigated. To begin, primers were designed. Then, genomic DNA was extracted from the specimens and amplified using polymerase chain reaction (PCR). All PCR samples were analyzed by agarose gel electrophoresis. The sequences were edited and aligned using the Geneious software. As a result, this study identified conserved repetitive amino acid sequences (TCTxSxxCxxAx) of antifreeze proteins in *Cylindrinotus charlesi, Odocnemis anatolica, Nalassus clavicornis,* and *Xanthomus ovulus* for the first time. Hypothetical 3D structures formed by these sequences were modeled and predicted using the Swiss-Model. The repeat motifs of Helopini AFPs whose sequences were determined, were analyzed using the MotifFinder. These AFPs were classified at the family levels using the SuperFamily database. In addition, the AFP sequences of Helopini species were compared to those of other beetle species available in the public database using BLAST analysis to assess sequence similarity.

Keywords: Antifreeze protein, Gene amplification, Helopini, Tenebrionidae

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Introduction

The survival of insects in cold climates is made possible by a range of adaptive mechanisms that mitigate the harmful effects of physiological stress caused by low temperatures. Among these adaptations, supercooling, freeze tolerance, and the accumulation of cryoprotectant compounds are particularly prominent [1-3]. Supercooling prevents ice crystal formation by lowering the freezing point of body fluids [4-6]. Species employing freeze tolerance strategies relocate approximately 65% of their intracellular fluids to the extracellular space, thereby increasing hemolymph osmolality and preventing intracellular ice formation. During extracellular freezing, ice nucleation is regulated by the presence of cryoprotectants and protective proteins, which stabilize cellular structures and limit ice propagation [7]. The accumulation of low molecular weight compounds such as glycerol, urea, glucose, sorbitol, trehalose, and proline [8], along with the synthesis of antifreeze proteins (AFPs), enables freeze-tolerant organisms to withstand prolonged exposure to subzero temperatures [7,9,10]. These physiological mechanisms, in conjunction with habitat selection, support insect adaptation to extreme environmental conditions.

Antifreeze proteins were first reported in Antarctic fish, where the presence of these molecules in their hemolymphs enabled survival at sub-zero temperatures [11]. AFPs have also been found in a wide range of organisms, including plants [12], terrestrial arthropods [5,13], bacteria and fungi [14].

AFPs stabilize cell membranes by binding to the water molecules on the membrane surface and preventing the growth of ice crystals at sub-zero temperatures [15]. AFPs, which protect the organisms against cold by increasing freezing tolerance, have two main functions. The first is the reduction of ice recrystallization, while the second is thermal hysteresis activity, which lowers the freezing point of body fluids [10,16]. The difference between the melting and hysteric freezing point is known as thermal hysteresis, a parameter that reflects the activity level of AFPs [9]. Each AFP has its own characteristic thermal hysteresis value [10]. Insects, which often live in areas with very low temperatures, have hyperactive AFPs with a high thermal hysteresis value [17].

Ice-binding proteins, which ensure the survival of organisms under hypothermic conditions by maintaining the structural integrity of cells and tissues, were known as antifreeze proteins in fish and thermal hysteresis proteins (THP) in insects [18,19]. Today, the terms AFP and THP are presented as alternative names in studies and databases [19-22].

Within the class of Insecta, 65% of known antifreeze proteins belong to the order Coleoptera [13]. The first insect AFPs were discovered in *Tenebrio molitor* (Tenebrionidae) and *Dendroides canadensis* (Pyrochoridae), both members of Coleoptera [6]. Similar insect antifreeze proteins are known to contain conserved amino acid units of 12-mer repeats (TCTxSxxCxxAx / ACTxSxxCxxAx), with regularly repeating T-C-T (Thr-Cys-Thr) amino acids occurring in these repeat units [6,22-25]. The AFPs of *Tenebrio molitor* and *Dendroides canadensis* species are also known to carry the aforecited 12-mer repeat sequences and show homology with 40-66% amino acid similarity [22,25,26].

Tenebrionid species have an annual life cycle. In this cycle, adults die shortly after mating and laying eggs. Hatching larvae complete their development under the soil. For the most species, they spend the cold period in the pupal stage, and with the onset of spring, they pass into the adult form [27]. *Cylindrinotus charlesi* Nabozhenko, 2015, *Odocnemis anatolica* Pic, 1899, *Nalassus clavicornis* Allard, 1876, and *Xanthomus ovulus* Seidlitz, 1895 (Coleoptera, Tenebrionidae) are exposed to sub-zero temperatures at certain times of the year during their developmental stages and are generally able to live under stones near snow or buried in cold dune areas in winter or early spring. Recent studies [28-31] indicate that *Cylindronotus charlesi* inhabits alpine zones and higher altitudes, while *Odocnemis anatolica* is typically found from the subalpine to lower alpine zones. *Nalassus clavicornis* occurs in forested habitats of low mountainous regions, particularly during the cold season. In contrast, *Xanthomus ovulus* is a dune beetle that resides at sea level and exhibits seasonal activity during colder periods, especially in winter and early spring. Despite their altitudinal differences, all four species share a common ecological trait: they are active during cold weather at specific times of the year.

Although antifreeze protein studies have been carried out on various Coleoptera species (Supplementary Table 1), there is an absence of such studies focusing on Helopini species, despite their frequent presence in cold and humid habitats. The main aim of this study was to reveal the presence of antifreeze proteins in *Cylindrinotus charlesi*, *Odocnemis anatolica*, *Nalassus clavicornis*, and *Xanthomus ovulus* species that prefer cold habitats.

Materials and Methods

Sampling and DNA Isolation

In addition to adult Helopini species, *Tenebrio molitor* species were also included in the study material for comparison. Information on the species is presented in Table 1. Genomic DNA was isolated using the Promega Kit (A1120 Wizard® Genomic DNA Purification Kit) following the manufacturer's protocol.

Table 1. Collection regions, altitudes, and dates of the samples.

*MSL Code	Species Names	Collection Region	Coordinate	Altitude	Collection Date
E5	*Cylindronotus charlesi* (Nabozhenko, 2015)	Arpaçay, Kars	40° 55' 9" N 43° 14' 41" E	2091 m	28.06.2020
B1	*Nalassus clavicornis* (Allard, 1876)	Hatila, Artvin	41° 10' 43.6" N 41° 44' 11.3" E	490 m	27.05.2012
G10	*Odocnemis anatolica* (Pic, 1899)	Hasan Dağ, Aksaray	38° 07' 07.5" N 34° 15' 27.2" E	1900 m	14.04.2014
E1	*Xanthomus ovulus* (Seidlitz, 1895)	Altınkum, Çeşme, İzmir	38° 16' 14" N 26° 15' 34" E	3 m	05.02.2021
D1	*Tenebrio molitor* (Linnaeus, 1758)	Bornova, İzmir	38° 45' 96.2" N 27° 23' 20.5" E	10 m	08.12.2021

*MSL (Molecular Systematics Laboratory)

Primer Design

Antifreeze and thermal hysteresis proteins were searched separately in model organisms of the Tenebrionidae family on the NCBI platform [20]. Among the tenebrionids with whole genome data recorded, THP was only recorded in *Tribolium madens* and *Tenebrio molitor* species, while AFP was only recorded in *Tenebrio molitor* species. Therefore, it aimed to design a primer with reference to the *Tenebrio molitor* AFP gene. Antifreeze proteins in the *Tenebrio molitor* genome were found to repeat very frequently and have very similar base sequences. For the primer pair to be designed; the 302 nucleotide coding antifreeze protein gene region between the bases 14172892 - 14173194 was selected (CAH1377342). This region was BLAST searched against all other Tenebrionidae genomes. A 93% similarity was found with *Tribolium madens* THP (NW_025401006_LOC123007010) gene. A primer was designed with the "pick primer" option among the tools of BLAST. The sequence and melting temperatures (Tm) of the primers are given in Table 2.

Table 2. Primers used for PCR analysis and their melting temperatures.

Primer Names	Primer Sequence (5'–3')	Length	Tm Degree	Amplified Sequence Length
Forward primer (TmadF)	ACTGGSGGTGCTGATTG	17	55.2 °C	243 bp
Reverse primer (TmadR)	TCCGGGACATCCTGTTG			

The designed primer pair was tested by in silico PCR in the *Tribolium madens* genome and amplified 243 base pair regions of the genome (NW_025401006.1), ranging from 4317391 to 4317633. In addition, the TmadF - TmadR primer pair was tested on the OligoCalc platform for potential hairpin structure, the presence of 3' complementary sequences and self-annealing. According to the result, synthesis of the primer pair was found appropriate (Supplementary Figure 1).

PCR Analyses and Agarose Gel Electrophoresis

In the PCR stage, Master Mix was prepared with a final volume of 25 µl. 12 µl Trehalose, 2,5 µl NH4 Buffer, 1,25 µl MgCl2, 0,5 µl Forward Primer, 0,5 µl Reverse Primer, 0,5 µl dNTP, 0,1 µl Taq, 2 µl genomic DNA, and ddH2O was added to the remaining amount according to the final volume. Touchdown PCR conditions for TmadF - TmadR were: 95°C for 5 min, 8 cycles of 60°C to 56°C, and 32 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. In PCR applications; MSL codes were used to represent the species. These codes are presented as D1 (*Tenebrio molitor*), B1 (*Nalassus clavicornis*), E1 (*Xanthomus ovulus*), E5 (*Cylindronotus charlesi*), and G10 (*Odocnemis anatolica*).

The bands obtained as a result of PCR applications were colorless and more than one band was seen in the D1 sample, so isolation from the gel was planned (Supplementary Figure 2). Each band was individually cut, placed in 1.5 ml microcentrifuge tubes and kept overnight at -24°C. Tubes removed from the freezer were quickly centrifuged at 14000 rpm for 5 minutes at room temperature. After centrifugation, approximately 3 µl of liquid supernatant accumulated at the bottom was taken as 'template DNA'. PCR was performed again with the template DNAs and visualized after 1% agarose gel electrophoresis (Figure 1).

Figure 1. Bands obtained after isolation from agarose gel.

A total of six PCR products (B1 *Nalassus clavicornis*, G10 *Odocnemis anatolica*, *Tenebrio molitor* D1_1, *Tenebrio molitor* D1_2, E1 *Xanthomus ovulus*, and E5 *Cylindronotus charlesi*) were submitted to Eurofins Scientific (Ebersberg, Germany) for Sanger sequencing.

Molecular and Data Analyses

The sequences were edited using Geneious 10.2.6 program. Multiple sequence alignments were performed using the "MUSCLE" algorithm implemented using the same program. Antifreeze proteins (AFPs) were classified at the superfamily and family levels using the SuperFamily database, which utilizes Hidden Markov Models (HMMs) derived from the "SCOP (Structural Classification of Proteins)" framework [32]. AFP motif repeats were analyzed on the MotifFinder platform [33]. Motif analysis was conducted on amino acid sequences using the "Pfam" database, applying an E-value cut-off threshold of 0.01 to ensure statistical significance. During the analysis, options to "skip frequently matching (unspecific) profiles" and to include "PROSITE Profile" searches were selected to enhance specificity and functional annotation. Hypothetical model prediction on 3D protein with homology modeling on Swiss-Model platform [34]. Finally, a similarity search was performed with the AFP nucleotide sequences against the nucleotide archive in the NCBI database [20].

Results

The nucleotide (Supplementary Table 2) and amino acid (Supplementary Table 3) sequences of Helopini AFP and *Tenebrio molitor* AFP in fasta format are presented with their lengths.

Conserved amino acid repeats in the sequences of the aligned Helopini AFPs (TCTxSxxCxxAx / ACTxxxxCxxAx) are shown using the Geneious program (Figure 2).

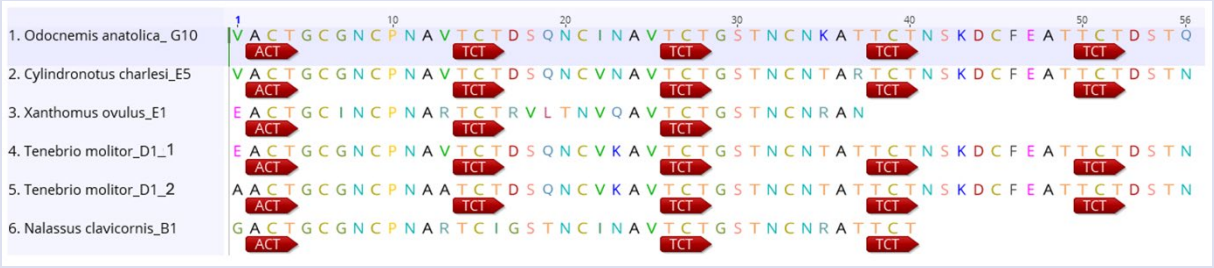


Figure 2. TCT repeat regions of the amplified Antifreeze proteins.

The amplified *Tenebrio molitor* and Helopini AFPs were identified using the SuperFamily database [32] as belonging to the insect cysteine-rich antifreeze protein family (Figure 3).

Sequence	Start Residue	End Residue	Superfamily	Superfamily E-value	Family	Family E-value
G10_Odocnemis_anatolica	1	68	Insect cysteine-rich antifreeze protein	6.15e-30	Insect cysteine-rich antifreeze protein	2.23e-05
B1_Nalassus_clavicornis	1	50	Insect cysteine-rich antifreeze protein	5.20e-20	Insect cysteine-rich antifreeze protein	0.00010
D1_1_Tenebrio_molitor	1	80	Insect cysteine-rich antifreeze protein	5.93e-34	Insect cysteine-rich antifreeze protein	4.96e-06
E1_Xanthomus_ovulus	1	46	Insect cysteine-rich antifreeze protein	3.95e-15	Insect cysteine-rich antifreeze protein	0.00038
D1_2_Tenebrio_molitor	1	79	Insect cysteine-rich antifreeze protein	2.20e-33	Insect cysteine-rich antifreeze protein	5.93e-06
E5_Cylindronotus_charlesi	1	66	Insect cysteine-rich antifreeze protein	5.49e-28	Insect cysteine-rich antifreeze protein	1.74e-05

Figure 3. Family predictions of the amplified Antifreeze proteins.

Repetitive 'AFP motifs' present in the amino acid sequences of Helopini AFPs and *Tenebrio molitor* AFP were determined using the MotifFinder platform, a tool for searching motifs in the sequence [33] (Figure 4).

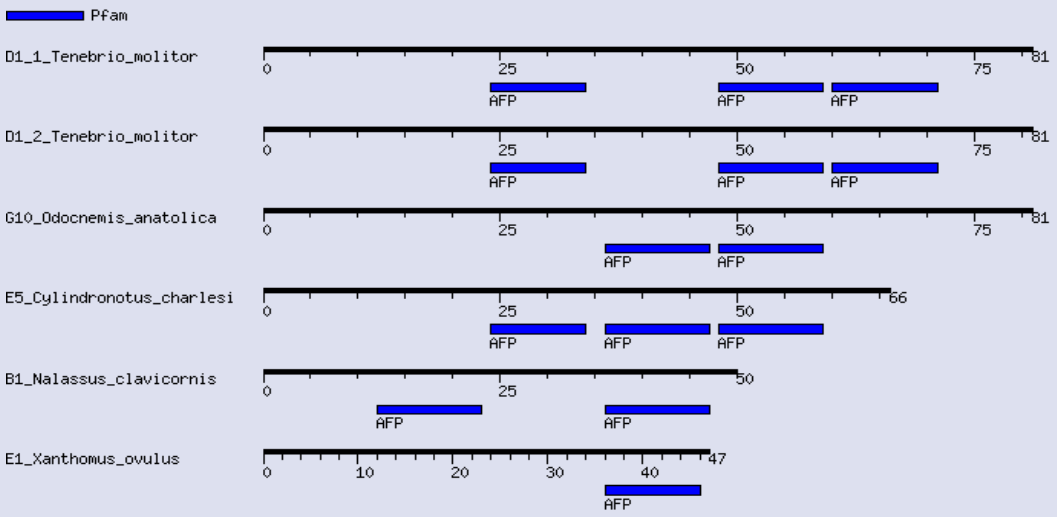
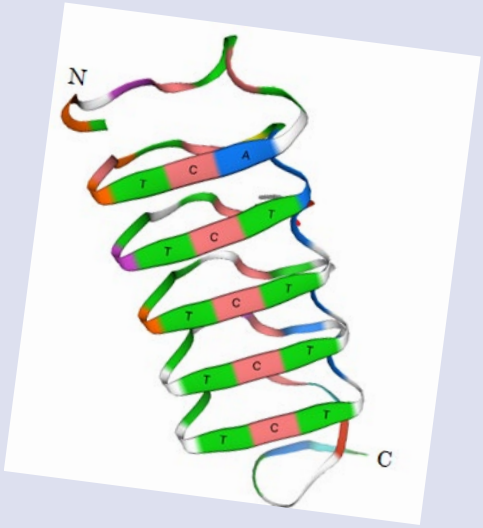


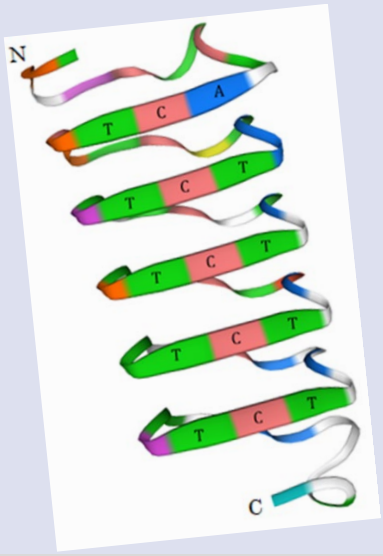
Figure 4. Antifreeze protein motifs shown with the MotifFinder tool.

For *Tenebrio molitor, Odocnemis anatolica, Cylindronotus charlesi, Xanthomus ovulus,* and *Nalassus clavicornis* antifreeze proteins, A-C-T / T-C-T repeats are shown on hypothetical models presented with 3D structure prediction using the Swiss-Model platform [34] (Figure 5). Hypothetical AFP models were found to have a β-sheet structure and conserved A-C-T / T-C-T amino acid repeats were located on this β-sheet. In all hypothetical models presented in Figure 5, the A-C-T motif seen in the first helix starting from the N terminal is followed by a T-C-T motif until the C terminal. Only in the *Nalassus clavicornis* antifreeze protein, the amino acid corresponding to the 2nd helix region was observed to change from Threonine (Thr-T) to Isoleucine (Ile-I).

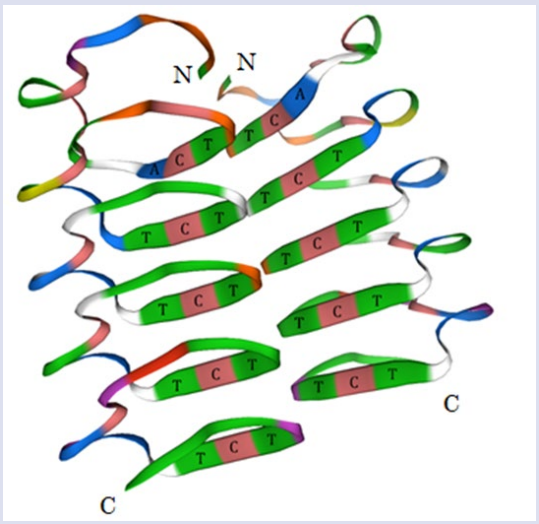
Tenebrio molitor



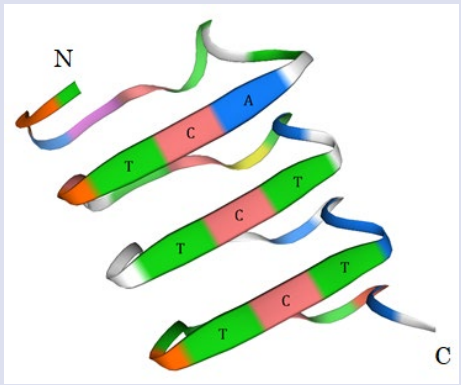
Odocnemis anatolica



Cylindronotus charlesi



Xanthomus ovulus



Nalassus clavicornis

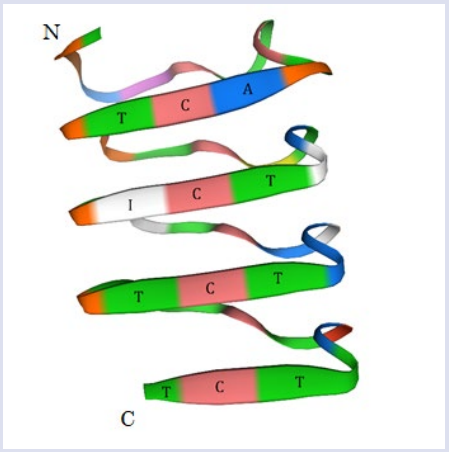


Figure 5. Hypothetical models of *T. molitor* and Helopini AFPs with 3D structure prediction.

Finally, a similarity table was created for each species by comparing the Helopini species antifreeze protein sequences with the beetle antifreeze protein sequences in the databases (Supplementary Table 4-7). Accordingly, Helopini AFP sequences were found to be similar to Tenebrionidae (*Tenebrio molitor*, *Blaps kashgarensis*, *Microdera dzhungarica punctipennis*), Lucanidae (*Dorcus parallelipipedus*, *Dorcus rectus rectus*, *Dorcus hopei binodulosus*), Carabidae (*Harpalus rubripes*), Curculionidae (*Dendroctonus ponderosae*), and *Xanthostigma xanthostigma* (Raphidiidae; Neoptera). In addition to the AFPs and THPs already registered in the database, the hits found to be similar include sequences that have not yet been associated with any protein, restricted to the level of chromosome data. Furthermore, Helopini AFPs showed high sequence similarity (99% - 75%) with AFPs and THPs registered in the database.

Discussion

As a result of homology-based screening, the presence of antifreeze proteins were identified for the first time in *Cylindrinotus charlesi*, *Odocnemis anatolica*, *Nalassus clavicornis*, and *Xanthomus ovulus* (Tenebrionidae, Coleoptera), all of which are adapted to cold environments. Hypothetical 3D structures of these proteins were predicted with the amino acid sequences amplified. Sequence similarity between the AFPs of these Helopini species and those of other beetle species available in the public databases were compared. The discovery of AFP sequences in Helopini species- whose genomes have not yet been sequenced- suggests that the primers designed in this study may serve as candidate primers in the amplification of other antifreeze protein isoforms in the future research. In addition, since the antifreeze protein sequences obtained from *Cylindrinotus charlesi*, *Odocnemis anatolica*, *Nalassus clavicornis*, and *Xanthomus ovulus* species will be registered in international bioinformatics databases, making them universally accessibe for use in other studies.

The conserved ACTxSxxCxxAx - TCTxSxxCxxAx amino acid repeats previously reported by Duman (2015), Davies and Sykes (1997), Graether et al. (2000), and Graham et al. (2007) were also identified in the antifreeze protein sequences of *Cylindrinotus charlesi*, *Odocnemis anatolica*, *Nalassus clavicornis*, and *Xanthomus ovulus* species [6,23-25].

D1_1 and D1_2 sequences isolated from *Tenebrio molitor* (D1), produced multiple bands in PCR applications, were found to have similar sequences. Replication of more than one similar sequence from the same individual may be explained by heterozygosity, where an individual carries two different alleles for a particular gene. Furthermore, the multilocal positioning of *Tenebrio molitor* antifreeze proteins in the genome, as reported in Graham et al. (2007), may lead to amplification of multiple similar sequences [25].

Homology modeling can be helpful way to understand structures of unknown proteins. *Tenebrio molitor* and Helopini AFPs, whose hypothetical 3D structures were predicted by homology modeling on the Swiss-Model platform [34], were found to have a β-sheet structure. Amino acid repeats corresponding to the same orientation of the β-sheets start from the N terminal and continue to the C terminal. In all AFPs except *Nalassus clavicornis*, the A-C-T motif is seen in the first helix, followed by a repeating T-C-T motif. Liou et al. (1999) and Graham et al. (2007) also reported that the β-sheets of each of the conserved amino acid repeats in insect antifreeze proteins correspond to the same orientation and that T-C-T amino acid repeats form the ice binding surface [22,25].

It is known that the antifreeze proteins of the Coleoptera species; *Tenebrio molitor* (Tenebrionidae) and *Dendroides canadensis* (Pyrochoridae) species carry 12-mer repeat sequences and show homology with 40-66% amino acid similarity [22,25,26]. It was observed that both antifreeze proteins have a β-sheet structure and these AFPs also showed homology by carrying the A-C-T / T-C-T repeats. Similarly, the Helopini antifreeze proteins (AFPs) identified in this study contain the conserved repetitive amino acid motifs ACTxSxxCxxAx and TCTxSxxCxxAx, showing homology with the AFPs of *Tenebrio molitor* and *Dendroides canadensis*.

Protein modeling includes various bioinformatics methods and tools used to predict the three-dimensional structure of proteins. Determining the accuracy of protein modeling is performed by a combination of multiple methods and tools (Ramachandran Plot Analysis, ProSA, Verify3D GROMACS, AMBER etc.). At the end of each validation process, the biological significance and reliability of the model is determined. The use of these methods helps to model protein structures more accurately and reliably. Therefore, future studies aim to determine the accuracy of the hypothetical 3D models of amplified Helopini AFPs.

The amplified Helopini AFPs showed homology with the "insect cysteine-rich antifreeze protein family". The cysteine (Cys-C) amino acid has a sulfur atom (-SH) in its side chain. These -SH groups lose H, resulting in the formation of S-S bonds (disulfide bridges). The formation of these bonds is a reversible reaction [35]. In particular, proteins that are subjected to a lot of physical and chemical influence outside the cell protect their structures with disulfide bridges. Disulfide bonding is known to play a role in protein stabilization and is important for high thermal hysteresis activity [36]. It has also been observed that the addition of disulfide bonds to proteins reduces thermal unfolding and denaturation by increasing the activation energy barrier, thus preserving the hydrophobic structure [37]. In a study on disulfide mapping of antifreeze proteins in *Tenebrio molitor* AFP [38], it was indicated that the entire cysteine residue in antifreeze proteins is disulfide bridged and is internally linked by disulfide bridges at 12-mer amino acid repeats. It is likely that disulfide bridges are also present in the Helopini AFPs amplified in this study. In fact, it is already known that most hyperactive antifreeze proteins contain more than one disulfide bond, thus being one of the factors contributing to the stability and thermal hysteresis activity of the protein [37].

In conclusion, this study is consistent with the existing research in the literature and supports the relevant theoretical framework. In this study, the conserved repetitive motifs in the antifreeze protein sequences of *Cylindrinotus charlesi*, *Odocnemis anatolica*, *Nalassus clavicornis*, and *Xanthomus ovulus* were identified for the first time. Thus, the presence of antifreeze protein in the tribe Helopini species were revealed

also for the first time in this study. The findings contribute to the understanding of the cold tolerance mechanisms of these species and provide new data compatible with the existing studies in the literature.

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Ethical Approval Statement

This work does not require ethics committee approval.

Conflicts of interest

There are no conflicts of interest in this work.

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