Discovery of Hyperactive Antifreeze Protein from Phylogenetically Distant Beetles Questions Its Evolutionary Origin

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Abstract: Beetle hyperactive antifreeze protein (AFP) has a unique ability to maintain a supercooling state of its body fluids, however, less is known about its origination. Here, we found that a popular stag beetle Dorcus hopei binodulosus (Dhb) synthesizes at least 6 isoforms of hyperactive AFP (DhbAFP). Cold-acclimated Dhb larvae tolerated −5 °C chilled storage for 24 h and fully recovered after warming, suggesting that DhbAFP facilitates overwintering of this beetle. A DhbAFP isoform (~10 kDa) appeared to consist of 6–8 tandem repeats of a 12-residue consensus sequence (TCTxSxNCxxAx), suggesting that DhbAFP facilitates overwintering of this beetle. A DhbAFP isoform (~10 kDa) appeared to consist of 6–8 tandem repeats of a 12-residue consensus sequence (TCTxSxNCxxAx), which exhibited 3 °C of high freezing point depression and the ability of binding to an entire surface of a single ice crystal. Significantly, these properties as well as DNA sequences including the untranslated region, signal peptide region, and an AFP-encoding region of Dhb are highly similar to those identified for a known hyperactive AFP (TmAFP) from the beetle Tenebrio molitor (Tm). Progenitor of Dhb and Tm was branched off approximately 300 million years ago, so no known evolution mechanism hardly explains the retainment of the DNA sequence for such a long divergence period. Existence of unrevealed gene transfer mechanism will be hypothesized between these two phylogenetically distant beetles to acquire this type of hyperactive AFP.

Keywords: antifreeze protein (AFP); thermal hysteresis (TH); stag beetle; ice binding; freeze avoidance; supercooling; tandem repeat; evolutionary origin

1. Introduction

Stag beetles include approximately 1200 species of insects belonging to the family Lucanidae, characterized by a pair of long horns, which resemble antlers of a stag, protruding from the mandibles of the male (Figure 1A) [1,2]. Stag beetles established immense popularity in Japan [3,4] as evidenced by cartoons, trading card games, fan websites, or the Japanese Samurai helmet of the 1500s, which was decorated with an ornament called “Kuwagata” designed after the stag beetle (Supplementary Material, Figure S1). Among them, Dorcus hopei binodulosus (Dhb) (Figure 1A,B) is especially popular because of its stout large body [5]. The Dhb beetle is found in northeastern China, Korea, and Japan that have an icy winter season, while it uniquely possesses a long-life overwintering nature; larvae live for 1–2 years until pupation in a rotten tree or underground, and the adult lives for 3–5 years on the ground [2,5]. Because hyperactive antifreeze protein (AFP) discovered...
in fishes, insects, and bacteria is known to facilitate their cold-survival [6], we speculated whether the Dhb beetle produces this protein in its body fluids. Since only four beetles, namely Tenebrio molitor, Dendroides canadensis, Anatolica polita, and Microdera punctipennis, are known to synthesize hyperactive AFP composed of a consensus sequence [7–10] and all of them belong to the same superfamily Tenebrionoidea, less is known about the origins of this protein, while the Dhb beetle belongs to a different superfamily Scarabaeoidea [2]. Therefore, if Dhb contains hyperactive AFP, the discovery provides a first clue to understanding the origin of this protein as well as new resources (i.e., stag beetles) to explore more new species of hyperactive AFP.

Figure 1. A stag beetle Dorcus hopei binodulosus (Dhb) and a bursting ice-crystal growth observed for its hemolymph at the freezing point. (A) An adult form of Dhb and (B) its final instar larva. The adult is uniquely equipped with a pair of antlers like a stag. (C) A photomicroscope image of a single ice crystal in ordinary supercooled water (−0.1 °C), which shows a rounded disk-like morphology. (D,E) The disk expanded when the temperature was held, implying that there is no antifreeze substance. (F) A photomicroscope image of a single ice crystal prepared in larval hemolymph, which kept unchanged even if the temperature was changed between −5.0 and 0 °C because of the ice-binding of hyperactive AFPs. The hyperactive AFP-bound ice crystal is uniquely modified into a lemon-like shape. (G) Bursting ice growth occurred from a tip of the lemon crystal when it further lowered the temperature to freezing point (−5.1 °C). (H) The bursting ice growth continues to show a vein-like pattern, which is also a known phenomenon for hyperactive AFPs.

Aqueous solutions that remain in liquid phase by lowering the temperature below the melting point are called supercooled solutions, in which numerous “embryo” ice crystals are present dispersively [11,12]. When the temperature is further lowered and thermal movement of embryo ice crystals decreases, they aggregate rapidly to form a large cluster to nucleate numerous ice particles, thereby changing the water molecules into a multi-crystalline state, which is the general ice crystal structure. Note that a substance other than water molecules sometimes catalyzed the aggregation process [11]. The overwintering, cold-adapted insects have long been known for their ability to stabilize such a supercooled state of their body fluids at subzero temperatures, leading to protection from freezing [13]. The supercooled state, or semi-frozen state, is also stabilized with low molecular weight solutes in the body fluids, such as polyhydroxy alcohol (ex. glycerol), sugars (ex. trehalose), amino acids (ex. proline), and/or glycolipids. Insects that equip such cold-survival mechanisms are called freeze-avoidance species, which cannot recover once their body fluids are frozen entirely. In contrast, freeze-tolerant species can recover from the freezing of their body fluids, though usually only extracellular fluids are thought to be frozen by secreting proteinaceous ice nucleators that protects inside of cells from freezing [13]. The hyperactive AFP synthesized in the freeze-avoidance insects strongly arrests the growth of embryonic single ice crystals generated in supercooled water [7]. This mechanism indeed prevents aqueous solutions from freezing down to approximately −5 °C. This protein is therefore regarded as a novel cryoprotectant to realize quality preservation of vaccines, cells, and organs around −5 °C without formation of ice blocks [14]. Note that the AFP’s ability to stabilize the supercooling state is evaluated by a difference between freezing and melting points of the AFP solution, which is called thermal hysteresis (TH) [15].
The hyperactive AFP, *Tm*AFP, was first identified in 1997 from the hemolymph of the beetle *T. molitor* (*Tm*), which exhibited a significant TH activity (~5 °C) [7]. *Tm*AFP is a mixture of nine isoforms (Mw = 8.4–12 kDa), consisting of 7–11 tandem repeats of a 12-residue consensus sequence, namely TCTxSxNCxxAx, where x is any amino acid residue [16]. A homologous AFP isoform (*Dc*AFP), containing the “TCT sequence,” was identified from another beetle *D. canadensis* that shares 46%–66% sequence identity with *Tm*AFP isoforms, and is composed of the same 12-residue sequence repeats with occasional insertions of additional amino acids [13]. This 12-residue sequence is not identified in either fish-, plant-, fungi-, nor bacteria-derived AFP. The spruce budworm *Choristoneura fumiferona* is not a beetle, but synthesizes AFP (SbwAFP, 9 kDa) composed of the TxT sequence and exhibits a high TH activity (5 °C) [17]. Extensions of TxT, such as TxTxTxT, were identified for other hyperactive AFPs from the longhorn beetle *Rhagium inquisitor* (*Ri*AFP, Mw = 12.8 kDa) [18] and the inchworm of the geometer moth *Campaea perlata* (*iw*AFP, Mw = 3.5–8.3 kDa) [19]. Hence, TxT is considered a key structural motif for significant TH activity. The AFP from snow flea *Hypogastrura harveyi* Folsom (*sf*AFP, Mw = 6.5–15.7 kDa) [20], a primitive arthropod with six legs and no wings, is an exception. It consists of a repetitive glycine-rich sequence instead of TxT, which also exhibits high TH activity (5.8 °C).

We repeatedly bred the *Dhb* beetle for years to obtain the final (3rd) instar larvae, which were used for cold-survival experiments and detection of hyperactive AFP synthesis in their hemolymph. We found that *Dhb* larvae tolerate −5 °C-chilled preservation for 24 h and synthesize at least six isoforms of hyperactive AFP (*Dhb*AFPs), for which we performed mitochondrial DNA and amino acid sequence analysis, recombinant-protein preparation, TH activity measurement, structural modeling, and phylogenetic analysis. Significantly, all the data obtained for *Dhb*AFP exhibited significant similarity to those identified for a known hyperactive AFP from a phylogenetically distant beetle, the *T. molitor* (*Tm*AFP).

2. Results and Discussion

2.1. Hemolymph of Dorcus Hopei Binodulosus (Dhb) Exhibits Antifreeze Activity

The stag beetle examined in this study (Figure 1A) was originally named *Dorcus curvidens binodulosus* [21] which was classified based on morphology, ecology, and karyology. Re-evaluating its detailed morphology and genome-based classification based on mitochondrial DNA (mtDNA) suggested that this beetle should be named *Dorcus hopei binodulosus* [2]. This new classification received a consensus from the scientific community and has been adopted [2,22]. Here, we extracted mtDNA from an adult sample (Supplementary Material, Figure S2) and determined its 16S ribosomal RNA (16S rRNA) sequence, which comprises 981 base pairs. This sequence exhibited 99.2% identity with the 16S rRNA sequence of *Dorcus curvidens binodulosus* (GenBank accession no. AB178292.1) [21] and was therefore designated *Dorcus hopei binodulosus*.

AFP secretion was examined in 0.8 µL hemolymph extracted from the final instar larvae of *Dhb*, which were raised according to the procedures shown in Supplementary Material, Figure S3. Of these, 40 were cold-acclimated at 4 or 10 °C in the last 2 months of breeding, and 11 were non-cold-acclimated at 25 °C during the last 2 months. The hemolymph droplet was flash frozen on the Linkam 10,002 L photomicroscope stage to form an assembly of numerous single ice crystals, from which we reduced to one single ice crystal by manipulation of the stage temperature near 0 °C [23]. We then applied slow temperature lowering (−0.1 °C min−1) on this ice crystal, which modified it into a lemon-like morphology (Figure 1C) and caused a vein-like bursting growth (Figure 1D,E). These are typical observations for a solution containing hyperactive AFP [6]. Notably, such changes were detected for +4 and 10 °C-acclimated larvae, while not for 25 °C-incubated larvae, indicating that *Dhb* does not synthesize AFP without cold-acclimation. The temperature at which the bursting ice growth occurred was evaluated as the freezing point. The melting point of the lemon-like single ice crystal was also evaluated by increasing stage temperature, which allowed TH evaluation [23]. The TH values of the hemolymph
were variable according to a development stage and a larval size, and maximal values of 1.7 ± 0.3 °C and 4.4 ± 0.2 °C were determined for +4 and 10 °C-acclimated final instar larvae, respectively. No significant TH value (0 °C) was detected for the hemolymph of non-cold-acclimated larvae. Graham et al. (1977) reported a detection of comparable TH activity (~5 °C) for the TmAFP-containing hemolymph [7]. The present observations hence suggest that Dhb preferably synthesizes hyperactive AFP (DhbAFP) by 10 °C-cold acclimation. The T. molitor larvae maximize TmAFP synthesis when acclimated at 4 °C [24], whereas 4 °C-acclimated Dhb larvae exhibited poor activity. We speculate that the Dhb larva finds it challenging to maintain its metabolic rate at 4 °C because of its large body (~20 g) compared with the T. molitor larva (<0.2 g), which leads to decreasing DhbAFP production at lower temperatures.

2.2. Dhb Larvae Tolerate −5 °C-Preservation for 24 Hours

To examine cold survivability of final instar Dhb larvae acclimated at 10 °C, we performed chilled preservation of Dhb larvae at −5 and −10 °C for 24 h. A dry plastic box (15 cm × 10 cm × 5 cm) containing naked larvae was placed in an incubator (model LTI-601SD, EYELA, Tokyo, JPN) preset at the required temperature (Supplementary Material, Figure S3). We took out the chilled vessel after the 24 h preservation period to evaluate larval recovery after warming them to room temperature (24 °C). All 10 larvae remained unfrozen by maintaining the supercooled state during the −5 °C-preservation and fully recovered after warming (Supplementary Material, Video S1). By contrast, at −10 °C, all 10 larvae froze and did not recover. We next performed the −5 °C-experiment with water droplets attached to larval skin. This droplet seeds ice crystals on the larval body and freezes them. The ice-seeding froze all 3 larvae during the 24-h preservation at −5 °C, and the frozen larvae never recovered. Note that reproducibility of these data was verified by using additional final instar larvae raised from different male-female pairs of Dhb captured in Osaka Japan. Asahina and Ohyama (1969) reported that larvae of some Japanese stag beetles overwinter within a small space of rotten wood, whose temperature was evaluated at −7 °C, whereas outside air was chilled to approximately −15 °C in the middle of winter [25]. They reported that beetle larvae remained unfrozen, whereas larvae of moths, namely Arctiidae and Noctuidae, froze in the same space. Although data on ambient temperatures of the Dhb larval habitat in the middle of winter are non-existent, the results suggest that Dhb tolerating up to −5 °C can be categorized as a freeze-avoiding species, where cold-protective substances of the hemolymph, including AFP, prevents freezing for cold-survival.

2.3. DhbAFP and Tenebrio Molitor AFP Show Significant Similarities

We constructed a cDNA library of Dhb according to the procedures described in Materials and Method. We synthesized primers based on the DNA sequences encoding TmAFP and DcAFP [8,16]. Partial sequence determination of the DNA amplified using the forward- and reverse-primers and extension of that sequence using an improved version of the primers enabled us to identify at least 6 isoforms of AFP in the final instar larva of Dhb (Figure 2). Alignment of the amino acid sequence with known beetle AFP isoforms is shown in Supplementary Material, Figure S4. The data revealed that Dhb synthesizes six isoforms of DhbAFP, which are thought to exist as a mixture in the hemolymph, similar to T. molitor and D. canadensis. Many organisms, including fish, plants, and fungi, also synthesize multiple isoforms of AFP, a mix of which exhibits higher TH activity than any single isoform [26,27]. The first 28 residues of all DhbAFP isoforms were designated as the signal peptide, similar to TmAFP, suggesting that DhbAFP isoforms are secreted into an extracellular space. Notably, this 28-residue signal peptide was also conserved in TmAFP, but not in DcAFP. The nucleotide sequence of DhbAFP1 encoding the signal peptide especially shares 98.8% identity with the TmAFP isoform Tq (accession no. DQ229126.1.) [28] and their amino acid sequences are 100% identical. DhbAFP isoforms are composed of repetitive peptides from the 12-residue consensus sequence TCTxSxNCxxAx (x is any
amino acid residue) (Figure 2B), which differentiates the number of repetitions. A BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 5 May 2020)) revealed that the top 20 polypeptides whose nucleotide sequences shared significant similarity with DhbAFP isoforms were TmAFP isoforms, of which DhbAFP6 exhibited highest identity (90%) with the TmAFP isoform Tq. DNA sequence similarity between the 6 DhbAFP isoforms and 25 TmAFP isoforms was 82% ± 5% on average, whereas that between the 6 DhbAFP isoforms and 10 DcAFP isoforms was 67% ± 2%; DhbAFP isoforms are similar to TmAFP isoforms. The Blast search using DNA sequence of DhbAFP3 against the T. molitor genome (GCA_014282415.1) further showed that 3'-untranslated region (UTR) of these two DNAs exhibited 81.9% of high sequence identity (122/149 base). The other 5' and 3'-UTR sequences of DhbAFP also exhibited 80–85% of high sequence identity with those identified in the TmAFP genome. It should be noted that intron does not exist in the TmAFP genome sequence [29].

![Figure 2.](https://example.com/figure2.png)

**Figure 2.** Amino acid sequence of Dorcus hopei binodulosus (Dhb) AFP. (A) Sequence comparison between the six DhbAFP isoforms. Each consists of 6–8 tandem repeats of a 12-residue consensus sequence, in which “TCT” locating the 3 ranks of ice-like waters are highlighted with cyan. N-glycosylation sites (NxT/S, x is any residue) are highlighted with green. (B) The WEBLOGO plot (http://weblogo.berkeley.edu/) showing the consensus amino acid sequence based on the alignment of all DhbAFP sequences. The 12-residue consensus sequence TCTxSxNCxxAx was deduced from this plot. (C) Biochemical properties of the six DhbAFP isoforms. Number of tandem repeats, estimated molecular weights, and isoelectric points were evaluated for each isoform.

The 108-residue isoform DhbAFP1 is composed of eight tandem repeats of the 12-residue consensus sequence (Figure 2A). Moreover, it possesses the highest isoelectric point of all six isoforms (pI = 8.51, Figure 2C) due to the presence of five arginine residues, which may increase its activity with an activity enhancer [30]. DhbAFP2 and DhbAFP3 are composed of seven repeats of the consensus sequence. DhbAFP2 is the largest isoform (11.2 kDa) and contains 109 residues. The C-terminus of DhbAFP2 contains the 12-residue segment VLLLSKIIEHDD, which deviates from the repetition rule and does not exist in other AFPs. A highly similar sequence, namely VLLLSKIIEHDD, is also located in the C-terminus of the DhbAFP4 isoform. The presence of this sequence in only two isoforms indicates that it does not play a crucial role in ice-binding and works as a capping structure to stabilize the tertiary fold of these isoforms. In the 6th and 7th repeat of DhbAFP3, the conserved tripeptide sequence Thr-Cys-Thr (TCT) is replaced with Thr-Cys-Ile (TCI), which is not found in other TmAFP isoforms. DhbAFP 5 and 6 are composed of six repeats of the
consensus sequence. DhbAFP5 exhibited 79% similarity to the 84-residue TmAFP isoform denoted 4–9 [16], which is the most abundant isoform (~50%) in T. molitor. The smallest isoform DhbAFP6 (8.2 kDa) shares the highest similarity (80%) with another TmAFP isoform denoted 2–14. Another feature of DhbAFP isoforms is that the N-glycosylation site composed of the tripeptide NxT/S (x is any residue) exists within the last repeat of all DhbAFP isoforms (Figure 2A, highlighted in green). More glycosylation sites exist in the N-terminal region of DhbAFP1 and 6th repeat of both DhbAFP1 and DhbAFP3. The glycans do not cause steric interference during ice-binding in the other AFP species [16]. No significant effect of glycan on TH activity was verified for our recombinant isoform of DhbAFP2 that contains no glycan, as it was prepared with Escherichia coli expression system (Figure 3).

2.4. A DhbAFP Isoform Equips the Properties of Hyperactive AFP

The production of a small protein (<10 kDa) is often difficult; however, tagging it with thioredoxin (Trx) sometimes improved yield, since the resultant fusion protein tends to become more stable and soluble [31]. Here, recombinant DhbAFP2 (rDhbAFP2) was prepared as a 26 kDa fusion protein with a Trx-tag (Figure 3A). The “His-tag,” consisting of six consecutive histidine residues, was inserted between Trx and DhbAFP2. The His-tag binds rDhbAFP2 selectively to a Ni-NTA column, enabling its elution with imidazole buffer.

![Figure 3](image-url)

Figure 3. Preparation of a recombinant Dorcus hopei binodulosus (Dhb)AFP2 isoform as a fusion protein. (A) A schematic representation of rDhbAFP2 composed of thioredoxin (Trx), histidine tag (His), and the 109-residue DhbAFP2 isoform connected in tandem. (B) The UV absorbance (280 nm) profile of rDhbAFP2 obtained with High-Q anion-exchange chromatography using an NaCl gradient (0–70 mM) at 25 °C. The lemon-like ice crystal and thermal hysteresis (TH) was detected for the “active” peak. (C) SDS-PAGE of the rDhbAFP2 sample purified with Ni-column chromatography. (D) Concentration dependence of TH values of rDhbAFP2. The measurement was performed in triplicate to draw error bars representing standard deviation. (E) A photograph of a single ice-crystal hemisphere, on which fluorescent (orange) rDhbAFP2 adsorbs entirely to illuminate the whole surface under UV light.

A soluble fraction containing rDhbAFP2 was obtained using the Ni-NTA column and dialyzed against Tris-HCl buffer (20 mM, pH 8.0) at 25 °C overnight. Notably, the solution exhibited no sign of TH activity before dialysis, but it was detected after dialysis (Figure 1C–E). This indicates that during dialysis, the 18 cysteines of DhbAFP2 were oxidized to generate disulfide bonds, leading to a properly folded protein. Such refolding was also observed when the recombinant TmAFP isoform was dialyzed [32], which depends on both the dialysis period and incubation temperature. Following dialysis, the active solution was applied to a High-Q anion-exchange column, which eluted three peaks shown in
Figure 3B using an NaCl gradient (0–300 mM). The second peak exhibited TH activity and was applied to the Superdex 200 gel-filtration column. This gave us a fraction containing purified rDhbAFP2, which was verified as a single band on 15% tricine SDS-PAGE (Figure 3C). Notably, a position mismatch on SDS-PAGE was reported for recombinant TmAFP [32]. Purified rDhbAFP2 exhibited hyperbolic TH-dependence on protein concentration, which has been reported for all AFP species [12]. A TH value of approximately 3 °C was evaluated at 150 µM (Figure 3D), which is consistent with that obtained for a hyperactive isoform of TmAFP (3 °C at 200 µM) [32]. A single ice crystal modified into lemon-like morphology within this hysteresis gap and exhibited a vein-like bursting growth pattern, similar to those observed for the Dhb hemolymph (Figure 1C–E). Again, they are typical observations for hyperactive AFPs. Furthermore, the target ice plane of rDhbAFP2 was examined by observing its binding onto a single ice-crystal hemisphere of a golf-ball size (φ = 30 mm), which was prepared by using a plastic pipe and refrigerant circulator [33]. This ice-crystal hemisphere was immersed in rDhbAFP2 (0.1 mM) labeled with the fluorescent detergent tetramethylrhodamine and held at −0.8 °C for 2 h. Then, the ice hemisphere was pulled and photographed under ultraviolet (UV) light. The illumination was observed entirely on the single ice-crystal hemisphere (Figure 3E), indicating that rDhbAFP2 can bind to multiple ice planes, including prism, pyramidal, and basal planes, similar to known hyperactive AFPs [33]. The rDhbAFP2 was hence identified as a hyperactive AFP regardless of the Trx-tagging and removal of the glycosylation ability.

Davies et al. determined a 1.4-Å resolution crystal structure of TmAFP isoform 2–14 (1EZG.pdb) that appeared to form an extremely regular right-handed β-helix (Figure 4A) [34]. Each coil consists of the 12-residue consensus sequence T1stCTxSxNCxxAx12th, of which side-chain OH-groups of T1st and T3rd are aligned with another string of waters trapped in a trough, leading to the construction of three ranks of oxygen atoms along the β-helical axis [35]. This enabled us to construct the model structure of DhbAFP6 (Figure 4B,C) because it shares 80% sequence similarity with TmAFP isoform 2–14 [16] (Supplementary Material, Figure S4). By using the structural coordinates of TmAFP (PDB ID: 1EZG) as a template, the model structure of this DhbAFP6 isoform was constructed using the software MODELLER (http://salilab.org/modeller/ (accessed on 5 May 2020), Univ of California, San Francisco, CA, USA). PYMOL and CHIMERA (http://www.cgl.ucsf.edu/chimera/ (accessed on 5 May 2020)) were also used for visualizing and drawing the model. As shown in Figure 4B,C, a rounded rectangular-shaped molecule composed of 6-turns right-handed β-helices was readily modeled for DhbAFP6, on which the location of the 3 ranks of oxygen atoms were speculated to be similar to TmAFP, though their atom positions were not finely postulated. Formation of the 8 disulfide bonds (C8−C18, C15−C21, C27−C33, C39−C45, C51−C57, C63−C69, and C75−C81), which are thought to stabilize-helical formation were also assumed for DhbAFP6 (Figure 4B,C), on which sidechains of the consensus sequence are thought to be oriented toward “out1st-in-out-in-out-in-out-out-in-out12th” directions (Figure 4C). Among them, inner pointing residues C2nd, S3th, C6th, and A11th comprised a core region together with six disulfide bonds between C2nd and C8th. The two-dimensional array of oxygen atoms on TmAFP (Figure 4A) exhibited a perfect position match to the water’s oxygen atoms, constructing multiple ice-crystal planes [34], which can also be speculated for DhbAFP6. These structural features were adopted for the larger DhbAFP isoforms (Figure 2), as the TmAFP structure is artificially elongated by the addition of more coils. Marshall et al. (2004) observed TH activity for a series of artificially prepared β-helical AFPs consisting of 6–11 tandem repeats and reported a 10–100-fold gain in activity upon going from 6 to 9 repeats [36]. The activity however decreased for 10 and 11 repeats, ascribed to imperfections of the position match between the ice-binding site and ice lattice, which occurs upon addition of too many coils.
similarity to those identified for TmAFP (Supplementary Material, Figure S4).

Figure 4. A model structure of Dorcus hopei binodulosus (Dhb) AFP6. (A) The X-ray crystal structure of an insect AFP from the beetle Tenebrio molitor (TmAFP, PDB code = 1EZG) determined by Graham et al. [7]. Tandem repeats of TCT-containing sequence locate 3 ranks of oxygen atoms on side-chains T1st and T3rd as well as surface-bound waters (BW) trapped in a trough. (B) A structural model of the DhbAFP6 isoform constructed using the TmAFP structure as a template. DhbAFP6 and TmAFP share 80% sequence identity. (C) A structural view down the model of DhbAFP6 from N- to C-terminus. The inner core is assumed to form six disulfide bonds between C2nd and C8th and organize the 3 ranks of oxygen atoms on the TCT sequence.

2.5. Unrevealed Gene Transfer Mechanism May Exist between Dhb and Tm

The hyperactive AFPs with the TCTxSxNCxxAx sequence have been identified for four beetle species: T. molitor, D. canadensis, A. polita, and M. punctipennis [7–10]. The present examined Dhb should be a new member. In addition, our preliminary results suggest that another stag beetle, Dorcus rectus rectus (Drr), synthesizes at least 14 isoforms of hyperactive AFP (Supplementary Material, Figure S5), which also exhibit the same repetitive property to DhbAFP. Notably, Dhb and Drr belong to the Scarabaeoidea superfamily, whereas the above four beetles with the 12-residue consensus sequence belong to Tenebrionoidea (Figure 5). To investigate the hypothetical evolutionary relationship among these beetles, we prepared a maximum likelihood phylogenetic tree (Figure 5A) based on a total of 29 mRNA sequences of their AFP isoforms registered in the National Center for Biotechnology Information (NCBI) GenBank. In Figure 5A, Scarabaeoidea Dhb (6 red dots) is the closest to Tenebrionoidea T. molitor (11 cyan squares), which is consistent with the present identified similarities between DhbAFP and TmAFP. However, such hypothetical relationship is not supported by the most updated version of the fossil-calibration-based beetle phylogenetic tree (Figure 5B) [37]. The Figure 5B shows that superfamilies Scarabaeoidea and Tenebrionoidea belong to distant lineages, which branched off 250–300 million years ago and corresponds to the Permian glaciation period in the geological time scale [38]. One may speculate that AFPs predate speciation 300 million years ago and that most of the diverged beetles contain AFP genes. However, no DhbAFP-like sequence exists in the genome of another Scarabaeoidea beetle, Trypoxylus dichotomus, for example (NCBI Genbank code: BNES01000010.1). Again, this type of hyperactive AFP was only identified for 4 beetle species in the past decades [7–10]. DNA analysis further questions the evolutionary origin of DhbAFP and TmAFP. Namely, the DNA sequence of Dhb including untranslated region, signal peptide region, and a DhbAFP-encoding region exhibited a significant similarity to those identified for TmAFP (Supplementary Material, Figure S4).
Figure 5. Phylogenetic relationship between the beetles synthesizing the hyperactive AFP composed of the consensus sequence TCTxSxNCxxAx. (A) Maximum likelihood phylogram showing a hypothetical relationship among the beetles based on analysis of a mRNA sequence alignment. The tree was created with MEGA7 software (http://www.megasoftware.net/ (accessed on 5 May 2020)) based on the HKY + G model. It used 29 mRNA sequences of beetle hyperactive AFP isoforms whose NCBI accession codes are AB264317.1–AB264322.1 (D. h. binodulosus), AF1591144.1–AF160497.1 (T. molitor), GU358703.1–GU358704.1 (A. polita), AY821792.1–AY821793.1 (M. punctipennis), and AF179408.1–AF179416.1 (D. canadensis). The bootstrap values for 500 replications (<30 are not shown) was shown at the nodes. (B) The fossil-calibration-based phylogenetic relationship between beetle superfamilies presented in Emmanuel, F. A. et al. (2017) [37]. The T. molitor, D. canadensis, A. polita, and M. punctipennis [7–10] belong to Tenebrionoidea. The ones examined in this study, namely D. h. binodulosus (Dhb) and D. r. rectus (Drr), belong to the phylogenetically distant superfamily Scrabaeeoidea. Note that another beetle consisting of extensions of the TxT sequence, Rhagium inquisitor, belong to Chrysomeleoidea.

Such similarity of the DNA sequences does not support the ordinary vertical evolution, since the sequences should not be retained in each superfamily for an ultimately long divergence period of approximately 300 million years. Indeed, no sign of sequence identity was detected for non-coding region of DNA in two different Collembola species, which were speculated to have diverged in the Permian glaciation period [39].
It has been shown that different lineages of organisms sometime synthesize similar genes through “convergent evolution”, which occurs when similar traits are acquired under selective pressure [40]. An example is the antifreeze glycoprotein (AFGP) discovered in two unrelated polar fishes, Antarctic notothenioid and Arctic cod. In Antarctic notothenioid fish, AFGP appears to be derived from a small fragment of a pancreatic trypsinogen gene, whereas in the Arctic cod, the DNA sequence is different [41,42]. Thus, the DNA sequences in two different organisms were also not retained in convergent evolution.

A remaining possibility to explain the DNA and protein similarities between the two phylogenetically distant beetles will be an unrevealed kind of horizontal gene transfer, the movement of genetic material between unicellular and/or multicellular organisms [43]. Recent studies based on genome sequencing revealed the presence of foreign DNA sequences in the genetic material of several species of Lepidoptera [44]. Indeed, pro- and eukaryotic genes that moved through the horizontal gene transfer are expressed in such insect genomes [45]. Hence, it might be speculated that progenitors of Dhb and T. molitor have acquired foreign DNA encoding hyperactive AFP through unrevealed gene transfer mechanism mediated by an organism, despite Blast searches not showing any sign of hyperactive AFP synthesis in plants, fungi, nor bacteria DNA. One way to advance this study would be to discover more AFP-containing beetles and to clarify DNA sequence encoding their AFPs. Examples of the stag beetles that are popular and their breeding techniques are established include Dorcus titanus, Dorcus reboformoratus, Lucanus maculiformoratus, and Prosopocoilus inclinatus.

3. Materials and Methods

3.1. Construction of cDNA Library from Dhb Larva

A final instar larva of the stag beetle Dorcus hopei binodulosus (Dhb) acclimated at 10 °C was flash frozen with liquid nitrogen and homogenized with a mortar and pestle. The homogenate suspension (200 mg) was prepared using TRI reagent (2 mL, MERCK, Darmstadt, Germany) and Qiashredder (QIAGEN, Hilden, Germany). After removing the insoluble fraction from the suspension by centrifugation at 11,000 × g for 20 min, chloroform (400 µL) was added to the supernatant to precipitate unwanted proteins and DNA. All RNAs, denoted “total RNA,” of the beetle was obtained in the aqueous phase. Total RNA was precipitated by adding 2-propanol (1 mL) and recovered with DEPC-treated water, which prevents RNA degradation. The Oligotex-dT30 mRNA purification kit (TaKaRa, Shiga, Japan) was used to extract mRNAs from total RNA. Then, reverse transcription was performed using oligo (dT) primers and obtained mRNAs for 60 min at 42 °C. This primer selectively binds the poly adenine (A) region of mRNAs, leading to the generation of single strand cDNAs (ss-cDNAs) that are complementary to mRNA sequences. The obtained ss-cDNAs were then reacted with RNase H and DNA polymerase I for 2.5 h at 16 °C to form double strand cDNAs. We utilized the ZAP-cDNA synthesis kit (TOYOBO, Osaka, Japan) for these experiments. We did not know which cDNAs encode isoforms of DhbAFP at this stage. To identify them in the next step, a DNA segment called “Adaptor,” whose nucleotide sequence is mentioned in the kit, was specifically ligated to 5'- and 3'-ends of each cDNA by T4 DNA ligase overnight at 8 °C.

3.2. Sequence Determination of cDNA Encoding DhbAFP

The cDNA sequence that encodes an isoform of DhbAFP is described as follows: (I) 5'-Adap–UTR1–Met–signal sequence–DhbAFP–stop codon–UTR2–poly (A)–Adap-3', where Adap, UTR 1 and 2, and Met denote the Adaptor, untranslated region 1 and 2, and methionine, respectively. DNA→protein translation is initiated from Met. A mature DhbAFP is produced by the removal of a signal sequence located upstream (left-hand side) of the DhbAFP sequence. On the basis of known DNA sequences that encode hyperactive AFGPs from beetles Tenebrio molitor and Dendroides canadensis (TmAFP and DcAFP) [8,16], we initially prepared the following primer: (a) 5'-TGACTGGDGSTBCYGAYTGYMVHDSKTGYAC-3' (forward primer). This primer (a) should bind to “DhbAFP” in the sequence (I), if it
is a correct nucleotide sequence that can encode a protein whose cDNA exhibits high sequence similarity to that encoding TmAFP and/or DcAFP. We next prepared primer (b), which was designed to bind to the segment composed of “poly (A)–Adap” region in the sequence (I). (b) 5′-GAGAGAATCTGTCTCGAGTTT-3′ (reverse primer). Polymerase chain reaction (PCR) was performed with primers (a) and (b) and the cDNA library (see previous paragraph) using Ex taq DNA polymerase (TaKaRa Bio Inc, Tokyo, Japan) on our PCR instrument (GeneAmp PCR system 9700, Applied Biosystems, Foster city, USA). The following reaction cycle was used: 94 °C (1 min)–(94 °C (1 min)=50 °C (1 min)=72 °C (1 min)) × 30–72 °C (5 min)=4 °C (hold). Various cDNAs amplified with these primers, which are the promising candidates for cDNAs encoding DhbAFP isoforms, were obtained using agarose gel electrophoresis (a similar example shown in Figure S3-G). The obtained cDNAs were ligated to the pGEM-T Easy vector (Promega, Madison, USA) using the Mighty TA-cloning kit (TaKaRa). The resultant plasmid, including a cDNA candidate, was then transformed into competent Escherichia coli JM109. Following colony PCR with universal M13RV and M13M4 primers on the agarose gel, the plasmid was collected from positive clones using the QIAPrep Spin Miniprep kit (QIAGEN). We then determined their nucleotide sequence by employing BigDyeTM Terminator v3.1 cycle sequencing kit and ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA). We found that cDNAs collected at this stage contained partial sequences, composed of half-way portion to the stop codon–UTR2 segment, but did not include the signal sequence described in (I).

On the basis of the partial sequence of cDNA candidates that encode DhbAFP isoforms, we prepared the following primers: (c) 5′-TCGGGAATTCGGCACGAGG-3′ (forward primer), (d) 5′-ATAGCGGCCGCGGATCCTTAATGTCCGGGACATCCTG-3′ (reverse primer). Primer (c) was designed to bind to the upstream “5′-Adap” region in the cDNA sequence (I). Primer (d) binds to a region including the “stop codon–UTR2”. With these two primers, PCR performed using the cDNA library according to the procedures described in the previous paragraph. A problem in this experiment was that the primer (c) binds not only to “5′-Adap,” but also to the “Adap-3′” region of the sequence (I), leading to the amplification of a large number of cDNAs. Hence, the amplified DNA fragments in the size range of 500–700 bp were purified using agarose gel electrophoresis, which was the estimated size of the cDNA encoding DhbAFP isoforms. Sequence analysis of our obtained samples in this time gave us revealed information, including the signal sequence, of cDNAs.

Because we could obtain information about the cDNA candidates, including the signal sequence, encoding DhbAFP isoforms, we prepared the following primer: (e) 5′-GGAAACATATGCGCATCCTAAAGCTGTGCT-3′ (forward primer). This primer was designed to bind to the signal sequence of cDNAs encoding DhbAFP isoforms, whose start codon is indicated with underlined text. Hence, final PCR was performed against the cDNA library with primers (e) and (b). The original reaction cycle was slightly modified to 94 °C (1 min)–(94 °C (1 min)=56 °C (1 min)=72 °C (1 min)) × 30–72 °C (5 min)=4 °C (hold). We obtained 650 bp PCR products in this final PCR. By employing the sequence determination procedures described in the last half of the 1st paragraph of this section, we could determine at least six nucleotide sequences encoding DhbAFP isoforms, which were translated into amino acid sequences shown in Figure 2 and Supplementary Material, Figure S4.

3.3. Expression and Purification of rDhbAFP2 Isoform

A codon optimized DNA sequence encoding DhbAFP2 isoforms (Figure 2) tagged with thioredoxin (Trx) plus six consecutive histidines (His) (rDhbAFP2) was synthesized to include NdeI and NotI cleavage sites at 5′ and 3′ sites, respectively. Coexpression with the Trx-tag is known to facilitate expression of the beetle AFP from Anatolica polita [46]. We used this strategy to prepare rDhbAFP2 in a similar manner. Histidine residues enabled affinity chromatography with the Ni-NTA column. Following the digestion of the two cleavage sites, the obtained DNA fragment encoding rDhbAFP2 was inserted into the pET20b vector.
This vector was then transformed into *E. coli* BL21 (DE3). Transformants selected on antibiotic plates were inoculated into 20 mL LB medium containing kanamycin and cultured at 37 °C for 16 h. Harvested cells were transferred into LB medium (1 L) containing kanamycin and cultured at 37 °C for 2–3 h. When its OD600 value reached 0.4–0.8, the expression of rDhbAFP2 was induced by isopropyl-β-D-thiogalactopyranoside (0.5 mM). The obtained cells were further cultivated at 15 °C for 24 h, collected by centrifugation, resuspended in Tris-HCl buffer (0.1 M, pH 8.0) containing NaCl (0.1 M), and disrupted by sonication for 45 min. A soluble fraction of the cell lysate was obtained by centrifugation and loaded into the Ni-NTA column (QIAGEN) equilibrated with Tris-HCl (20 mM) containing NaCl (0.5 M). The rDhbAFP2 was eluted by the same buffer containing 250 mM imidazole. The collected fraction was dialyzed against Tris-HCl buffer (20 mM, pH 8.0) overnight and purified using anion-exchange High-Q (Bio-Rad) column chromatography. The column-bound protein was eluted using a linear gradient of NaCl (0–300 mM). The active fraction obtained from this anion-exchange chromatography was dialyzed against and purified by gel-filtration Superdex 200 (GE-Healthcare, Amersham, UK) chromatography. The purity of the protein was confirmed by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3C). Purified DhbAFP was dialyzed against Tris-HCl buffer (20 mM, pH 8.0) and stored at −20 °C until use.

3.4. Thermal Hysteresis (TH) Measurement for rDhbAFP2

The procedure to evaluate TH activity was described previously [26]. Briefly, hemolymph (0.8 µL) was placed in the middle of a HIRSCHMANN minicaps DE-M 18 glass capillary (φ = 0.92 mm) (HIRSCHMANN, Eberstadt, Germany). It was then soaked into a house-made capillary holder to set into a Linkam 10002L temperature-controlled photomicroscope stage (Linkam Science, London, UK) and observed under a Leica DMLB 100 photomicroscope system (Leica Microsystems AG, Wetzlar, Germany). The sample was flash frozen once to form a polycrystalline state of ice crystals by temperature reduction to −25 °C, and then warming to obtain a single ice crystal in that solution. Following 3-min incubation, this ice crystal was cooled again at the rate of −0.1 °C min⁻¹ until bursting ice-crystal growth occurred, whose temperature was determined as the non-equilibrium freezing point [14]. The measurement was performed at least three times and averaged values were evaluated with error bars.

3.5. Fluorescence-Based Ice Plane Affinity (FIPA) Measurement for rDhbAFP2

The FIPA analysis was performed according the published procedures [33]. Briefly, a single ice crystal (φ = 3 cm) was prepared with a cylindrical mold. After determining its c-axis using a polarizer, a half-cut of the cylindrical ice crystal was mounted on a hollow copper tube (φ = 15 mm), in which −0.8 °C coolant was circulated by a refrigerant pump (Hitachi AMS-007, Hitachi, Japan). The “ice pitting method” [33] generated a six-sided star mark on the polar region of the single ice-crystal hemisphere, which indicates the a1—a3 directions of the hexagonal ice unit under atmospheric pressure [47]. The hemisphere with known orientation is then mounted onto the −0.8 °C chilled probe to face down the desired ice plane. Following 1–2 h incubation with a 0.1 mg/mL solution of a fluorescence-labeled AFP sample, the FIPA pattern illuminated on the ice-crystal hemisphere was observed under UV light. The fluorescence dye used was tetramethylrhodamine (5(6)-TAMRA-X, SE) (Thermo Fisher Scientific, Waltham, USA), which attaches to a lysine residue (K103) of the rDhbAFP2 isoform.

4. Conclusions

The present study revealed that the popular stag beetle *Dorcus hopei binodulosus* (Dhb) synthesizes hyperactive AFP. Cold-acclimated Dhb larvae were not frozen after −5 °C-chilled preservation for 24 h and recovered after warming, thus the survival strategy of Dhb is freeze-avoidance. The larvae synthesize at least 6 hyperactive AFP isoforms (DhbAFPs), which are tandem repeat peptides of a 12-residue consensus sequence. The
DhbAFPs exhibited significant similarities to a known hyperactive AFP from *T. molitor* (*Tm*AFP), where progenitors of *Dhb* and *Tm* have diverged approximately 300 million years ago. Hence, any known evolution mechanism hardly explains the retainment of the DNA sequence, suggesting the existence of a recent gene transfer between these two beetles to share the hyperactive AFP.

**Supplementary Materials:** The following is available online at https://www.mdpi.com/article/10.3390/ijms22073637/s1.

**Author Contributions:** Conceptualization, Y.C.S. and S.T.; methodology, T.A., A.Y., A.M. and Y.N.; software, T.A.; validation, H.K., Y.C.S. and S.T.; formal analysis, T.A. and A.Y.; investigation, T.A. and A.Y.; resources, A.M. and H.K.; data curation, T.A.; writing—original draft preparation, T.A. and A.Y.; writing—review and editing, S.T.; visualization, H.K. and S.T.; supervision, Y.C.S. and S.T.; project administration, Y.C.S. and S.T.; funding acquisition, Y.C.S. and S.T. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by KAKENHI grant numbers 19H02529 and 19K22989 from Japan Society for the Promotion of Science (JSPS) (for S.T.).

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and carried out in accordance with relevant guidelines and regulations of the National Institute of Advanced Industrial Science and Technology (AIST), Japan. All experiments involving animals were conducted with approved methods designated in Guidelines for Proper Conduct of Animal Experiments, Science Council of Japan (Law No. 105, 1973).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The mRNA sequences of antifreeze protein isoforms from *Dorcus curvidens binodulosus*, which was later revised to *Dorcus hopei binodulosus*, are available at the National Center for Biotechnology Information (NCBI) GenBank (https://www.ncbi.nlm.nih.gov/genbank/ (accessed on 5 May 2020)) (Accession codes: AB264317.1–AB264322.1) and at the DNA Data bank of Japan (DDBJ) (https://www.ddbj.nig.ac.jp/ddbj/index-e.html (accessed on 5 May 2020)) (Accession codes: LC603133–LC603137). The mRNA sequences of 14 antifreeze protein isoforms from *Dorcus rectus rectus* are also available in DDBJ (Accession codes: LC598940–LC598953).

**Acknowledgments:** We acknowledge Peter L. Davies in Queen’s university for careful reading and giving critical comments on our original manuscript. We also thank Ahmed Al-Baloul for English editing on the final version of the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| AFP | Antifreeze protein |
| Dhb | *Dorcus hopei binodulosus* |
| Tm | *Tenebrio molitor* |
| TH | Thermal hysteresis |
| FIPA | Fluorescence-based ice plane affinity |
| NCBI | National Center for Biotechnology Information |

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