



IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF A SERINE PROTEASE INHIBITOR GENE IN THE KHAPRA BEETLE *Trogoderma granarium*

Aslı DAĞERİ^{1*}


¹Necmettin Erbakan University, Faculty of Science, Department of Molecular Biology and Genetics, 42090, Konya, Türkiye

Abstract: Insect serine protease inhibitors (ISPis) are essential for regulating various protease-mediated activities and play crucial roles in metabolism, metamorphosis, reproduction, and immunity. As a member of the ISPis, serpins are recognized as the most essential protease inhibitor family in higher eukaryotes, encompassing a diverse array of biological functions. They are involved in the Toll pathway, the prophenoloxidase cascade, development, immunity, and reproduction in all insects. In this study, a serpin from the khapra beetle, *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae) was identified and characterized using both transcriptomic and bioinformatics methodologies. The BGISEQ-500 platform was used to construct a cDNA library from *T. granarium*, which led to the identification and characterization of a novel Serine Protease Inhibitor gene (*TgSPI*). Sequence analysis confirmed TgSPI's classification within the serine protease inhibitor (SPI) superfamily. It has conserved features, including a Reactive Center Loop (RCL) close to the C-terminal end, which is essential for protease inhibition. Phylogenetic analysis and 3D structure modeling of TgSPI were performed using MEGA6 software and the Phyre2 Protein Fold Recognition Server, respectively. The phylogenetic analysis positioned TgSPI within a cluster of coleopteran insect SPIs (ISPis), supporting its evolutionary lineage. Predicted tertiary structure modeling of TgSPI revealed similarity to conserpin in the latent state. This study provides foundational information on the evolutionary patterns and structural-functional aspects of TgSPI in the khapra beetle and highlights probable role of TgSPI as a promising target for further genetic and functional studies aimed at sustainable pest control strategies.

Keywords: Gene characterization, Insect serine protease inhibitor, Khapra beetle, Serpin

*Corresponding author: Necmettin Erbakan University, Faculty of Science, Department of Molecular Biology and Genetics, 42090, Konya, Türkiye

E mail: adageri@erbakan.edu.tr (A. DAĞERİ)

Aslı DAĞERİ  <https://orcid.org/0000-0001-8564-9922>

Received: July 21, 2024

Accepted: September 05, 2024

Published: September 15, 2024

Cite as: Dağeri A. 2024. Identification and molecular characterization of a serine protease inhibitor gene in the Khapra beetle *Trogoderma granarium*. BSJ Eng Sci, 7(5): 982-987.

1. Introduction

Approximately one-third of all characterized proteolytic enzymes are classified as serine proteases, distinguished by the nucleophilic Serin residue at their active site (Hedstrom, 2002). Serine protease inhibitors (SPIs) are a superfamily of protease inhibitors essential for regulating various protease-mediated proteolysis activities in many organisms (Yang et al., 2017; Shakeel, 2021). These inhibitors play crucial roles in metabolism, metamorphosis, reproduction, and immunity (Yang et al., 2017; Li et al., 2022). Insect serine protease inhibitors (ISPis) are categorized as canonical/non-canonical SPIs, α -macroglobulins, and serpins (Li et al., 2022).

Serpins are a superfamily of comparatively large proteins, generally 350–500 amino acids long with a molecular weight of 45–50 kDa, characterized by three β -sheets and seven to nine α -helices (Shakeel et al., 2019). They engage in a suicide inhibition mechanism within the Reactive Center Loop (RCL), leading to the irreversible and permanent inactivation of both the serpin and the protease (Meekins et al., 2017). Following cleavage between the P1 and P1' residues, the serpin experiences a swift conformational change, incorporating the N-

terminal segment of the RCL into its core structure (Loebermann et al., 1984; Huntington et al., 2000). During this process, the protease, covalently attached to the RCL, is translocated, causing the distortion and inactivation of its active site; consequently, the resultant stable protease-serpin complex is then targeted for degradation (Stratikos and Gettins, 1999; Huntington and Carrell, 2001; Sanrattana et al., 2021).

Compared to higher mammals, insects possess a sophisticated innate immune system governed by continuous reaction cascades and developed through evolution to combat pathogen invasion. Serpins are crucial in modulating the proteolytic cascade, thereby enhancing the effectiveness of the insect's immune system when serine proteases are unnecessary (Liu et al., 2024). Distinct serpins have been identified and characterized from numerous insects, illustrating their critical roles in insect physiology. Shakeel (2021) reported that the fat body of *Helicoverpa armigera* (Lepidoptera: Noctuidae) is the primary expression site of serpin, with fluctuations observed during developmental stages. They also noted relatively high expression level during the prepupal stage, suggesting its prominent function at the wandering stage.



In another study, it was found that up-regulated serpin in *H. armigera* contributes to *Bacillus thuringiensis* (Bt) resistance by inhibiting trypsin and chymotrypsin activities (Zhang et al., 2022). Similarly, a Serpin from *Musca domestica* (Diptera: Muscidae) (MDSPI16) has been demonstrated to possess inhibitory activity against elastase and chymotrypsin (Tang et al., 2016). Furthermore, Serpin6 from *Bombyx mori* (Lepidoptera: Bombycidae) has been shown to regulate prophenoloxidase activity and the expression of antimicrobial proteins, suggesting its involvement in silkworm immune responses (Li et al., 2017).

The khapra beetle, *Trogoderma granarium* (Everts) (Coleoptera: Dermestidae) is a destructive quarantine pest that infests stored cereals and grain derivatives worldwide, particularly in South Asia and Africa, as well as in Mediterranean countries such as Türkiye and Cyprus (EPP0, 2024). It can enter long-term diapause, lasting up to eight years, which complicates management efforts. Furthermore, the development of insecticide resistance necessitates the exploration of alternative management methods. Specific genes expressed under various stress conditions, such as cold- and starvation-induced diapauses, have been reported to enhance the khapra beetle's tolerance, thereby identifying these genes as potential targets for pest management strategies (Dageri et al., 2023; Dageri, 2024).

To date, only a few serpin studies have been carried out in Coleoptera, such as *Tenebrio molitor* (Coleoptera: Tenebrionidae) and *Tribolium castaneum* (Coleoptera: Tenebrionidae) (Jiang et al., 2011), and no studies on any SPIs from *T. granarium* have been conducted yet. In *Tenebrio molitor*, 62 serine protease inhibitor (SPI) genes have been detected and classified into serpin, canonical SPI, and α -macroglobulins families, demonstrating roles in immunity, development, and digestion with stage-specific and tissue-specific expression patterns (Li et al., 2022). In the present study, a *serine protease inhibitor* gene (*TgSPI*) was identified from the cDNA library of the khapra beetle. Using various bioinformatics tools, the gene was characterized and found to belong to the serpin superfamily. Phylogenetic and tertiary structure analyses were conducted to provide a comprehensive understanding of this gene and its putative amino acid sequence.

2. Materials and Methods

2.1 RNA Isolation and cDNA Library Construction

RNA isolation was carried out from the khapra beetle using the NucleoZOL reagent (Machery-Nagel GmbH, Düren, Germany). The extracted RNA was evaluated using an Agilent 2100 Bioanalyzer instrument (Agilent Technologies Inc.) with the RNA6000 kit, which provided measurements of total RNA concentration, RNA integrity, 28S/18S ratios, and fragment length distribution. Transcriptome sequencing was conducted by Beijing Genomics Institute (BGI) China on a BGISEQ-500 platform sequencer following the manufacturer's instructions. Briefly, the sequencing process included mRNA

enrichment, RNA fragmentation and reverse transcription, end repair: "A" tailing and adaptor ligation, PCR amplification, denaturation and cyclization, sequencing on BGISEQ-500, sequencing reads filtering, *de novo* assembly and unigene functional annotation. Contigs were annotated via BLAST2Go suite (Conesa et al., 2005).

2.2. Bioinformatic Analysis

A full-length unigene cDNA encoding *serine protease inhibitor* gene (*TgSPI*) was detected from the transcriptome database of khapra beetle. BLASTX tool was used to search the similarity of *TgSPI* to other genes (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). To determine open reading frame of the nucleotide sequence of *TgSPI*, ORFFinder tool was utilized (<https://www.ncbi.nlm.nih.gov/orffinder>). The isoelectric point and molecular weight of the predicted protein were evaluated using pI/Mw tool (https://web.expasy.org/compute_pi/). SignalP-6.0 server was employed to detect predicted signal peptide (<https://services.healthtech.dtu.dk/services/SignalP-6.0/>). Putative phosphorylation residues were predicted using the NetPhos 3.1 server (<https://services.healthtech.dtu.dk/services/NetPhos-3.1/>). Multiple sequence alignment of the SPIs from different coleopteran insects was performed using Kalign (<https://www.ebi.ac.uk/jdispatcher/msa/kalign>) (Madeira et al., 2022). BoxShade tool was utilized to shade the conserved regions in amino acid sequences (<https://junli.netlify.app/apps/boxshade/>). Phyre2 was utilized to visualize the predicted secondary and tertiary protein structures (<http://www.sbg.bio.ic.ac.uk/phyre2/html>).

2.3. Phylogenetic Analysis

The evolutionary history was deduced using the Neighbor-Joining method (Saitou and Nei, 1987). The phylogenetic tree was drawn to scale in MEGA6 (Tamura et al., 2013), with branch lengths in the same units as the evolutionary distances used to infer it. The Poisson correction method (Zuckerandl and Pauling, 1965) was used to calculate evolutionary distances, which were measured in the units of the number of amino acid substitutions per site. The positions including gaps and missing data were eliminated. The percentage of replicate trees is displayed next to branches (Felsenstein, 1985).

3. Results and Discussion

As members of the ISPI family, serpins are found in nearly all eukaryotes and are occasionally present in microorganisms such as fungi and bacteria (Irving et al., 2000). They play a crucial role in the innate immunity of insects (Liu et al., 2024). Thus far, the number of studied coleopteran serpins remains limited (Jiang et al., 2011). In this study, the full-length cDNA of the serine protease inhibitor gene (*TgSPI*) was identified for the first time from the cDNA library of the khapra beetle, containing a predicted ORF of 1910 nucleotides encoding 550 amino acids. *TgSPI* showed 59.30% nucleotide identity to *serine protease inhibitor 28Dc* from another coleopteran insect,

Zophobas morio (Coleoptera: Tenebrionidae) Predicted amino acid sequence of TgSPI has an approximate molecular weight of 61.23 and a pI of 8.75. It includes a signal peptide of 17 amino acid residues (MLLKVLIFLAFCGFLEG). The amino acid sequence of TgISP was analyzed, revealing the presence of putative phosphorylation sites. The sequence was found to include potential phosphorylated residues at tyrosine (Y), serine (S), and threonine (T) positions. This suggests that TgISP may undergo phosphorylation, which could play a significant role in its functional regulation and interaction within the cell. Identifying these putative sites provides valuable insight into the potential regulatory mechanisms governing TgISP activity and its involvement in cellular processes. Furthermore, the putative TgSPI possesses a domain belonging to the serine protease inhibitor (serpin) superfamily, confirming its classification within this superfamily (Figure 1). Serpins are large proteins with conserved structures,

consisting of three β -sheets and seven to nine α -helices, and include signature motifs such as a Reactive Center Loop (RCL) that attaches to the active protease site, as well as a predicted P1-P1' cleavage site (Shakeel, 2021). Following the P1-P1' cleavage, the serpin goes through a substantial conformational shift caused by the insertion of the RCL into the central β -sheet A, leading to the inactivation of the protease through the distortion of its structure (Chamankhah et al., 2003). An RCL was found near the C-terminal end of the TgSPI, with the P1-P1' residues being highly conserved among coleopteran species, including *T. granarium* (Figure 2). This conservation might suggest a vital functional role for the RCL in *T. granarium*, potentially related to its involvement in protease inhibition. Furthermore, the existence of these conserved structural elements in TgSPI not only verifies its classification as a serpin but also implies its probable significance in the physiology and immune response of *T. granarium*.



Figure 1. Deduced amino acid sequence of TgSPI. The predicted signal peptide is marked by an underscore. Serpin domain is shown in a blue box (66-544 residues). Putative phosphorylated tyrosine (Y), serine (S), and threonine (T) residues are indicated by blue-shaded frames.

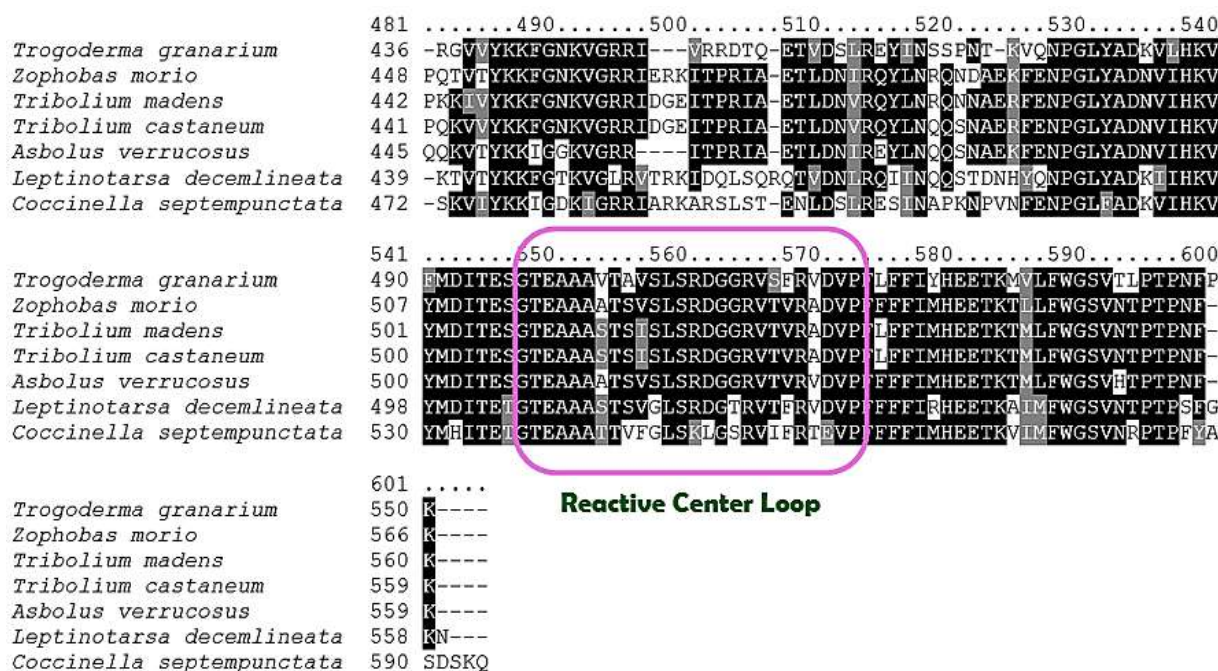


Figure 2. Multiple partial sequence alignment of the deduced amino acids of the coleopteran ISPIs, including TgSPI. The conserved Reactive Center Loop (RCL) was highlighted in a pink box.

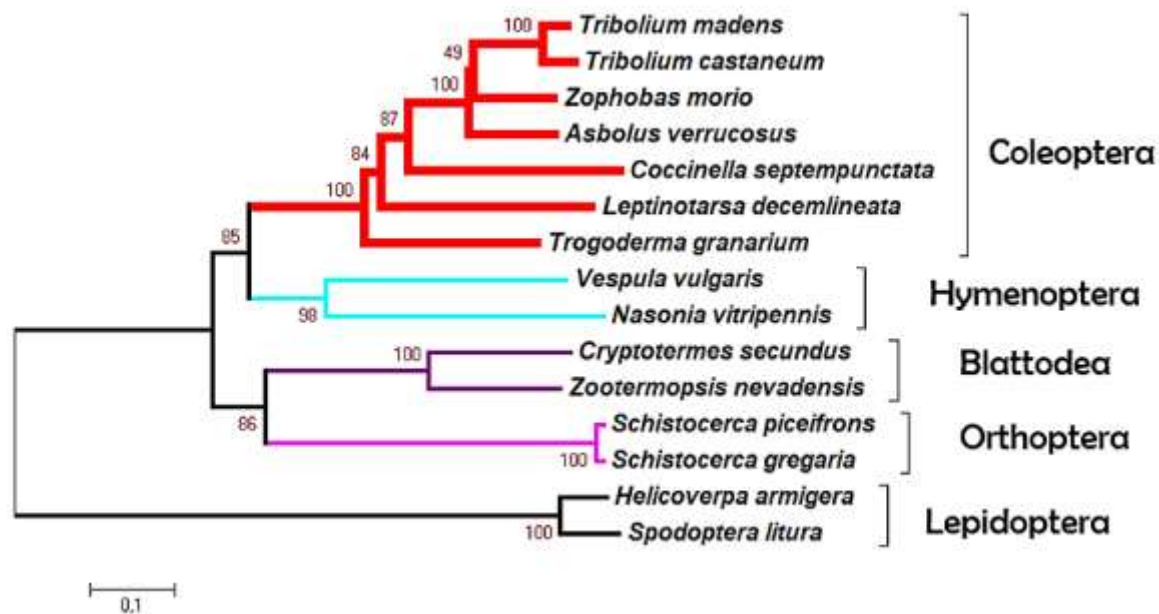


Figure 3. Phylogenetic tree of serine protease inhibitors from various insect species constructed using MEGA6. Each different order was indicated using different colors. GenBank Accession numbers of each species are as follow: *Tribolium madens* Serine protease inhibitor 28Dc XP_044254319.1; *Tribolium castaneum* Serine protease inhibitor 28Dc XP_008191787.1; *Zophobas morio* Serine protease inhibitor 28Dc XP_063914317.1; *Asbolus verrucosus* Serpin B12 RZC34043.1; *Coccinella septempunctata* Serine protease inhibitor 28Dc isoform X2 XP_044748515.1; *Leptinotarsa decemlineata* Serine protease inhibitor 28Dc-like XP_023026529.1; *Vespula vulgaris* Serine protease inhibitor 28Dc-like isoform X3 XP_050854871.1; *Nasonia vitripennis* Serine protease inhibitor 28Dc isoform X2 XP_001603946.3; *Cryptotermes secundus* Serine protease inhibitor 28Dc XP_023720181.1; *Zootermopsis nevadensis* Serine protease inhibitor 28Dc-like XP_021926289.1; *Schistocerca piceifrons* Serpin B6-like XP_047105853.1; *Schistocerca gregaria* Serpin B6-like XP_049854892.1; *Helicoverpa armigera* Serine protease inhibitor XP_021182965.2; *Spodoptera litura* Serine protease inhibitor-like XP_022837600.1.

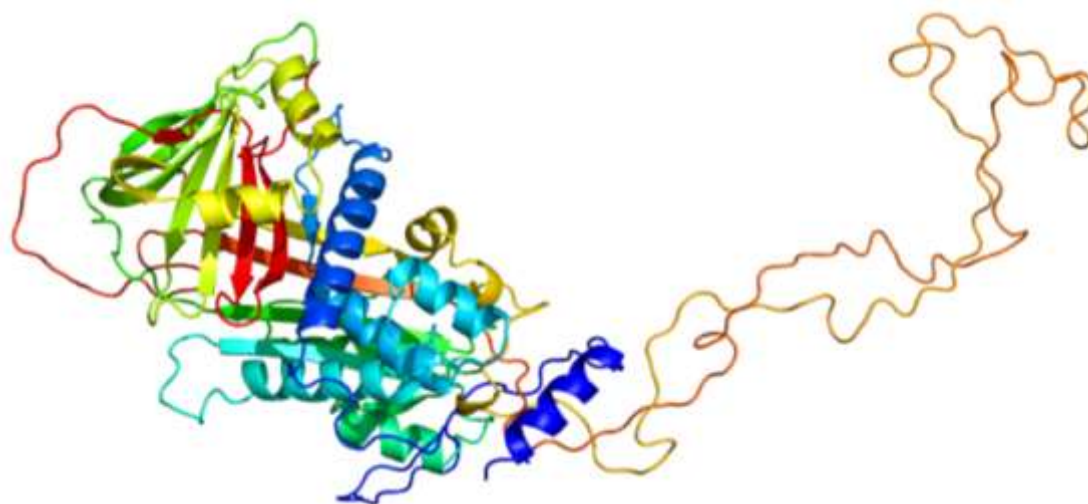


Figure 4. Predicted 3D formation of TgSPI.

The constructed phylogenetic tree of ISPIs consists of two main branches. One main branch comprises ISPIs from Lepidoptera, while the other contains ISPIs from Orthoptera, Blattodea, Hymenoptera, and Coleoptera, including the TgSPI. TgSPI clustered together with coleopteran ISPIs, indicating consistency with the BLAST result (Figure 3).

The tertiary structure of TgSPI was predicted using the "c5cdzyA_" model with 100% confidence and 66%

coverage (Figure 4). It showed highest identity to crystal structure of conserpin in the latent state with 37%.

Identifying and characterizing genes such as *TgSPI* is essential for pest management strategies. Saadati and Bandani (2011) found that incorporating serine protease inhibitors such as SBTI, TLCK, TPCK, and a combination of SBTI and TPCK into the diet of *Eurygaster integriceps* (Hemiptera: Scutelleridae) at concentrations of 1% and 4% of dietary protein resulted in notable detrimental

impacts on growth, development, and gut proteinases. This research highlighted the potential of these inhibitors as a pest management tool in *E. integriceps*, emphasizing the importance of understanding the functional roles of protease inhibitors in pest species. Han et al. (2014) identified and characterized three *serpins* in *Plutella xylostella* (Lepidoptera: Plutellidae), revealing their highest expression in the fat body and hemolymph of the 4th larval stage. They demonstrated that RNAi-mediated knockdown of these genes led to significantly lower expression levels and increased mortality and immune responses in the presence of destruxin A. In another study, knockdown of the *Spn5* gene by RNAi in *Drosophila melanogaster* (Diptera: Drosophilidae) resulted in a complete defect in wing unfolding and expansion in freshly eclosed mutant flies, despite not affecting early embryogenesis (Charron et al., 2008). These studies indicated the potential of genetic manipulation of *serpin* genes to enhance pest control strategies. Characterizing genes such as *TgSPI* can assist to discover novel targets for genetic manipulation, leading to the development of effective, sustainable pest control methods. This approach can not only reduce reliance on chemical pesticides but also minimize environmental impact by contributing to more sustainable agricultural practices. The limitations of this study include the absence of direct experimental validation of *TgSPI*'s functional role in *T. granarium*, such as through gene knockdown or overexpression studies, which would help confirm its involvement in protease inhibition and its impact on insect physiology. Additionally, the study does not account for environmental factors like diapause, temperature, humidity, or pathogen exposure that could influence *TgSPI* expression and function. These factors are essential for fully understanding the gene's role in the insect's immune response and its potential as a target for pest control strategies.

4. Conclusions

This study presents the first characterization, phylogenetic analysis, and predicted 3D structure of the putative TgSPI protein in *T. granarium*. This information could significantly contribute to understanding the evolutionary patterns of serpins in insects. Moreover, the *TgSPI* gene may emerge as a promising candidate for genetic control strategies such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and RNA interference (RNAi). Further comprehensive studies are required to elucidate the mechanisms of action and regulatory pathways involving *TgSPI* in the physiology and immune response of *T. granarium*, potentially paving the way for novel pest management strategies.

Author Contributions

The percentages of the author contributions are presented below. The author reviewed and approved the final version of the manuscript.

	A.D
C	100
D	100
S	100
DCP	100
DAI	100
L	100
W	100
CR	100
SR	100
PM	100
FA	100

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The author declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

References

- Chamankhah M, Braun L, Visal-Shah S, O'Grady M, Baldwin D, Shi X, Hegedus DD. 2003. *Mamestra configurata* serpin-1 homologues: cloning, localization and developmental regulation. *Insect Biochem Mol Biol*, 33(3): 355-369.
- Charron Y, Madani R, Combepine C, Gajdosik V, Hwu Y, Margaritondo G, Vassalli JD. 2008. The serpin Spn5 is essential for wing expansion in *Drosophila melanogaster*. *Int J Dev Biol*, 52(7): 933-942.
- Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21(18): 3674-3676. <https://doi.org/10.1093/BIOINFORMATICS/BT1610>
- Dageri A, Kadir ML, Guz N, Oğreten A, Arshad M. 2023. The involvement of Antifreeze protein maxi-like and Cold-shock domain-containing protein genes in cold-induced larval diapause and cold-shock treatment of khapra beetle. *J Stored Prod Res*, 101: 102074.
- Dageri A. 2024. Molecular characterization and expression analysis of six small heat shock protein genes in *Trogoderma granarium* during cold and starvation-induced larval diapause. *J Stored Prod Res*, 108: 102368.
- EPPO. 2024. European and Mediterranean plant protection organization. EPPO global data base. *Trogoderma granarium*. URL=<https://gd.eppo.int/taxon/TROGGA/distribution> (accessed date: July 01, 2024).
- Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39: 783-791.
- Han P, Fan J, Liu Y, Cuthbertson AG, Yan S, Qiu BL, Ren S. 2014. RNAi-mediated knockdown of serine protease inhibitor genes increases the mortality of *Plutella xylostella* challenged by destruxin A. *PLoS One*, 9(5): e97863.
- Hedstrom L. 2002. Serine protease mechanism and specificity.

- Chem Rev, 102(12): 4501-4524.
- Huntington JA, Read RJ, Carrell RW. 2000. Structure of a serpin-protease complex shows inhibition by deformation. *Nature*, 407(6806): 923-926.
- Huntington JA, Carrell RW. 2001. The serpins: nature's molecular mousetraps. *Sci Prog*, 84(2): 125-136.
- Irving JA, Pike RN, Lesk AM, Whisstock JC. 2000. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Res*, 10(12): 1845-1864.
- Jiang R, Zhang B, Kurokawa K, So YI, Kim EH, Hwang HO, Lee BL. 2011. 93-kDa twin-domain serine protease inhibitor (Serp1) has a regulatory function on the beetle Toll proteolytic signaling cascade. *J Biol Chem*, 286(40): 35087-35095.
- Li B, Yu HZ, Ye CJ, Ma Y, Li X, Fan T, Xu JP. 2017. *Bombyx mori* Serpin6 regulates prophenoloxidase activity and the expression of antimicrobial proteins. *Gene*, 610: 64-70.
- Li GY, Yang L, Xiao KR, Song QS, Stanley D, Wei SJ, Zhu JY. 2022. Characterization and expression profiling of serine protease inhibitors in the yellow mealworm *Tenebrio molitor*. *Arch Insect Biochem Physiol*, 111(3): e21948.
- Liu T, Chu J, Wang Q, Wang Y, Zhang X, Liu D, Wang. 2024. Role of serpin-25 in prophenoloxidase activation and expression of antimicrobial peptide genes in the silkworm *Bombyx mori*. *J Asia-Pac Entomol*, 27(2): 102222.
- Loebermann H, Tokuoka R, Deisenhofer J, Huber R. 1984. Human α 1-proteinase inhibitor: crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. *J Mol Biol*, 177(3): 531-557.
- Madeira F, Pearce M, Tivey AR, Basutkar P, Lee J, Edbali O, Lopez R. 2022. Search and sequence analysis tools services from EMBL-EBI in 2022. *Nucleic Acids Res*, 50(W1): W276-W279.
- Meekins DA, Kanost MR, Michel K. 2017, February. Serpins in arthropod biology. *Sem Cell Devel Biol*, 62: 105-119.
- Saadati F, Bandani AR. 2011. Effects of serine protease inhibitors on growth and development and digestive serine proteinases of the Sunn pest, *Eurygaster integriceps*. *J Insect Sci*, 11(1): 72.
- Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406-425.
- Sanrattana W, Sefiane, T, Smits S, van Kleef ND, Fens MH, Lenting PJ, de Maat S. 2021. A reactive center loop-based prediction platform to enhance the design of therapeutic SERPINs. *Proc Natl Acad Sci*, 118(45): e2108458118.
- Shakeel M, Xu X, De Mandal S, Jin F. 2019. Role of serine protease inhibitors in insect- host-pathogen interactions. *Arch Insect Biochem Physiol*, 102(3): 1-8. <https://doi.org/10.1002/arch.21556>
- Shakeel M. 2021. Molecular identification, characterization, and expression analysis of a serine protease inhibitor gene from cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Braz J Biol*, 81(3): 516-525.
- Stratikos E, Gettins PG. 1999. Formation of the covalent serpin-proteinase complex involves translocation of the proteinase by more than 70 Å and full insertion of the reactive center loop into β -sheet A. *Proc Natl Acad Sci*, 96(9): 4808-4813.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*, 30: 2725-2729.
- Tang Y, Wang Y, Pei Z, Li W, Zhang D, Liu L, Kong L, Liu S, Jiang X, Ma H. 2016. A serine protease inhibitor from *Musca domestica* larva exhibits inhibitory activity against elastase and chymotrypsin. *Biotechnol Lett*, 38(7): 1147-1153. <https://doi.org/10.1007/s10529-016-2089-0>
- Yang L, Mei Y, Fang Q, Wang J, Yan Z, Song Q, Lin Z, Ye G. 2017. Identification and characterization of serine protease inhibitors in a parasitic wasp, *Pteromalus puparum*. *Sci Rep*, 7(1): 1-13. <https://doi.org/10.1038/s41598-017-16000-5>
- Zhang C, Wei J, Naing ZL, Soe ET, Tang J, Liang G. 2022. Up-regulated serpin gene involved in Cry1Ac resistance in *Helicoverpa armigera*. *Pestic Biochem Physiol*, 188: 105269.
- Zuckerkindl E, Pauling L. 1965. Evolutionary divergence and convergence in proteins. Edited in *Evolving Genes and Proteins* by V. Bryson and HJ. Vogel. Academic Press, New York, US, pp: 97-166.