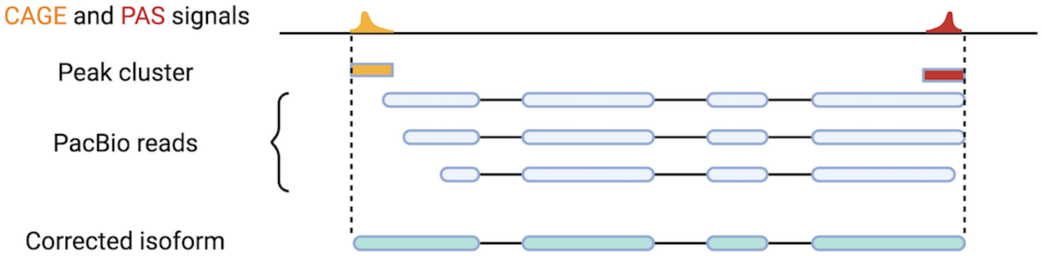
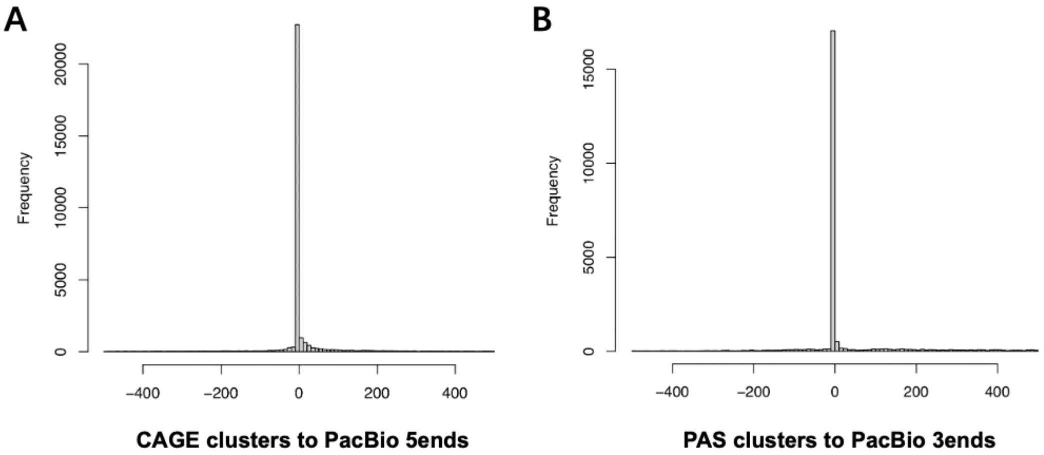
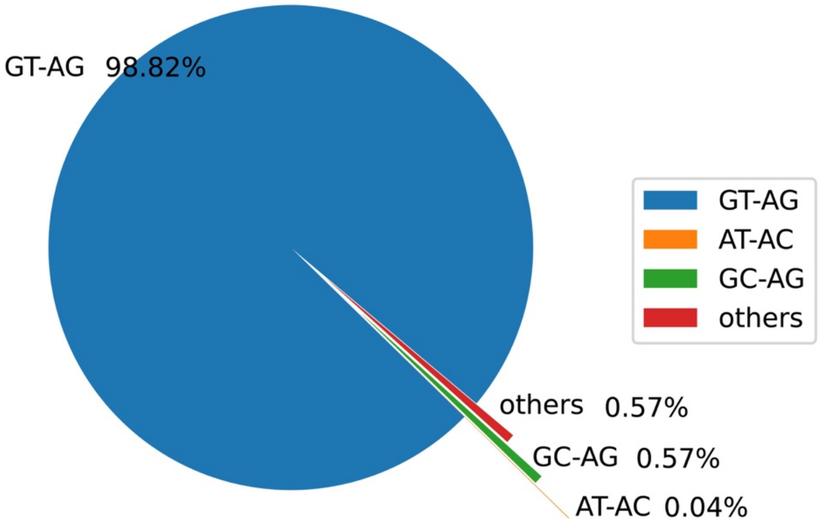
Supplemental Figures



Supplemental Fig S1. A schematic figure for the correction of ends of isoforms by CAGE and PAS peaks. Due to incomplete cDNA synthesis or mRNA degradation, the primary isoforms we obtained directly from PacBio Iso-Seq may include some fragments of mature mRNAs. To refine the 5′-end and 3'-end boundaries of transcripts, we analyzed the data as previously described by Sun et al., 2021. In brief, we first identified CAGE and PAS peak clusters that represented reliable TSS and TES signals, respectively. We then corrected the 5′-end and 3'-end of the isoforms obtained from Iso-Seq according to these TSS and TES signals.

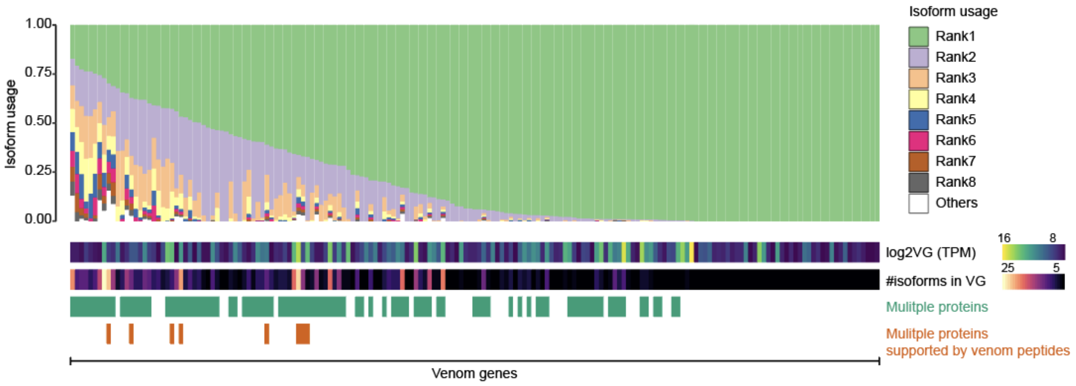


Supplemental Fig S2. The distances of 5’-end and 3’end of the isoforms from the PacBio Iso-Seq with peaks identified by CAGE-seq (A) and PAS-seq (B), respectively.



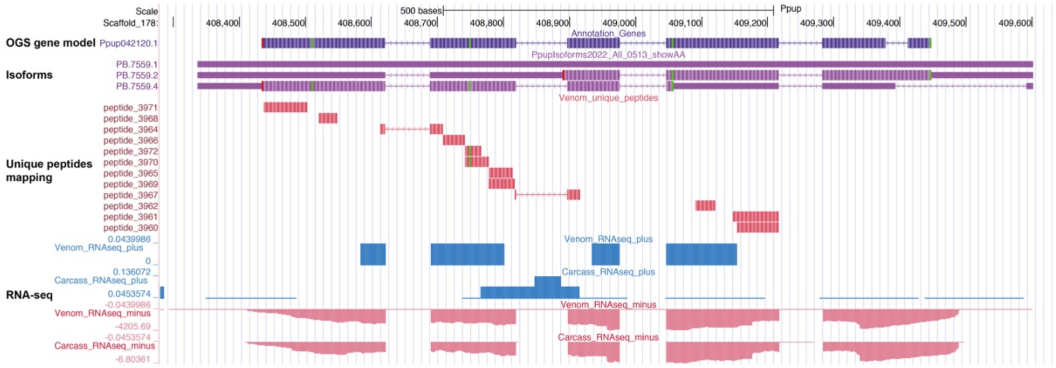
Supplemental Fig S3. Pie chart showing the percentage of the splicing donor-acceptor di-nucleotide utilization among all transcripts in *P. puparum*. In total, we found 141,589 GU-AG splice sites, 820 GC-AG splice sites, 53 AU-AC splice sites, and 823 other splice sites.

Supplemental Fig S4. Overview of the isoforms of the longitudinals lacking (*lola*) gene in *P. puparum*.

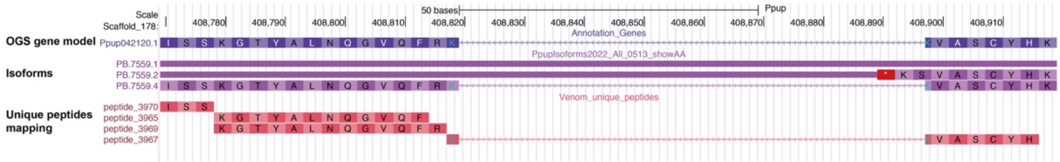


Supplemental Fig S5. Isoform usage of venom genes. Only venom gland expressed isoforms were included, and the isoforms with TPM values higher than 1 in at least one repetition of venom gland sample were defined as expressed isoforms. Usages of isoforms in each gene were ranked (Rank1 represented the most used isoform in a gene) and showed in different colors. Venom genes with various isoforms that encoded multiple proteins were indicated, and the genes with multiple venom proteins supported by mass spectrometry-based proteomics were highlighted.

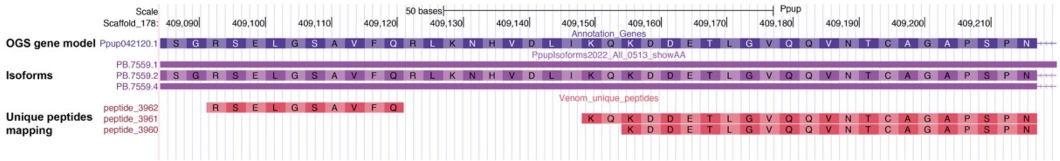
A Ppup042120: Venom allergen 3



B Unique peptides mapping supporting the isoform PB.7559.4



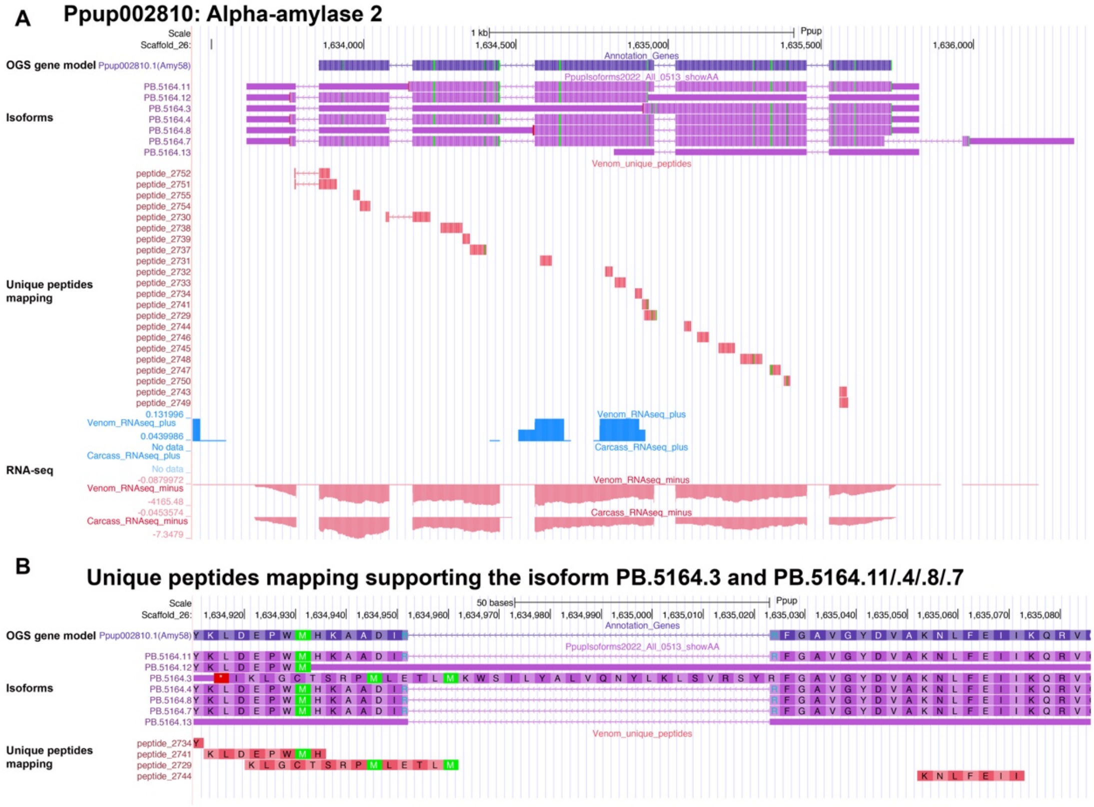
C Unique peptides mapping supporting the isoform PB.7559.2



Supplemental Fig S6. Venom allergen 3 gene produces diverse venom proteins through alternative splicing, which supported by mass spectrometry. (A) Genome browser view of the venom allergen 3 gene. From top to bottom, OGS gene model, Isoforms, Unique venom peptides mapping results, RNA-seq. (B) Zoomed view showing the peptide supporting information of the venom PB.7559.4 protein. (C) Zoomed view showing the peptide supporting information of the venom PB.7559.2 protein.

Supplemental Fig S7. Lipase-like gene produces diverse venom proteins through alternative splicing, which supported by mass spectrometry. Genome browser view of the lipase-like gene. From top to bottom, OGS gene model, Isoforms, Unique venom peptides mapping results, RNA-seq.

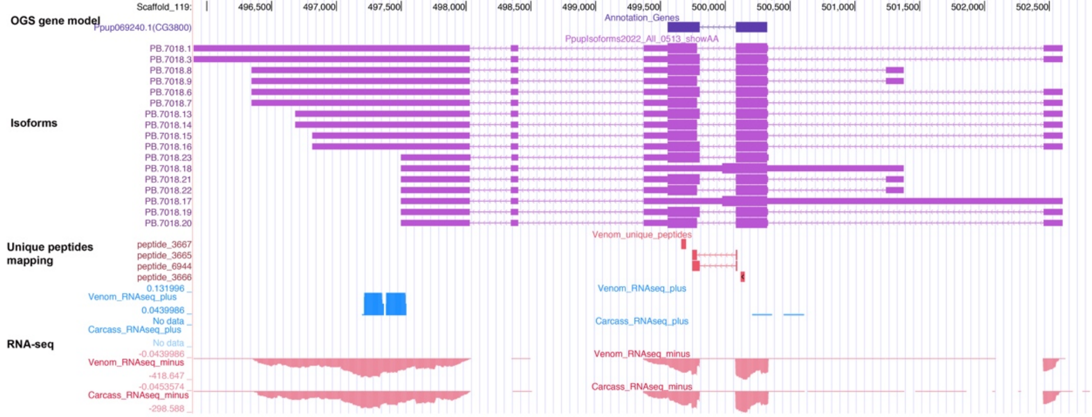
Supplemental Fig S8. Zoomed view showing the peptide supporting information of the venom proteins encoded by the lipase-like gene (geneid: novelGeneAS210).



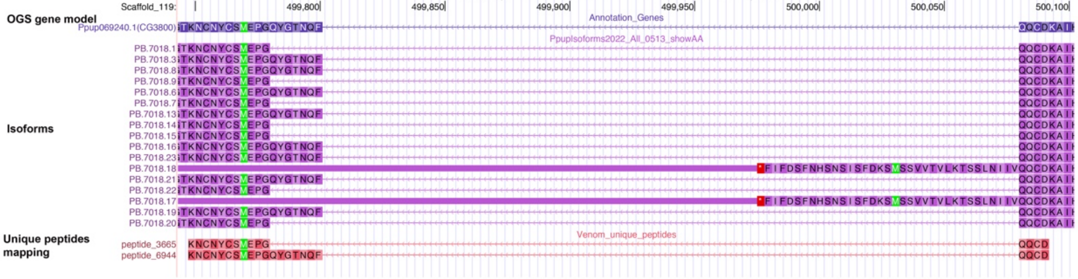
Supplemental Fig S9. Alpha-amylase 2 gene produces diverse venom proteins through alternative splicing, which supported by mass spectrometry. (A) Genome browser view of the alpha-amylase 2 gene. From top to bottom, OGS gene model, Isoforms, Unique venom peptides mapping results, RNA-seq. (B) Zoomed view showing the peptide supporting information of the venom proteins.

Supplemental Fig S10. Venom acid phosphatase Acph-1 gene produces diverse venom proteins through alternative splicing, which supported by mass spectrometry. (A) Genome browser view of the venom acid phosphatase Acph-1 gene. From top to bottom, OGS gene model, Isoforms, Unique venom peptides mapping results, RNA-seq. (B) Zoomed view showing the peptide supporting information of the venom PB.5722.5 protein. (C) Zoomed view showing the peptide supporting information of the venom proteins encoded by PB. 5722.12-17/.19.

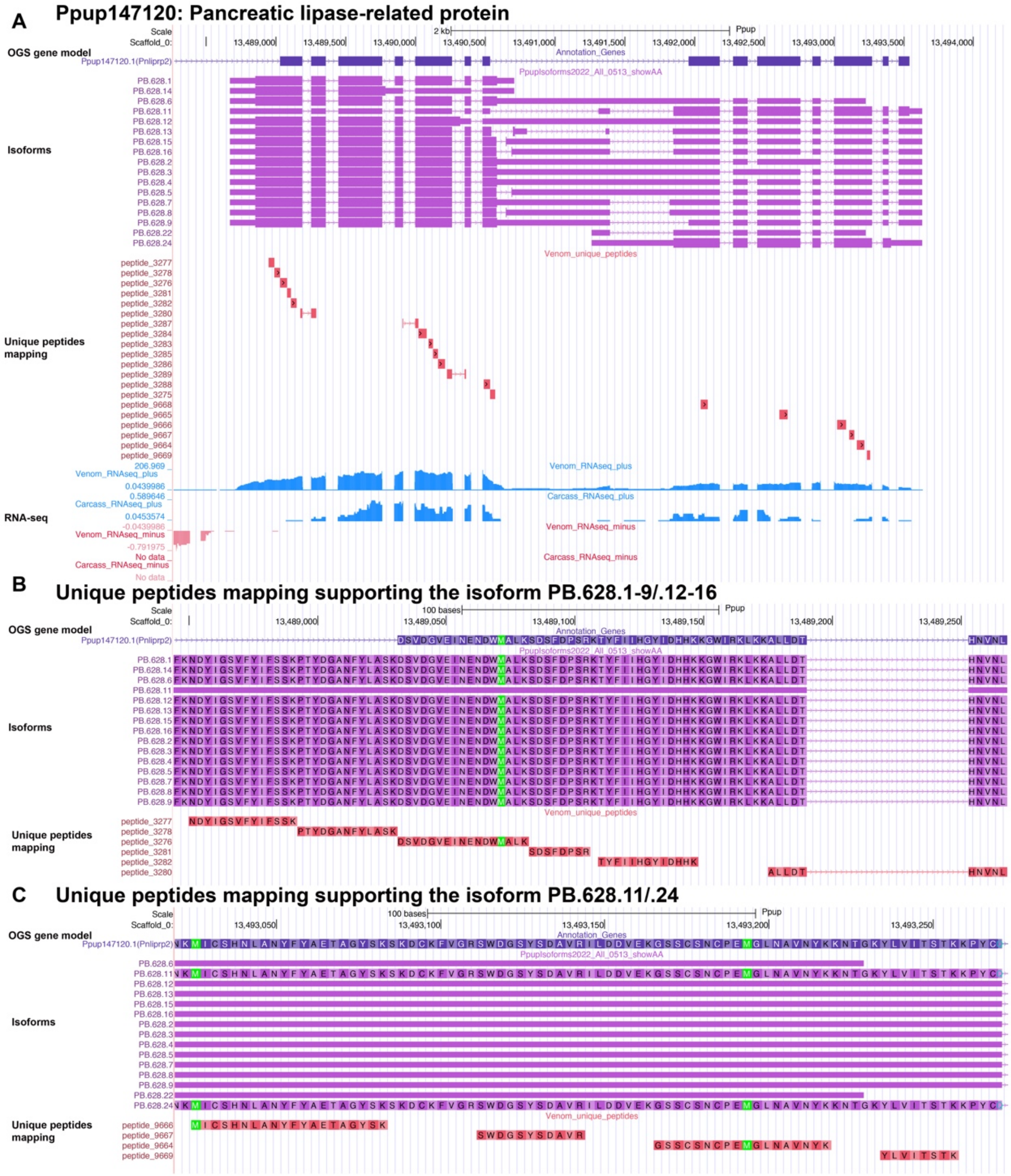
A Ppup069240: CCHC-type zinc finger protein



B Unique peptides mapping supporting the isoform PB.7018.1/.3/.6-9/.13-16/.19-23

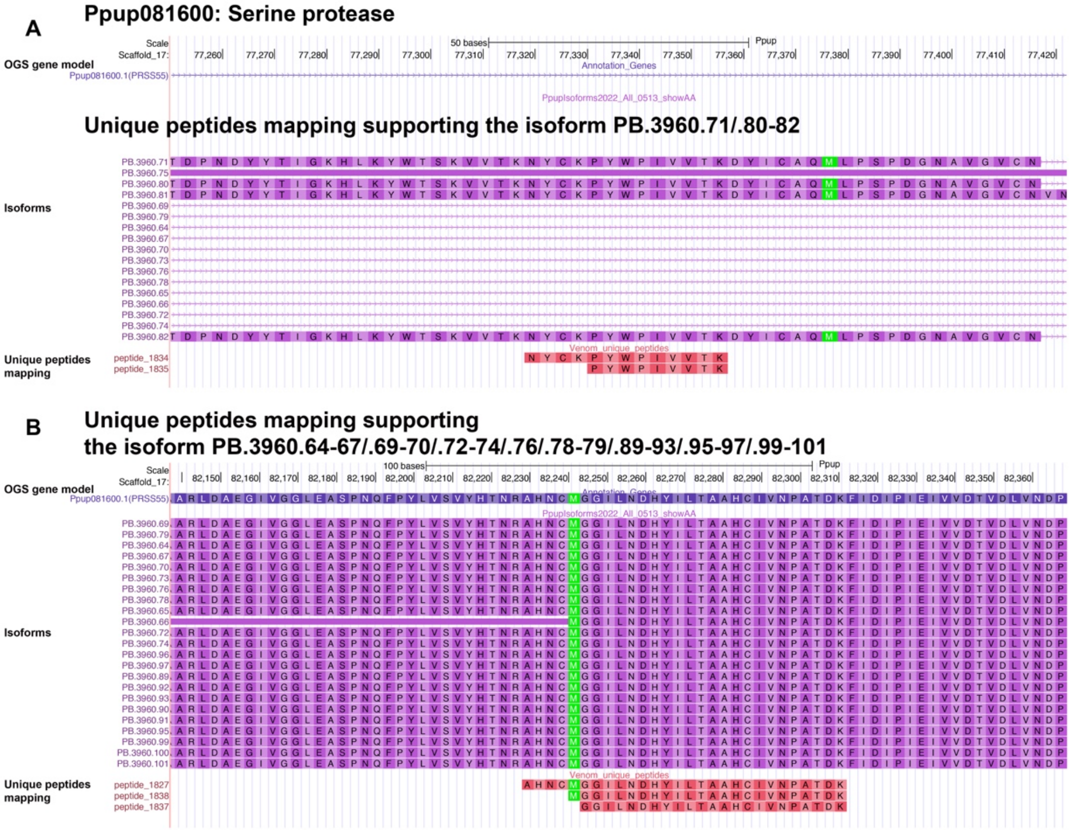


Supplemental Fig S11. CCHC-type zinc finger protein gene produces diverse venom proteins through alternative splicing, which supported by mass spectrometry. (A) Genome browser view of the CCHC-type zinc finger protein gene. From top to bottom, OGS gene model, Isoforms, Unique venom peptides mapping results, RNA-seq. (B) Zoomed view showing the peptide supporting information of the venom proteins.



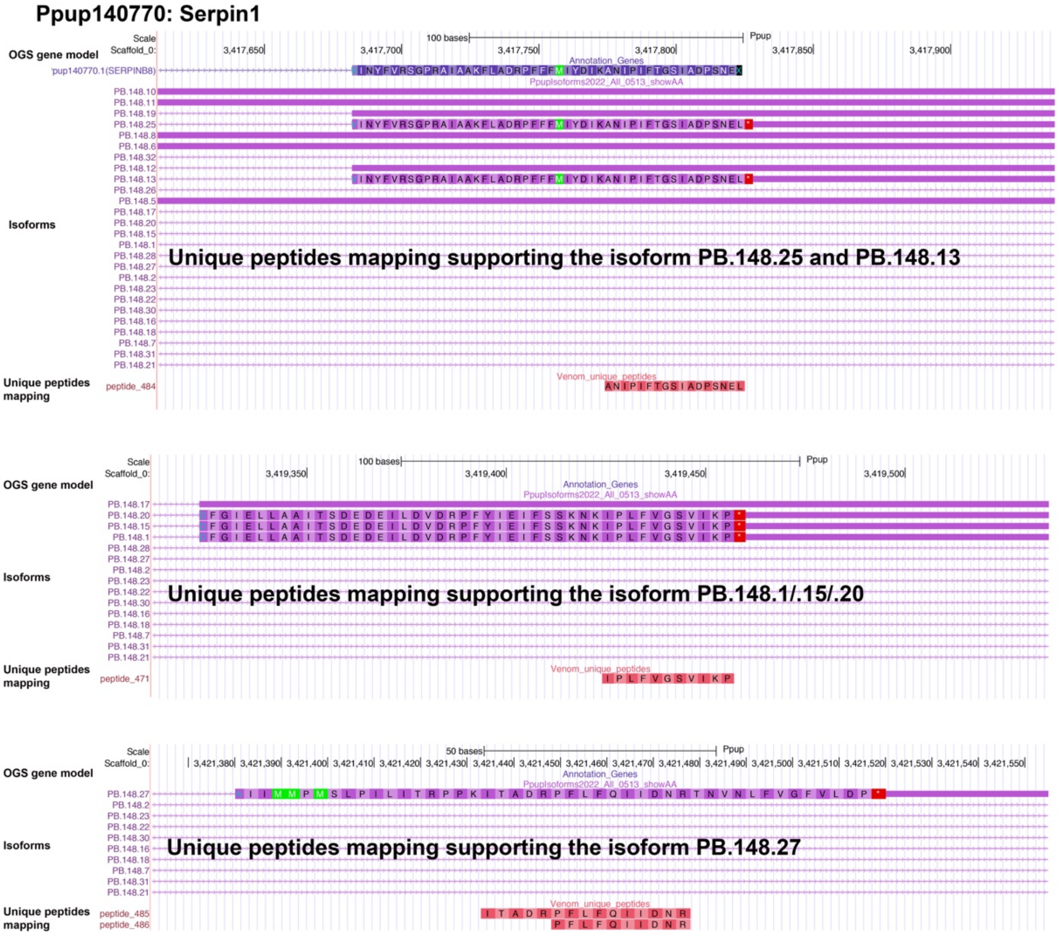
Supplemental Fig S12. Pancreatic lipase-related protein gene produces diverse venom proteins through alternative splicing, which supported by mass spectrometry. (A) Genome browser view of the Pancreatic lipase-related protein gene. From top to bottom, OGS gene model, Isoforms, Unique venom peptides mapping results, RNA-seq. (B) Zoomed view showing the peptide supporting information of the venom proteins encoded by PB.628.1-9/.12-16. (C) Zoomed view showing the peptide supporting information of the venom proteins encoded by PB.628.11/.24.

Supplemental Fig S13. Serine protease gene produces diverse venom proteins through alternative splicing, which supported by mass spectrometry. Genome browser view of the serine protease gene. From top to bottom, OGS gene model, Isoforms, Unique venom peptides mapping results, RNA-seq.

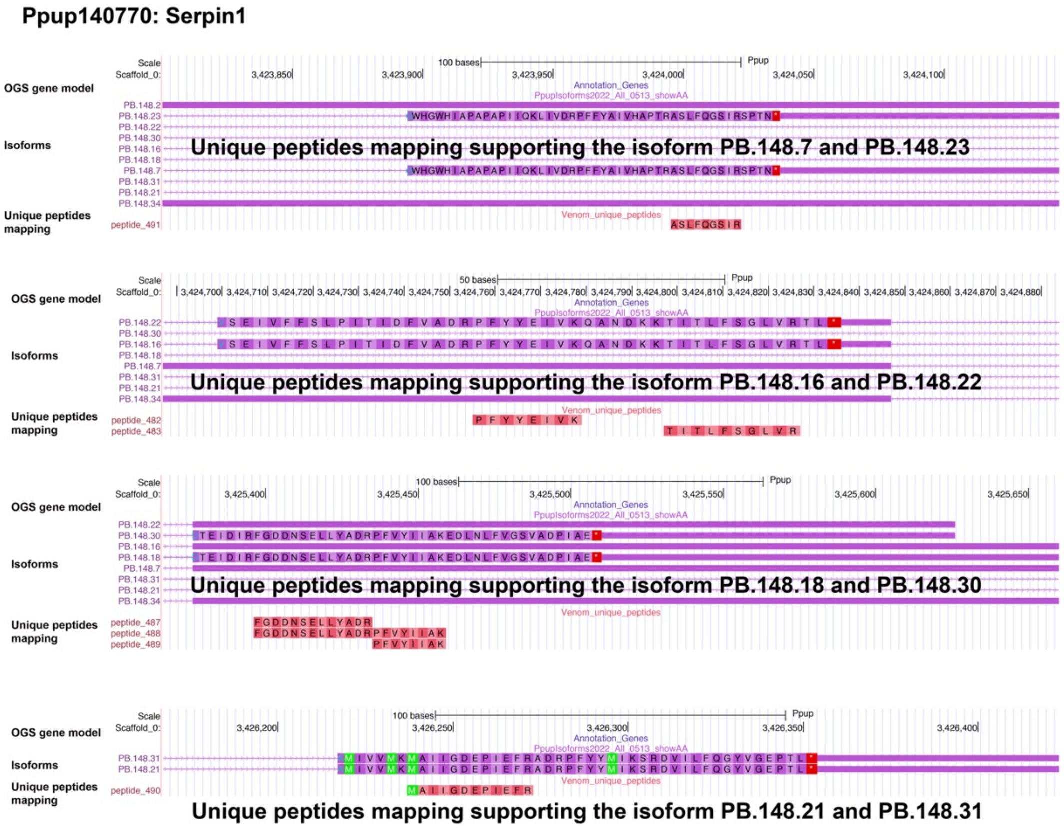


Supplemental Fig S14. Zoomed view showing the peptide supporting information of the venom proteins encoded by the serine protease gene (geneid: Ppup081600).

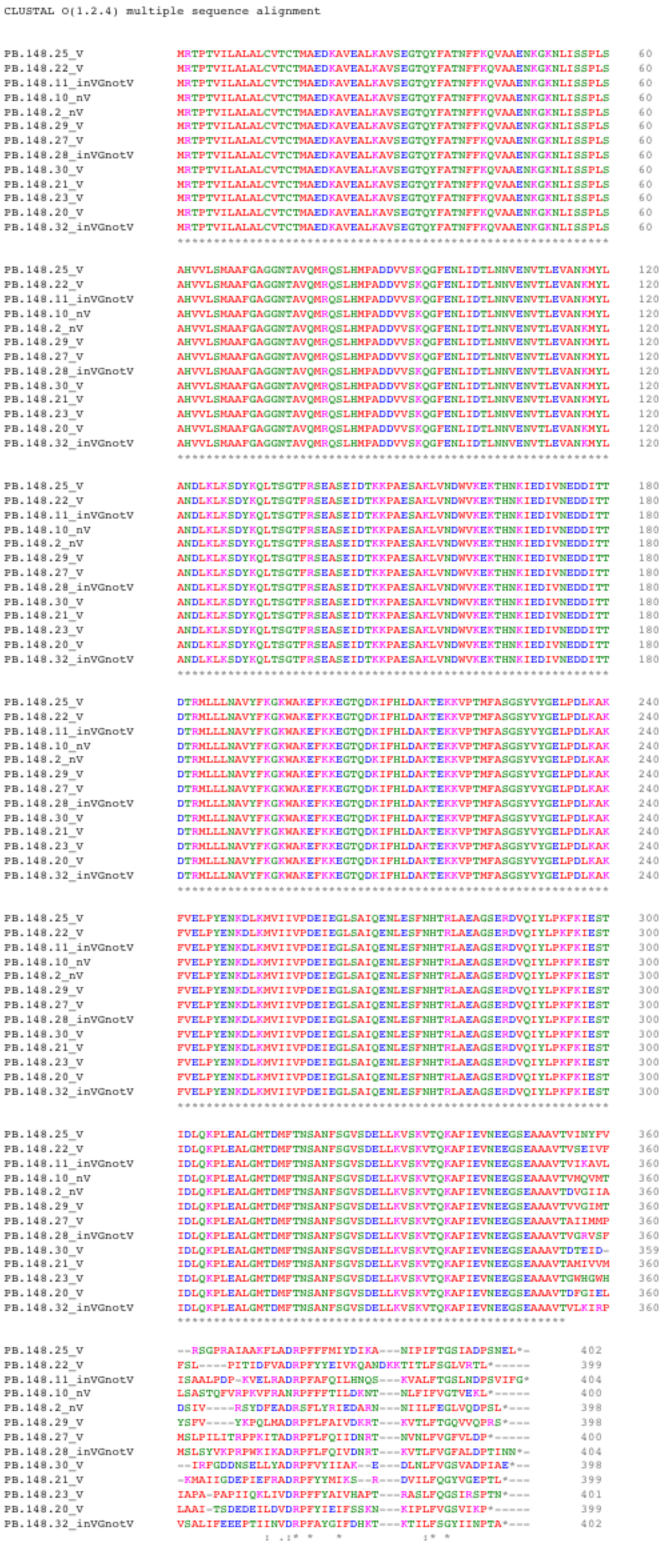
Supplemental Fig S15. *Serpin1* gene produces diverse venom proteins through alternative splicing, which supported by mass spectrometry. Genome browser view of the *serpin1* gene. From top to bottom, OGS gene model, Isoforms, Unique venom peptides mapping results, RNA-seq.



Supplemental Fig S16. Zoomed view showing the peptide supporting information of the venom proteins encoded by the *serpin1* gene (geneid: Ppup140770). This figure showed that peptide supporting evidence of three venom proteins that encoded by different isoforms of *serpin1* gene.



Supplemental Fig S17. Zoomed view showing the peptide supporting information of the venom proteins encoded by the *serpin1* gene (geneid: Ppup140770). This figure showed that peptide supporting evidence of four venom proteins that encoded by different isoforms of *serpin1* gene.



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CLUSTAL O(1.2.4) multiple sequence alignment


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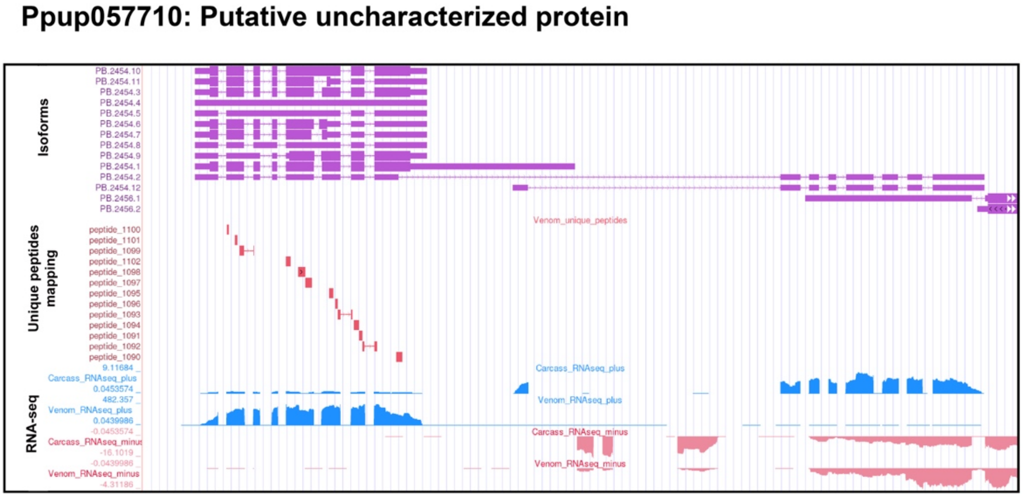
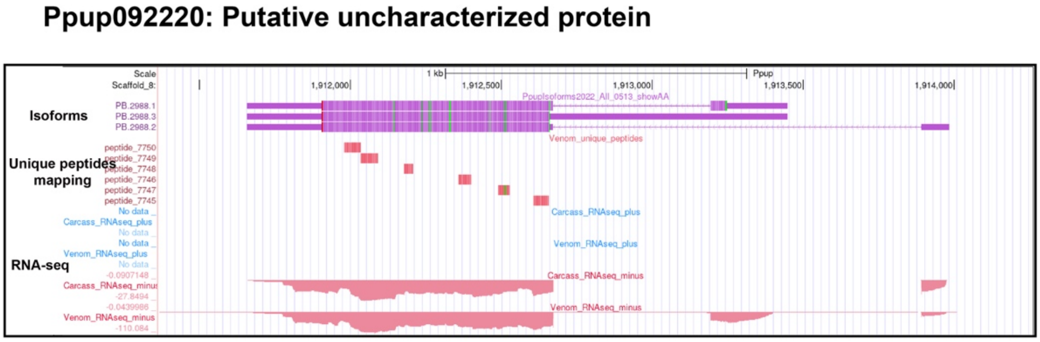
Supplemental Fig S18. Multiple sequence alignment of Serpin proteins encoded by *PpSerpin1* gene.

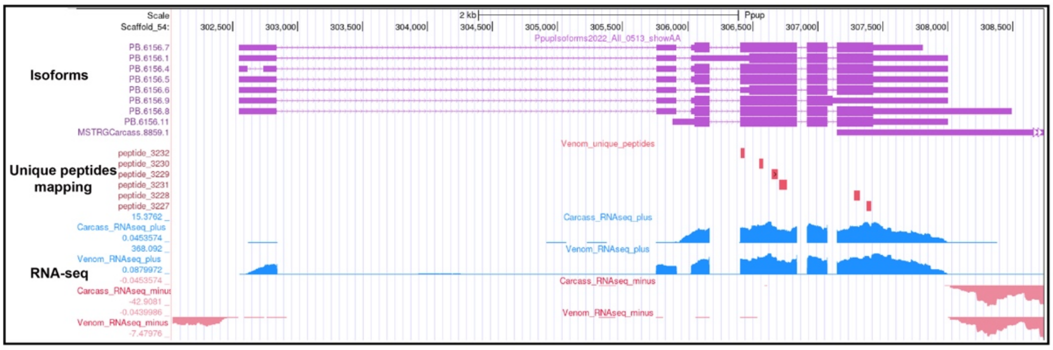


Supplemental Fig S19. Ppup057710 venom gene shows isoform usage shifts between venom gland and carcass. From top to bottom, Isoforms, Unique venom peptides mapping results, RNA-seq (Blue represents Watson strand mapping reads; Red represents Crick strand mapping reads).



Ppup092220: Putative uncharacterized protein

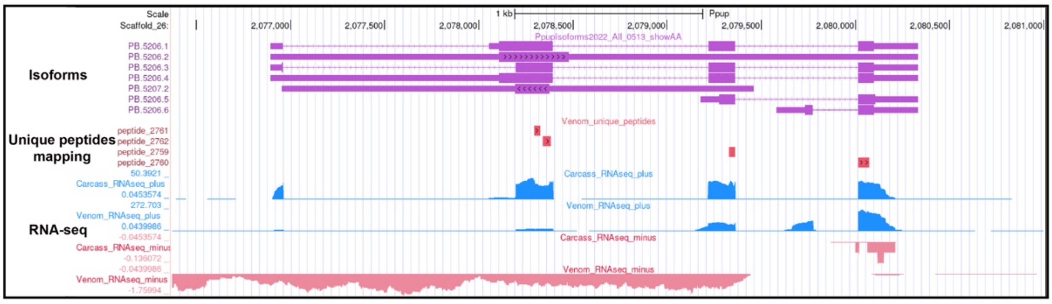
Supplemental Fig S20. Ppup092220 venom gene shows isoform usage shifts between venom gland and carcass. From top to bottom, Isoforms, Unique venom peptides mapping results, RNA-seq (Blue represents Watson strand mapping reads; Red represents Crick strand mapping reads).



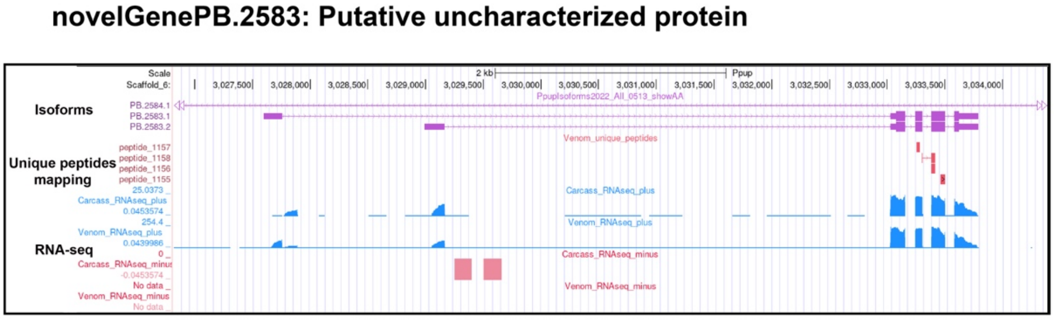
Ppup035100: 2-oxoglutarate and iron-dependent oxygenase domain-containing protein 3

Supplemental Fig S21. Ppup035100 venom gene shows isoform usage shifts between venom gland and carcass. From top to bottom, Isoforms, Unique venom peptides mapping results, RNA-seq (Blue represents Watson strand mapping reads; Red represents Crick strand mapping reads).

Ppup003240: Natterin-3



Supplemental Fig S22. Ppup003240 venom gene shows isoform usage shifts between venom gland and carcass. From top to bottom, Isoforms, Unique venom peptides mapping results, RNA-seq (Blue represents Watson strand mapping reads; Red represents Crick strand mapping reads).



novelGenePB.2583: Putative uncharacterized protein

Supplemental Fig S23. novelGenePB.2583 venom gene shows isoform usage shifts between venom gland and carcass. From top to bottom, Isoforms, Unique venom peptides mapping results, RNA-seq (Blue represents Watson strand mapping reads; Red represents Crick strand mapping reads).

Supplemental Fig S24. Mass spectrometry data supports presence of the serpin-C protein in venom. (A) Genome browser view of the *serpin3* gene. This is a zoomed view of the Fig. 4A in the main text. From top to bottom, OGS gene model, Isoforms, Unique venom peptides mapping results. (B-E) Zoomed view showing the peptide supporting information.

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CLUSTAL O(1.2.4) multiple sequence alignment


PB.7110.1       MLLVVALCCAVVAGIQASPISPQREIIPLNAEAFAQPEYVQALANELEKEGVAELKSQAE      60
PB.7110.3       MLLVVALCCAVVAGIQASPISPQREIIPLNAEAFAQPEYVQALANELEKEGTTTESSLNI      60
                ***************************************************.:  .*

PB.7110.1       DHLTRNAPNDFDSPDYGHNQKFAGHRKPDSTHHSVNKGFSETIIFPSDIIEYTNQKRNFD      120
PB.7110.3       RR-KRGYPVHVYEPVFPS--LFNNEKLTTTT----------TTVAP----------KKYR      97
                 : .*. * .. .* :     * ..:   :*          * : *          :::

PB.7110.1       EIDRNG------FSGFSKRNFDEID-R-TGFSGFSKRNFDEIDRTGFSGFNKKRNFDEID      172
PB.7110.3       EIHSGGNLDHIGGSNLLRRSVSRSPDKKAADSGRVRTKRDVLYNP-Y--YAMVPLFPDIP      154
                **. .*       *.: :*....   : :. **  : : * : .  :  :     * :*

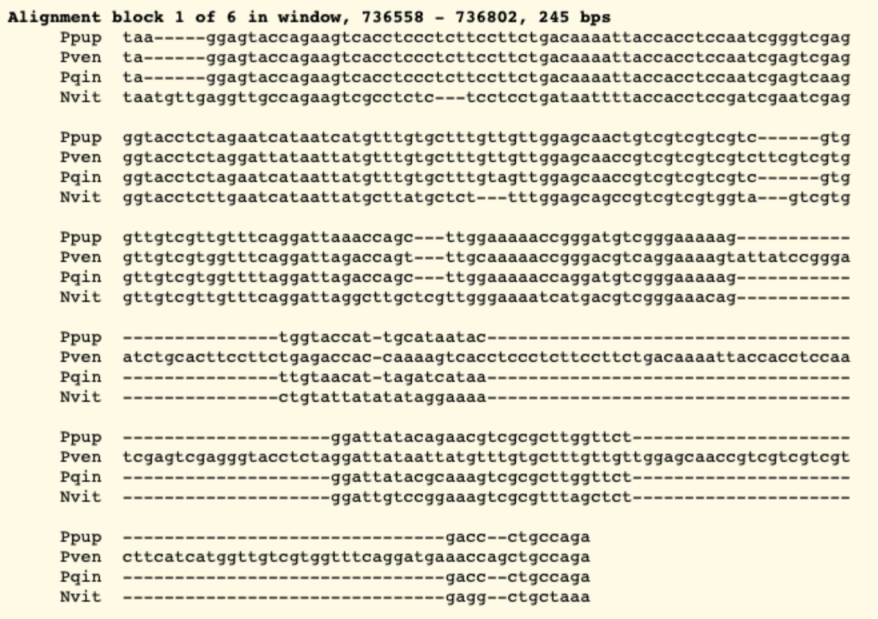
PB.7110.1       RTGFSGF-NKRNFDEIDRNGFSGFSKRNFDEIDRTGFSGFNKKRNFDEIDRNGFSGFSKR      231
PB.7110.3       --VFPSWFNPETTTTTTTTTVAPTTKHK---------HDYDSRGTLDPIGGG---NFVRR      200
                   * .: * ..      . .:  :*::          .::.: .:* *. .   .* :*

PB.7110.1       NFDEIDRTGFSGFNKRNFDEIDRTGFSGFNKRNFDEIDRSGFSGFNKKRNFDEIDRSGFS      291
PB.7110.3       K-----REVTSGTP*---------------------------------------------      209
                :     *   **

PB.7110.1       GFSRKRNFDEIDRSGVPGFAKRSIASSRSGKSQRTH*    327
PB.7110.3       -------------------------------------    209
```

Supplemental Fig S25. Multiple sequence alignment of the two proteins that are encoded by *PpOrcokinin* gene.

Supplemental Fig S26. Mass spectrometry data supports presence of the PB.7110.3 protein in venom. (A) Genome browser view of the *orcokinin* gene. This is a zoomed view of the Fig. 5A in the main text. From top to bottom, OGS gene model, Isoforms, Unique venom peptides mapping results. (B and C) Zoomed view showing the peptide supporting information.



```
Alignment block 1 of 6 in window, 736558 - 736802, 245 bps
     Ppup  taa-----ggagtaccagaagtcacctccctcttccttctgacaaaattaccacctccaatcgggtcgag
     Pven  ta------ggagtaccagaagtcacctccctcttccttctgacaaaattaccacctccaatcgagtcgag
     Pqin  ta------ggagtaccagaagtcacctccctcttccttctgacaaaattaccacctccaatcgagtcaag
     Nvit  taatgttgaggttgccagaagtcgcctctc---tcctcctgataattttaccacctccgatcgaatcgag

     Ppup  ggtacctctagaatcataatcatgtttgtgctttgttgttggagcaactgtcgtcgtcgtc------gtg
     Pven  ggtacctctaggattataattatgtttgtgctttgttgttggagcaaccgtcgtcgtcgtcttcgtcgtg
     Pqin  ggtacctctagaatcataattatgtttgtgctttgtagttggagcaaccgtcgtcgtcgtc------gtg
     Nvit  ggtacctcttgaatcataattatgcttatgctct---tttggagcagccgtcgtcgtggta---gtcgtg

     Ppup  gttgtcgttgtttcaggattaaaccagc---ttggaaaaaccgggatgtcgggaaaaag-----------
     Pven  gttgtcgtggtttcaggattagaccagt---ttgcaaaaaccgggacgtcaggaaaagtattatccggga
     Pqin  gttgtcgtggttttaggattagaccagc---ttggaaaaaccaggatgtcgggaaaaag-----------
     Nvit  gttgtcgttgtttcaggattaggcttgctcgttgggaaaatcatgacgtcgggaaacag-----------

     Ppup  ---------------tggtaccat-tgcataatac-----------------------------------
     Pven  atctgcacttccttctgagaccac-caaaagtcacctccctcttccttctgacaaaattaccacctccaa
     Pqin  ---------------ttgtaacat-tagatcataa-----------------------------------
     Nvit  ---------------ctgtattatatataggaaaa-----------------------------------

     Ppup  --------------------ggattatacagaacgtcgcgcttggttct---------------------
     Pven  tcgagtcgagggtacctctaggattataattatgtttgtgctttgttgttggagcaaccgtcgtcgtcgt
     Pqin  --------------------ggattatacgcaaagtcgcgcttggttct---------------------
     Nvit  --------------------ggattgtccggaaagtcgcgtttagctct---------------------

     Ppup  -------------------------------gacc--ctgccaga
     Pven  cttcatcatggttgtcgtggtttcaggatgaaaccagctgccaga
     Pqin  -------------------------------gacc--ctgccaga
     Nvit  -------------------------------gagg--ctgctaaa
```

Supplemental Fig S27. The zoomed view of the alignment of the exon3 of PB.7110.3 showing a specific DNA insertion in *P. venustus*.