nature portfolio

Peer Review File

A Newly Evolved Small Secretory Peptide Enhances Mechanical Properties of Spider Silk

Corresponding Author: Professor Yi Wang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The mamnuscript entitled "A Newly Evolved Small Secretory Peptide Enhances Mechanical Properties of Spider Silk" by Anqiang Jia1 et al., deals with the investigation of the evolution of silk glands in Araneoidea spiders through comprehensive genomic and transcriptomic mining. ly was reported numerous new genes with simplified structures and constrained expression patterns, expressed predominantly in silk glands and coexpressed with ancient genes to drive the evolution of silk glands.

Amongst these new genes it was identified the SpiCE-DS8, which is a newly evolved small secretory peptide, which in turn seems to be incorporated into dragline silk and is involved in the MaSp network. SpiCE24 DS8 originated de novo from intergenic regions of ancient genes and is an orphan gene unique to the Nephilinae subfamily. This peptide was suggested to interacts with the N-terminal region of MaSp1b aiding in silk protein solidification. In vitro wet-spinning experiments demonstrated that SpiCE-DS8 significantly improved fiber properties. The results of the present study are indicating that the central role of new lineage-specific genes in silk gland evolution, providing new insights into the evolutionary mechanisms associated with foraging webs and for advancing the recombinant synthesis silk material development.

The study was well conducted, using suitable experimental protocols. The manuscript was well written, with careful presentation of the results. The figures were very relevant for data presentation, The present investigation my bring a nice contribution for the knowledge about the role of the web fibers in prey capture, as well for the development of of novel protocols for the production of recombinant spider web fibers more resistant.

Despite to this, the authors could have described in more details the protocol of proteomic analysis, since the results generated by this specific approach was fundamental for validating the expression of SpiCE-DS8, The authors did not described / characterized the database used for proteíns idenntification. There was only a brief mention to a custon nr DB, and nothing else.

The manuscript mention the interacts with the N-terminal region of MaSp1b, potentially aiding in silk protein solidification; however, the text is not clear about how this result was got. Was it obtained from molecular modeling ? Please, make it clear.

Reviewer #2

(Remarks to the Author)
This is a really interesting manuscript studying the evolution of silk glands in Araneoidea spiders. The authors use a broad range of approaches mining the genomes. In particular, the authors identified SpiCE-DS8 as a newly evolved secretory peptide, which is expressed in the silk gland, and thus incorporated into the dragline silk. The authors demonstrate how this peptide affects the protein secondary structure of the spidroins via AlphaFold, which showed an increase its tendency to form β-sheets, an important precursor to high mechanical performance of the silk fibers.
Interestingly, the authors combine this analysis with some experimental work, where the influence of this peptide on the mechanical properties of spun silks fibers is tested. However, this work was carried out using silk proteins from silkworms and from spidroin-expressing silkworms. The sequences of silkworm proteins (fibroins) are very different from the MaSp1

proteins studied in most other parts of the manuscript. And unfortunately, this appears to be not discussed at all by the authors, which is a significant omission. Similarly, the authors do not discuss any details of the silk from the spidroin-expressing silkworm. What is the sequence of this silk? What's its molecular weight? What are the major and minor differences relative to the MaSp1 spidroins discussed in other places of the manuscript? Without further discussion and explanation, it is unclear whether all other findings and analyses on MaSp1 readily apply to the experiments that are based silkworm silk and spidroin-expressed silk from silkworms.
Moreover, the spinning mechanism used in this manuscript is relatively primitive, compared to the highly sophisticated process found in the natural spinneret, which triggers multiple physico-chemical effects, which are critical to obtaining a fiber with the known outstanding properties of spider silk. . Accordingly, the fibers obtained by the researchers feature a performance that is only a fraction of what is observed in native silk fibers, which feature higher breaking strains, higher Young's moduli, and higher toughness.
While it is encouraging that the authors find an increase in mechanical performance and an increase in β-sheet content, the authors need to make it clearer why they think this would also apply to natural silk spun under more ideal conditions. To further corroborate this correspondence, the authors could carry out AlphaFold work for the experimentally studied proteins (and the effect of the SpiCE-DS8 peptide on them). If this reveals similarities in the behavior of the proteins when exposed to the new peptide, the authors' conclusion would be somewhat better motivated and supported.

Reviewer #3

(Remarks to the Author)
Manuscript Report:
The authors of this manuscript investigate the molecular evolution and gene expression in silk glands of Araneoidea spiders– a group that utilizes ecologically and functionally diverse silks– to answer fundamental questions about the role of novel lineage-specific genes in phenotypic evolution across lineages. Using a genomic and transcriptomic approach, the authors trace back the evolutionary age of various genes using phylostratigraphic (PS) levels, from most ancestral to most recently derived. They also examine expression patterns of the genes associated with the different PS levels with tissue specificity. The authors use publicly available genomic and transcriptomic resources from several sources to conduct their analyses, with a robust taxonomic sampling. The authors find that relatively young genes show patterns of gene expression that localize to specific tissues (silk glands), while more ancestral genes have a more widespread correlation rather than tissue specificity. The authors also investigate co-expression networks in younger genes, finding integration with more ancestral genes. Using comparative phylogenomic methods, the authors find a set of novel genes (~200) that are uniquely expressed and correlated to the silk glands, and two genes that show potential for correlation to dragline silk function. For the two novel genes associated with dragline function, the authors assess the potential functional roles using Alpha Fold to predict 3D structure of sequence data and demonstrate areas that have potential for interaction with other spidroin proteins. To link these findings to silk fiber function, the authors both quantified the mechanical properties through spectroscopy, and experimentally introduce the novel protein to the fiber for mechanical comparison to standard fibroin. They show improvement in strength and toughness when SpICE-DS8 was introduced to silk fibers. This manuscript is well written, and the results demonstrate multiple convincing elements for the discovery, correlation and understanding of how novel silk genes and expression variation can contribute to the molecular and phenotypic evolution of silks. The authors create clear and visually engaging figures for visualizing the PS results, structural descriptions, and gene expression. Finally, the authors provide clear and reproducible analyses with providing their code, accession numbers and associated datasets. My main critique to the authors is to add language using existing references about the existing body of literature on silk gene expression and tissue specificity, since there is minimal in the introduction. Additionally, to discuss potential limitations or alternative explanations to their interpretations in the gene orthology assessments.

Comments and Minor Suggestions:
- Line 74: "have" to "has"
- Line 130: May also mention limitations of this interpretation (i.e missing functional annotations due to lack of homology, or incomplete databases)
- Line 153-157: For the sentence starting with "Notably...", I suggest rewording this to clarify what synergistic interactions you're referring to. As written, it can be interpreted as Leg, Epi and Ven expression patterns being correlated to Ma and Mi expression patterns. Rather, I think you're trying to convey that similarly to Ma and Mi tissues have co-expression due to collaboration in function, and that Leg/Epi/Ven show similar co-expression patterns as these two tissues. Or am I misinterpreting?
- Lines 196-197: Would suggest utilizing some of the existing citations and add a sentence about what is already known specifically about differential gene expression in silk glands in the introduction, to reflect the existing body of research (such as Chaw et al. 2021 or Clarke et al. 2017).
- Lines 214-215: It would be interesting to look at hybridization chain reaction (HCR) methods for precisely differentiating gene expression for these novel genes across the Ma silk glands. This method would help ensure that the separation of glands accurately correlates to the differences in expression. Could look at something similar to Goodheart et al., 2024 for HCR gene expression profiling, for future studies.
- Line 243-245: The structure of the Masp gland morphology (duct, sac, tail) is similar to that of some holometabolous insect silk glands (e.g. lepidopterans with anterior silk gland, median silk gland, and posterior silk gland) where insect silks use sericins and lubricant proteins to interact with the core fiber. It would be interesting to compare the similarities in interactions of MaSp/SpiCE-DS8 to that of H-Fibroin/Sericin as an interesting case of convergence.
- Line 574: add “R” before package

- Figure 1c: Would recommend changing the text color in the Venn diagram to black for visibility. It's difficult to read the yellow-on-yellow lettering, and blue-on-blue lettering.
- Figure 2a: If possible, I would recommend a less directionally dependent way of showing the orientation of the heat maps. Perhaps labeling PS1-PS16 on each of their respective heat blocks would help avoid confusion, and keeping the legend indicating the tissue type?
- Figure 4d: The text for transcripts, information conveying PS levels/New Gene status, and the phylogeny on the left are fairly small and compressed, making it difficult to decipher patterns outside of general clustering of level/gene type. I would suggest offering a stretched-out version of this figure in the supplement if possible.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The current version of the revised manuscript brings interesting results, with the more details of the methodology, attending the suggestions of reviewers, making the experimental protocols more easy to be reproduced. Still, its important to remind that the protein database used in the present study should be publically available.

Several aspects of discussion were expanded making the results more robust and reliable.

Apparently, the authors made all the corrections indicated by the reviewers, and clarified all the aspects of manuscript raising concerns; thus, the manuscript became reliable, using suitable methodology, and presenting reesults that support the hypothesis of work.

It is recommended the publications of the manuscript in the present format.

Reviewer #2

(Remarks to the Author)
The authors have addressed all issues noted in my original review in a satisfying manner. No further issues are raised.

Reviewer #3

(Remarks to the Author)
Similar to my first review of this manuscript, I thought the authors used robust and diverse methodology to answer novel evolutionary questions about silk production in Araneoidea spiders. My initial feedback suggested minor revisions, including to highlight existing literature on orb-weaver silk gene expression, and to contextualize some of their findings in the broader arthropod-silk producing context (inclusive of convergence). The authors thoroughly addressed all feedback by citing additional publications, producing additional visual elements, and clarifying language in the figures and figure legends.

Original Report: The authors of this manuscript investigate the molecular evolution and gene expression in silk glands of Araneoidea spiders– a group that utilizes ecologically and functionally diverse silks– to answer fundamental questions about the role of novel lineage-specific genes in phenotypic evolution across lineages. Using a genomic and transcriptomic approach, the authors trace back the evolutionary age of various genes using phylostratigraphic (PS) levels, from most ancestral to most recently derived. They also examine expression patterns of the genes associated with the different PS levels with tissue specificity. The authors use publicly available genomic and transcriptomic resources from several sources to conduct their analyses, with a robust taxonomic sampling. The authors find that relatively young genes show patterns of gene expression that localize to specific tissues (silk glands), while more ancestral genes have a more widespread correlation rather than tissue specificity. The authors also investigate co-expression networks in younger genes, finding integration with more ancestral genes. Using comparative phylogenomic methods, the authors find a set of novel genes (~200) that are uniquely expressed and correlated to the silk glands, and two genes that show potential for correlation to dragline silk function. For the two novel genes associated with dragline function, the authors assess the potential functional roles using Alpha Fold to predict 3D structure of sequence data and demonstrate areas that have potential for interaction with other spidroin proteins. To link these findings to silk fiber function, the authors both quantified the mechanical properties through spectroscopy, and experimentally introduce the novel protein to the fiber for mechanical comparison to standard fibroin. They show improvement in strength and toughness when SpICE-DS8 was introduced to silk fibers. This manuscript is well written, and the results demonstrate multiple convincing elements for the discovery, correlation and understanding of how novel silk genes and expression variation can contribute to the molecular and phenotypic evolution of silks. The authors create clear and visually engaging figures for visualizing the PS results, structural descriptions, and gene expression. Finally, the authors provide clear and reproducible analyses with providing their code, accession numbers and associated datasets.

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In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.
The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.
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RESPONSE TO Reviewer COMMENTS:

We sincerely thank the Reviewers for their time and effort dedicated to carefully reviewing our manuscript and for their insightful comments. Following the Editor's suggestions, we have further improved the manuscript by refining the formatting requirements, enhancing the accessibility of data and code, and completing the nr-reporting-summary. Point-by-point responses to all comments and modifications to the manuscript are listed below. The Reviewers' comments are in plain text and our responses in blue. The cross-references to the manuscript are bold and underlined. (Line numbers mentioned in the responses may not coincide with the original line numbers.)

Reviewer #1 (Remarks to the Author):

The mamnuscript entitled "A Newly Evolved Small Secretory Peptide Enhances Mechanical Properties of Spider Silk" by Anqiang Jia1 et al., deals with the investigation of the evolution of silk glands in Araneoidea spiders through comprehensive genomic and transcriptomic mining. Iy was reported numerous new genes with simplified structures and constrained expression patterns, expressed predominantly in silk glands and coexpressed with ancient genes to drive the evolution
of silk glands.

Amongst these new genes it was identified the SpiCE-DS8, which is a newly evolved small secretory peptide, which in turn seems to be incorporated into dragline silk and is involved in the MaSp network. SpiCE24 DS8 originated de novo from intergenic regions of ancient genes and is an orphan gene unique to the Nephilinae subfamily. This peptide was suggested to interacts with the N-terminal region of MaSp1b aiding in silk protein solidification. In vitro wet-spinning experiments demonstrated that SpiCE-DS8 significantly improved fiber properties. The results of the present study are indicating that the central role of new lineage-specific genes in silk gland evolution, providing new insights into the evolutionary mechanisms associated with foraging webs and for advancing the recombinant synthesis silk material development.

The study was well conducted, using suitable experimental protocols. The manuscript was well written, with careful presentation of the results. The figures were very relevant for data presentation, The present investigation my bring a nice contribution for the knowledge about the role of the web fibers in prey capture, as well for the development of of novel protocols for the production of recombinant spider web fibers more resistant.

Response: We sincerely appreciate the Reviewer's positive evaluation of our experimental methodology, manuscript presentation, and the potential significance of our findings. We are particularly encouraged by their recognition of our work's contribution to understanding spider web fiber mechanics and its applications in developing enhanced recombinant silk materials. These constructive comments affirm the value of our research direction, and we will continue to explore these important aspects in our future investigations.

Question 1. Despite to this, the authors could have described in more details the protocol of proteomic analysis, since the results generated by this specific approach was fundamental for validating the expression of SpiCE-DS8, The authors did not described / characterized the database used for proteíns idenntification. There was only a brief mention to a custon nr DB, and nothing else.

Response: We thank the Reviewer for the comments and agree with the suggestions. As the Reviewer rightly noted, the "Proteomic analysis" section indeed lacked sufficient detail. We have expanded this section in the "Proteomics" subsection of the Materials and Methods: "**The identified peptides were quantified using MaxQuant v1.3.0.5 against a custom protein sequence database comprising 37,607 entries from *T. clavata.* The search parameters included a minimum peptide length of 7 aa and allowed a maximum of two missed cleavages. Both peptide and protein identifications were filtered at a 1% false discovery rate (FDR), with protein identification requiring at least one unique peptide. All reverse database matches and potential contaminants were systematically excluded from the final dataset to ensure data quality**". We identified SpiCE-DS8 expression in both the Sac and Tail regions through proteomic analysis (Fig. 4b). A custom database was constructed incorporating: 1) Protein sequences from the NCBI-Nr database; 2) de novo assembled transcripts from this study, and 3) Additional protein sequences collected from other databases, including ScienceDB, GigaDB, Figshare, Dryad, and BIGD. This custom database was primarily employed for progressive alignment to determine the phylogenetic strata (PS) levels of *T. clavata* genes.

Question 2. The manuscript mention the interacts with the N-terminal region of MaSp1b, potentially aiding in silk protein solidification; however, the text is not clear about how this result was got. Was it obtained from molecular modeling ? Please, make it clear.

Response: We sincerely appreciate the Reviewer's insightful comments regarding the mechanistic evidence of SpiCE-DS8 interaction with the N-terminal domain of MaSp1b. The conclusion that "SpiCE-DS8 interacts with the N-terminal region of MaSp1b, potentially contributing to silk protein solidification" was drawn based on: 1) Prior established research (Gaines et al.[1]; Heiby et al.[2]) demonstrating that the N-terminal domain of MaSp plays a regulatory role in controlled self-assembly and solidification of spider silk fibers; 2) Our current findings where combined analytical and experimental results demonstrate SpiCE-DS8-MaSp1b N-terminal interactions, with *in vitro* spinning assays confirming mechanical enhancement of silk fibers. In response to similar queries from other Reviewer, we have further strengthened the evidence by: we conducted additional *in vitro* protein interaction assays (Materials and Methods): "***In vitro* protein interaction assay monitored by bright-field microscopy**"). Key experimental details: "**SpiCE-DS8-L protein was dissolved in ultrapure water, with complete dissolution confirmed by centrifugation (no observable precipitation). SP2-KI and fibroin powders were independently dissolved in 9.3 M LiBr and dialyzed (14 kDa MWCO) against deionized water for 48 h (conductivity < 10 µS/cm). For the interaction assay, equimolar mixtures of SpiCE-DS8-L with either SP2-KI or fibroin were incubated at 23°C for 30 min, followed by bright-field microscopic analysis (10 µL aliquots)**". The results demonstrated that SpiCE-DS8-L promoted the formation of solid protein aggregates in both fibroin and SP2-KI systems, with particularly pronounced effects on fibroin (Fig. R1 corresponds to Supplementary Fig. 11). Fibroin represents the natural silkworm silk protein, whereas SP2-KI is a recombinant MaSp2 fused with the N-terminal domain of silkworm fibroin. This new experimental evidence, combined with our above findings, provides stronger support for the role of SpiCE-DS8 in facilitating silk protein solidification through potential N-terminal interactions.

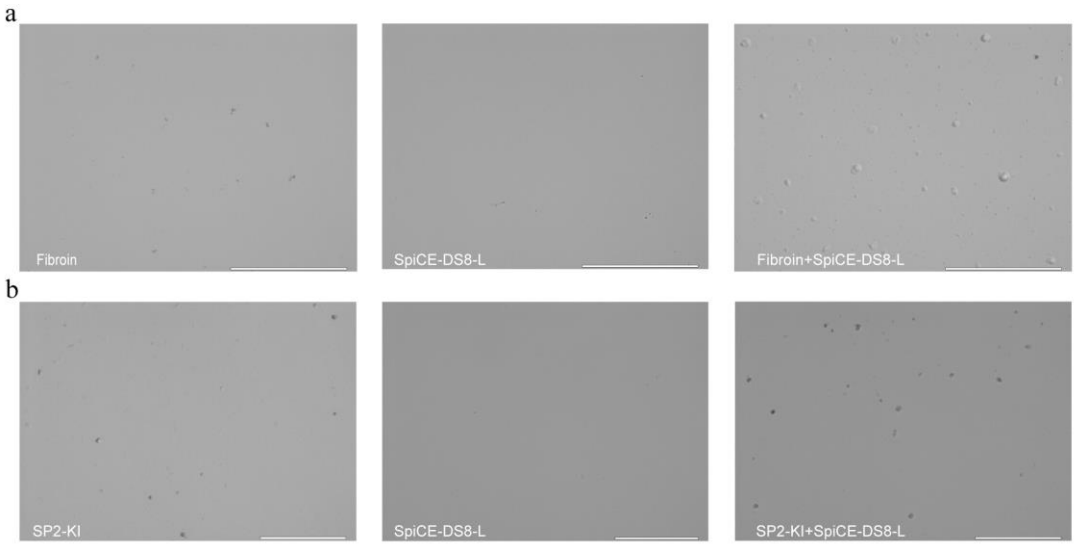

Fig. R1 | Protein-solidification assay by bright-field microscopy. a, A mixture of fibroin and SpiCE-DS8-L incubated at 23℃ resulted in extensive formation of protein aggregates, as observed by bright-field microscopy. **b**, SP2-KI combined with SpiCE-DS8-L under identical conditions similarly results in protein aggregation. The white scale bars represent 100 μm in (**a**) and 50 μm in (**b**).

Reviewer #2 (Remarks to the Author):

This is a really interesting manuscript studying the evolution of silk glands in Araneoidea spiders. The authors use a broad range of approaches mining the genomes. In particular, the authors identified SpiCE-DS8 as a newly evolved secretory peptide, which is expressed in the silk gland, and thus incorporated into the dragline silk. The authors demonstrate how this peptide affects the protein secondary structure of the spidroins via AlphaFold, which showed an increase its tendency to form β-sheets, an important precursor to high mechanical performance of the silk fibers.

Response: We sincerely thank the Reviewer for their positive comments on the interesting findings in our manuscript regarding silk gland evolution and SpiCE-DS8's function.

Question 1. Interestingly, the authors combine this analysis with some experimental work, where the influence of this peptide on the mechanical properties of spun silks fibers is tested. However, this work was carried out using silk proteins from silkworms and from spidroin-expressing silkworms. The sequences of silkworm proteins (fibroins) are very different from the MaSp1 proteins studied in most other parts of the manuscript. And unfortunately, this appears to be not discussed at all by the authors,

which is a significant omission. Similarly, the authors do not discuss any details of the silk from the spidroin-expressing silkworm.

Response: We thank the Reviewer for the comments. We have first added detailed descriptions regarding the expression of MaSp2 in silkworms in the Materials and Methods section: "**Briefly, transgenic expression of the spider gene *MaSp2* in silkworms was achieved using the BmFibH-R system[3], which produced a 160 kDa MaSp2 protein (GenBank accession ID: AF350276). In this system, endogenous *BmFibH* expression was completely silenced, while *MaSp2* expression was driven by the regulatory elements of BmFibH. The repetitive sequence of BmFibH was replaced with that of MaSp2, whereas the N- and C-terminal retained the native BmFibH sequence. Homozygous transgenic silkworms were successfully generated, with MaSp2 accounting for 51.02% of the total cocoon silk**".

Question 2. Moreover, the spinning mechanism used in this manuscript is relatively primitive, compared to the highly sophisticated process found in the natural spinneret, which triggers multiple physico-chemical effects, which are critical to obtaining a fiber with the known outstanding properties of spider silk. . Accordingly, the fibers obtained by the researchers feature a performance that is only a fraction of what is observed in native silk fibers, which feature higher breaking strains, higher Young's moduli, and higher toughness. While it is encouraging that the authors find an increase in mechanical performance and an increase in β-sheet content, the authors need to make it clearer why they think this would also apply to natural silk spun under more ideal conditions.

Response: We sincerely thank the Reviewer for their insightful comments on our spinning methodology. We completely agree that our artificial spinning system is relatively primitive compared to the sophisticated natural spinning process. Our laboratory currently only possesses this wet-spinning equipment, which cannot replicate the natural silk-spinning process. However, the theoretical design, experimental rationale, and results from this study fully support our hypothesis and conclusion that "SpiCE-DS8 can significantly enhance the mechanical properties of silk fibers". To further validate this finding, we have conducted additional experiments using "***In vitro* protein interaction assay monitored by bright-field microscopy**". These results demonstrate that even without the spinning process, the addition of SpiCE-DS8 enhances the fibroin assembly capability of both silkworm and spider silk proteins (Fig. R1 corresponds to Supplementary Fig. 11). This provides additional evidence

supporting our conclusions and helps compensate for the limitations of our spinning methodology.

Question 3. What is the sequence of this silk? What's its molecular weight? What are the major and minor differences relative to the MaSp1 spidroins discussed in other places of the manuscript? Without further discussion and explanation, it is unclear whether all other findings and analyses on MaSp1 readily apply to the experiments that are based silkworm silk and spidroin-expressed silk from silkworms. To further corroborate this correspondence, the authors could carry out AlphaFold work for the experimentally studied proteins (and the effect of the SpiCE-DS8 peptide on them). If this reveals similarities in the behavior of the proteins when exposed to the new peptide, the authors' conclusion would be somewhat better motivated and supported.

Response: We thank the Reviewer for these comments. Notably, these comments are crucial to the findings and conclusions of this study. We have added a detailed discussions of the sequence characteristics of spider MaSp and silkworm fibroin heavy chain (FibH, the dominant component of silkworm cocoon silk). Additionally, following the Reviewer's suggestions, we employed AlphaFold3 to analyze potential interactions between SpiCE-DS8 and FibH, which may provide a plausible explanation for how SpiCE-DS8 could also enhance the mechanical properties of silkworm silk. The corresponding modifications in the manuscript are as follows: "**The Ma gland of spider and the silk gland of silkworm share morphologically similar spinning apparatuses and convergent silk-secretion characteristics. Notably, the mechanical properties of silkworm cocoon silk and spider dragline silk are primarily attributed to FibH and MaSp proteins, respectively. Although FibH and MaSp exhibit almost no sequence homology, they share a common architectural organization: a conserved N-terminal domain, a highly variable repeat region, and a conserved C-terminal domain (Fig. R2a corresponds to Supplementary Fig. 13a). Crucially, while their N-terminal sequences differ significantly (Fig. R2b corresponds to Supplementary Fig. 13b), AlphaFold3 predictions revealed that the N-terminal domain of fibroin (FibH-N) can also interact with SpiCE-DS8 (Fig. R2c-e corresponds to Supplementary Fig. 13c-e). This finding strongly suggests that SpiCE-DS8 may similarly enhance the mechanical properties of silkworm silk. Based on this evidence, we further validated the functional role of SpiCE-DS8 using both native silkworm silk and transgenic MaSp-expressing silkworm silk**".

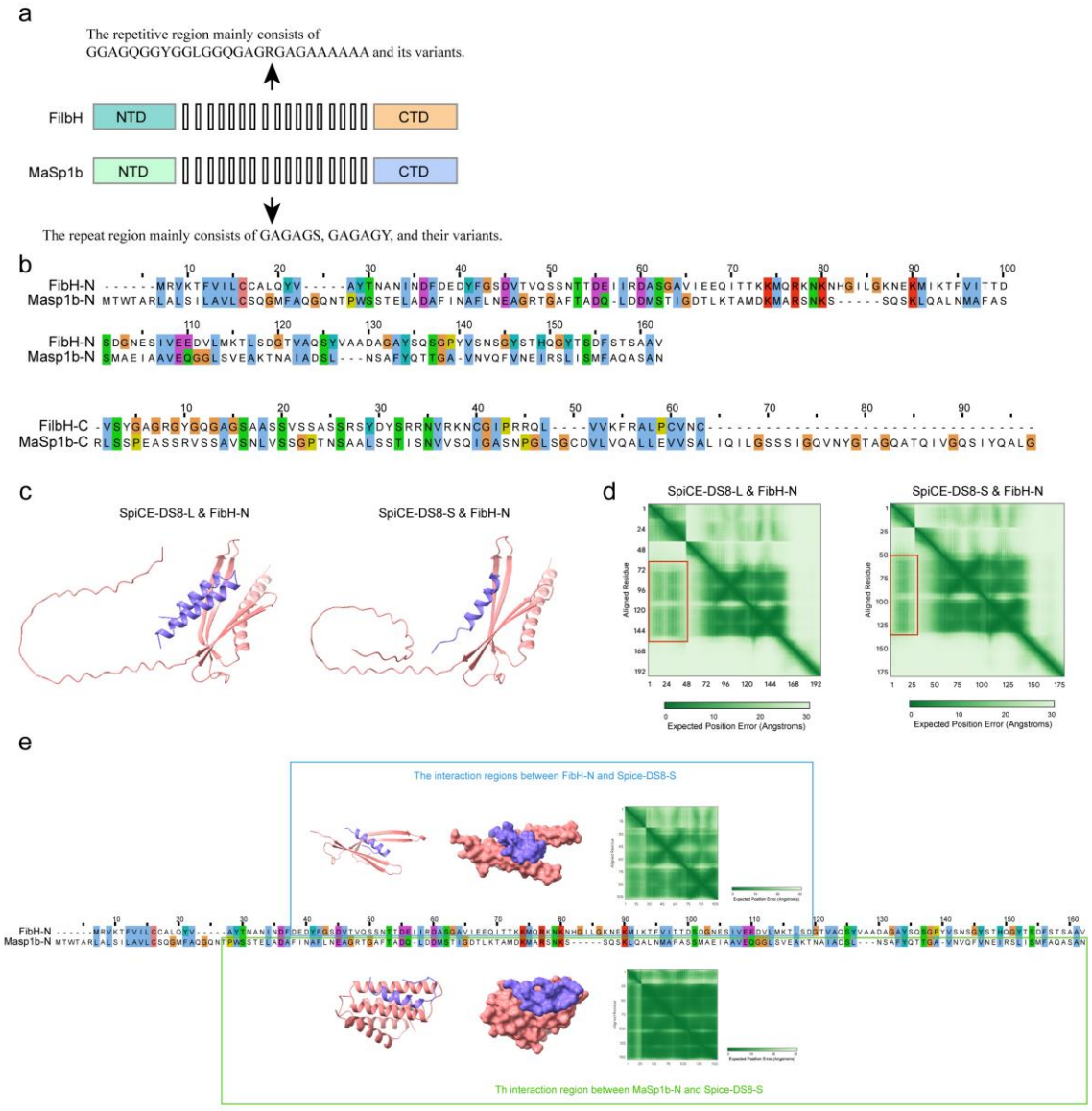

Fig. R2 | Schematic representation and predicted interaction models of FibH and MaSp1b with SpiCE-DS8-L. a, Sequence architecture of FibH and MaSp1b. Schematic diagrams displaying the structural composition (not to scale). **b**, Sequence alignment of the N-terminal domains of FibH and MaSp1b. **c**, Predicted 3D interaction interfaces between SpiCE-DS8-L and the N-terminal domains of FibH (left) and MaSp1b (right). **d**, Interaction heatmaps of SpiCE-DS8-L with the N-terminal domains of FibH (left) and MaSp1b (right). **e**, Predicted 3D interfaces and interaction heatmaps between SpiCE-DS8-L and truncated N-terminal domains, with corresponding sequence alignment.

Reviewer #3 (Remarks to the Author):

Manuscript Report:

The authors of this manuscript investigate the molecular evolution and gene expression in silk glands of Araneoidea spiders– a group that utilizes ecologically and functionally diverse silks– to answer fundamental questions about the role of novel lineage-specific genes in phenotypic evolution across lineages. Using a genomic and transcriptomic approach, the authors trace back the evolutionary age of various genes using phylostratigraphic (PS) levels, from most ancestral to most recently derived. They also examine expression patterns of the genes associated with the different PS levels with tissue specificity. The authors use publicly available genomic and transcriptomic resources from several sources to conduct their analyses, with a robust taxonomic sampling. The authors find that relatively young genes show patterns of gene expression that localize to specific tissues (silk glands), while more ancestral genes have a more widespread correlation rather than tissue specificity. The authors also investigate co-expression networks in younger genes, finding integration with more ancestral genes. Using comparative phylogenomic methods, the authors find a set of novel genes (~200) that are uniquely expressed and correlated to the silk glands, and two genes that show potential for correlation to dragline silk function. For the two novel genes associated with dragline function, the authors assess the potential functional roles using Alpha Fold to predict 3D structure of sequence data and demonstrate areas that have potential for interaction with other spidroin proteins. To link these findings to silk fiber function, the authors both quantified the mechanical properties through spectroscopy, and experimentally introduce the novel protein to the fiber for mechanical comparison to standard fibroin. They show improvement in strength and toughness when SpICE-DS8 was introduced to silk fibers. This manuscript is well written, and the results demonstrate multiple convincing elements for the discovery, correlation and understanding of how novel silk genes and expression variation can contribute to the molecular and phenotypic evolution of silks. The authors create clear and visually engaging figures for visualizing the PS results, structural descriptions, and gene expression. Finally, the authors provide clear and reproducible analyses with providing their code, accession numbers and associated datasets.

Response: We sincerely thank the Reviewer for their thoughtful evaluation of our work and their positive comments regarding the manuscript's clarity, figures, and data accessibility. We are particularly grateful for their recognition of our efforts to provide comprehensive and reproducible analyses through open data sharing.

Question 1. My main critique to the authors is to add language using existing references about the existing body of literature on silk gene expression and tissue specificity, since there is minimal in the introduction.

Response: We thank the Reviewer for the suggestions. Indeed, this suggestion is crucial for our discussion of silk gland gene expression. Accordingly, we have added the following statement to the Introduction section: "**Comparative transcriptomic studies of silk glands in *Argiope argentata* and cobweb-weaving spiders have revealed lineage-specific gene expression patterns, including silk gland-specific transcripts (SSTs) and divergent expression profiles between fiber- and glue-producing glands, highlighting the crucial role of gland-specific expression components in silk synthesis and evolution [3,4]**".

Question 2. Additionally, to discuss potential limitations or alternative explanations to their interpretations in the gene orthology assessments.

Response: We sincerely appreciate the Reviewer's thoughtful comments regarding potential limitations in orthology assessments. We fully acknowledge these concerns and have implemented multiple safeguards in our analysis:

(1) While Blastp, as the most widely used method for homology detection, may introduce biases in distant homology searches, our stringent e-value cutoff ($1e^{-3}$) effectively minimizes false positives. Although prior studies note that homology-based approaches may introduce temporal estimation biases in gene age dating, our focus here is specifically on identifying genes emerging after Araneoidea divergence, not on determining their precise origin timing.

(2) To reduce potential bias through expanded datasets, we employed the following resources: the Nr database, de novo assembled transcriptomes from more than 1,000 spiders, and 30 published arthropod genomes. Compared with previous studies that relied on fewer species for gene assignment, our comprehensive dataset significantly minimizes false positives.

(3) We implemented a progressive alignment method: (i) Orthology assignment of genes to phylogenetic strata (PS); (ii) Sequential alignments initiating from PS1, and (iii) Iterative exclusion of hits at each stratum until PS16 (Fig. R3). Compared with best-hit-only approaches, this method demonstrably reduces misclassification.

(4) For uncertain phylogenetic relationships, we reconstructed the phylogenetic tree to ensure the

accuracy of the gene assignments.

We acknowledge that no orthology assessment method is perfect, but our comprehensive approach, which incorporates stringent homology filtering, phylogenetic tracing, and expanded datasets, effectively minimizes potential false positives. As evidenced by the Venn diagram in Figure 1c, none of the 6,868 Araneoidea-specific genes were annotated with functional domains (potentially due to lack of homology or complete databases), further validating the robustness of our gene assignment strategy.

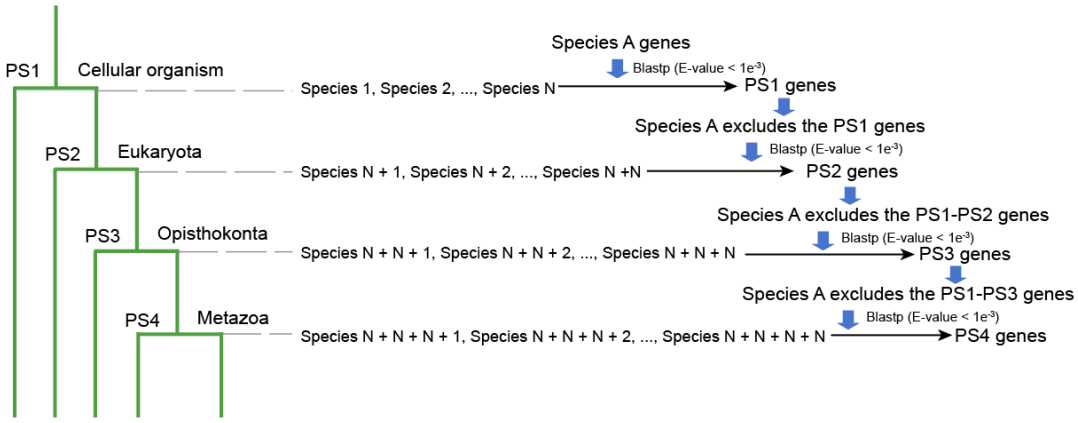

Fig. R3 | Progressive alignment strategy across phylogenetic strata. Only 4 PS levels are shown; the other levels follow the same protocol.

Comments and Minor Suggestions:

- Line 74: "have" to "has"

Response: We thank the Reviewer for this suggestion. We have corrected "have" to "**has**" in the manuscript.

- Line 130: May also mention limitations of this interpretation (i.e missing functional annotations due to lack of homology, or incomplete databases)

Response: We thank the Reviewer for this suggestion. As you correctly noted, the lack of functional annotations could indeed stem from current limitations in arachnid genomic data, including insufficient homology information or incomplete database coverage. Accordingly, we have incorporated the explanation "**potentially due to lack of homology or incomplete database coverage**" in the manuscript.

- Line 153-157: As written, For the sentence starting with "Notably...", I suggest rewording this to clarify what synergistic interactions you're referring to. it can be interpreted as Leg, Epi and Ven expression patterns being correlated to Ma and Mi expression patterns. Rather, I think you're trying to convey that similarly to Ma and Mi tissues have co-expression due to collaboration in function, and that Leg/Epi/Ven show similar co-expression patterns as these two tissues. Or am I misinterpreting?

Response: We thank the Reviewer for this suggestion. Indeed, the term "synergistic interactions" could be ambiguous in this context. We have modified the text accordingly: "**Strong expression correlations were observed among Ped, Leg, and Epi, as well as between Ma and Mi, suggesting potential functional or developmental linkages. For instance, the Ma and Mi glands share similar morphological features and collaborate in prey capture through their combined secretions, which may underlie their coordinated gene expression patterns**".

- Lines 196-197: Would suggest utilizing some of the existing citations and add a sentence about what is already known specifically about differential gene expression in silk glands in the introduction, to reflect the existing body of research (such as Chaw et al. 2021 or Clarke et al. 2017).

Response: We sincerely appreciate the Reviewer's comment. As you correctly noted, describing differential gene expression in silk glands is indeed crucial for this study. Accordingly, we have added the following statement to the introduction: "**Comparative transcriptomic studies of silk glands in Argiope argentata and cobweb-weaving spiders have revealed lineage-specific gene expression patterns, including silk gland-specific transcripts (SSTs) and divergent expression profiles between fiber- and glue-producing glands, highlighting the crucial role of gland-specific expression components in silk synthesis and evolution[4,5]**".

- Lines 214-215: It would be interesting to look at hybridization chain reaction (HCR) methods for precisely differentiating gene expression for these novel genes across the Ma silk glands. This method would help ensure that the separation of glands accurately correlates to the differences in expression. Could look at something similar to Goodheart et al., 2024 for HCR gene expression profiling, for future studies.

Response: We sincerely appreciate the Reviewer's suggestion. We had not previously considered the

recent work by Goodheart et al. (2024) utilizing hybridization chain reaction (HCR) methods for high-resolution gene expression localization, and we thank you for bringing this to our attention. The approach of using synthetic gene probes to capture spatial expression patterns is indeed fascinating. Notably, this method requires careful tissue sectioning to ensure proper capture of target regions. For Ma silk glands, the complex three-dimensional morphology of silk glands presents technical challenges: the Tail region exhibits elongated coiling, the Sac shows significant dilation, and the Duct maintains a slender morphology, making it particularly difficult to capture all three segments within a single plane. In our previous work, we achieved this goal only after numerous sectioning attempts, enabling spatial transcriptomic analysis that functionally resembles electronic in situ hybridization for observing region-specific gene expression patterns (Fig. R4a corresponds to Supplementary Fig. 9a). While our spatial transcriptomics data successfully demonstrated *SpiCE-DS8* expression in the Sac region adjacent to the Duct, thereby validating our segmental bulk RNA-seq results, we acknowledge that the resolution may not match the precision offered by HCR approaches. We are truly grateful for this suggestion and agree that the HCR methodology could be highly valuable for future investigations of novel gene expression patterns, particularly when studying the functional roles of newly identified genes. This approach will be considered in our subsequent research.

- Line 243-245: The structure of the Masp gland morphology (duct, sac, tail) is similar to that of some holometabolous insect silk glands (e.g. lepidopterans with anterior silk gland, median silk gland, and posterior silk gland) where insect silks use sericins and lubricant proteins to interact with the core fiber. It would be interesting to compare the similarities in interactions of MaSp/SpiCE-DS8 to that of H-Fibroin/Sericin as an interesting case of convergence.

Response: We sincerely appreciate the Reviewer's insightful suggestion. The silk-spinning apparatuses of lepidopteran insects (*B. mori*) and spider silk glands (major/minor ampullate glands) exhibit remarkable morphological similarities. In our previous studies[6], we systematically analyzed the morphology, cell types, segment-specific gene expression patterns, GO functional annotations, and silk protein and metabolite profiles of silkworm silk and spider silk. These comprehensive analyses consistently demonstrated their convergent evolutionary features.

Particularly intriguing is the widely recognized consensus among biologists and material scientists that the posterior regions (PSG in silkworms and Tail in spiders) serve as the core production sites for

silk fibroin/spidroin, whereas the middle regions (MSG in silkworms and Sac in spiders) function primarily in storage and lubrication, with the anterior regions (ASG in silkworms and Duct in spiders) dedicated to fiber formation. This conserved spinning mechanism presents exciting opportunities for engineering spider silk production in silkworms. These established findings provide the foundation for our current investigation of SpiCE-DS8 function. On the basis of its spatially restricted expression pattern, we hypothesize that SpiCE-DS8 may interact with MaSp and contribute to silk fiber performance. Accordingly, we have revised the manuscript: "**Based on the process of spidroin synthesis, secretion, and fiber formation in the silk gland[31], as well as the convergent silk fiber formation mechanism in the silkworm silk gland (Fig. R4b corresponds to Supplementary Fig. 9b). We hypothesize that the MaSp proteins are synthesized in the Tail and Sac, and then flow through the Sac and Duct where they interact with SpiCE-DS8**". To enhance reader engagement, we have added comparative diagrams of silkworm and spider silk glands in Supplementary Fig. 9b to highlight these evolutionary convergences.

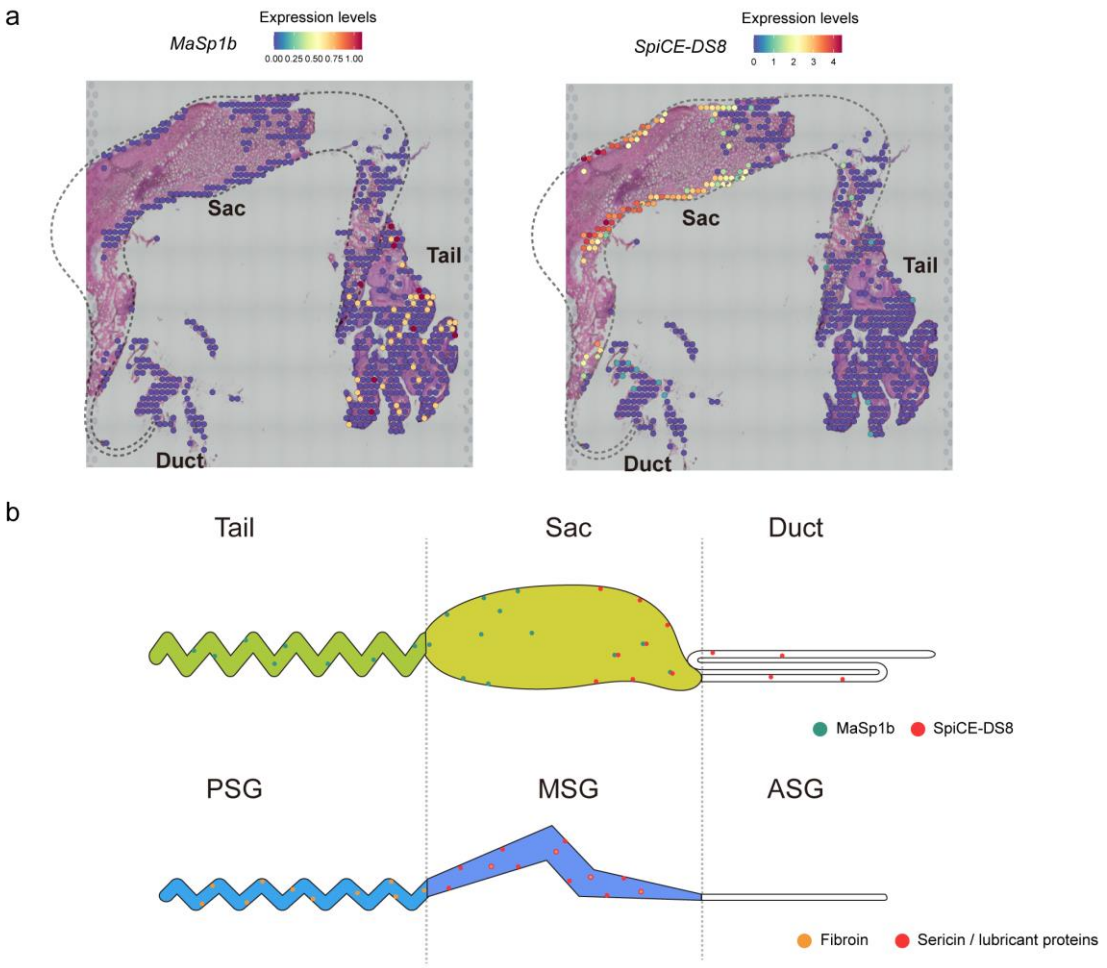

Fig. R4 | Spatial expression of *MaSp1b* and *SpiCE-DS8* and their proposed interaction schematic in Ma. a, Spatial transcriptomic (ST) spots of *MaSp1b* and *SpiCE-DS8*. The dotted lines depict the outline of the Ma gland. **b**, Comparative secretion models of silk components in spider (top) and silkworm (bottom) glands. Top: MaSp1b-SpiCE-DS8 interaction in the major ampullate lumen. Bottom: Fibroin and sericin/lubricant protein interactions in the silk gland lumen.

- Line 574: add “R” before package

Response: We thank the Reviewer for this suggestion. We have added “**R**” before the package in the manuscript.

- Figure 1c: Would recommend changing the text color in the Venn diagram to black for visibility. It’s difficult to read the yellow-on-yellow lettering, and blue-on-blue lettering.

Response: We thank the Reviewer for this suggestion. We have modified all text elements to use black

font for improved clarity in both Fig. 1c and Supplementary Fig. 3c.

- Figure 2a: If possible, I would recommend a less directionally dependent way of showing the orientation of the heat maps. Perhaps labeling PS1-PS16 on each of their respective heat blocks would help avoid confusion, and keeping the legend indicating the tissue type?

Response: We thank the Reviewer for this suggestion. In Fig. 2a and Supplementary Fig. 5a-b, each heatmap is labeled with its corresponding PS1-PS16. The figure legend clarifies that "**Tissue order in each PS heatmap (top-to-bottom, left-to-right) follows the arrow-indicated order in the legend**".

- Figure 4d: The text for transcripts, information conveying PS levels/New Gene status, and the phylogeny on the left are fairly small and compressed, making it difficult to decipher patterns outside of general clustering of level/gene type. I would suggest offering a stretched-out version of this figure in the supplement if possible.

Response: We thank the Reviewer for this suggestion. To the best of our understanding, the comment likely refers to Fig. 3d. We have included an expanded version of Fig. 3d as Figure R5/Supplementary Fig. 7, where the heatmap has been elongated and the gene IDs are now arranged in two staggered columns to prevent overlap, thereby improving the visibility of the gene IDs, functional categories, and PS-level annotations.

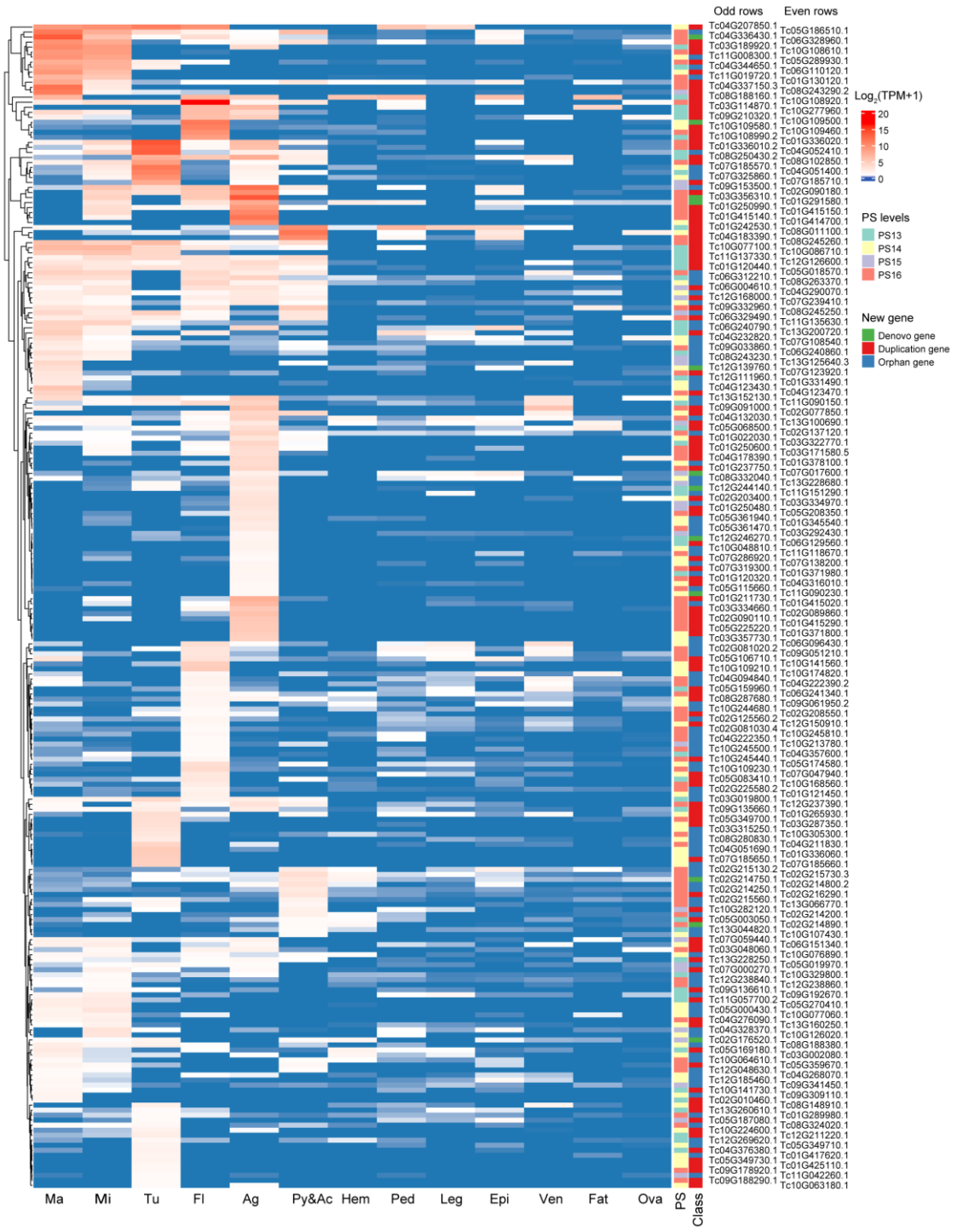

Fig. R5 | Expression patterns of 232 Araneoidea-specific genes across 13 tissues of *T. clavata*. Ma: major ampullate gland; Mi: minor ampullate gland; Tu: tubuliform gland; Fl: flagelliform gland; Ag: aggregate gland; Ac&Py: mixed aciniform and pyriform gland; Hem: hemolymph; Ped: pedipalp; Leg: legs; Epi: epidermis; Ven: venom gland; Fat: fat body; Ova: ovary.

1. Gaines, W. A., Sehorn, M. G. & Marcotte, W. R. Spidroin N-terminal Domain Promotes a pH-dependent Association of Silk Proteins during Self-assembly. *J. Biol. Chem.* **285**, 40745–40753 (2010).
2. Heiby, J. C., Goretzki, B., Johnson, C. M., Hellmich, U. A. & Neuweiler, H. Methionine in a protein hydrophobic core drives tight interactions required for assembly of spider silk. *Nat. Commun.* **10**, 4378 (2019).
3. Yu, Y. *et al.* Custom-designed, mass silk production in genetically engineered silkworms. *Pnas nexus* **3**, pgae128 (2024).
4. Clarke, T. H. et al. Evolutionary shifts in gene expression decoupled from gene duplication across functionally distinct spider silk glands. *Sci. Rep.* **7**, 8393 (2017).
5. Chaw, R. C., Clarke III, T. H., Arensburger, P., Ayoub, N. A., & Hayashi, C. Y. Gene expression profiling reveals candidate genes for defining spider silk gland types. *Insect Biochem. Mol. Biol.* **135**, 103594 (2021).
6. Hu, W. et al. A molecular atlas reveals the tri-sectional spinning mechanism of spider dragline silk. *Nat. Commun.* **14**, 837 (2023).