

# Pervasive convergent evolution of sperm conjugation across the Arthropoda tree of life

Corresponding Author: Dr Scott Pitnick

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

**Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Dear Authors,

Your manuscript is very interesting and presents valuable new information about the evolution of aggregated sperm in Arthropods. You have done relevant work, including transcriptomic and molecular phylogenetic analyses, as well as a thorough bibliographic review of this particular sperm phenomenon in Arthropods.

Overall, I find the manuscript well formulated and well written. I have made specific suggestions in the text and in the main and supplementary figures. The most important points to address are summarized below (see the other comments and suggestions in the attached files):

Main text

Crucial point: In several parts of the text and figures, you mention an estimated divergence time of approximately 500 mybp as a reference to explain the emergence of aggregated sperm. Using the exact time estimates from your temporal calibration would allow you to provide explicit divergence dates and confidence intervals for the estimated ages. I suggest including these exact data and the probability of the character in the Results section.

A deeper exploration of the calibrated evolutionary times and their relationship to the evolution of sperm aggregation for each subphylum is needed, especially for Hexapoda. At present, nothing is mentioned beyond the approximate age of 500 mybp has origin to sperm aggregation. Including these additional data would be very relevant for your manuscript.

The results of the phylogenetic time calibration and the confidence intervals for each node should be shown and included in the supplementary material.

According to Figure 1, the MRCA between Hemiptera and Coleoptera might be estimated at more than 400 mybp, which is not consistent with Figure 4. I emphasize again the importance of showing the exact age estimates with their confidence intervals. Because the phylogenetic tree is so collapsed, it is difficult to identify the MRCA of these two orders.

Figures

Figure 1: Add the names of the orders next to each icon. This information would be useful and informative for readers who are not familiar with each group.

Figure 2a: The figure is too small, making it impossible to read the terminal labels and posterior probabilities on the branches, which makes interpretation difficult. I suggest enlarging the figure or dividing the tree into two panels. You could reduce the size of Figure 2b. The double temporal legend in both directions may cause confusion; it would be clearer to separate each subphylum visually using soft shading or to mark the origin of each clade with a dot or arrow. In its current form, it is difficult to know where each clade begins and ends.

Please also see my comments on the supplementary figures in the annotated PDF.

## Methods

I am not fully convinced about the phylogenetic reconstruction methodology, particularly the grafting approach. Although it is a practical strategy when no single tree includes all species of interest, it has important limitations—especially when used for quantitative evolutionary analyses such as diversification or ancestral state reconstruction. The result is a hybrid supertree combining information from different sources, often without shared molecular data among species, which may not always be comparable or congruent. Internal relationships may therefore be inconsistent with the true evolutionary history. This limitation should be explicitly mentioned in the Methods section.

In addition, I believe that this part of the methodology does not provide enough detail to allow reproduction of the analysis. I strongly recommend that the phylogenetic trees generated using the grafting method be made available to readers, including the backbone tree that was used. This is important because grafting is a manual method, and the points where other phylogenies are inserted into the backbone tree can directly influence the results obtained in the ancestral state reconstruction and in the temporal calibration.

In the Bayesian analysis, did you check the Effective Sample Size (ESS) for each statistic calculated in BEAST? This was not mentioned. Ensuring  $ESS > 200$  (in BEAST or other Bayesian phylogenetic programs) is essential to verify the reliability and convergence of the estimated tree (branch lengths, times, and rates). This can be checked, for example, using Tracer.

Besides you should to show exactly are phylogenetically placed the fossils used to calibration.

## Supplementary Material

It would be very informative to include the higher-level taxonomic information (class, order, and family) for the taxa listed in the supplementary table (t1vd1d). The species name alone is not very informative and does not guide the reader to find the precise information in the phylogenetic tree. Since you are showing a phylogeny across multiple taxonomic levels, including detailed taxonomy would improve clarity.

Please also include in the supplementary material the Bayesian tree showing the calculated node ages with confidence intervals and posterior probabilities. This will allow readers to visualize the temporal estimates for each clade concretely. The details currently available in the figures are minimal.

In summary, I believe the manuscript can be improved and completed with the adjustments mentioned above. These revisions are important and, once addressed, could substantially enhance the quality of the study. After these corrections, the manuscript could be considered for publication in the journal.

Best regards,

Reviewer #2

(Remarks to the Author)

Review of Gomez et al., "Pervasive convergent..."

The authors survey literature to examine the origins and evolution of sperm conjugation. The authors postulate Cambrian origin and multiple reversals and regains.

The construction of the base arthropod phylogeny appears to have been more or less by hand. This is unacceptable. Either perform an analysis based on published data (there are many) or use a published phylogeny with the taxa used in the manuscript as exemplars (and perhaps with some level of missing data). The phylogeny employed follows general arthropod phylogenetics (e.g. crustacean paraphyly with respect to insects) but the specifics need to be supported by data and analysis.

Other comments:

How was the 2/3 of evolutionary history of arthropods calculated? 2/3 of the time arthropods have been around there has been conjugated sperm? Or of all time in all lineages, 2/3 of lineage time has conjugated sperm? This is unclear. The first is not very meaningful, the second would not be simple to calculate and given the Bayesian analysis—would have a distribution. Please clarify.

True bugs and beetles as "deep homology" (could not be deeper than Eumetabola) is a rather restricted sample both numerically and phylogenetically. The handful of proteomes is not very informative.

P16 I352. There really doesn't have to be an immediate selective advantage for any trait. A trait's presence can be purely due to historical factors.

### Reviewer #3

(Remarks to the Author)

1. In this paper, the authors have exhaustively reviewed the vast literature on sperm ultrastructure in arthropods to examine sperm conjugation, meanwhile the authors have reconstructed the phylogenetic and time trees of arthropods based on nearly all available genomic or gene data given the arthropod backbone trees. The results showed that sperm conjugation of arthropods first likely arose before the Cambrian Period at about more than 600 million years ago, and the character of sperm conjugations was estimated to occur at about two-thirds of its timespan, with its evolutionarily lost and gained approximately about 45 times; in addition, the results indicated that the common ancestor of all insects likely had conjugated sperm along with the origin of extracellular sperm-associated material (SAM); furthermore, comparative proteome analyses of spermatostyles of representative taxa indicated that a common genetic toolkit was likely to be used to convergently shape the deep homology for the evolution of complex ejaculate adaptations of arthropods. The work attempted to shape the evolutionarily dynamic history of ejaculate form and function in arthropods and obtained lots of remarkably interesting results of morphological evolution of sperms in a very important animal group and provided a very good example for this study areas.
2. The phylogenetic analysis methods in the manuscript was not fully clear stated, for example, "within the scaffolding of these well-supported higher-level clades, we grafted additional tips onto the tree based upon more targeted molecular phylogenetic studies, typically at the ordinal level or below, while prioritizing more recent comprehensive phylogenomic or multi-locus studies over older single gene studies." and in this tree grafting of shallow-level clade onto higher-level clade, the grafting was direct or not? and if not what evolutionary models was used in both higher and shallower-level tree reconstruction? in addition, the divergence time estimation of this work was mainly conducted according to the results of previous work, and should be conducted by the authors on the grounds of more suitable fossil records and priors of more arthropod taxa.
3. The references 69~87 of manuscript was not listed or shown in the draft, and thus the authors were suggested to add these references in the revised manuscript carefully.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Dear Authors,

Thank you for the answer to my suggestions and comments. Most of them were well answer and included in this new version of the manuscript. I did few more suggestions to improve even more your manuscript and that I considered important.

- I added other few comments and suggestions in the Supplementary material and main text (see below and in the attached files).
- I understand there is not enough space in the main body of the manuscript to include a large Fig.2 a, however I insist that Supplementary Figure 2 continue to be not legible and not so useful to the readers. I recommend you at least in Supplementary material divided this fig. tree in 2 parts (e.g., Hexapoda; others clades) so as not to compress the names of taxa and age data in the nodes. This could be more informative and best presented allow valorise the data in the phylogenetic tree.
- 654438\_1\_data\_set\_12132588\_t8wyk7 table: missing description of State1 in: "ConjugationMulti / Conjugation when present occurs in 5 discrete types (0 unconjugated sperm, 2 paired, 3 ensheathed, 4 aggregate, 5 rouleaux, 6 spermatostyle)"  
If they are unconjugated sperm why you pass from state 0 to 2? Can you clarify this point?
- Pag 8 line 170 main text : "Imprecision" word I suggested changing this word.  
I consider that this "agreggate" type is precise in its function and design, or well the "agreggate" is designed to perform a specific function.
- Then, what are the structural specializations of SAM that you mentioned is not in "agreggate" as in spermatostyle? Since SAM is present in "agreggates", as mentioned in the supplementary table, could you clarify this point?

Reviewer #2

(Remarks to the Author)

The authors have addressed all my comments.

Reviewer #3

(Remarks to the Author)

1. The authors have responded point-by-point to the reviewers and accordingly revised the manuscript carefully according to the reviewer's comments, especially about the phylogeny reconstruction as well as the molecular dating, with more supplementary figures and data files, thus improving the quality of the manuscript a lot for acceptance of publication.

2. The authors have done this valuable work about the evolution of aggregated sperm in Arthropods and related animal groups through multiple disciplinary criteria, and revised the estimate of Hexapod's initial sperms aggregated conjugates at about 508.5-452.6 myr across the Cambrian and Ordovician. So further studies about the molecular mechanism, especially the SAM in "proto-conjugates" facilitating subsequent diversification of multiple sperm conjugate types, using multi-omics data of the representative living hexapod taxa coupled with relevant geological events are expected in the near future.

Reviewer #4

(Remarks to the Author)

Gomez et al. "Pervasive convergent evolution of sperm conjugation across the 1 Arthropoda tree of life".

The authors present an evolutionary analysis of sperm conjugation across arthropods. Conjugation comes in several forms – aggregates, pairs, rouleaux, spermatostyles, and ensheathed. By mapping these states on a phylogeny, the authors learned a great deal about how such a complex trait evolves.

This is an excellent paper. If the only thing this paper did was report the dataset collected across 600 species, it would have a huge impact. But they go to make insights into the evolution of this very interesting trait. It is surprising how widespread this phenomenon is and how labile it is. There is clear directionality – SAM evolves first, then aggregation, then more complicated forms of conjugation. It really is a very interesting paper. The conjugation phenotype is complicated, but the authors have made reasonable choices about how to score them.

Figure 1 is beautiful!

I only have one absolutely crucial comment. All the rest can be considered minor.

Main comment:

1) A central claim is true bugs and beetles use a "common genetic toolkit" in their spermatostyles even though they have arisen independently. The key argument comes in the paragraph that begins on line 306. They say that 21.7% of the identified proteins were present in both beetles and bugs, significantly greater than expected. My understanding of how they created the null expectation is that they randomly drew from the pool of 4 bug + 6 beetle + *Drosophila* reference proteome. I am underlining *Drosophila* reference proteome because that is going to be an enormous set of protein orthogroups. When they randomly sample from that huge set, of course they will not see much overlap in any of their permutations – this almost guarantees that 21.7% is higher than expected. What they've really asked is "are spermatostyle proteomes random draws from a huge universe of proteins". It is almost impossible to not reject that hypothesis – however, I would guess you would see the same result if you tested any tissue, not just spermatostyles. Thus a "shared genetic toolkit" is not a strong conclusion, or at least not unexpected given the construction of the null. What we care about is whether there is more sharing among independently derived spermatostyles compared to some other trait that is single-origin. Another reason this is not the right null is because it assumes orthogroups are recruited independently. But orthogroups in the observed proteomes are most certainly not independent – i.e., some co-occur often because they participate in similar pathways.

One last concern: orthogroup has been binarized to present/absent. However, some orthogroups are going to be more abundant than others, and we might have more confidence in abundant orthogroups, compared to rare ones that might simply drop out because of stochastic sampling.

This concern does not undermine the overall paper, but the claim of a shared genetic toolkit should be tempered or reframed, or they need to devise a different null.

Minor comments:

2) It seems like most sperm phenotypes were scored from dissections of males. I realize this won't exist for all species, but are there data to suggest how similar/different sperm conjugates are in the male RT vs. after being transferred to the female RT? Around line 170 they mention this, but I would have liked some more details/discussion.

3) The Introduction wanders quite a bit. As just one example, why talk about spider silk to such detail? I recommend tightening up the Introduction.

4) Fig. 3 b shows that cicadas have a very different curve than bugs or beetles. One way this could happen is if the cicada transcriptome (or whatever they search against for their mass spectra) is poor quality. In other words, it is just more difficult to match spectra to a proteome if the underlying data is poorly assembled or annotated. This would be a methodological artifact. Can the authors address this?

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)

I was reviewer #4 originally. This remains an excellent paper and should definitely be published in Nature Communications. The vast size of the dataset, and the amount of insight gained from their analyses is high impact. The main comment in my original review focused on their simulation. I appreciate their clarifications, but I still don't think this test leads to a "shared genetic toolkit" conclusion. Their test shows that spermatostyles are not random protein collections, but this is likely true for any cell-type's proteome.

I am comfortable with the analysis remaining as is, but they would need to temper the "shared genetic toolkit" conclusion. Or at least pose the right experiment (comparing overlap to single-origin cell-type) as potential future work. As a hypothetical, what if this approach was repeated for bug and beetle heads? If the overlap observed in spermatostyles were comparable to, or even lower than, that observed for another tissue, then the current result would not support a "shared genetic toolkit" interpretation. Or maybe more accurately... we would conclude that spermatostyles are like other tissues in that they express non-random proteomes.

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## Point-by-point Response to Reviewers

### Reviewer #1 (Remarks to the Author):

*Your manuscript is very interesting and presents valuable new information about the evolution of aggregated sperm in Arthropods. You have done relevant work, including transcriptomic and molecular phylogenetic analyses, as well as a thorough bibliographic review of this particular sperm phenomenon in Arthropods.*

*Overall, I find the manuscript well formulated and well written. I have made specific suggestions in the text and in the main and supplementary figures. The most important points to address are summarized below (see the other comments and suggestions in the attached files):*

Thank you for your very useful comments. Your detailed feedback has helped us improve the manuscript tremendously. We addressed all of your comments and implemented most of your suggestions. If you wish to follow precisely what changes have been made, we have provided a copy of the manuscript with tracked changes. The most substantive changes include: (1) we more richly detailed how the phylogeny was constructed and divergence times inferred, including the addition of a new Supplementary Figure (Supplementary Figure 2) that shows the 95% HPD node ages, fossil priors, and posterior distributions of conjugate type, (2) we added taxonomic information to Supplementary Data files (e.g., Supplementary Data 1 includes Subphylum, Class, Order, and Family for all 621 tips, and (3) we report age ranges for many of our results in addition to point estimates for the timing of origins of conjugate type.

### Main text

*Crucial point: In several parts of the text and figures, you mention an estimated divergence time of approximately 500 mybp as a reference to explain the emergence of aggregated sperm. Using the exact time estimates from your temporal calibration would allow you to provide explicit divergence dates and confidence intervals for the estimated ages. I suggest including these exact data and the probability of the character in the Results section.*

We agree with this being crucial point and addressing it has substantially improved the manuscript. In the Abstract, Figure 2 caption, and throughout the text, the 500 mybp point estimate has been replaced by an age range based on our modeling, nodal ages, and time calibrated phylogeny. To be clear, the “500 mybp” value was based on the crown-group age of Hexapoda in our phylogeny (median height = 495 mybp, to be exact), which is likely to have had aggregates at or near its root. As suggested, we now report age ranges using both the information contained in the tree’s nodal ages and the results from stochastic mapping, which precisely correspond to the divergence times from a summary of the ancestral state reconstructions. Specifically, we report the ages of nodes in mybp (millions of years before the present) when a clade was reconstructed as having a posterior probability of the character state between 50-90 across 1000 simulated mappings (Methods updated; p. 29, lines 627 – 630). Following these criteria, we now report an estimate of 508.5 – 452.6 mybp for when the Hexapoda initially evolved aggregated conjugates.

*A deeper exploration of the calibrated evolutionary times and their relationship to the evolution of sperm aggregation for each subphylum is needed, especially for Hexapoda. At present, nothing is mentioned beyond the approximate age of 500 mybp has origin to sperm aggregation. Including these additional data would be very relevant for your manuscript.*

Done. Thank you for the suggestion. We appreciate the need for ranges over individual estimates, and we implemented your suggestion to report result per subphylum. We now report ranges (min – max) of divergence time estimates for the clades highlighted in the Results, including a revised Supplemental Figure (Fig. S2). We highlight the most inclusive clades possible for independent origins of distinct conjugate types that include two or more species. We also added subphylum text labels to our main Figure 2. Additionally, we provide several files to address the need for more explicit details about how the tree was constructed and branch lengths inferred. These include the following: First, the complete 621 tip time-calibrated ultrametric phylogeny; more specifically, the phylogeny with median nodal ages summarized from 10,000 posterior trees from a BEAST analysis using 16 calibrated nodes. Second, the 621-tip topology in Nexus format with clade colors corresponding to taxonomic order to allow visualization of the entire topology including the ‘backbone’ phylogeny. Third, the xml input for BEAST including starting tree topology, substitution model, clock model, and fossil priors. Fourth, the 10,000 posterior trees that were summarized to generate the tree. Fifth, the log file that reports the Effective Sample Sizes of all run parameters. In addition, to help the reader visualize age estimates, we have provided a new Supplemental Figure (Fig. S2) of the ultrametric phylogeny with node bars indicating 95% highest posterior density of node ages and their locations corresponding to node calibrations, which are explicitly defined in the xml file, methods, and cited literature.

*The results of the phylogenetic time calibration and the confidence intervals for each node should be shown and included in the supplementary material.*

Done. See new Supplemental Figure S2 and the additional tree files (e.g., the phylogeny in Newick format, BEAST xml file, topology, etc) supplied as extended data files.

*According to Figure 1, the MRCA between Hemiptera and Coleoptera might be estimated at more than 400 mybp, which is not consistent with Figure 4. I emphasize again the importance of showing the exact age estimates with their confidence intervals. Because the phylogenetic tree is so collapsed, it is difficult to identify the MRCA of these two orders.*

This point is well taken. We agree that it can be difficult to discern the exact timing of nodes, especially for large trees. The widths of the branches in Fig. 4, does make it difficult to identify the MRCA of Hemiptera and Coleoptera. We have thus added 95% HPD bars for nodal ages and their median ages. Additionally, we have now provided additional tree files with full details on branch lengths, a Supplemental Figure (Fig. S2) with the nodal age heights (95% HPD) for all nodes, and the nodal age height ranges for the three specific nodes indicated in the right subtree in Figure 4a.

## **Figures**

*Figure 1: Add the names of the orders next to each icon. This information would be useful and informative for readers who are not familiar with each group.*

This is a fantastic suggestion. Wish we had thought of it. Done.

*Figure 2a: The figure is too small, making it impossible to read the terminal labels and posterior probabilities on the branches, which makes interpretation difficult. I suggest enlarging the figure or dividing the tree into two panels. You could reduce the size of Figure 2b. The double temporal legend in both directions may cause confusion; it would be clearer to separate each subphylum visually using soft shading or to mark the origin of each clade with a dot or arrow. In its current form, it is difficult to know where each clade begins and ends.*

Thank you for the suggestion. Although your point is well taken, we chose not to modify this figure for the following reasons. First, the arc tree is already a full page wide, and rendering it vertically would take up more space without substantively enhancing readability. Second, we would like to retain the double temporal legend for clarity. Nevertheless, we added several additional tree files and figures to help visualization of the results. Importantly, we also added a supplemental figure that includes nodal ages, calibrated nodes, and posterior probabilities of multistate conjugate type (Supp Fig). We also provide the phylogeny ancestral state estimates for all nodes for our binary and multistate schemes. These additions collectively provide far more contextualization for Fig. 2a. Finally, as you suggested, we have added labelled arrows to the figure to indicate the origin of each major clade (Hexapoda, Crustacea, Myriapoda, Chelicerata).

*Please also see my comments on the supplementary figures in the annotated PDF.*

Thank you for the additional comments. We addressed all of them and accepted nearly all of your suggestions. Full documentation of those changes is contained within versions of the main manuscript and supplementary materials with tracked changes.

### **Methods**

*I am not fully convinced about the phylogenetic reconstruction methodology, particularly the grafting approach. Although it is a practical strategy when no single tree includes all species of interest, it has important limitations—especially when used for quantitative evolutionary analyses such as diversification or ancestral state reconstruction. The result is a hybrid supertree combining information from different sources, often without shared molecular data among species, which may not always be comparable or congruent. Internal relationships may therefore be inconsistent with the true evolutionary history. This limitation should be explicitly mentioned in the Methods section.*

- Done. We further articulated details regarding how the phylogeny was obtained (p. 23 – 24; lines 503 – 568). Note that although this approach is undoubtedly imperfect – a point we now make explicitly in our Methods (see p. 23; lines 486 – 502) - our phylogeny has more biologically realistic branch lengths than many trees used in comparative analyses, which often rely on arbitrary ultrametric methods (e.g., Grafen branch lengths).

*In addition, I believe that this part of the methodology does not provide enough detail to allow reproduction of the analysis. I strongly recommend that the phylogenetic trees generated using the grafting method be made available to readers, including the backbone tree that was used. This is important because grafting is a manual method, and the points where other phylogenies are inserted into the backbone tree can directly influence the results obtained in the ancestral state reconstruction and in the temporal calibration.*

Done. We provide a much more comprehensive explanation of our methodology to improve the transparency and reproducibility of our study. The ‘backbone’ topology is included as part of the complete topology as a Nexus tree file. This tree is additionally color-coded by taxonomic order, which tend to be robustly supported by multiple lines of evidence. This tree was used as input for the Bayesian analysis using BEAST, and the input xml file is provided as well as the resulting posterior trees and summary tree. Finally, estimates of the most probable ancestral state are provided for all nodes for both our binary and multistate data.

*In the Bayesian analysis, did you check the Effective Sample Size (ESS) for each statistic calculated in BEAST? This was not mentioned. Ensuring  $ESS > 200$  (in BEAST or other Bayesian phylogenetic programs) is essential*

to verify the reliability and convergence of the estimated tree (branch lengths, times, and rates). This can be checked, for example, using Tracer.

Done. We discuss this in the Methods (see p. 25 – 26; lines 541 – 563). Further discussion of the Bayesian MCMC sampling procedure is now in the Methods, including details regarding ESS. Briefly, although there is some evidence of insufficient mixing for some parameters (based on the “rule of thumb” of >200 ESS), our biologically realistic approach to dating (birth death model and relaxed clock) is significantly better than simpler models. The parameters that do have low ESS are not integral to the goals of the study, and the nodes ages fall within expectations based on the fossil literature. We provide all the necessary files to repeat our Bayesian analysis from start to finish, including log and tree files.

Besides you should to show exactly are phylogenetically placed the fossils used to calibration.

Done. This is included in our new Supporting Figure (S2).

### **Supplementary Material**

*It would be very informative to include the higher-level taxonomic information (class, order, and family) for the taxa listed in the supplementary table (t1vd1d). The species name alone is not very informative and does not guide the reader to find the precise information in the phylogenetic tree. Since you are showing a phylogeny across multiple taxonomic levels, including detailed taxonomy would improve clarity.*

Done. We provide subphylum, order, and family for all 621 tips in our Supplementary Data 1 and 2.

*Please also include in the supplementary material the Bayesian tree showing the calculated node ages with confidence intervals and posterior probabilities. This will allow readers to visualize the temporal estimates for each clade concretely. The details currently available in the figures are minimal.*

Done. We provide a visualization of the 95% HPD nodal ages in our final ultrametric phylogeny used for downstream analyses, which is also provided as a separate Newick tree file.

*In summary, I believe the manuscript can be improved and completed with the adjustments mentioned above. These revisions are important and, once addressed, could substantially enhance the quality of the study. After these corrections, the manuscript could be considered for publication in the journal.*

Thanks! Your input was incredibly helpful and improved the manuscript tremendously.

### **Reviewer #2 (Remarks to the Author):**

*The authors survey literature to examine the origins and evolution of sperm conjugation. The authors postulate Cambrian origin and multiple reversals and regains.*

*The construction of the base arthropod phylogeny appears to have been more or less by hand. This is unacceptable. Either perform an analysis based on published data (there are many) or use a published phylogeny with the taxa used in the manuscript as exemplars (and perhaps with some level of missing data). The phylogeny employed follows general arthropod phylogenetics (e.g. crustacean paraphyly with respect to insects) but the specifics need to be supported buy data and analysis.*

We appreciate your feedback and have substantially expanded our documentation of our methodology (see p. 23 – 26, lines 458 – 568; also see Supplementary Information and responses to Reviewer #1). We apologize for not making this explicitly clear in the original submission. Contrary to what you have suggested, we have in fact used state-of-art phylogenetic approaches to obtain our phylogeny. We used Bayesian analysis with BEAST and calibrated nodes based on well-vetted fossils as priors to obtain biologically realistic age estimates of arthropods (Wolfe et al. 2016). More specifically, we combined multiple published topologies to build the backbone topology, which reflected our current understanding of higher-level relationships enabled by phylogenomic data. This topology among all arthropods is supported by the preponderance of DNA data and is therefore as robust as possible given currently available molecular and fossil data. Furthermore, branch lengths (i.e., divergence times) were inferred using formal dating analyses that include 15 carefully chosen priors based on the fossil literature (see Methods p. 25, Lines 529 – 533 and Supplementary Figure 2). Details of our methodology have also been greatly expanded in response to Reviewer #1. Briefly, this includes full documentation of divergence time analyses (e.g., necessary software files to replicate the Bayesian analyses) as well as revised figures (e.g., new Supplementary Figure 2) to aid in the communication of Bayesian posterior distributions. We also note that basing our analysis on published phylogenies, as suggested, is not possible for our extensive sampling of sperm conjugation because some species have never been included in a formal phylogenetic analysis.

Wolfe, J. M., Daley, A. C., Legg, D. A. & Edgecombe, G. D. Fossil calibrations for the arthropod Tree of Life. *Earth Sci. Rev.* **160**, 43-110 (2016).

**Other comments:**

*How was the 2/3 of evolutionary history of arthropods calculated? 2/3 of the time arthropods have been around there has been conjugated sperm? Or of all time in all lineages, 2/3 of lineage time has conjugated sperm? This is unclear. The first is not very meaningful, the second would not be simple to calculate and given the Bayesian analysis—would have a distribution. Please clarify.*

Done. We did employ a Bayesian approach and the details of this can now be found on p. 6, Lines 126 –128. We are specifically referring to the mean time spent across a set of mappings for the entire phylogeny (technically, the sum of all branches in a conjugated state as a proportion of the total tree length). Thanks for pointing this out.

*True bugs and beetles as “deep homology” (could not be deeper than Eumetabola) is a rather restricted sample both numerically and phylogenetically. The handful of proteomes is not very infomative.*

We appreciate the fact that our proteomic characterization of spermatostyles is numerically restricted in relation to the scope of our phylogenetic analysis. Nonetheless, we contend that this molecular investigation represents an important initial investigation into the genomic basis of “deep homology” and provides an informative complement to our comparative phylogenetic results. Furthermore, we would like to emphasize that obtaining these data is not trivial. It is extremely costly and labor intensive. Having said that, we have tried to be circumspect in our interpretation of these results and explicitly acknowledge the inherent uncertainties associated with these data (see p. 17; Lines 357 – 358).

*P16 l352. There really doesn't have to be an immediate selective advantage for any trait. A traits presence can be purely due to historical factors.*

Yes, we agree and have revised the sentence to acknowledge this fact.

**Reviewer #3 (Remarks to the Author):**

*1. In this paper, the authors have exhaustively reviewed the vast literature on sperm ultrastructure in arthropods to examine sperm conjugation, meanwhile the authors have reconstructed the phylogenetic and time trees of arthropods based on nearly all available genomic or gene data given the arthropod backbone trees. The results showed that sperm conjugation of arthropods first likely arose before the Cambrian Period at about more than 600 million years ago, and the character of sperm conjugations was estimated to occur at about two-thirds of its timespan, with its evolutionarily lost and gained approximately about 45 times; in addition, the results indicated that the common ancestor of all insects likely had conjugated sperm along with the origin of extracellular sperm-associated material (SAM); furthermore, comparative proteome analyses of spermatostyles of representative taxa indicated that a common genetic toolkit was likely to be used to convergently shape the deep homology for the evolution of complex ejaculate adaptations of arthropods. The work attempted to shape the evolutionarily dynamic history of ejaculate form and function in arthropods and obtained lots of remarkably interesting results of morphological evolution of sperms in a very important animal group and provided a very good example for this study areas.*

Thank you so much for your positive feedback.

*2. The phylogenetic analysis methods in the manuscript was not fully clear stated, for example, "within the scaffolding of these well-supported higher-level clades, we grafted additional tips onto the tree based upon more targeted molecular phylogenetic studies, typically at the ordinal level or below, while prioritizing more recent comprehensive phylogenomic or multi-locus studies over older single gene studies." and in this tree grafting of shallow-level clade onto higher-level clade, the grafting was direct or not? and if not what evolutionary models was used in both higher and shallower-level tree reconstruction? in addition, the divergence time estimation of this work was mainly conducted according to the results of previous work, and should be conducted by the authors on the grounds of more suitable fossil records and priors of more arthropod taxa.*

To address concerns of how the supertree phylogeny was obtained (also see responses to Reviewer #1), we added more details to the methods on supertree topology construction, including details on direct grafting to join multiple topologies (p. 23; lines 503 – 516). Additionally, we provide several new tree files to allow for inspection of the topology, posterior trees, input xml, and final ultrametric tree used for comparative analyses.

With regard to divergence times, your comment suggests there may be a misunderstanding. This is difficult to pinpoint since the suggestion was made to use more suitable fossil taxa without providing more precise feedback as to why our list of well-vetted arthropod calibrations (Wolfe et al. 2016, in addition to two priors for beetles based on previous dating analyses) is insufficient. To help visualize the location of fossils and their priors, we added these details to a new supporting online figure (S2).

Wolfe, J. M., Daley, A. C., Legg, D. A. & Edgecombe, G. D. Fossil calibrations for the arthropod Tree of Life. *Earth Sci. Rev.* **160**, 43-110 (2016).

*3. The references 69~87 of manuscript was not listed or shown in the draft, and thus the authors were suggested to add these references in the revised manuscript carefully.*

Per the journal guidelines, the Methods sections and all the associated references (i.e., those in question) are to be included with the Supplementary Information.

NCOMMS-25-74522A

We thank the reviewers for the extensive and helpful comments, including the additional, fourth reviewer. The reviewer comments have improved the manuscript. We respond to each comment below. In addition to the revised manuscript, our resubmission includes a version with "tracked changes" to easily view all changes between versions.

**[Original comments in black] [Author responses in blue]**

**Reviewer #1 (Remarks to the Author):**

Thank you for the answer to my suggestions and comments. Most of them were well answer and included in this new version of the manuscript. I did few more suggestions to improve even more your manuscript and that I considered important.

- I added other few comments and suggestions in the Supplementary material and main text (see below and in the attached files).

Thank you. We have incorporated these comments (see the revised manuscript file with tracked changes).

- I understand there is not enough space in the main body of the manuscript to include a large Fig.2 a, however I insist that Supplementary Figure 2 continue to be not legible and not so useful to the readers. I recommend you at least in Supplementary material divided this fig. tree in 2 parts (e.g., Hexapoda; others clades) so as not to compress the names of taxa and age data in the nodes. This could be more informative and best presented allow valorise the data in the phylogenetic tree.

Done. We split Supplementary Figure 2 into two full-page figures as suggested (Supplementary Figures 2a and 2b). This was a good suggestion as it does greatly enhance clarity.

- 654438\_1\_data\_set\_12132588\_t8wyk7 table: missing description of State1 in: "ConjugationMulti / Conjugation when present occurs in 5 discrete types (0 unconjugated sperm, 2 paired, 3 ensheathed, 4 aggregate, 5 rouleaux, 6 spermatostyle)"  
If they are unconjugated sperm why you pass from state 0 to 2? Can you clarified this point?

Thank you for catching this error in the key. We originally coded the unconjugated species as "0" and later changed it to "1" without updating the key. The data file has now been corrected, and the key has been modified to make clear that this character captures both

conjugation absence/presence and type. Note: the data, analyses and results were unaffected.

- Pag 8 line 170 main text : “Imprecision” word I suggested changing this word. I consider that this “agreggate” type is precise in its function and design, or well the “agreggate” is designed to perform a specific function.

We agree with you about adaptive design, and it was not our intention to infer that any form of conjugation is less "adaptive" than any other. We also agree that relative "precision" was not the ideal terminology to capture this variation of interest across conjugation types. As recommended, we have removed all references to conjugate "precision" and "imprecision" and now provide a clearer description of the conjugate state "specialization."

- Then, what are the structural specializations of SAM that you mentioned is not in “agreggate” as in spermatostyle? Since SAM is present in “aggregates”, as mentioned in the supplementary table, could you clarify this point?

Excluding the sperm "trains" of muroid rodents (not part of this study), conjugation requires some binding or cementing material to link together multiple sperm. This is why the reviewer observes that in the supplementary table species with conjugation are also coded as having SAM. In other words, no species with conjugation lacks SAM, but there are species with SAM that do not conjugate their sperm (e.g., many spiders).

The point we are trying to make in this section (page 8, lines 168 – 196) is that the SAM in aggregates has been remodeled in many insect species, leading to dramatic examples of phenotypic variation in conjugates from a common starting point of ancestral aggregates. We clarified what we mean here.

## **Reviewer #2 (Remarks to the Author):**

The authors have addressed all my comments.

Thank you.

### **Reviewer #3 (Remarks to the Author):**

1. The authors have responded point-by-point to the reviewers and accordingly revised the manuscript carefully according to the reviewer's comments, especially about the phylogeny reconstruction as well as the molecular dating, with more supplementary figures and data files, thus improving the quality of the manuscript a lot for acceptance of publication.

Thank you.

2. The authors have done this valuable work about the evolution of aggregated sperm in Arthropods and related animal groups through multiple disciplinary criteria, and revised the estimate of Hexapod's initial sperms aggregated conjugates at about 508.5-452.6 myr across the Cambrian and Ordovician. So further studies about the molecular mechanism, especially the SAM in "proto-conjugates" facilitating subsequent diversification of multiple sperm conjugate types, using multi-omics data of the representative living hexapod taxa coupled with relevant geological events are expected in the near future.

We appreciate the kind words and agree that more investigation of the molecular composition and significance of SAM are needed in diverse taxa.

### **Reviewer #4 (Remarks to the Author):**

The authors present an evolutionary analysis of sperm conjugation across arthropods. Conjugation comes in several forms – aggregates, pairs, rouleaux, spermatostyles, and ensheathed. By mapping these states on a phylogeny, the authors learned a great deal about how such a complex trait evolves.

This is an excellent paper. If the only thing this paper did was report the dataset collected across 600 species, it would have a huge impact. But they go to make insights into the evolution of this very interesting trait. It is surprising how widespread this phenomenon is and how labile it is. There is clear directionality – SAM evolves first, then aggregation, then more complicated forms of conjugation. It really is a very interesting paper. The conjugation phenotype is complicated, but the authors have made reasonable choices about how to score them.

Figure 1 is beautiful!

I only have one absolutely crucial comment. All the rest can be considered minor.

Thank you very much for the positive feedback.

Main comment:

1) A central claim is true bugs and beetles use a “common genetic toolkit” in their spermatostyles even though they have arisen independently. The key argument comes in the paragraph that begins on line 306. They say that 21.7% of the identified proteins were present in both beetles and bugs, significantly greater than expected. My understanding of how they created the null expectation is that they randomly drew from the pool of 4 bug + 6 beetle + *Drosophila* reference proteome. I am underlining *Drosophila* reference proteome because that is going to be an enormous set of protein orthogroups. When they randomly sample from that huge set, of course they will not see much overlap in any of their permutations – this almost guarantees that 21.7% is higher than expected. What they’ve really asked is “are spermatostyle proteomes random draws from a huge universe of proteins”. It is almost impossible to not reject that hypothesis – however, I would guess you would see the same result if you tested any tissue, not just spermatostyles. Thus a “shared genetic toolkit” is not a strong conclusion, or at least not unexpected given the construction of the null. What we care about is whether there is more sharing among independently derived spermatostyles compared to some other trait that is single-origin. Another reason this is not the right null is because it assumes orthogroups are recruited independently. But orthogroups in the observed proteomes are most certainly not independent – i.e., some co-occur often because they participate in similar pathways.

One last concern: orthogroup has been binarized to present/absent. However, some orthogroups are going to be more abundant than others, and we might have more confidence in abundant orthogroups, compared to rare ones that might simply drop out because of stochastic sampling. This concern does not undermine the overall paper, but the claim of a shared genetic toolkit should be tempered or reframed, or they need to devise a different null.

Thank you for this exceptionally thoughtful input. It reveals a slight misunderstanding about our simulation approach, which is on us for not being sufficiently clear. Hence, this comment has led to dramatic improvement of the paper. We have clarified the Results (in addition to making our interpretation more circumspect), and we have expanded the relevant Methods section to clarify our simulation approach.

First, we would like to briefly clarify the simulation approach. The simulation was conducted by randomly sampling from the transcriptome of a single bug species at a time, rather than using the combined proteins across the 11 species you mention (this was not explicitly stated). *Drosophila melanogaster* was only used as an outgroup in the orthology relationship determination and played no part in the simulation. Additionally, the six beetle species were not part of the simulation process either. A single conserved beetle spermatostyle proteome was used as the point for comparison for the simulated bug data.

As such, the sampling space was much more restricted. We apologize for the not being clearer in the original submission, and we have clarified this in the Methods section (Line 738-744). Again, we are indebted for you bringing this clarity to essential methodology. We also would like to point out that proteome similarities between bugs and beetles extend to the level of protein abundance and are not solely restricted to binarized presence/absence (see Line 325-343).

Nevertheless, we agree with you about there being limitations to our underlying null hypothesis. The idea to compare levels of proteome convergence to other “single origin” traits is excellent. Unfortunately, we do not have any empirical data in these taxa to serve as a point of comparison at this time, and it is important to note that conservation/divergence among “single origin” traits is also likely to be quite variable. We also agree that recruitment is almost certainly not random and likely to be influenced by gene regulation and participation in relevant pathways. These are important, unanswered questions of relevance to the concept of deep homology. Unfortunately, we know very little about the developmental, molecular and genetic processes that govern recruitment, and so it is not possible for us to realistically model this within the scope of this current study. We have revised the manuscript to be explicit and circumspect about this analytical constraints:

*Line 323-325: “We note, however, that this analysis does not account for the fact that proteins could be co-recruited as spermatostyle components based on shared patterns of expression or functionality.”*

*Line 452-455: “Alternatively, it is possible that S-LAPs are more likely to be recruited as novel spermatostyle proteins given their enhanced expression in the testis. We currently have a limited understanding of how shared functionality or expression may influence the co-recruitment of genes underlying novel phenotypes.”*

Minor comments:

2) It seems like most sperm phenotypes were scored from dissections of males. I realize this won't exist for all species, but are there data to suggest how similar/different sperm conjugates are in the male RT vs. after being transferred to the female RT? Around line 170 they mention this, but I would have liked some more details/discussion.

This comment reveals how well you understand the core biology of these traits and just how much your interests align with our own. Understanding the evolution of sperm, seminal fluid and other ejaculate traits is all about complex male x female interactions, which is why this is the core of our groups research program. Indeed, we have a project in progress that examines the changes to the proteome of spermatostyles of a whirligig beetle across protracted storage within the FRT. (Note: there are no published studies that compare conjugate structure before and after insemination, other than their dissociation.) However, the data we analyzed for the current manuscript were mined from the literature which almost exclusively focusses on sperm isolated from male seminal vesicles.

Nonetheless, we highlight conjugate persistence and our unpublished observations (Line 402-417).

3) The Introduction wanders quite a bit. As just one example, why talk about spider silk to such detail? I recommend tightening up the Introduction.

We also appreciate this suggest your feedback on the Introduction. However, we respectfully disagree on this point and ask your indulgence. The Introduction is actually relatively concise, being just four paragraphs, and the narrative was crafted to accomplish several goals. Only the first paragraph addresses spider silk and other secretions for which diversification has critically shaped biodiversity. Similar studies have not been done with reproductive secretions and that context is essential for readers. Note also that this manuscript (addressing spermatostyles) is the first of a larger research program and series of papers on the evolution of secreted ejaculate structures, including spermatophores, mating plugs, seminal fluid inclusions and parasperm. Hence, this Introduction will prove of increasing, emergent value to the scientific literature as part of that larger body of work.

4) Fig. 3 b shows that cicadas have a very different curve than bugs or beetles. One way this could happen is if the cicada transcriptome (or whatever they search against for their mass spectra) is poor quality. In other words, it is just more difficult to match spectra to a proteome if the underlying data is poorly assembled or annotated. This would be a methodological artifact. Can the authors address this?

This is an important point, and one that we had carefully considered. The cicada transcriptome is well assembled and of similar quality to our other, high quality insect transcriptomes. The Cicada BUSCO and Transrate scores are 97.7% and 0.22, respectively, which are both very good and within the ranges of our other bug transcriptomes (BUSCO range 84.6 – 98.1%; Transrate range 0.20 – 0.23). This information, as well as our additional analyses of the divergence of the cicada spermatostyle proteome are provided in Supplementary Information. Readers are directed to this information in the following sentence:

*“Given the nested phylogenetic position of the cicada relative to the other sampled true bug species (see asterisk in Fig. 4d), the most parsimonious explanation is dramatic, lineage-specific molecular evolution of the cicada spermatostyle (see Supplementary Information for further details).”*

We thank the one reviewer for their additional comments:

I was reviewer #4 originally. This remains an excellent paper and should definitely be published in Nature Communications. The vast size of the dataset, and the amount of insight gained from their analyses is high impact. The main comment in my original review focused on their simulation. I appreciate their clarifications, but I still don't think this test leads to a "shared genetic toolkit" conclusion. Their test shows that spermatostyles are not random protein collections, but this is likely true for any cell-type's proteome.

I am comfortable with the analysis remaining as is, but they would need to temper the "shared genetic toolkit" conclusion. Or at least pose the right experiment (comparing overlap to single-origin cell-type) as potential future work. As a hypothetical, what if this approach was repeated for bug and beetle heads? If the overlap observed in spermatostyles were comparable to, or even lower than, that observed for another tissue, then the current result would not support a "shared genetic toolkit" interpretation. Or maybe more accurately... we would conclude that spermatostyles are like other tissues in that they express non-random proteomes.

Our response:

We appreciate the reviewer's cautionary perspective on our interpretation of a shared genetic toolkit. We share this concern, especially since it is a general consideration and challenge of all studies applying this "deep homology" and "shared genetic toolkit" hypothesis.

The reviewer comments, while well intentioned, are slightly misguided with regard to our study in two important regards. First, they wrote "*Their test shows that spermatostyles are not random protein collections, but this is likely true for any cell-type's proteome.*" However, it is an important consideration that spermatostyles are not cells or tissues. Rather, they are structures formed from secretions within an extracellular lumen of the male reproductive tract. Second, the reviewer suggests "*As a hypothetical, what if this approach was repeated for bug and beetle heads? If the overlap observed in spermatostyles were comparable to, or even lower than, that observed for another tissue, then the current result would not support a "shared genetic toolkit" interpretation.*" However, this suggestion is not a valid control or point of comparison, as beetle and bug heads, including eyes, mouthparts, brains, etc, are all homologous structures. In contrast, our paper convincingly demonstrates that the spermatostyles of bugs and beetles are not homologous; they have evolved

convergently and represent independent evolutionary events. Hence, we stand behind our interpretation.

Nevertheless, we agree with the reviewer's general sentiment, and thus have revised the relevant section of the Discussion to be more circumspect by (1) using terms such as "putatively," (2) acknowledging our limited understanding, and (3) stating that more studies are required.

Here is the relevant text (lines 420 - 439):

Our proteomic analysis of independently derived spermatostyles in true bugs and beetles represents a new example of convergent evolution, putatively through the deployment of a common genetic toolkit<sup>37</sup>. It thus expands the relevance of deep homology to the evolution of social cooperation among gametes<sup>63</sup>. Both taxa recruited S-LAPs, which have been predicted to have gained a structural function in *Drosophila*<sup>64</sup>, as part of the developmental origin of spermatostyles. As is the case more generally of LAPs functioning outside of reproduction<sup>65</sup>, S-LAPs appear to have notable multifunctionality and may represent a broader class of “common toolkit” genes with high evolutionary potential. Alternatively, it is possible that S-LAPs are more likely to be recruited as spermatostyle proteins given their enhanced expression in the testis. We currently have a limited understanding of how expression may influence the co-recruitment of genes and gene regulatory modules underlying convergent phenotypes. Still, cicada spermatostyle composition revealed that alternative evolutionary solutions were also possible (see Supplementary Note 1). Additional comparative studies of the developmental basis and molecular composition of different conjugate forms and other ejaculate structures are required before more general conclusions can be drawn about the contribution of deep homology to the convergent evolution of ejaculate adaptations. In particular, SAM warrants intense investigation as a highly evolvable and multifunctional substrate for the development of ejaculate structures and the diversification of extended gamete phenotypes.

24  
 25 Theory suggests that ejaculates should evolve heightened functionality through  
 26 integration of their component parts: spermatozoa, seminal fluid and ejaculate  
 27 structures (e.g., mating plugs, spermatophores, spermatostyles and seminal inclusions).  
 28 Here, we exhaustively review the vast literature on sperm ultrastructure in arthropods to  
 29 examine sperm conjugation - a form of social cooperation among sperm - in addition to  
 30 its relationship with an ostensible antecedent: membrane-bound, extracellular sperm-  
 31 associated material (SAM). Our results suggest that sperm conjugation first arose  
 32 during the Cambrian Period (i.e., > 500 million years ago). Since then, the Arthropoda  
 33 tree of life has spent an estimated two-thirds of its time in a state of sperm conjugation,  
 34 which has been both evolutionarily lost and gained approximately 45 times. Notably, the  
 35 common ancestor of all insects likely had conjugated sperm. Most evolutionary  
 36 derivations of conjugation from an unconjugated state occurred following the origin of  
 37 SAM, with SAM in "proto-conjugates" facilitating subsequent diversification of multiple  
 38 conjugate types. Finally, comparative analyses of proteomes of the convergently  
 39 derived spermatostyles (specialized rods of SAM to which sperm attach) of true bugs  
 40 and beetles indicate parallel utilization of a common genetic toolkit that may draw upon  
 41 deep homology for the evolution of complex ejaculate adaptations. Our analyses reveal  
 42 an ancient, pervasive and dynamic history of evolutionary experimentation with  
 43 ejaculate form and function.

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ADD the specific taxon to this sentence

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 in Arthropods

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**S** Author: redacted Subject: Cross-Out Date: 20/10/2025 11:10:41  
 Suggestion: Arthropods are estimated to have

Exp: the tree of life is only a graphical representation but the subject are the arthropods>

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**S** Author: redacted Subject: Cross-Out Date: 20/10/2025 11:12:48  
 their evolutionary history with

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## Introduction

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Coleoptera:

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Hemiptera:

widespread distribution of sperm conjugation. Our analysis indicates that sperm conjugation occurs in all extant arthropod subphyla<sup>37</sup> (1 of 2 classes of Chelicerata, 1 of 4 classes of Myriapoda, 4 of 11 classes of "Crustacea," and in the Hexapoda) and in more than one-third of sampled orders (34 of 88, or 38.63%).

We next examined the evolutionary history of conjugation by reconstructing ancestral character states and estimating transition parameters while treating conjugation as a binary trait (i.e., present or absent). Stochastic character mapping determined that unconjugated sperm was the ancestral state and present in the backbone of the Arthropoda with high posterior probability (pp > 0.99; Supplementary Fig. 1). Nevertheless, sperm conjugation exhibits a dynamic evolutionary history, having been independently gained from unconjugated sperm throughout the phylum 34 - 63 times (95% Highest Posterior Density (HPD) = 39 - 56 times). An informative measure of the evolutionary prevalence of a trait is the amount of time spent in a particular character state, which can be estimated by stochastic simulations of exact character histories<sup>38,39</sup>. Despite unconjugated sperm being the likely ancestral state, conjugation was estimated to have been present across approximately two-thirds of the evolutionary history of arthropods (66.57% of the time conjugated; Supplementary Fig. 1). This striking prevalence of conjugation is primarily attributable to its propensity for independent derivation rather than to its evolutionary persistence. Although there are clades for which conjugation is common (e.g., Odonata; Hymenoptera; adepghan beetles, auchenorrhynch true bugs, and spiders) or nearly universal (e.g., Lepidoptera, Isopoda, and Scorpiones), the number of estimated evolutionary losses of conjugation (29 - 77 times; 95% HPD = 34 - 54 times) is comparable the number of gains.



162 co-occur with other specializations such as the precisely aligned rows of  
 163 intramembranous particles found in the attachment point of some pairs<sup>48,49</sup>. In the case  
 164 of spermatostyles, SAM forms slender hyaline rods (Fig. 2 and Supplementary Fig.  
 165 2b,c) to which sperm heads attach, and these can persist in the FRT after sperm  
 166 dissociation<sup>29,43,50</sup>. Finally, for ensheathed conjugates, SAM form a coat surrounding  
 167 groups of sperm (Fig. 2 and Supplementary Fig. 2h-i), in which case conjugates are  
 168 immotile.

169 Aggregates appear to be the least specialized form of conjugation as judged by  
 170 the imprecision commonly observed in their design. For example, they often exhibit  
 171 marked intra-ejaculate variation in the number of sperm per conjugate and they lack  
 172 both the structural specialization of SAM present in spermatostyle and ensheathed  
 173 conjugates and the morphological adaptations of sperm heads observed in pairs and  
 174 rouleaux conjugates. We coupled ancestral state reconstruction with multistate  
 175 character modeling to explore the evolutionary relationships among conjugation states  
 176 while testing the related predictions that (1) aggregates will be the most common type of  
 177 conjugation, (2) most independent origins of conjugation from unconjugated sperm will  
 178 be of the aggregate type, and (3) most derivations of other, more structurally complex  
 179 conjugate types will first pass through the aggregate conjugation state. **This analysis**  
 180 **reinforced the conclusion that the ancestral sperm of all arthropods, and ancestors of**  
 181 **the Chelicerata, Myriapoda and Pancrustacea were unconjugated (Supplementary Fig.**  
 182 **1).** However, the ancestor of the Hexapoda likely had conjugated sperm in the form of  
 183 aggregates (Fig. 2 and Supplementary Fig. 2), which is consistent with aggregates  
 184 being widespread in the backbone of the Insecta clade, including members of several

185 early diverging taxa such as Diplura and Zygentoma, Palaeoptera, and  
186 Polyneoptera<sup>44,47</sup>. The multistate analysis also reiterated a dynamic evolutionary history  
187 of sperm form across the Arthropoda (Fig. 2 and Supplementary Fig. 2 and 3;  
188 Supplementary Table 2). Stochastic mapping revealed an average of 161.2 character  
189 state changes per stimulation across the entire tree, with 9 - 44 (95% HPD = 15 - 34)  
190 origins of conjugation, 45 -148 (95%; HPD = 57-115) losses of conjugation, and 19 -  
191 105 (95% HPD = 30 - 74) transitions among conjugate types. All five conjugate types  
192 were observed among distantly related taxa and thus are likely to have arisen  
193 independently through convergent evolution (Fig. 2 and Supplementary Fig. 2 and 3;  
194 Supplementary Table 2).

195 We found strong support for our predictions regarding the role of sperm  
196 aggregates in the origin and diversification of conjugation. First, of the total accumulated  
197 time that the arthropod tree spent in a state of sperm conjugation, 49.00% of the time  
198 was spent as aggregates, whereas sperm were conjugated as pairs 6.98% of the time,  
199 as rouleaux 10.71% of the time, as spermatostyles 16.53% of the time, and as  
200 ensheathed 16.73% of the time. Second, estimates of transitions between conjugation  
201 states under optimal “all rates different” maximum likelihood models (Supplementary  
202 Fig. 3) indicate that aggregates evolved from unconjugated sperm more often than all  
203 other conjugate types combined (55.90% of all independent origins of conjugation), with  
204 rouleaux and spermatostyles never directly evolving from unconjugated sperm (Fig. 2;  
205 Supplementary Table 2). Third, aggregates are the only form of conjugation with a  
206 propensity to evolve into every other conjugate form (Fig. 2 and Supplementary Fig. 3;  
207 Supplementary Table 2). In addition, when all non-aggregate conjugate types undergo

208 an evolutionary transition, they have a high probability of evolving into either aggregate  
 209 conjugates or into unconjugated sperm; all of the possible transitions between different  
 210 specialized forms of conjugation were rare-to-nonexistent (Fig. 2 and Supplementary  
 211 Fig. 2 and 3; Supplementary Table 2).

212 Because transitions between different specialized forms of conjugation were rare  
 213 in the arthropod tree of life, we closely examined a rare exception that occurred in  
 214 ground beetles (family Carabidae). Stochastic mapping supports spermatostyle  
 215 conjugates as ancestral to this family, following an independent origin from aggregates  
 216 around 250 mybp (pp = 1.0; see \* in Fig. 2). Within carabids, a transition from  
 217 spermatostyle to ensheathed conjugation was indicated with high probability, as seen in  
 218 some species of *Clivina* and *Paraclivina*. In *C. fossor* and *P. bipustulata*, the sperm are  
 219 ensheathed within the cavity of a unique elongate structure<sup>50</sup> (Fig. 3). *Clivina* nest within  
 220 the family Carabidae, and their nearest relatives make spermatostyle conjugates that  
 221 vary in the extent to which the sperm flagella are enveloped by the spermatostyle  
 222 material (e.g., <sup>50,51</sup>). We postulate the series of morphological modifications illustrated in  
 223 Fig. 3 as representing the progressive series of intermediate forms between  
 224 spermatostyle and ensheathed conjugation along this lineage.

225

## 226 Evolutionary relationships between conjugation and sperm-associated material 227 (SAM)

228 SAM, when present, is readily observable and explicitly described in most ultrastructural  
 229 investigations of sperm (Fig. 1 and 2 and Supplementary Fig. 4 and Supplementary  
 230 Data 2). Notably, SAM is not limited to conjugated sperm, as numerous taxa across the

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 no italic

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 you mention these species but where are these in the phylogeny showed? in which of these showed clades?

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 3b

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 what do you mean ? other genus of Carabidae other spp in the same genus, the sister group of Carabidae ?  
 \* Explain

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 So in these gre in the second clade of Carabidae showed in your phylogeny fig 3?

**H** Author: redacted Subject: Highlight Date: 28/10/2025 19:41:39  
 Explain even more this.  
 So we can think that Ensheathed form is a most derivative character to Carabidae?  
 For this group when this feature appeared following your time phylogeny ? This can seen keys for that.

pairwise comparisons between true bugs and beetles). This pattern is largely attributable to sperm-leucyl aminopeptidases (S-LAPs) which were consistently among the most abundant proteins in spittlebug, leafhopper and whirligig beetle spermatostyles (Fig. 4c). Phylogenetic reconstruction of the S-LAP protein family further revealed that spermatostyle S-LAPs in both true bugs and beetles are members of the same S-LAP subclass (Fig. 4c). Further, protein abundance in spermatostyles was significantly correlated between the two taxa ( $P = 2.2 \times 10^{-16}$  for protein families in the top 25% by abundance). Finally, true bug spermatostyles were significantly enriched for proteolytic functions (Supplementary Fig. 8), including metalloexopeptidase activity (GO:0008235;  $P = 0.003$ ) and aminopeptidase activity (GO:0004177;  $P = 0.001$ ), which further supports functional coherence with beetle spermatostyles (see Supplementary Online Materials for additional details; Supplementary Fig. 9). Taken together, we conclude that the independent evolution of spermatostyles in beetles and true bugs involved the co-option of many of the same proteins and that these ejaculate structures possess a highly convergent molecular architecture.

In light of the parallel co-option of S-LAPs into both true bug (excluding cicada) and whirligig beetle spermatostyles, we investigated whether S-LAPs might also be present in SAM. To do so, we examined both *Scolops pungens*, a dictyopharid true bug that has unconjugated sperm with SAM and is closely related to true bugs with spermatostyles sampled in this study (Supplementary Fig. 7), and *Gyrinus natator*, a whirligig beetle with aggregate conjugates that is closely related to the examined *Dineutus* species with spermatostyles<sup>29</sup>. In both cases, immunohistochemistry using an S-LAP antibody confirmed that S-LAPs are present within SAM (Supplementary Fig. 9).

Although it is not possible to directly assess the ancestral molecular composition of SAM, these results confirm the presence of S-LAPs in the SAM of both true bugs and beetles. We thus postulate that the presence of S-LAPs in SAM facilitated their parallel recruitment during the independent evolution of spermatostyles in these distinct lineages.

## Discussion

With limited evidence for haploid selection on sperm or intra-ejaculate competition among sperm<sup>33,53,54</sup>, ejaculates are expected to function with highly evolved integration among component parts. Nevertheless, studies of ejaculate quality, functionality and competitiveness have almost exclusively restricted their focus to single component ejaculate phenotypes, such as sperm number, sperm length, and the function of individual seminal fluid proteins, rather than more integrative components and emergent properties. A striking exception has been resolving the interactions between sperm, Sex Peptide and the female<sup>30,55,56</sup>. One of the only other exceptions is sperm conjugation, although our understanding of the developmental origins, functional biomechanics and adaptive value of this complex trait is still limited<sup>33–35,57</sup>.

Whereas it is intuitive that selection should persistently favor social cooperation among sperm within ejaculates, sperm conjugation was believed to be a relatively rare phenomenon (albeit widespread in particular taxonomic groups<sup>35,57</sup>). In striking contrast, our comparative analysis of the Arthropoda reveals that sperm conjugation has been a pervasive and dynamic aspect of evolutionary experimentation. Conjugation likely first arose in arthropods during the Cambrian Period (Fig. 1 and 2) and was subsequently

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evolutionary history of arthropods

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so you can add (Hexapoda).

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346 both lost and gained approximately 45 times. Moreover, we estimate that across its  
347 entire ~~history the arthropod tree of life~~ has spent two-thirds of its time in a state of sperm  
348 conjugation (Fig. S1). The common ancestor of ~~all insects (and non-insect hexapods)~~ is  
349 estimated to have had conjugated sperm. Throughout this history, conjugation has  
350 adopted several discrete types and massive variation in sizes, from sperm pairs to  
351 thousands of sperm precisely organized into rouleaux.

352       The adaptive value of conjugation is not known for any taxon, and the selective  
353 benefits and costs of conjugation may differ among lineages or over time<sup>35</sup>. Most  
354 authors have focused on enhanced swimming speed and its benefits in sperm  
355 competition<sup>33–35</sup>. The number of sperm per conjugate is expected to positively correlate  
356 with swimming velocity due to enhanced force generation with less than additive drag  
357 (due to sperm alignment), provided that flagellar beating is synchronous to minimize  
358 interference<sup>35,58</sup>. Indeed, a study of bull sperm, which lacks conjugation, showed that  
359 when sperm traveling in the same direction contact one another, phase of beat  
360 frequency synchronizes and velocity increases beyond that of either individual sperm<sup>59</sup>.  
361 Hence, any novel origin of sperm adhering to one another by SAM (i.e., proto-  
362 conjugation) could immediately result in a motility benefit - a condition that best  
363 describes aggregate conjugates prior to more specialized adaptations of SAM and/or  
364 sperm morphology to facilitate conjugation. This hypothetical scenario is supported by  
365 our phylogenetic modeling showing that most novel evolutionary derivations of  
366 conjugation occurred following the origin of SAM. Unfortunately, few studies have  
367 quantified motility of conjugated sperm, and those that have done so provide  
368 inconclusive or mixed results<sup>35</sup>. The "enhanced motility" hypothesis also cannot explain

369 the evolution of immotile ensheathed conjugates, nor the widespread association of  
370 SAM and unconjugated sperm.

371 Results of the current study suggest a novel adaptive hypothesis for conjugation  
372 that is premised on the benefits of enhancing the (1) location and (2) persistence of  
373 seminal fluid constituents within the FRT. Seminal fluid proteins support sperm and  
374 mediate myriad important post-mating interactions between the sexes<sup>20</sup>. Yet, the vast  
375 majority of the several hundred seminal fluid proteins of *D. melanogaster* are  
376 undetectable in the FRT a few hours after insemination<sup>60</sup>. Only those few seminal fluid  
377 proteins that are physically associated with sperm accompany them from the site of  
378 insemination into the sperm-storage organs and exhibit longer persistence<sup>31</sup>. In the case  
379 of Sex Peptide, its efficacy can last for days because it is cleaved from sperm in a time-  
380 release manner<sup>32</sup>. As an alternative to seminal fluid proteins binding to sperm (with or  
381 without conjugation), selection may have favored males incorporating critical proteins  
382 into ejaculate structures to ensure their proper localization and to enhance their  
383 persistence. For example, spermatophores, spermatostyles and mating plugs can  
384 persist for days-months within the FRT<sup>27,28</sup> (Gomez, unpublished data). However, the  
385 only way to ensure that ejaculatory structures are transported to the sperm-storage  
386 organs may be through their physical association with sperm, which the female requires  
387 for fertilization.

388 The “seminal fluid delivery” hypothesis contends that sperm conjugation  
389 represents (at least in part) sperm cooperation for the regulated localization and  
390 persistence of SAM in the female sperm-storage organs<sup>29</sup>. For example, rather than  
391 spermatostyles having evolved as a structural mechanism facilitating the conjugation of

392 sperm, conjugation may have evolved as a mechanism of delivering spermatostyles to  
393 the female sperm-storage organs. This hypothesis is consistent with several  
394 observations. First, in many instances, the association between SAM and sperm can  
395 only serve to impede motility: (1) SAM can be bound to single sperm that transport it to  
396 the sperm-storage organs<sup>52</sup> (Fig. 1 and Supplementary Fig. 4); (2) the spermatostyles of  
397 some species have extensive bare regions without sperm head attachment, and so are  
398 longer than necessary to facilitate conjugation<sup>50</sup>; (3) ensheathed conjugates are  
399 immotile until the sheaths are shed within the bursa copulatrix or within sperm-storage  
400 organs<sup>61</sup>. Further, in lepidoptera, only the fertilizing sperm morph is ensheathed<sup>61</sup>.

401 Second, SAM in the form of spermatostyles is not composed of inert structural material  
402 as expected if functioning purely as scaffolding for conjugation. Our proteomic analyses  
403 of spermatostyles has revealed an enrichment of proteases in all true bugs and beetles  
404 species examined to date. Furthermore, in the case of the beetle *D. assimilis*, we have  
405 confirmed that at least some of these retain biochemically activity as spermatostyle  
406 components<sup>29</sup>.

407 Throughout its more than 500-million-year history, the Arthropoda have been  
408 remarkably successful, diversifying to occupy a vast array of habitats and ecological  
409 niches. Our study reveals that, across this same time span, arthropods likewise  
410 explored a vast phylomorphospace of ejaculate design that frequently featured intra-  
411 ejaculate sperm cooperation through conjugation<sup>33–35</sup>. We find evidence for evolutionary  
412 trajectories repeatedly driving lineages to convergently innovate one of five generalized  
413 conjugate blueprints: aggregates, pairs, ensheathed, rouleaux and spermatostyles. Our  
414 proteomic analysis of independently derived spermatostyles in true bugs and beetles



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How you do that. Add more details.



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Did you use the maximum and minimum age of each fossil? Add the details and you could list the fossils.

484 **Phylogeny.** We assembled a phylogeny for the 621 species using a custom supertree  
485 approach by manually constructing a topology within Mesquite v. 3.61<sup>68</sup> based upon a  
486 large set of phylogenetic studies (Supplementary Data 1 with phylogenetic references  
487 for all 621 tips). The backbone topology was derived from the most comprehensive  
488 phylogenomic analysis to date of the Pancrustacea<sup>69</sup>. Deeper nodes reflect recent  
489 phylogenomic studies of the Chelicerata, Myriapoda and Insecta (see Supplementary  
490 Data 1 and references therein). Within the scaffolding of these well-supported higher-  
491 level clades, we grafted additional tips onto the tree based upon more targeted  
492 molecular phylogenetic studies, typically at the ordinal level or below, while prioritizing  
493 more recent comprehensive phylogenomic or multi-locus studies over older single gene  
494 studies. All analyses are based on a phylogeny that was finalized on November 1, 2024.  
495 The topology was ultrametricized using a node-based calibration approach in BEAST v.  
496 2.6.3<sup>70</sup>. Calibrated nodes included 14 high-quality fossil calibrations for Arthropoda<sup>71</sup> in  
497 addition to crown-group priors for Coleoptera and Adephaga based on robust  
498 divergence time estimates from Baca et al.<sup>72</sup>. Nodes were calibrated with normal prior  
499 distributions, and the divergence time analysis was performed on a fixed tree topology  
500 with initial branch lengths obtained with R ape and the chronos function<sup>73</sup> based upon  
501 these same calibrations. The chain was run for 200 million iterations, sampling every  
502 20,000 iterations, and with the first 20 million samples discarded as burn-in. The  
503 phylogeny's branch lengths are based on the median estimate of the posterior  
504 distributions of nodal heights.

506 **Characterization of sperm traits.** We assembled a character state matrix of sperm  
507 traits for each of the 621 sampled species that included (1) presence versus absence of  
508 conjugation, (2) type of conjugation, and (3) presence versus absence of sperm-  
509 associated material (SAM). Sperm conjugation occurs when two or more sperm  
510 physically bind to one another. Conjugates form within the male reproductive tract and  
511 involve species-specific morphological and secretory adaptations. The presence of  
512 conjugation, structural mechanism of conjugation, and often the precise number of  
513 sperm per conjugate are clearly discernable in ultrastructural studies (Fig. 2b and  
514 Supplementary Fig. S2a-l). The presence or absence of SAM, which we operationally  
515 define as extracellular material that is bound to sperm, could also be unambiguously  
516 determined from ultrastructural studies (Fig. 2b and Supplementary Figs. 2a-l and 4).  
517 Because our understanding of trait variation (and hence morphological categorization;  
518 see below) became more refined as the study progressed, we reexamined every paper  
519 and reevaluated the tip data for every species at least once following an initial scoring of  
520 all 621 tips (Supplementary Data 2).

521 The manner by which sperm structurally bind to one another to form conjugates  
522 and the number of sperm per conjugate have functional implications for sperm  
523 performance within the FRT<sup>41,42</sup>. In one of the most extreme forms, conjugates are  
524 formed when groups of sperm are entirely ensheathed by SAM, which precludes  
525 motility. Conjugates were categorized into the following types: (1) pairs (2) ensheathed,  
526 (3) aggregate, (4) rouleaux, and (5) spermatostyle<sup>35</sup>. This organizational system focuses  
527 on the most distinctive and diagnosable aspects of conjugate morphology (i.e., variation  
528 in the number of included sperm and the organization of sperm within a conjugate), and

529 those characteristics most likely to dictate the functional biology of conjugates within the  
530 FRT. Paired conjugates comprise two sperm, typically with their heads joined together  
531 in a "crystalline" fashion (Fig. 2b and Supplementary Fig. 2a,g). Conjugates with three  
532 or more sperm were categorized depending on the nature of their association to each  
533 other and the degree of morphological specialization in sperm and/or binding material  
534 (i.e., SAM). Ensheathed conjugates occur when multiple sperm are completely  
535 enveloped together within a coat (Fig. 2b and Supplementary Fig. 2h-j). Aggregates  
536 typically contain variable numbers of sperm joined together principally via their heads,  
537 which are aligned in register and embedded in secreted material (Fig. 2b and  
538 Supplementary Fig. 2d-f). Rouleaux conjugates are orderly stacks of sperm, in which  
539 one sperm head nests within the head of another sperm in a repeated series that can  
540 include from a few to thousands of sperm<sup>41</sup> (Fig. 2b and Supplementary Fig. 2k,l).  
541 Hence, rouleaux require morphological adaptations such as a concavity or hollow  
542 pocket in the sperm head. Spermatostyles occur when the secretory material joining  
543 multiple sperm adopts an elongate, rod-like form and typically involves very precise  
544 sperm attachment (Fig. 2b and Supplementary Fig. 2b,c). The different conjugation  
545 states typically do not co-occur, but rare instances when they do so lend support to our  
546 recognition of discrete states (Supplementary Table 3). For example, the rouleaux  
547 conjugates of male *Stictonectes optatus* diving beetles also feature attached  
548 spermatostyle material (Supplementary Fig. 2k), and the complex rouleaux of some  
549 telemid spiders are ensheathed (Supplementary Fig. 2l). It should be noted that  
550 numerous papers mistakenly refer to ensheathed conjugates as spermatophores, which

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Suggestion: ADD Hexapoda order names in the phylogeny.

Exp: this is more informative for the readers

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- Extended figure of the phylogenetic tree. This: is very difficult to read the character states in the terminal branches. Then reduce the size of the microphotographs.

ADD and more extended figure also this in supplementary: you can use a linear phylogeny to better read the characters.

- in Fig 2: You could mark on the phylogeny with the letter of the micrograph, so that the reader has a more or less idea of where that taxon and that type of sperm conjugate is found.

- hard recommendation: add the orders names in the figure. Or at least complete this in Fig: 1.

-ADD what mean \* on the phylogentic tree

## Figure Legends

**Figure 1. Sperm conjugation and sperm associated material (SAM) have a deep and evolutionarily dynamic history across the Arthropoda tree of life.** Ultrametric fan phylogeny of 621 species of arthropods and outgroups with presence/absence data for sperm conjugation and SAM plotted at the tips. Time indicated in millions of years before present (mybp). Silhouette illustrations from PhyloPic (<http://phylopic.org>; CC0 1.0 license).

**Figure 2. Sperm conjugation occurs in five discrete states, all of which are estimated to have independently evolved multiple times, with sperm aggregates estimated to have arisen over 500 million years ago in the ancestor of all Hexapoda.** (a), Time-calibrated phylogeny of 621 species of arthropods and outgroups with conjugation states estimated for ancestral nodes (see also Supplementary Figs. 2 and 3) and time indicated in millions of years before present (mybp). (b), Photomicrographs depicting an example of each conjugation state (border colors match character state key); from upper left to bottom right: the unconjugated sperm of the millipede *Polyxenus lagurulus* are coiled and individually embedded within SAM at insemination (left), with SAM shed and sperm uncoiling inside the female reproductive tract (right) (reproduced with permission<sup>52</sup>), a sperm aggregate of the sawfly *Arge pagana* containing 240 - 350 sperm (reproduced with permission<sup>45</sup>), heads of a sperm pair of the silverfish *Thermobia domestica* (reproduced with permission<sup>47</sup>), a rouleaux comprising 32 plate-shaped spermatozoa arranged in 16 pairs of the solifugid *Eusimonia mirabilis* (reproduced with permission<sup>65</sup>), a spermatostyle conjugate of the ground beetle *Calathus montivagus* (reproduced with permission from <sup>51</sup>), and an

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conjugation

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what mean black arrows in the phylogeny?  
Explain  
-What is the clade in green ? sister group of Carabidae ??

873 ensheathed conjugate containing multiple sperm from the mite *Neomolgus littoralis*  
874 (reproduced with permission<sup>66</sup>).

875 **Figure 3. The sperm of ground beetles (family Carabidae) illustrate a probable**  
876 **progressive evolutionary transition from aggregate to spermatostyle to**  
877 **ensheathed conjugation states. (a),** Arthropod tree-wide posterior distributions of  
878 counts of three specific transitions between sperm character states each observed to  
879 have occurred with high probability within the Carabidae. **(b),** Simplified stochastic  
880 mapping of ancestral state reconstructions of conjugate form within the Carabidae,  
881 which reveals a high probability of gain of spermatostyles from aggregates, followed by  
882 the derivation of ensheathed conjugates. Specifically, a progressive series is observed  
883 in which the spermatostyle material (i.e., SAM) encapsulates increasing proportions of  
884 the sperm flagella, resulting in total ensheathment of the sperm as illustrated by *Clivina*  
885 *fossor* (far right image; note, blue indicates signal from DAPI-stained heads of sperm  
886 contained within a central cavity of the elongate sheath). Micrographs and illustrations  
887 depict the four character states shown in the tree. (photos copyright R Antonio Gomez;  
888 CC BY-SA).

889 **Figure 4. Evolutionarily independent origin of spermatostyle conjugates in true**  
890 **bugs (order Hemiptera) and beetles (order Coleoptera), with evidence of parallel**  
891 **recruitment of sperm leucyl aminopeptidases (S-LAPs). (a),** Ancestral character  
892 state reconstructions based on stochastic mapping (N = 1000 simulations) of the  
893 multistate conjugation data for all bugs, beetles and intervening taxa strongly supports  
894 beetles and bugs having independently derived spermatostyle conjugates. (left)  
895 Representative map illustrating the distribution of unconjugated, aggregate and

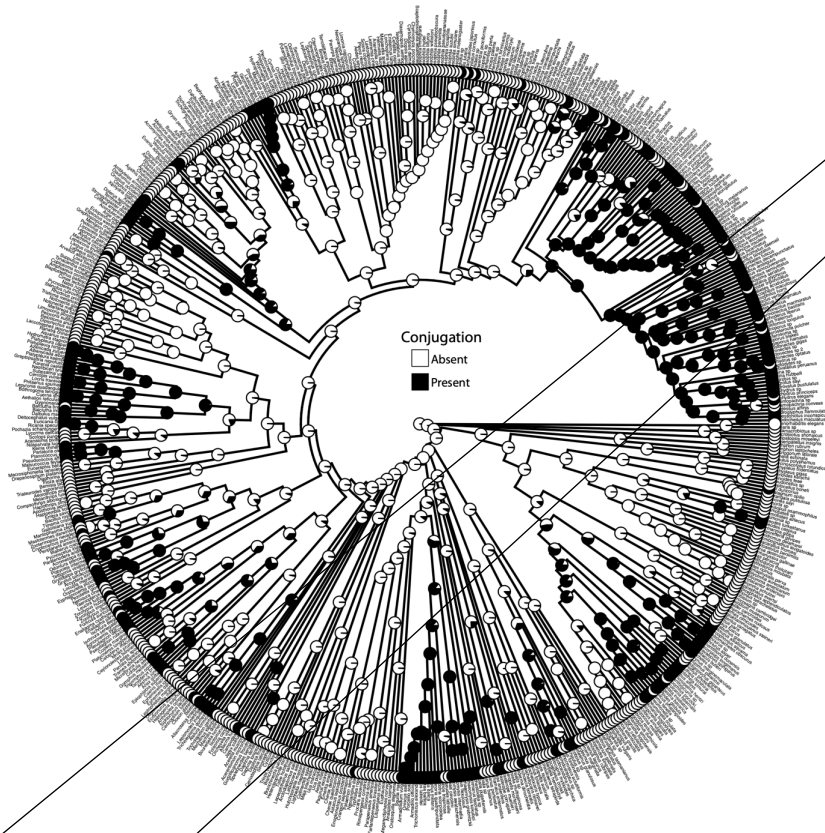
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ADD the names to the clades as Fig 1

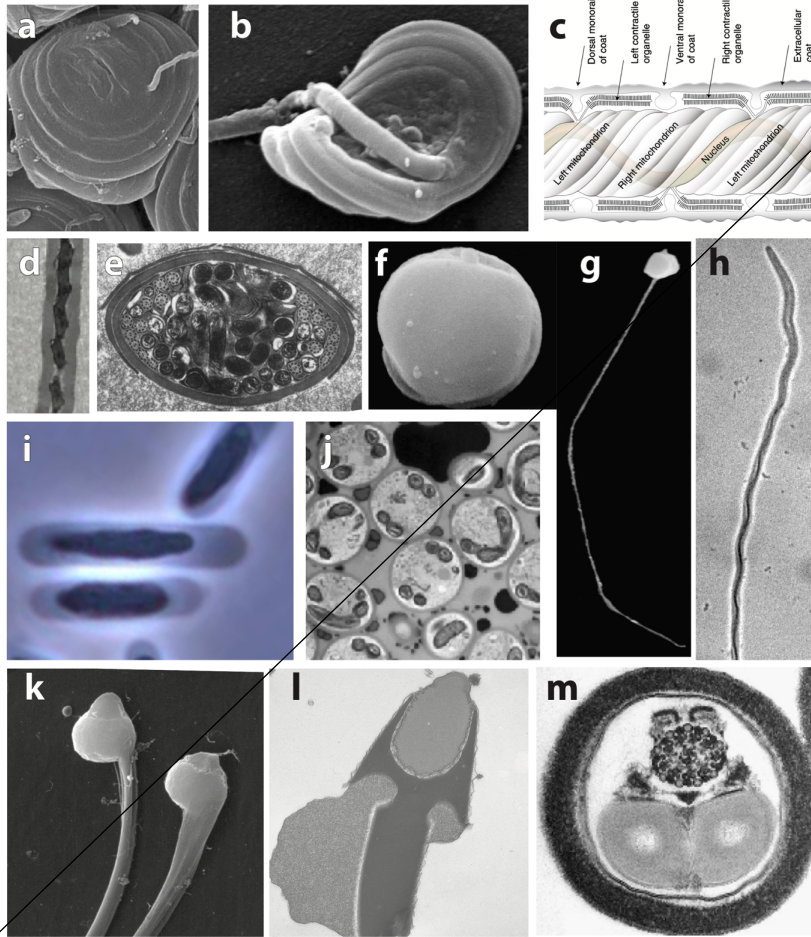
- Suggestion ADD here the phylogeny in vertical extent format. this could be better to read the characters. As this is extent phylogeny you can cut in two parts.

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ADD to additional list with the node number and the PP values because here the pie charts are not so informative.



**Supplementary Figure 1.** Phylogenetic reconstruction of conjugation evolution in arthropods and outgroup taxa based upon analysis of the univariate data. Pie charts at nodes depict posterior probabilities of sperm conjugation presence (black) or absence (white) from (N = 1000) stochastic mappings.



**Supplementary Figure 4.** Sperm Associated Material (SAM) is diverse and widespread in arthropods and commonly occurs in the absence of conjugation. **(a,b)**, Scanning electron micrographs (SEMs) of individual sperm of the collembolans **(a)** *Allacma fusca* and **(b)** *Orchesella villosa*, which are coiled around a disc of extracellular material (reproduced with permission<sup>97</sup>). **(c)**, Schematic of a portion of an unconjugated sperm in the cypridoidean ostracods, which possess a distinct extracellular coat that is shed inside the female reproductive tract (reproduced with permission<sup>98</sup>). **(d)**, Transmission electron



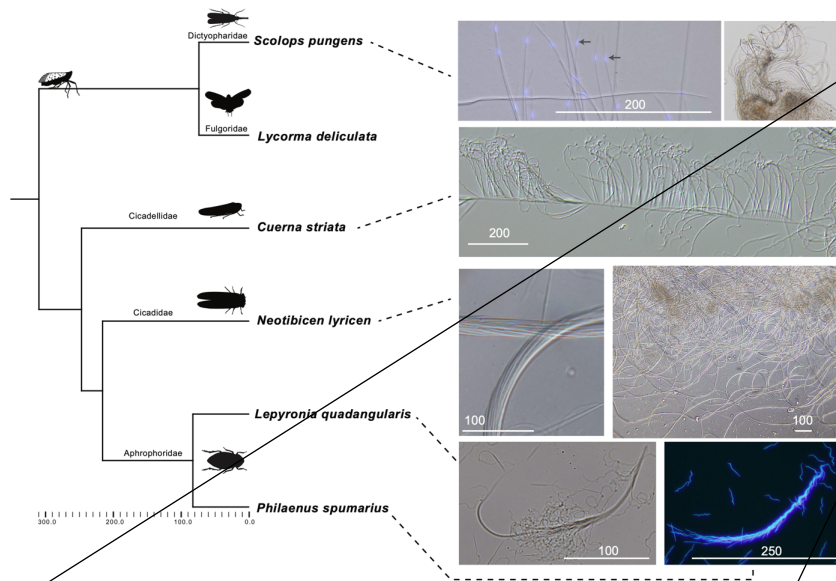
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- Mention the scale of the photomicrograph.
- The scale of the tree it evolutionary time? complete the details.
- ADD few descriptions concerning the photomicrographs.



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ADD here at least the insect Order



**Supplementary Figure 7.** Phylogeny (derived from Johnson et al.<sup>105</sup>) for true bugs sampled for spermatostyle proteomics, morphology, and S-LAP immunohistochemistry. Silhouette drawings of true bugs from PhyloPic (CC0 1.0 license; <http://www.phylopic.org>).