

## Supplementary Results, Figures, Tables and References for Gomez et al.

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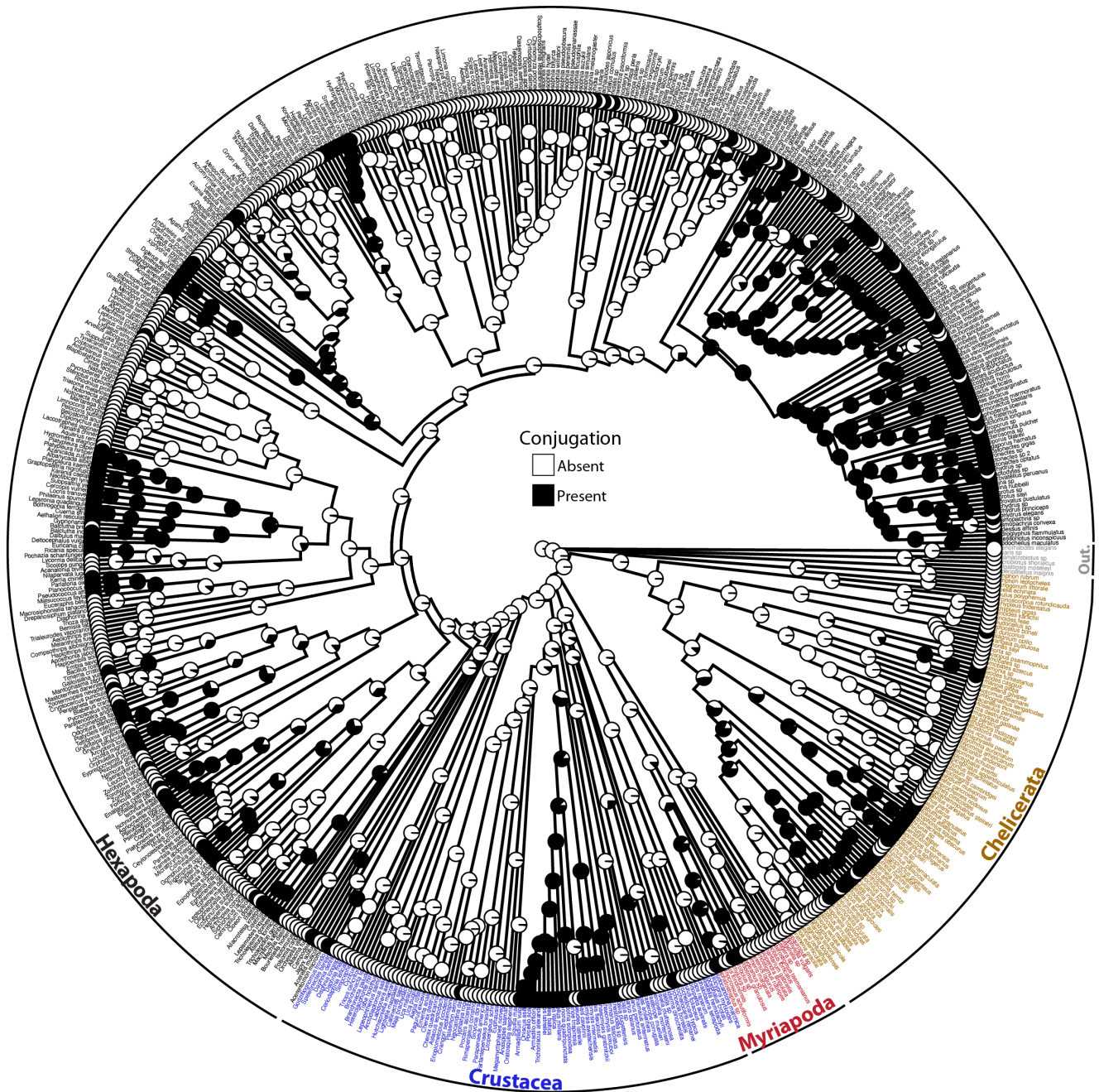
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## Supplementary transcriptomic and proteomic results for the cicada (*Neotibicen lyricen*)

We conducted additional analysis of our cicada transcriptomic and proteomic data because of the large-scale differences observed relative to the spermatostyle proteomes of other examined true bug and beetle<sup>30</sup> species. First, our *de novo* cicada transcriptome assembly score (0.22) was comparable to other species in this study (0.20-0.23)<sup>92</sup>, and a high BUSCO score (97%) indicates that the transcriptome is unlikely to have substantive gaps (missing <1% of single copy orthologs present in arthropods)<sup>93</sup>. Second, to address whether our results would improve with the use of other protein references, we repeated the MS/MS analysis of our cicada data (415,644 spectra across two biological replicates) using protein models derived from two additional cicada species: *Okanagana villosa* (SRA: SRS462915) and *Magiccicada septendecula*<sup>94</sup>. In all cases, the native reference for *Neotibicen lyricen* identified significantly more proteins indicating that our *de novo* reference was the optimal search database. Contrary to other true bugs, the cicada proteome lacked S-LAPs (Supporting Data 3), and, critically, their absence was not due to their absence from their transcriptome, which includes three unique S-LAP genes distributed in Cluster II and Cluster III (data not shown). We note that members of the S-LAP protein family were not identified in any of the searches using alternative cicada reference databases. Instead, their proteomes include a limited number of highly abundant uncharacterized proteins that are members of cicada-specific protein families. In fact, the 10% of cicada proteins in species-specific proteins families account for over 40% of spermatostyle protein abundance. Given the phylogenetic placement of cicadas (Supplementary Fig. 8), and that our protein coverage was robust (127,281 PSMs; 1% FDR) and highly reproducible between replicates (95.7% and 99% of proteins identified in each sample, respectively), the cicada spermatostyle proteome appears to have diverged substantially in a lineage-specific fashion.



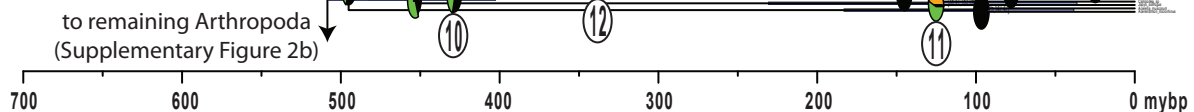
**Supplementary Figure 1.** Phylogenetic reconstruction of conjugation evolution in arthropods and outgroup taxa based upon analysis of the univariate binary data. Pie charts at nodes depict posterior probabilities of sperm conjugation presence (black) or absence (white) from (N = 1000) stochastic mappings. Ancestral state estimates of all nodes are also provided online as a separate text file with the tree file. The six outgroup Ecdysozoa tips species are referred to with the abbreviation “Out.”.

a

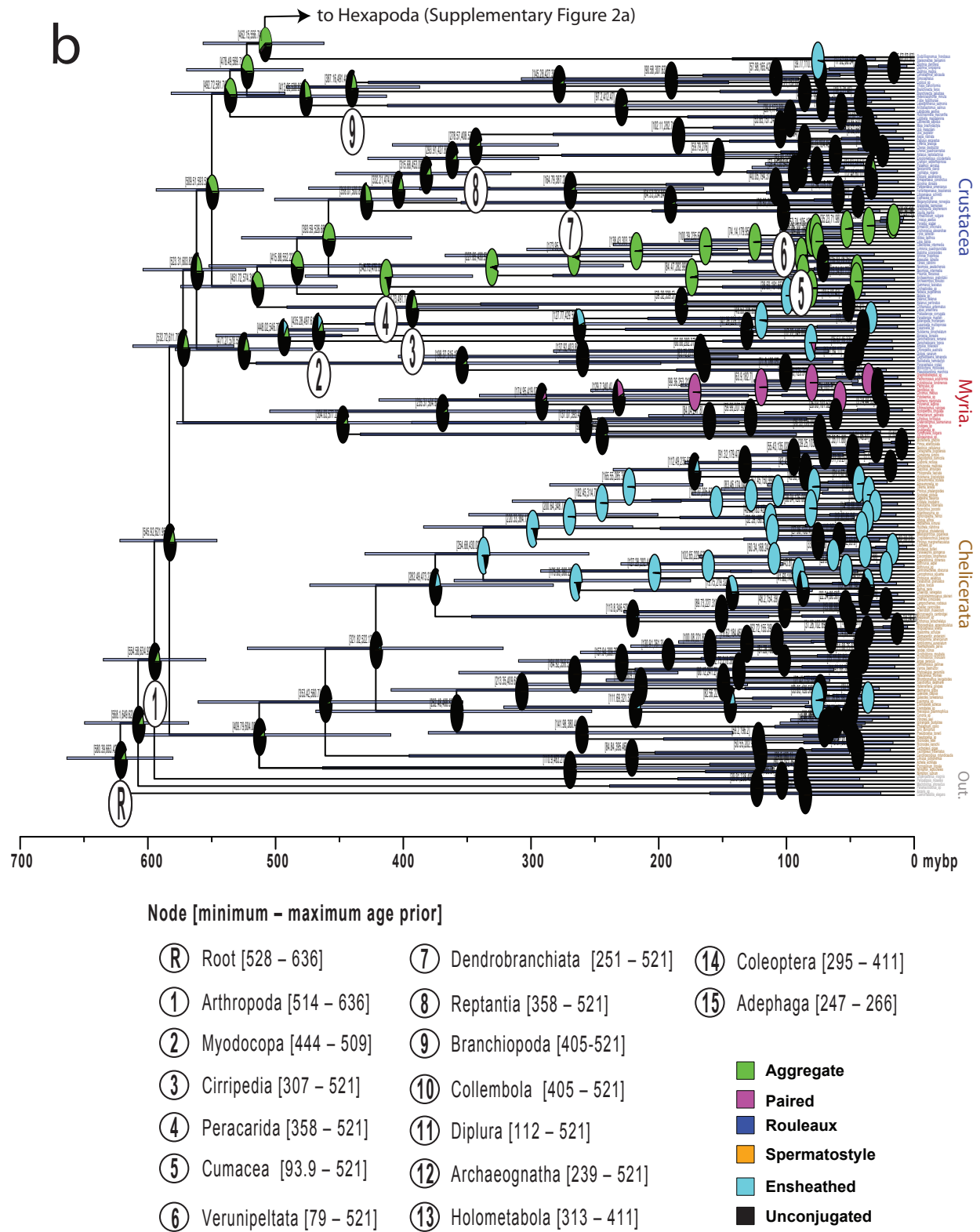
Node [minimum – maximum age prior]

- Ⓡ Root [528 – 636]
- ① Arthropoda [514 – 636]
- ② Myodocopa [444 – 509]
- ③ Cirripedia [307 – 521]
- ④ Peracarida [358 – 521]
- ⑤ Cumacea [93.9 – 521]
- ⑥ Verunipeltata [79 – 521]
- ⑦ Dendrobranchiata [251 – 521]
- ⑧ Reptantia [358 – 521]
- ⑨ Branchiopoda [405–521]
- ⑩ Collembola [405 – 521]
- ⑪ Diplura [112 – 521]
- ⑫ Archaeognatha [239 – 521]
- ⑬ Holometabola [313 – 411]
- ⑭ Coleoptera [295 – 411]
- ⑮ Adephaga [247 – 266]

- Aggregate
- Paired
- Rouleaux
- Spermatostyle
- Ensheathed
- Unconjugated

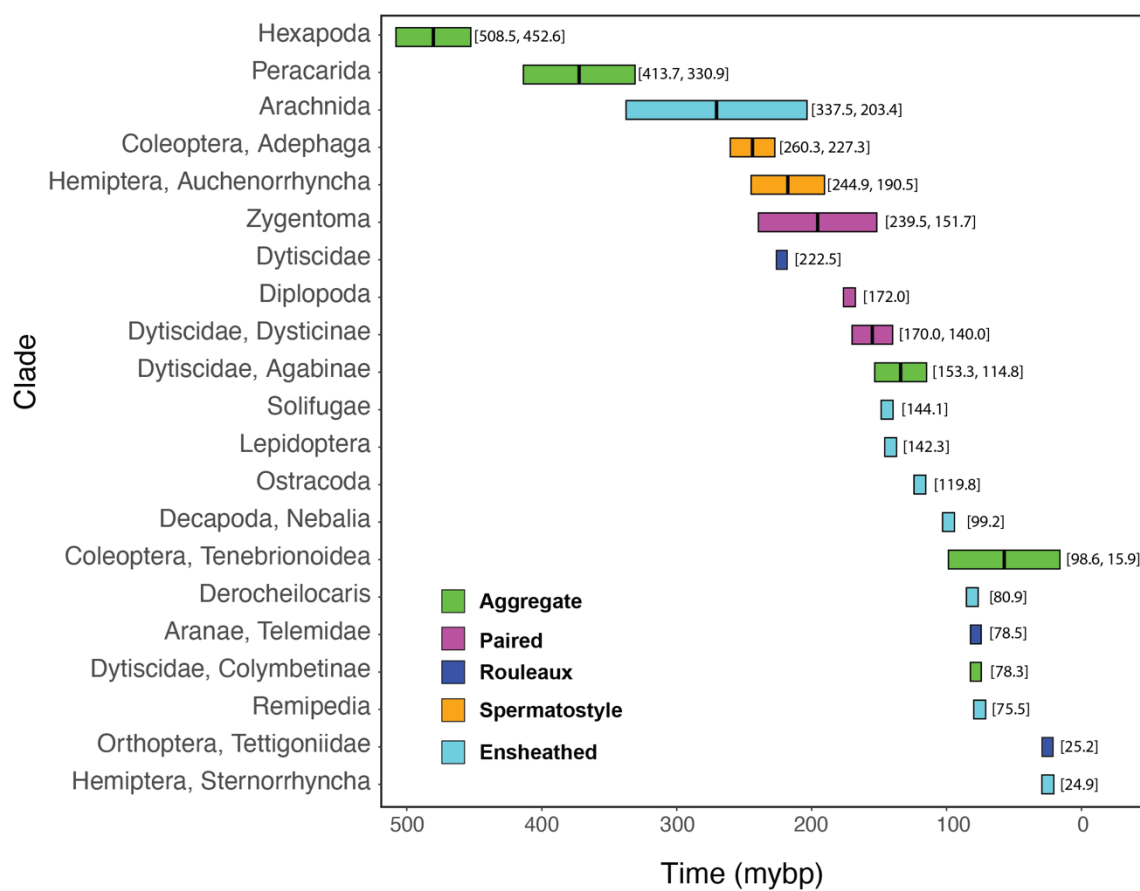
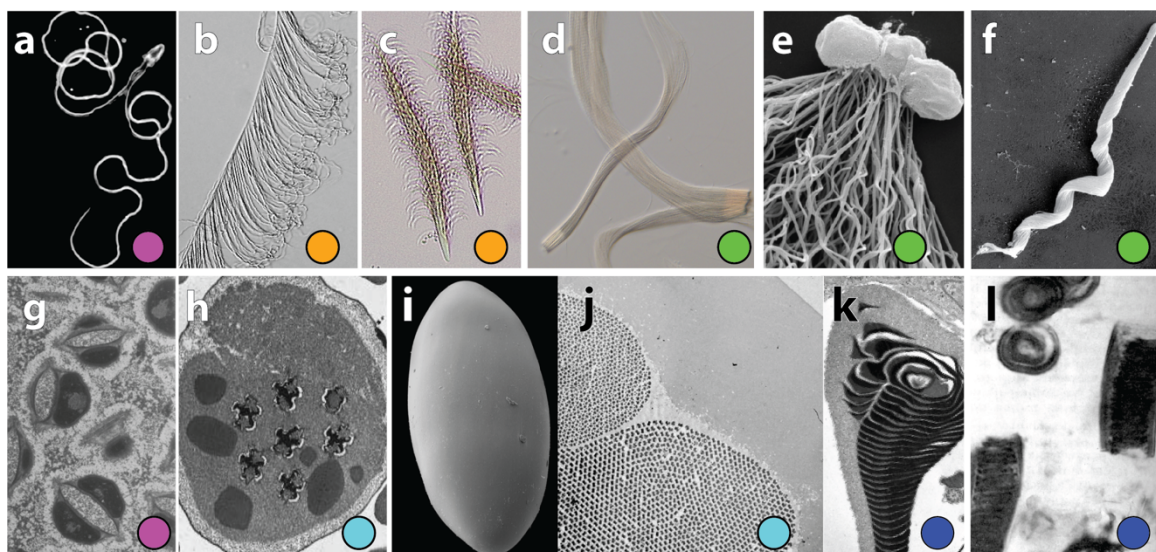


Hexapoda



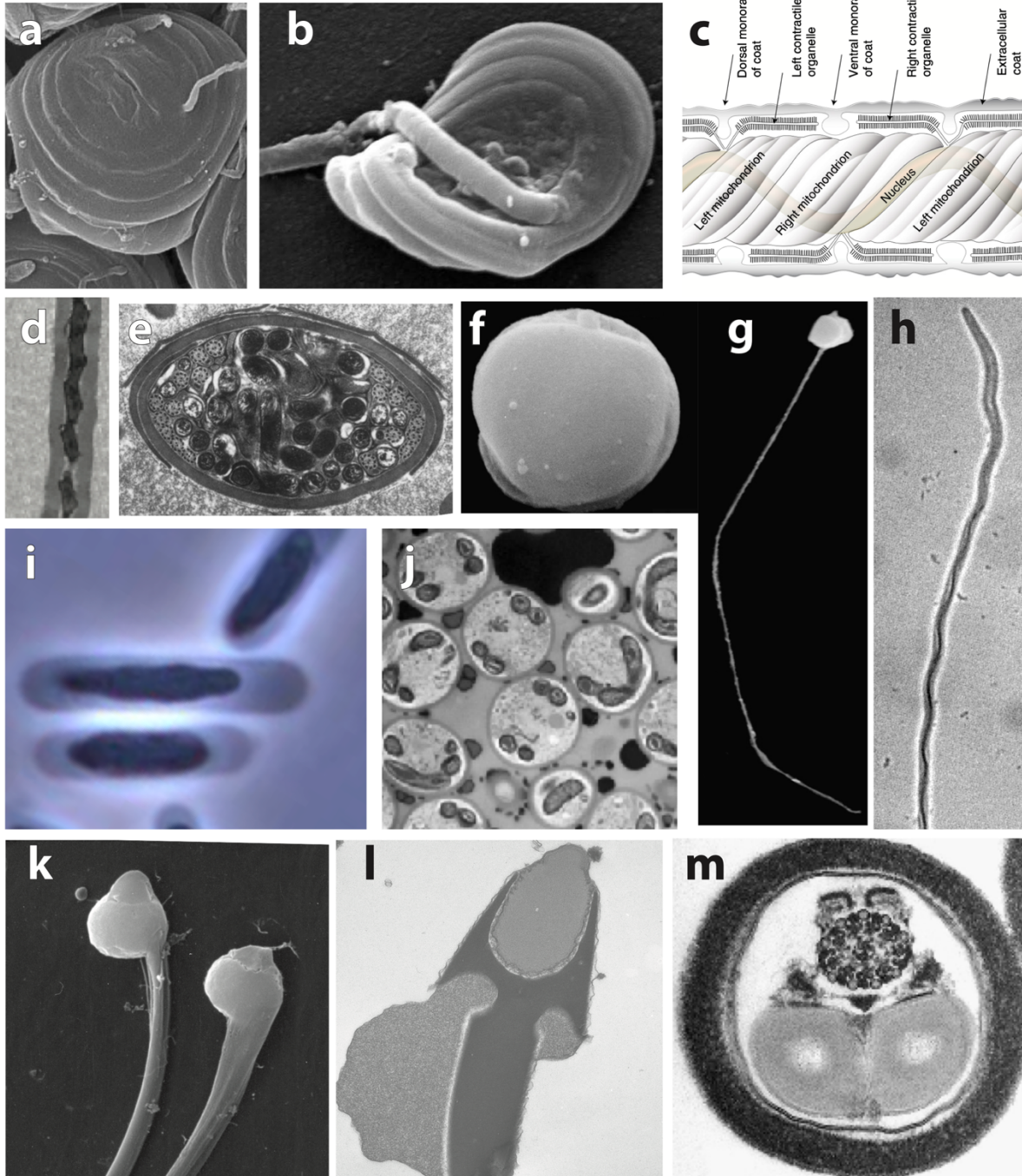
**Supplementary Figure 2.** Time-calibrated ultrametric phylogenetic tree of 621 Arthropoda and outgroup species: (a) the Hexapoda subtree and (b) the remaining non-hexapod

Arthropoda. Pie charts depict the most probable ancestral states for our multistate conjugation data summarized from  $N = 1000$  stochastic mappings and node ages from our Bayesian analysis of divergence times using fossil priors (see Methods, fossil details<sup>72</sup>, and online files). Node heights are the median node ages and node bars refer to 95% highest posterior density (HPD) of node ages (see Methods for more details and the online supporting materials for the tree and associated files from the BEAST dating analysis).



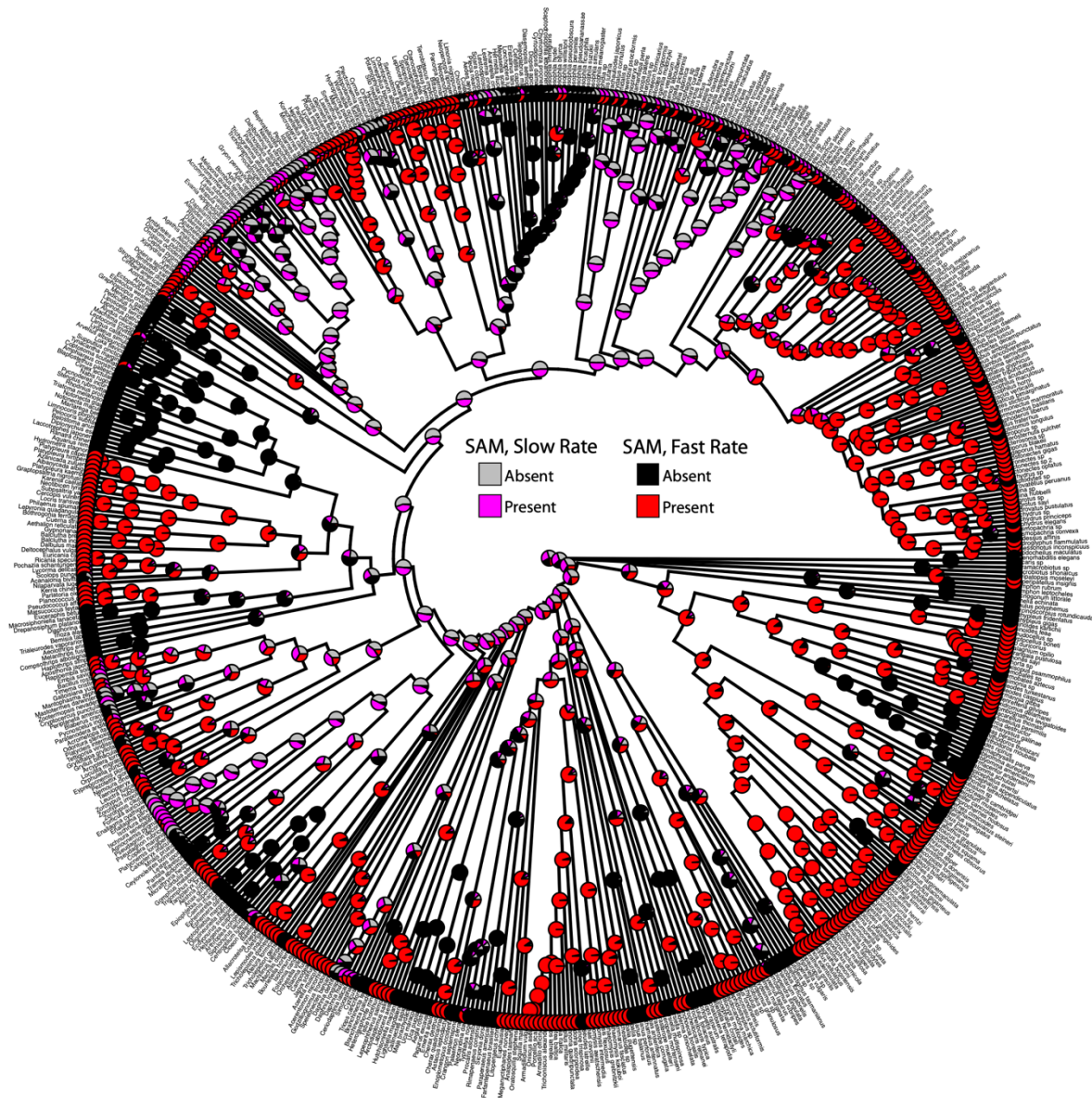
**Supplementary Figure 3.** Additional examples of the dramatic morphological variation in conjugation type across arthropods (upper panel) and their probable (posterior probability  $\geq 50$  to  $\geq 90$ ) times of origin (millions of years before present; mybp) in major taxonomic groups (lower panel). Age ranges based on a summary of  $N = 1000$  stochastic mappings and the node ages of our phylogeny (see Methods and Fig. S2); the vertical bars refer to the median of these ranges. Note: branches with only a single number represent clades in which there was a rapid transition from low ( $< 50$  pp) to high probability ( $> 90$  pp) of a given type). **(a)**, Darkfield micrograph of paired sperm of the diving beetle *Graphoderus liberus* (Coleoptera: Dytiscidae; reproduced with permission<sup>42</sup>). **(b)**, Micrograph of hundreds of sperm unilaterally bound to a spermatostyle in *Gypononana* sp. (Hemiptera: Cicadellidae). **(c)**, Micrograph of sperm evenly distributed across entire surface of the short, stout spermatostyles of the beetle *Blethisa oregonensis* (Coleoptera: Carabidae). **(d-f)**, Micrograph **(d)** and scanning electron micrographs (SEMs) **(e,f)** of sperm aggregates, which exhibit variable numbers of sperm bound together via their heads by SAM: **(d)**, *Gyrinus natator* (Coleoptera: Gyrinidae); **(e)**, *Cloeon dipterum* (Ephemeroptera: Baetidae; reproduced with permission<sup>95</sup>); **(f)**, *Galloisiana yuasai* (Grylloblattodea: Grylloblattidae). **(g)**, Transmission electron micrograph (TEM) of the aflagellate, paired and ensheathed sperm of *Polydesmus* millipedes (Diplopoda: Polydesmidae; reproduced with permission<sup>96</sup>). **(h)**, TEM of the aggregated (groups of 3-6) and ensheathed sperm of the silverfish *Atelura formicaria* (Zygentoma: Nicoletiidae; reproduced with permission<sup>97</sup>). **(i,j)**, Conjugates of *Galeodes caspius* (Solifugae: Galeodidae) include a thick sheath **(i, SEM)** surrounding hundreds of sperm **(j, TEM; reproduced with permission<sup>98</sup>)**. **(k)**, TEM of the precisely stacked sperm heads of the rouleaux of *Stictonectes optatus* diving beetle enveloped by a spermatostyle-like structure (reproduced with permission<sup>99</sup>). **(l)**, Micrograph of rouleaux of *Apneumonella* spiders (Araneae: Telemidae) that are also ensheathed (reproduced with permission<sup>100</sup>).



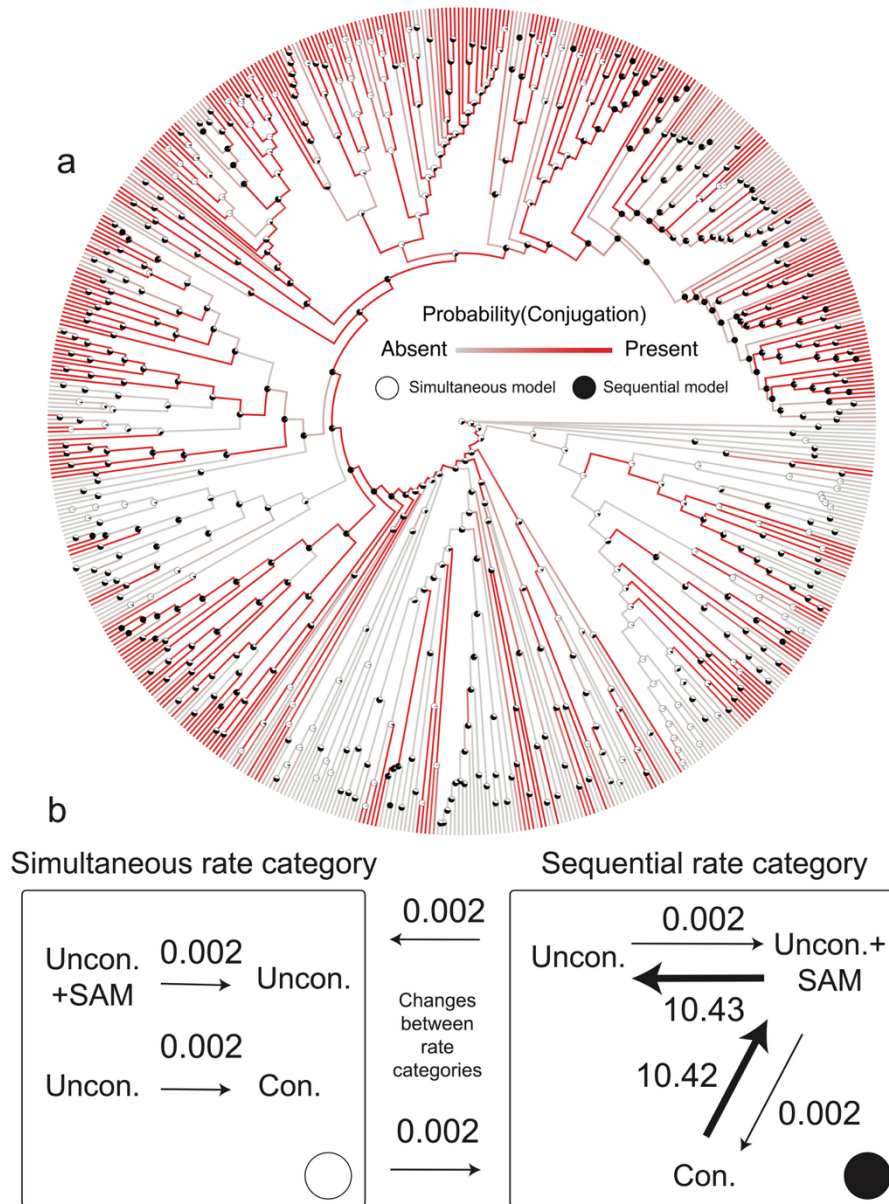


**Supplementary Figure 5.** Sperm Associated Material (SAM) is diverse and widespread in arthropods and commonly occurs in the absence of conjugation. (a,b), Scanning electron micrographs (SEMs) of individual sperm of the collembolans (Collembola) (a) *Allacma fusca* and (b) *Orchesella villosa*, which are coiled around a disc of extracellular material (reproduced with permission<sup>101</sup>). (c), Schematic of a portion of an unconjugated sperm in the cypridoid ostracods (Podocopida), which possess a distinct extracellular coat that

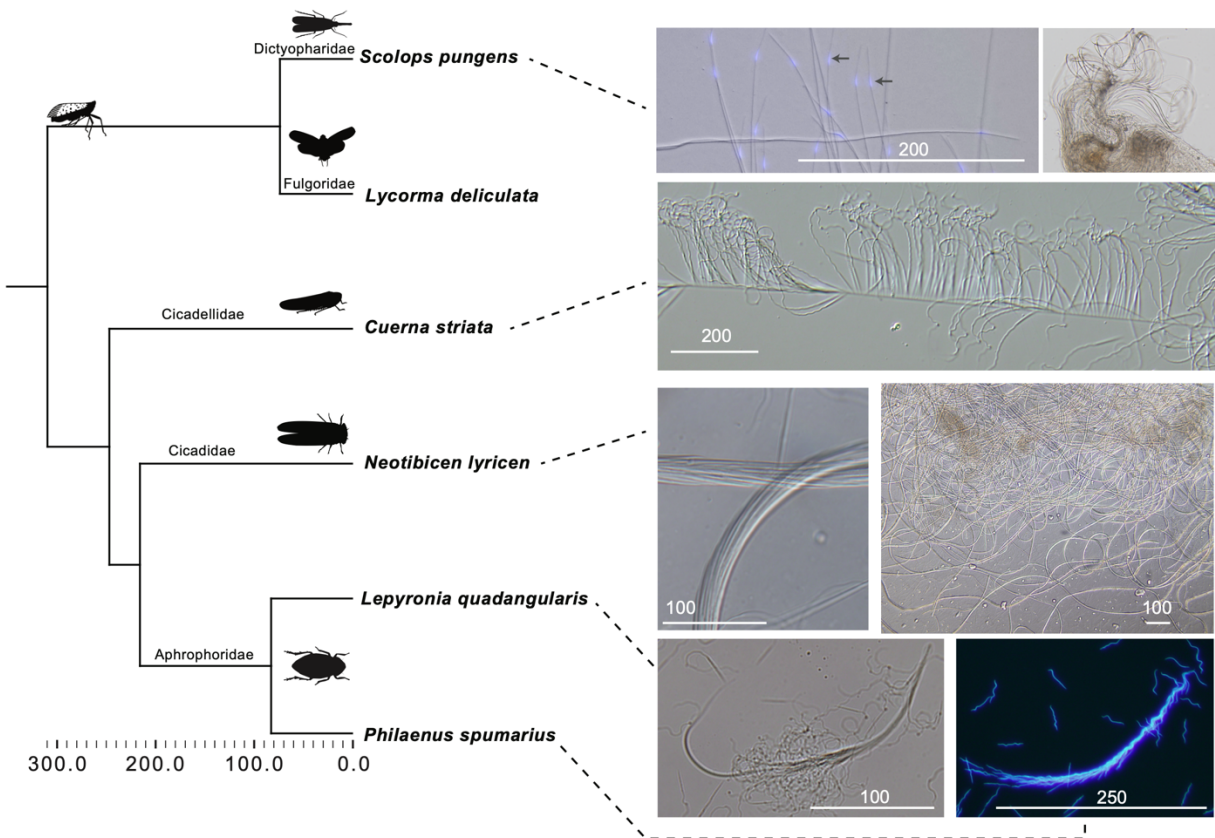
is shed inside the female reproductive tract (reproduced with permission<sup>102</sup>). **(d)**, Transmission electron micrograph (TEM) of an unidentified species of *Idarnes* wasp (Hymenoptera) showing an extracellular layer around the sperm head (reproduced with permission<sup>103</sup>). **(e-g)**, sperm of Ricinulei **(e)** TEM of *Cryptocellus* sp. and **(f-g)** SEMs of *Pseudocellus* sp. (Ricinulei) sperm, which are flagellated and coiled within individual sheaths (reproduced with permission<sup>104,105</sup>). **(h)**, micrograph of a sperm of the carabid beetle *Broscodera insignis* (Coleoptera) with an attached elongate mass of SAM that is reminiscent of spermatostyles (reproduced with permission<sup>51</sup>). **(i)**, micrograph of the aflagellate sperm of the water flea *Daphnia magna* (Anomopoda), which are surrounded by a thick capsule-like sheath (reproduced with permission<sup>106</sup>). **(j)**, TEM of *Pholcus phalangoides* (and numerous other spider species (Araneae) are unconjugated and ensheathed (reproduced with permission<sup>107</sup>). **(k,l)**, SEM and TEM, respectively, of a sperm in the web-spinner *Embia savignyi* (Embioptera), which possess additional bound SAM surrounding the head. **(m)**, TEM of a *Spiralizoros caudelli* (Zoraptera) sperm, which possesses a thick glycocalyx that is shed inside female reproductive tract (reproduced with permission<sup>108</sup>).



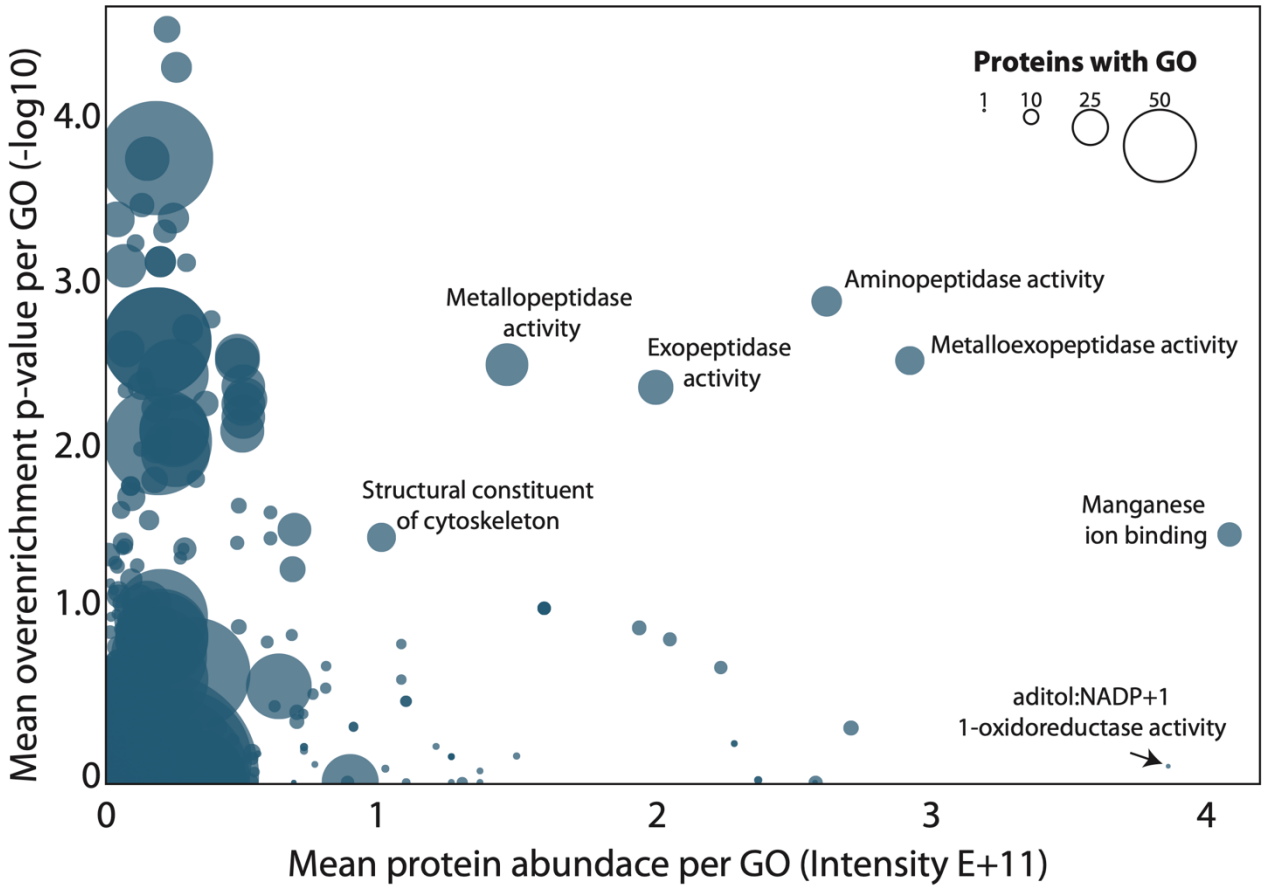
**Supplementary Figure 6.** Phylogenetic reconstruction of the evolution of SAM in arthropods and outgroup taxa based on analysis of the univariate data under the optimal model with two rate categories, both with equal transition rates. Pie charts at nodes depict posterior probabilities of SAM presence/absence in either rate category (see color key) from (N = 1000) stochastic mappings.



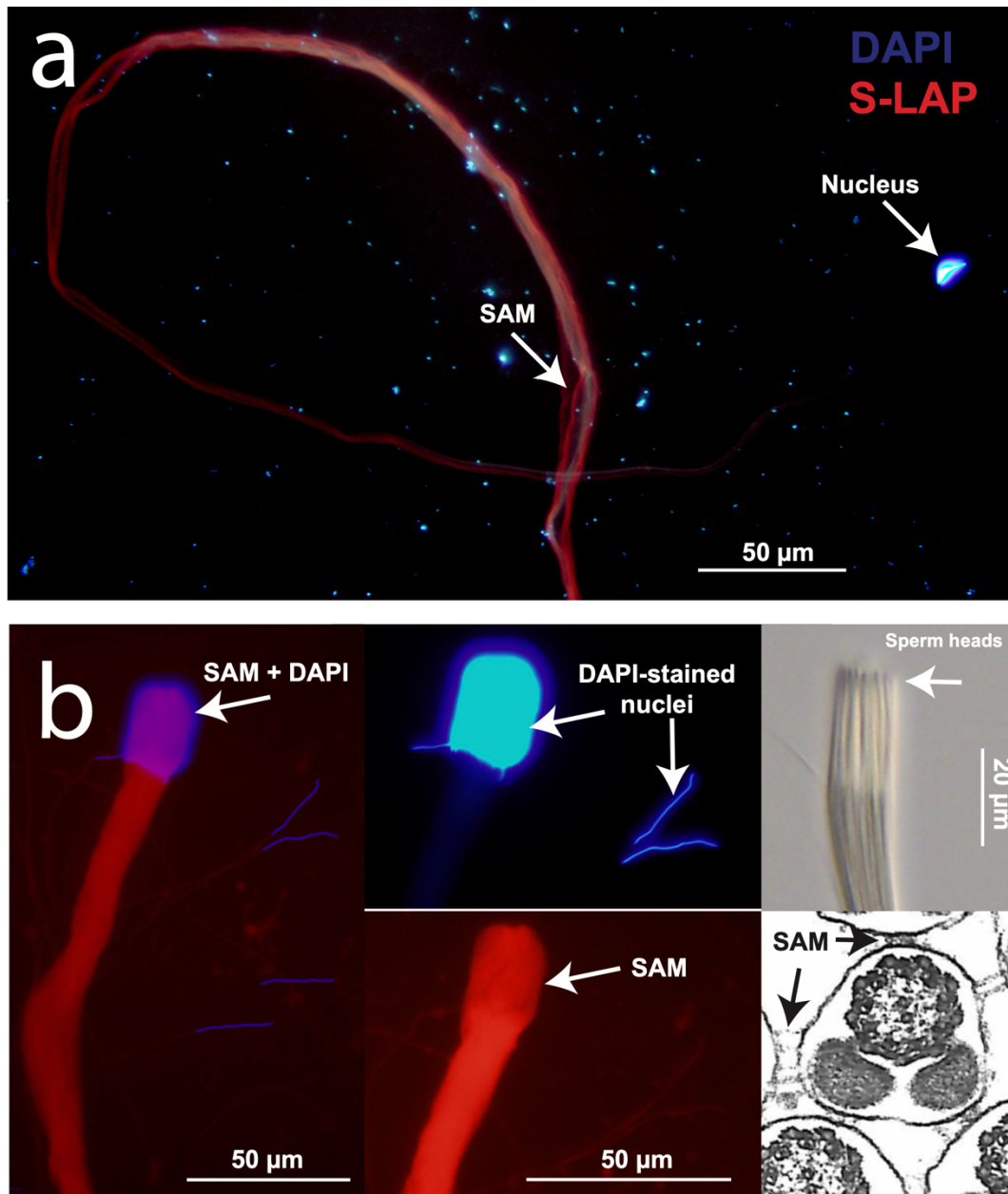
**Supplementary Figure 7.** Results of Bayesian probabilistic modeling and reversible-jump MCMC support SAM as an antecedent trait to conjugation gain. Conjugation states abbreviated as ‘Con.’ and ‘Uncon.’ for conjugated and unconjugated sperm, respectively. **(a)**, Arthropod phylogeny with branches colored according to posterior estimates of conjugation presence or absence; pie charts at nodes indicate ancestral state estimates of being in one of two possible rate categories. **(b)**, The Discrete Covarion model (see Methods<sup>76</sup>) fits optimally and allows species to transition between states and rate categories. Within the sequential rate category, gains of conjugation must follow gains of SAM and vice versa, whereas the simultaneous rate category allows dual gains of SAM and conjugation.



**Supplementary Figure 8.** Time-calibrated ultrametric phylogeny (derived from Johnson et al.<sup>109</sup>) for true bugs (Hemiptera) sampled for spermatostyle proteomics, morphology, and S-LAP immunohistochemistry. The scale of the phylogeny is in millions of years before the present and is based on divergence time analysis using phylogenomic data and 23 fossil calibrations<sup>109</sup>. Brief descriptions of sperm are as follows from top to bottom row: unconjugated sperm with sperm associated material are found in fulgoroids (Dictyopharidae and Fulgoridae) and can appear as filaments with additional surrounding material (black arrows indicate DAPI-stained nuclei), spermatostyles in planthoppers (Cicadellidae) can include unilateral sperm attachment, cicadas (Cicadidae) have large and distinctive spermatostyles, and spittle bugs (Aphrophoridae) make spermatostyles that appear similar to spermatostyles in some beetles (Coleoptera). Scales in light and fluorescence photomicrographs refer to 100 or 200 micrometers. Silhouette drawings of true bugs from PhyloPic (CC0 1.0 license; <http://www.phylopic.org>).



**Supplementary Figure 9.** GO term abundance, count, and over-enrichment for the true bug (Hemiptera) spermatostyle proteomes, excluding the cicada. Plotted terms include those annotated to proteins confidently identified in two of three true bug proteomes. GO terms with both high abundance and significant over-enrichment at the 5% significance level after multiple test correction (Intensity > 1E+11 and  $-\log P > 1.3$ ) are labeled, excluding the labeled term at the extreme bottom right indicated with a black arrow. The plot does not include the two following groups of outliers: 15 extremely lowly abundant GOs with very high over-enrichment  $P$  values ( $P > 4.5$ ), and two GOs present in spermatostyles in high abundance that were not significantly enriched (GO:0047939: L-glucuronate reductase activity; GO:0008106: alcohol dehydrogenase (NADP+) activity).



**Supplementary Figure 10.** By examining a close relative of the true bugs (Hemiptera; Fig. S8) and the whirligig beetles<sup>30</sup> (Coleoptera), respectively, for which we examined spermatostyle proteomes, we use an S-LAP antibody and immunofluorescence to show that S-LAPs localize to Sperm Associated Material (SAM) in species lacking spermatostyles. (a), Composite fluorescence micrograph of a sperm of *Scolops pungens*, a dictyopharid planthopper with unconjugated sperm bounded by SAM showing that S-LAPs localize to the SAM, which has a gossamer appearance. (b), (upper right) micrograph of apical end of an aggregate conjugate of the whirligig beetle *Gyrinus natator* (see also Fig.

S3d). (left) Fluorescent micrographs of *G. natator* aggregate, which reveal the presence of S-LAPs in the apices of their aggregates where both DAPI and S-LAP antibody signals co-localize. S-LAPs are abundant in flagella but are absent in dissociated (individual) sperm heads. We interpret the observed pattern as indicative of S-LAPs in the SAM binding the sperm together in aggregates, which is hypothesized to closely match prior descriptions of SAM in a congener, *G. marinus*, based on transmission electron microscopy (lower right; SAM, indicated by black arrows, appears as distinct filamentous bridges connecting sperm; reproduced with permission from<sup>110</sup>).

**Supplementary Table 1.** Multistate categorization of sperm conjugation: conjugation types and definitions, occurrence within class-level taxa (Ch = Chelicerata, M = Myriapoda, Cr = "Crustacea," and H = Hexapoda) and lower taxonomic distributions (taxa are included if conjugated sperm observed in any species), and ranges for reported number of sperm per conjugate. Full list of references provided in Supplementary Data 1.

| Conjugation Type | Definition                                                                                                         | Occurrence |   |    |   | Taxonomic distribution<br><small>Taxa are included if conjugation type observed in any species</small>                                                                                                                 | Sperm per conjugate |
|------------------|--------------------------------------------------------------------------------------------------------------------|------------|---|----|---|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
|                  |                                                                                                                    | Ch         | M | Cr | H |                                                                                                                                                                                                                        |                     |
| Aggregate        | Sperm heads (often variable in number) imprecisely embedded in SAM                                                 | ×          | × | ✓  | ✓ | Isopoda, Zygentoma, Odonata, Ephemeroptera, Polyneoptera (Blattodea, Grylloblattodea, Mantodea, Mantophasmatodea, Orthoptera, Zoraptera), Hemiptera, Hymenoptera, Megaloptera, Raphidioptera, Coleoptera <sup>1*</sup> | 3 - 1200            |
| Pairs            | Two sperm, typically with precise head alignment                                                                   | ×          | ✓ | ✓  | ✓ | Diplopoda, Mystacocarida, Remipedia, Zygentoma. Coleoptera (Noteridae, Dytiscidae)                                                                                                                                     | 2                   |
| Rouleaux         | Orderly stacks of sperm with head modifications facilitating precise juxtaposition                                 | ✓          | × | ✓  | ✓ | Solifugae, Araneae (Telemidae), Isopoda, Orthoptera (Tettigoniidae), Coleoptera (Carabidae, Dytiscidae*)                                                                                                               | 2 - 64              |
| Spermatostyle    | A distinctive rod-like structure with multiple sperm heads (and, in some cases, portions of flagella) are embedded | ×          | × | ×  | ✓ | Diplura, Odonata, Hymenoptera, Hemiptera*, Coleoptera (Carabidae, Gyrinidae, Halipidae, Dytiscidae)                                                                                                                    | 5 - 200             |
| Ensheathed       | Multiple sperm encased by a common sheath, coat or glycocalyx                                                      | ✓          | × | ✓  | ✓ | Scorpiones, Araneae, Solifugae, Opiliones*, Trombidiformes, Remipedia, Ostracoda, Isopoda, Decapoda, Zygentoma, Hemiptera, Lepidoptera <sup>1</sup> , Coleoptera (Carabidae)                                           | 2 - 256             |

\*Taxa with conjugates composed of multiple types of sperm (i.e., heterospermatozeugmata; Pitnick et al. 2009a)

<sup>1</sup>Taxa with ejaculates composed of both un- and conjugated sperm co-occurring with heteromorphism (e.g., most Lepidoptera transfer unconjugated parasperm and ensheathed eusperm)

**Supplementary Table 2.** Minimum (Min), maximum (Max) and 95% highest posterior distribution (HPD) estimates of the number of character state transitions from 1000 stochastic mappings.

| Transition                                | Min       | Max        | 95% HPD       |
|-------------------------------------------|-----------|------------|---------------|
| <b><i>Simple-&gt;Conjugated</i></b>       | <b>9</b>  | <b>39</b>  | <b>15-34</b>  |
| Simple->Aggregate                         | 5         | 24         | 8-19          |
| Simple->Pairs                             | 0         | 5          | 1-3           |
| Simple->Rouleaux                          | 0         | 0          | 0             |
| Simple->Spermatostyle                     | 0         | 0          | 0             |
| Simple->Ensheathed                        | 4         | 15         | 6-12          |
| <b><i>Conjugated-&gt;Simple</i></b>       | <b>45</b> | <b>148</b> | <b>57-115</b> |
| Aggregate->Simple                         | 39        | 95         | 46-80         |
| Pairs->Simple                             | 0         | 6          | 1-4           |
| Rouleaux->Simple                          | 2         | 11         | 4-8           |
| Spermatostyle->Simple                     | 2         | 16         | 3-11          |
| Ensheathed->Simple                        | 2         | 20         | 3-12          |
| <b><i>Conjugate Type A-&gt;Type B</i></b> | <b>19</b> | <b>105</b> | <b>30-74</b>  |
| <b><i>Aggregate-&gt;Other</i></b>         | <b>5</b>  | <b>32</b>  | <b>8-23</b>   |
| Aggregate->Pairs                          | 0         | 3          | 0-2           |
| Aggregate->Rouleaux                       | 1         | 7          | 2-5           |
| Aggregate->Spermatostyle                  | 4         | 14         | 5-11          |
| Aggregate->Ensheathed                     | 0         | 8          | 1-5           |
| <b><i>Pairs-&gt;Other</i></b>             | <b>1</b>  | <b>10</b>  | <b>2-6</b>    |
| Pairs->Aggregate                          | 0         | 5          | 1-3           |
| Pairs->Rouleaux                           | 0         | 0          | 0             |
| Pairs->Spermatostyle                      | 0         | 0          | 0             |
| Pairs->Ensheathed                         | 1         | 5          | 1-3           |
| <b><i>Rouleaux -&gt;Other</i></b>         | <b>3</b>  | <b>21</b>  | <b>7-16</b>   |
| Rouleaux ->Aggregate                      | 2         | 13         | 4-10          |
| Rouleaux ->Pairs                          | 1         | 8          | 3-6           |

|                                       |           |           |              |
|---------------------------------------|-----------|-----------|--------------|
| Rouleaux ->Spermatostyle              | 0         | 0         | 0            |
| Rouleaux ->Ensheathed                 | 0         | 0         | 0            |
| <b><i>Spermatostyle-&gt;Other</i></b> | <b>10</b> | <b>39</b> | <b>12-27</b> |
| Spermatostyle->Aggregate              | 10        | 32        | 12-23        |
| Spermatostyle->Pairs                  | 0         | 0         | 0            |
| Spermatostyle->Rouleaux               | 0         | 4         | 0-2          |
| Spermatostyle->Ensheathed             | 0         | 3         | 0-2          |
| <b><i>Ensheathed-&gt;Other</i></b>    | <b>0</b>  | <b>3</b>  | <b>1-2</b>   |
| Ensheathed->Aggregate                 | 0         | 0         | 0            |
| Ensheathed->Pairs                     | 0         | 0         | 0            |
| Ensheathed->Rouleaux                  | 0         | 3         | 1-2          |
| Ensheathed->Spermatostyle             | 0         | 0         | 0            |

**Supplementary Table 3.** The co-occurrence of multiple conjugation states is uncommon in arthropods and was observed in nine out of 621 (1.4%) sampled species. Sampled species with co-occurring conjugate types were coded as having 50/50 prior probability of being in one of two co-occurring states.

| Primary type  | Co-occurring type | Example taxa                                                                                 | Description                                                                | References                                                                           |
|---------------|-------------------|----------------------------------------------------------------------------------------------|----------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| Pairs         | Ensheathed        | <i>Derocheilocaris</i> spp. (Mystacocarida);<br><i>Godzilliognomus frondosus</i> (Remipedia) | Pairs are further enveloped in secretory material                          | Tuzet and Fize <sup>111</sup> ; Brown and Metz <sup>111</sup> ; Yager <sup>112</sup> |
| Rouleaux      | Ensheathed        | Telemidae spider conjugates                                                                  | Rouleaux are further ensheathed in material                                | Legendre and Lopez <sup>100</sup> ; Juberthie et al. <sup>114</sup>                  |
| Rouleaux      | Spermatostyle     | <i>Stictonectes optatus</i> (Dytiscidae);<br><i>Omophron</i> (Carabidae)                     | Rouleaux stacking of heads which are embedded in spermatostyle             | Gomez and Maddison <sup>51</sup> ; Mercati et al. <sup>99</sup>                      |
| Spermatostyle | Aggregate         | Some species of <i>Brachinus</i> (Carabidae)                                                 | Heteromorphic sperm with only one of the morphs attached to spermatostyles | Gomez and Maddison <sup>51</sup>                                                     |

**Supplementary Table 4.** Summarized state changes across stochastic mappings (N = 1000 simulations) of the binary SAM presence data for a phylogeny of 621 arthropods and outgroups under the optimal two-rate “equal rates” model as determined by corHMM. For simplicity, rates 1 and 2 are referred to as fast and slow, respectively, based upon their higher and lower relative character state transition probabilities.

| <b>SAM gain<br/>[fast]</b> | <b>SAM loss<br/>[fast]</b> | <b>SAM gain<br/>[slow]</b> | <b>SAM loss<br/>[slow]</b> | <b>% SAM<br/>absent<br/>[fast]</b> | <b>% SAM<br/>present<br/>[fast]</b> | <b>% SAM<br/>absent<br/>[slow]</b> | <b>% SAM<br/>present<br/>[slow]</b> |
|----------------------------|----------------------------|----------------------------|----------------------------|------------------------------------|-------------------------------------|------------------------------------|-------------------------------------|
| 846.825                    | 847.347                    | 20.989                     | 28.765                     | 44.43                              | 28.56                               | 13.39                              | 13.60                               |

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