How to align arthropod legs

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Abstract

How to align leg segments between the four groups of arthropods (insects, crustaceans, myriapods, and chelicerates) has tantalized generations of researchers, as this would answer over a century of speculation about the origins and homologies of the fascinating diversity of arthropod appendages and outgrowths. Here we compare the expression and loss-of-function phenotypes of leg patterning genes in crustaceans, insects, and arachnids using our own and previously published data. We find that all arthropod leg segments correspond to each other in a one-to-one fashion. This alignment suggests that chelicerates with seven leg segments incorporated a proximal leg segment into the body wall. In addition, this alignment suggests that insect and myriapod tracheae are convergent and homologous structures: each evolved via the independent internalization of an ancestral gill (respiratory exite) on the proximal-most leg segment of their shared ancestor. A framework for understanding the homologies of arthropod appendages opens up a powerful system for studying the origins of novel structures, the plasticity of morphogenetic fields across vast phylogenetic distances, and the convergent evolution of shared ancestral developmental fields.

Introduction

Arthropods are the most successful animals on the planet, in part due to the diversity of their appendages. How to align the legs of all arthropods has tantalized researchers for over a

century[1–8], as the solution would answer centuries of observation and speculation about arthropod structures. There are four groups of arthropods: chelicerates (spiders, etc.), myriapods (millipedes, etc.), crustaceans (shrimps, etc.), and insects (beetles, etc.). The difficulty in aligning or homologizing arthropod leg segments is due to the different numbers, shapes, and names of leg segments. Chelicerates can have either 7 or 8 leg segments, myriapods have either 6 or 7, insects have 6, and crustacean have 7 or 8 leg segments (Fig. 1)[4,8–11]. Since at least 1927, researchers have proposed many different theories to account for this variation, invoking leg segment deletions, duplications, and fusions to account for the different numbers of leg segments between arthropod taxa (see for example [3,4,7,8]). The incredible diversity of arthropod legs even contributed to some author's conclusions that the four arthropod groups arose independently, and therefore it is not possible to homologize and align their legs[12]. However, as molecular studies confirmed arthropod monophyly and mapped the topology of arthropod relationships with ever greater precision[13,14], and as loss-of-function studies of leg patterning genes have been conducted on more branches of the arthropod tree of life, this long sought model can now be brought to light.

Aligning the leg segments of crustaceans and insects

To align the leg segments of two arthropod groups, crustaceans and insects, Bruce and Patel 2020[15] compared the function of five leg patterning genes, *Distalless (Dll)*, *dachshund (dac)*, *Sp6-9*, *extradenticle (exd)*, and *homothorax (hth)*, in the amphipod crustacean *Parhyale hawaiensis* to previously published results in insects. By aligning the leg segment deletion phenotypes for these five genes, they found that the six distal leg segments of *Parhyale* and insects (leg segments 1 – 6, counting from the distal claw) aligned in a one-to-one fashion (Fig. 2). To align the proximal leg segments, they compared the expression of *pannier* (*pnr*) and the

Iroquois complex gene *araucan* (*ara*) in *Parhyale* and insects[15]. They found that, in both *Parhyale* and insects, the expression of *ara* distinguishes two proximal leg segments (leg segments 7 and 8; Fig. 2), while expression of *pnr* marks the true body wall (tergum). These data suggested that insects had incorporated two ancestral proximal leg segments, 7 and 8, into the body wall[16]. This work demonstrated that crustacean and insect legs had 8 leg segments could be homologized in a straightforward, one-to-one relationship. If insect and crustacean legs can be homologized, this model may extend to myriapods and chelicerates as well, in a generalizable model of appendages across all four groups of arthropods.

Aligning the leg segments of crustaceans, insects, and chelicerates

To align *Parhyale*, insect, and chelicerate legs, the leg segment deletion phenotypes in *Parhyale* and insects were compared to previously published results in chelicerates. Functional experiments in chelicerates have been performed for *Dll*, *Sp6-9*, *dac,* and *hth*. Based on the leg segment deletion phenotypes of these genes, the six distal leg segments of *Parhyale*, insects, and chelicerates (leg segments 1 – 6, counting from the distal claw) can be aligned in a one-to-one fashion, as follows.

In spiders, *Parhyale*, and insects, *Dll* is required for the development of leg segments 1 - 5, counting from the distal end of the leg (Fig. 3A-F)[15,17–22]. In spiders, *Parhyale,* and insects, *Sp6-9* is required for the development of leg segments 1 – 6 (Fig. 3G – L)[15,23–27]. In spiders, harvestman, *Parhyale*, and insects, *dac* is required to pattern leg segments 3 – 5 (in insects, *dac* function extends partway into leg segment 2)[15,28–31]. In spiders, harvestman, and *Parhyale*, a weak *dac* phenotype causes leg segment 4 to be truncated and fused onto leg segment 3 (Fig. 4A - F). In harvestman, *Parhyale*, and insects, a strong *dac* phenotype affects leg segments 3 – 5

(Fig. 4G - L). In harvestman, *Parhyale*, and insects, loss of hth affects the proximal leg segments
75 (Fig. 4M-R). In *Parhyale* and insects, loss of *hth* deletes the proximal leg segments, leaving only the distal 2 leg segments intact. In harvestman, reduction of *hth* shortens and fuses the proximal leg segments, leaving the distal segments unaffected[32]. It is not clear from the figures or text how many distal leg segments are unaffected - the most severely affected embryos did not survive to hatching and their cuticle was shriveled, thus obscuring what deformities are due to loss of *hth*
80 and what are due to the embryo not developing fully before hatching. However, leg segment 1 and at least the distal half of leg segment 2 appear unaffected. Thus, in harvestman, insects, and *Parhyale*, *hth* appears to function in all but the distal 2 leg segments. For the above comparisons, it is known that RNAi gives a range of partial knockdowns, but the above data focuses on what appear to be the null phenotypes.

85 To elucidate the composition of proximal leg segments in chelicerates, the expression of *pnr*, *ara,* and *Dll* was compared between *Parhyale*, *Tribolium*, and the tarantula *Acanthoscurria geniculata* (Fig. 5) [15]. Three orthologs of *pnr* were identified in *Acanthoscurria* that had closest homology to *Drosophila*, *Tribolium*, and *Parhyale pnr* (Fig. S1). However, only one of these was expressed at the stages examined and was therefore presumed to be *pnr*. An *Acanthoscurria*
90 *Iroquois* gene was identified which was the reciprocal best BLAST hit to *Drosophila*, *Tribolium*, and *Parhyale ara*. An *Acanthoscurria Dll* gene was identified which was the reciprocal best BLAST hit to *Drosophila*, *Tribolium*, and *Parhyale Dll*.

As in *Parhyale* and *Tribolium, Acanthoscurria Dll* was found to be expressed in leg segment 1 – 5, and *pnr* expressed in the most dorsal tissue (Fig. 5). Thus, it appears that *pnr*
95 marks the "true" body wall in all arthropods. In *Parhyale* and *Tribolium, ara* is expressed in three domains: a dorsal armband on proximal leg segment 8 that is adjacent to the *pnr* domain; a

second armband on proximal leg segment 7; and a dot of expression on the medial side of leg segment 6. *Parhyale* also expresses *ara* in the tip of the claw . In *Acanthoscurria*, at the embryonic stages examined, *ara* is expressed in three of these domains: a dorsal armband adjacent to the *pnr* domain; a second armband on proximal leg segment 7; and some expression in the tip of the claw. The dot of *ara* expression in leg segment 6 was not observed. Perhaps this domain is expressed at embryonic stages that were not examined, or it is not expressed in the *Acanthoscurria* lineage. However, as predicted by the leg segment alignment model, the two armbands of *ara* expression in *Acanthoscurria* bracket a region proximal to leg segment 7 (spider coxa) and adjacent to *pnr*. This suggests that *Acanthoscurria,* like *Parhyale* and *Tribolium*, also retains an ancestral, proximal 8th leg segment.

To test this hypothesis, the expression of *odd-skipped* was examined in *Acanthoscurria*. In *Drosophila*, the *odd-skipped* family of genes is expressed in the distal edge of each leg segment, where it induces cells to buckle and form the flexible joint[33]. An *odd-skipped* gene was identified in *Acanthoscurria* which was the reciprocal best BLAST hit to *Cupiennius* (spider) *odd-related* 3 (*odd-r3*)[34]. This *Acanthoscurria odd-r3* is expressed in the distal region of leg segments 1 – 7 but also in an additional ring proximal to leg segment 7 (Fig. 6). This additional ring of *odd-r3* notably occurs in the distal side of a leg-segment-like bulge. Given that *odd-skipped* is expressed in the distal side of leg segments[33], the ring of *odd-r3* expression on the distal side of the leg-segment-like bulge suggests that it is a bona fide leg segment. Together, the expression of *pnr*, *ara* and *odd-r3* and the presence of a leg-segment-like bulge suggest that *Acanthoscurria* has an additional proximal 8th leg segment.

Aligning the leg segments of myriapods

No functional data for leg patterning genes is available for myriapods. However, morphological and embryological evidence suggests that myriapods, like insects, have incorporated proximal leg segment(s) into the body wall[4,35,36]. In the embryos of both myriapods and insects, the proximal part of the developing leg ("subcoxa" in insects or "limb base" in myriapods) broadens and flattens to form the adult lateral body wall [36–45]. In insects, this subcoxa was shown to correspond to the two proximal-most leg segments of crustaceans[15], and the same may be true for myriapods[35]. Incorporation of proximal leg segments into the myriapod body wall would bring their leg segment count to 8, in agreement with other arthropods.

Discussion

The expression and embryological data shown here, in conjunction with the expression and functional data from previous publications, demonstrates that all arthropod legs can be aligned in a one-to-one fashion (Fig. 7). For example, the coxa of spiders, millipedes, and crustaceans are leg segment 7; the insect coxa, crustacean basis, and spider trochanter are leg segment 6; the insect trochanter, crustacean ischium, and spider femur are leg segment 5. In this model, arthropods ancestrally possessed a total of 8 leg segments. This fits well with the observation that arthropods in general have a maximum of 8 leg segments[4,8,9], as well as with the fossil data, where the ancestors of living arthropods also possessed 8 leg segments[51]. The canonical leg segment numbers in the four groups of arthropods are proposed to be due to the incorporation of proximal leg segments into the body wall, likely to support more of the body weight on the legs. Insects, which have 6 leg segments, have incorporated two proximal leg segments into the body wall (leg segments 7 and 8). Crustaceans with 7 leg segments, like *Parhyale*, have partially incorporated one leg segment into the body wall (leg segment 8).

Myriapods with 6 or 7 leg segments incorporated 2 or 1 leg segments into the body wall, respectively.

Chelicerate legs

Chelicerates with 8 free leg segments, such as sea spiders and solfugids, have not incorporated any leg segments into the body wall. However, in chelicerates with 7 leg segments, such as *Acanthoscurria*, a proximal leg segment is missing and must be accounted for. One hypothesis is that it was simply deleted. However, the expression of *Acanthoscurria pnr*, *ara*, and *odd-r3* presented here suggests that the proximal-most 8th leg segment of these chelicerates was incorporated into the body wall, similar to how insects incorporated proximal leg segments into their body wall[3,15,39,45]. Embryological evidence also supports this conclusion: a leg-like proximal 8th leg segment can be observed in embryos of the tarantula spider *Acanthoscurria* (Fig. 6b; also observable in [22,54]), as well as in *Cupiennius* embryos[55,56] and *Parasteatoda* embryos in [57,58]. Similarly, whip spiders (amblypygids) have a disconnected sliver of exoskeleton dorsal to the coxa, which appear to articulate to the coxa with condyle joints (Fig. S2), that may be the remnant of the proximal 8th leg segment.

Myriapod legs

When the morphological and embryological evidence in myriapods is incorporated into the above leg segment alignment model, two fascinating hypotheses become apparent: insect and myriapod respiratory systems may in fact be homologous; and insect wings may be homologous to myriapod “wings” (polydesmid paranota).

Myriapod and insect respiratory systems are astonishingly similar - a small circular spiracle associated with each leg leads to internally branching trachea[46] (Fig. S3). This contributed to entrenched support for their sister relationship[46]. Notably, both respiratory systems

appear to occur on the proximal-most leg segment (leg segment 8)[4,15,36,38]. Thus, while these two systems are not homologous as tracheae[13], the evidence suggests that insect and myriapod tracheae each evolved via the independent internalizing an ancestral gill (respiratory exite) on the proximal-most leg segment of their shared ancestor[47,48].

In support of this, the genes *trachealess (trh)* and *ventral veins lacking (vvl)*, which are expressed in and required for insect tracheal formation, are also expressed in the crustacean gill[49,50], an exite on the leg. This is expected if insect tracheae are an invaginated exite on the incorporated 8th leg segment. If insect tracheae are an invaginated exite on the 8th leg segment, then perhaps the morphologically and functionally similar myriapod tracheae are as well.

Some myriapods (polydesmid and platydesmid millepedes) have many wing-like "paranota" or "paratergites" (Fig. S3) along the side of the body. If these emerge from the proximal-most leg segment, then they would be positionally homologous to the *Parhyale* tergal plate and insect wing. Thus, millipedes and insects may have convergently evolved tracheae and "wings" from the same exites on the same leg segment. These structures would therefore be bizarrely both convergent and homologous.

To test these two hypotheses, the expression of *pnr*, *Iroquois* genes like *ara,* joint-markers like *serrate* or *odd-skipped*, flat exite-patterning genes like *vestigial*, and trachea-patterning genes like *ventral veins lacking*, and *trachealess* should be examined in millipede embryos. If the tracheae and paratergites are carried on leg segment 8, the following expression patterns are expected in millipede embryos: *pnr* will be expressed dorsal to the spiracle and paratergite; *Iroquois* genes like *ara* will bracket the spiracle and paratergite dorsally and ventrally; and joint-markers like *odd-skipped* and *serrate* will be expressed around the perimeter of the subcoxa that carries the spiracle and paratergite. If millipede trachea and paratergites are

patterned like their proposed corresponding structures in crustacean and insect, then paratergites

190 should express *vestigial* and *scalloped*, while the the tracheae should express *ventral veins lacking* and *trachealess.*

Materials and Methods

In situ HCR version 3.0

Acanthoscurria cDNA sequences were submitted to Molecular Instruments[59], and the probe sets are available from the company. In situs were performed as in Bruce et. al. 2021[60]

Imaging

Embryos imaged with Zeiss LSM880 confocal. Image processing done with Fiji-ImageJ. Fiji "Image Calculator > Subtract" method was used to remove high background from yolk autofluorescence. Figures processed using Adobe Photoshop 2020.

Data and materials availability: All data is available in the main text or the supplementary materials.

Competing interests: Authors declare no competing interests.

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Author contributions

HSB conceived, designed, and performed experiments and wrote manuscript. NHP edited manuscript.

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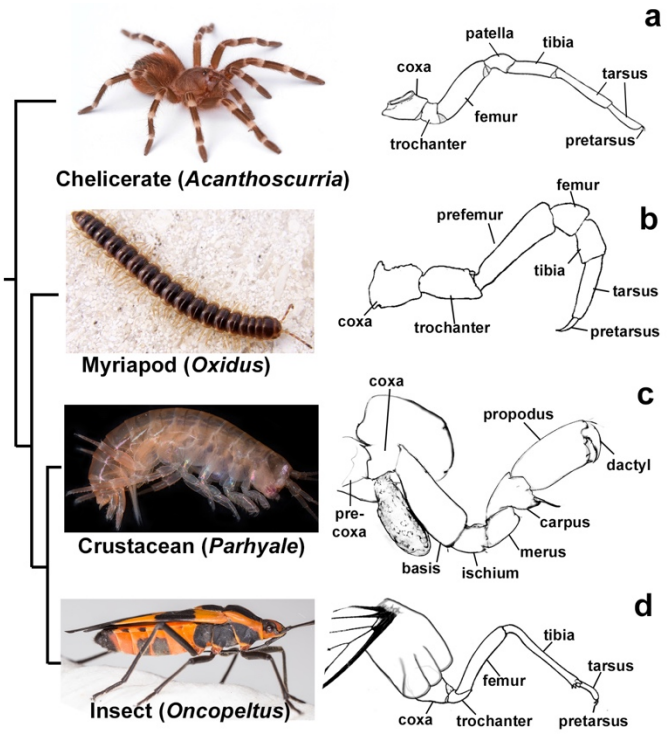

Fig. 1. Arthropod legs. Chelicerates, myriapods, crustaceans, and insects have different numbers, shapes, and names for their leg segments. Phylogeny based on Lozano 2019[13]. *Acanthoscurria* and *Oxidus* images from Wikipedia. Leg drawings adapted from Snodgrass 1952[9], except *Parhyale* leg, original image.

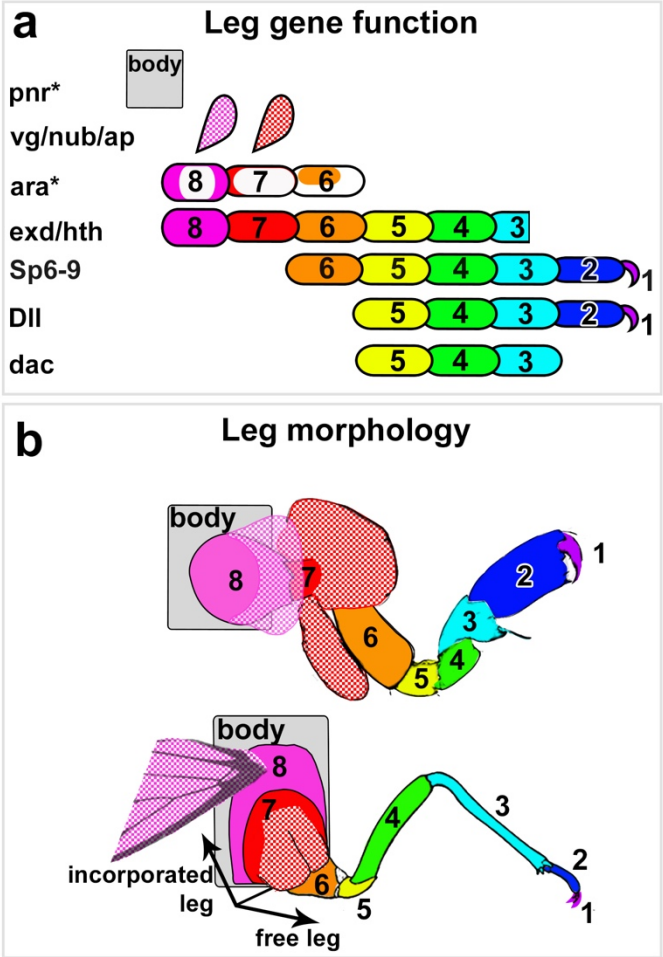

Fig. 2. Leg segment homologies (colors) between insects and crustaceans (from Bruce & Patel 2020 [15]). (a) The leg segments or structures in which each gene functions. (b) Leg segment
375 morphologies in *Parhyale* and insect.

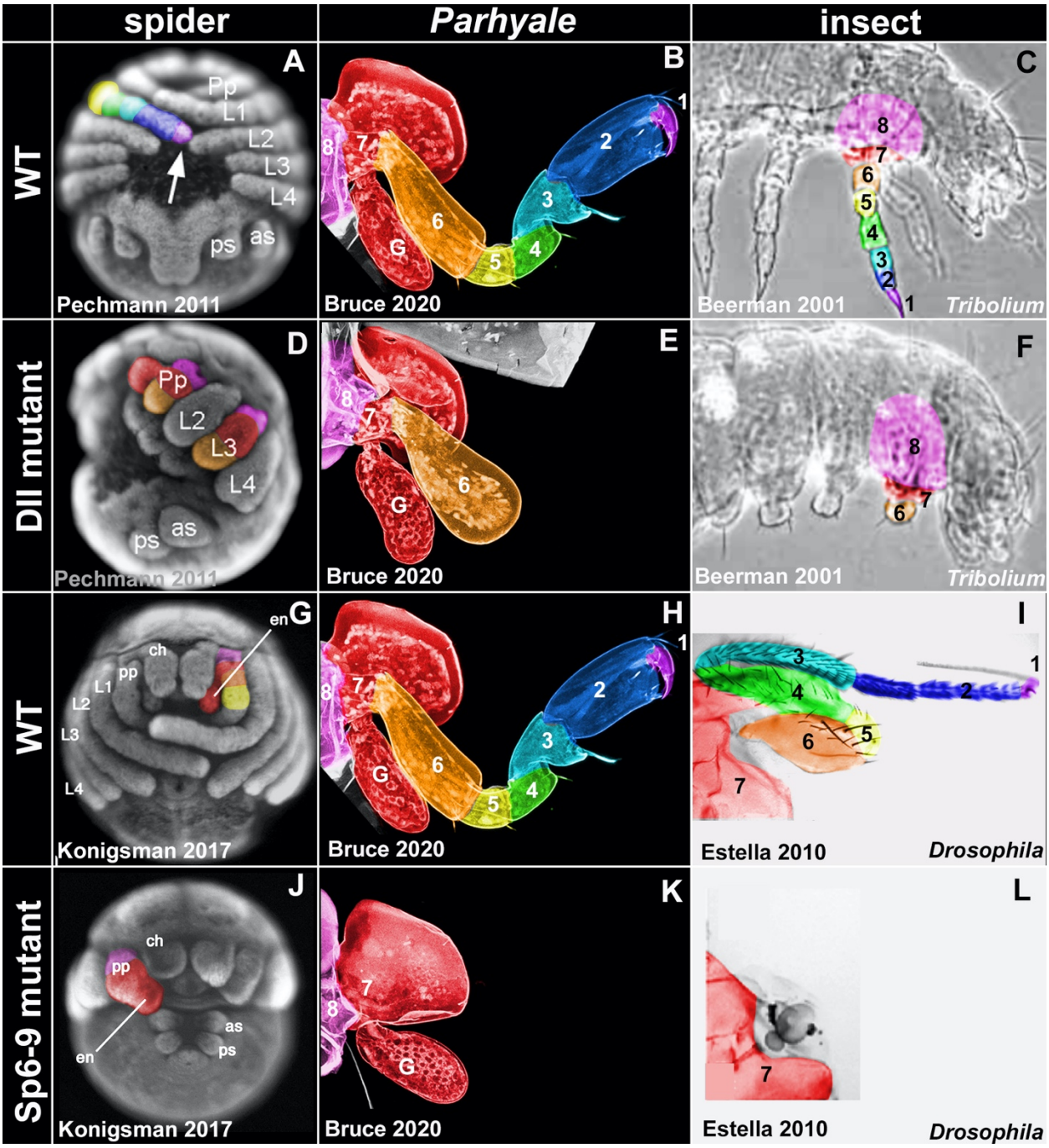

Fig. 3. *Dll* and *Sp6-9* function in arthropods. In spiders, *Parhyale*, and insects, *Dll* is required for the development of leg segments 1 - 5, counting from the distal end of the leg. A, D from Pechmann 2011. B, E, H, K from Bruce&Patel (?) 2020. C, F From Beerman 2001. G, J from Konigsman 2017. I, L from Estella 2010.

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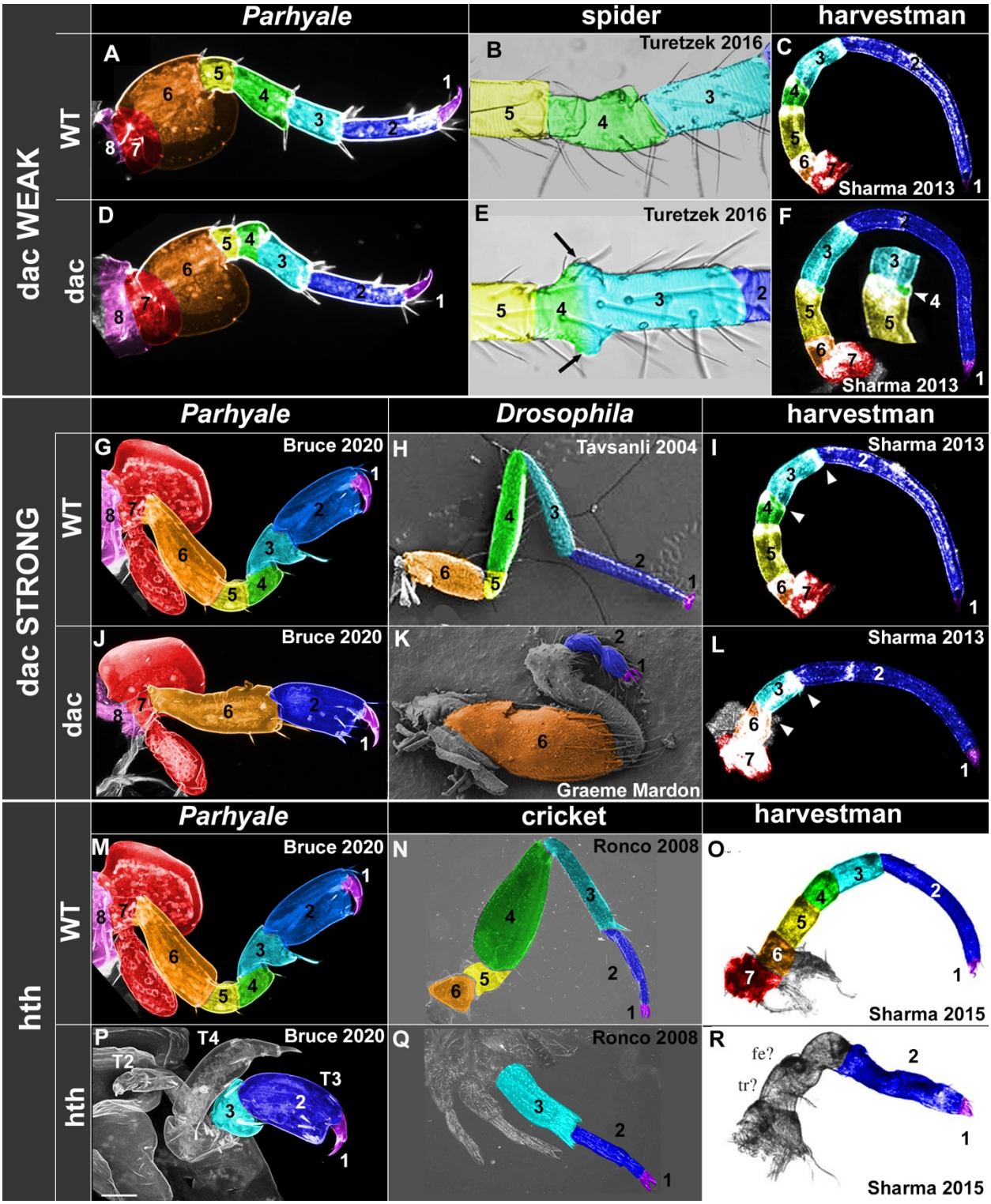

dac WEAK
WT
dac
Parhyale
spider
harvestman
A
B
Turetzek 2016
C
Sharma 2013
D
E
Turetzek 2016
F
Sharma 2013
dac STRONG
WT
dac
Parhyale
Drosophila
harvestman
G
Bruce 2020
H
Tavsanli 2004
I
Sharma 2013
J
Bruce 2020
K
Graeme Mardon
L
Sharma 2013
hth
WT
hth
Parhyale
cricket
harvestman
M
Bruce 2020
N
Ronco 2008
O
Sharma 2015
P
T4
Bruce 2020
T2
T3
Q
Ronco 2008
R
fe?
tr?
Sharma 2015

Fig. 4. *dac* and *hth* function in arthropods. In *Parhyale* and insects, loss of *hth* deletes the proximal leg segments, leaving only the distal 2 leg segments intact. In harvestman, reduction of *hth* shortens and fuses the proximal leg segments, leaving the distal segments unaffected[32]. It is not clear from the figures or text how many distal leg segments are unaffected - the most severely affected embryos did not survive to hatching and their cuticle was shriveled, thus obscuring what deformities are due to loss of *hth* and what are due to the embryo not developing fully before hatching. However, leg segment 1 and at least the distal half of leg segment 2 are clearly unaffected. Thus, in harvestman, insects, and *Parhyale*, *hth* appears to function in all but the distal 2 leg segments. In spiders, harvestman, and *Parhyale*, a weak *dac2* phenotype causes green leg segment 4 to be truncated and fused onto cyan leg segment 3. In harvestman, *Parhyale*, and *Drosophila*, a strong *dac2* phenotype affects leg segments 3 – 5.

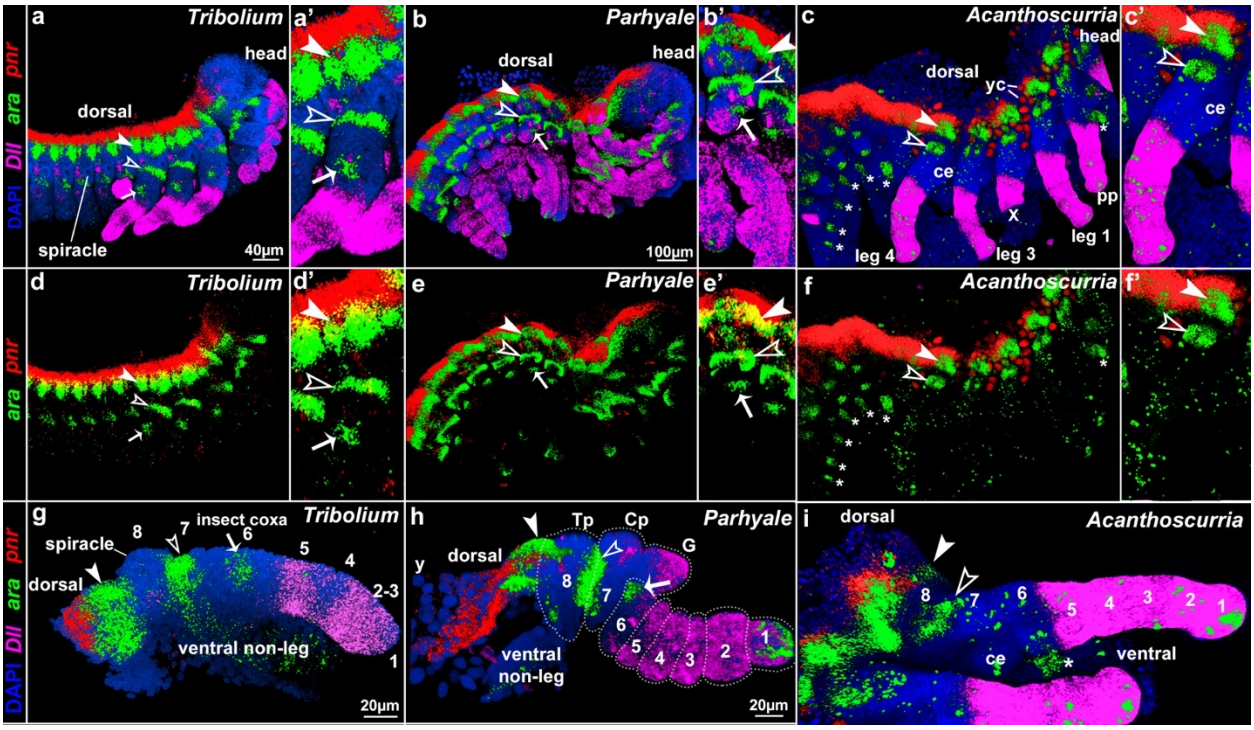


Fig. 5 Elucidating proximal leg segments in arthropods. (a, a', d, d') dissected right half of *Tribolium* embryo. a' and d' are zoomed in images of a and d respectively. (b, b', e, e'), dissected right half of *Parhyale* embryo. b' and e' are zoomed in images of b and e respectively. (c, c', f, f') dissected right half of *Acanthoscurria* embryo. c' and f' are zoomed in images of c and f respectively. (g) dissected leg of *Tribolium* embryo. (h) dissected T3 leg of *Parhyale* embryo. Large cells dorsal to *pnr* expression in b and f are extra-embryonic yolk (y) that exist prior to dorsal closure. (i) dissected leg of *Acanthoscurria* embryo. In all three arthropods, leg segments 1 through 5 are identified by *Dll* expression (magenta)[61]. In all three arthropods, the two *ara* (green) armband domains bracket a region proximal to leg segment 7. In all three arthropods, *pnr* (red) marks the most dorsal domain and is adjacent and partially overlapping the dorsal *ara* domain. In g, h, i, *ara* is expressed in a smattering of ventral non-leg cells. *Tribolium* larvae have a fused tibia and tarsus, the tibiotarsus, here labelled 2-3[37]. In *Acanthoscurria*, leg segment 7 is easily identified by the coxal endite (ce) that bulges medially. In both *Tribolium* (g) and *Parhyale* (h) legs, a muscle expressing *pnr* and *ara* that extends the length of leg segments 7 and 8 was masked to clearly show the ectodermal domains. Tp, tergal plate. Cp, coxal plate. G, gill. In *Acanthoscurria*, auto-fluorescent yolk cells (yc); ventral expression domain of ara (*). X in c indicates distal leg that was accidentally removed.

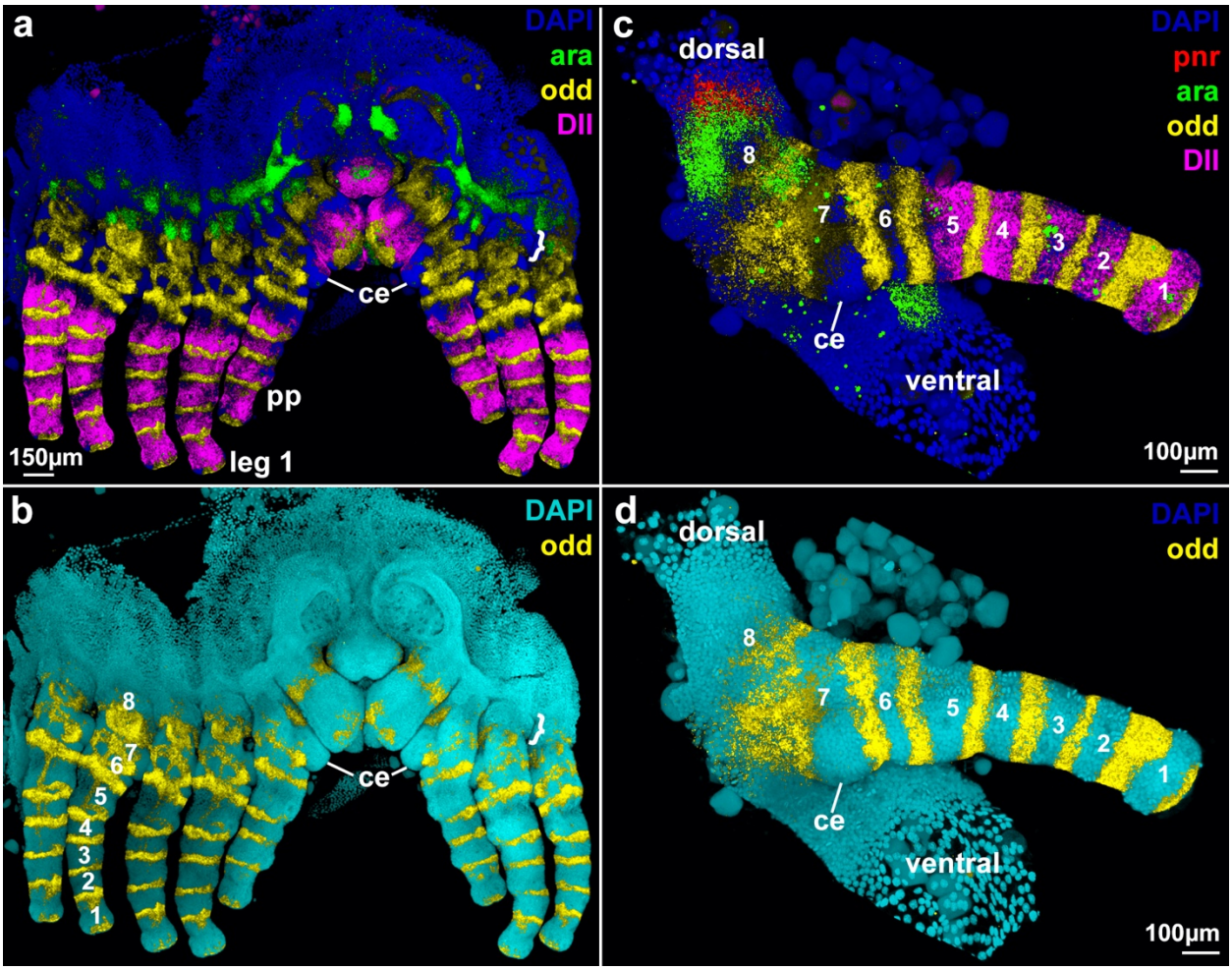
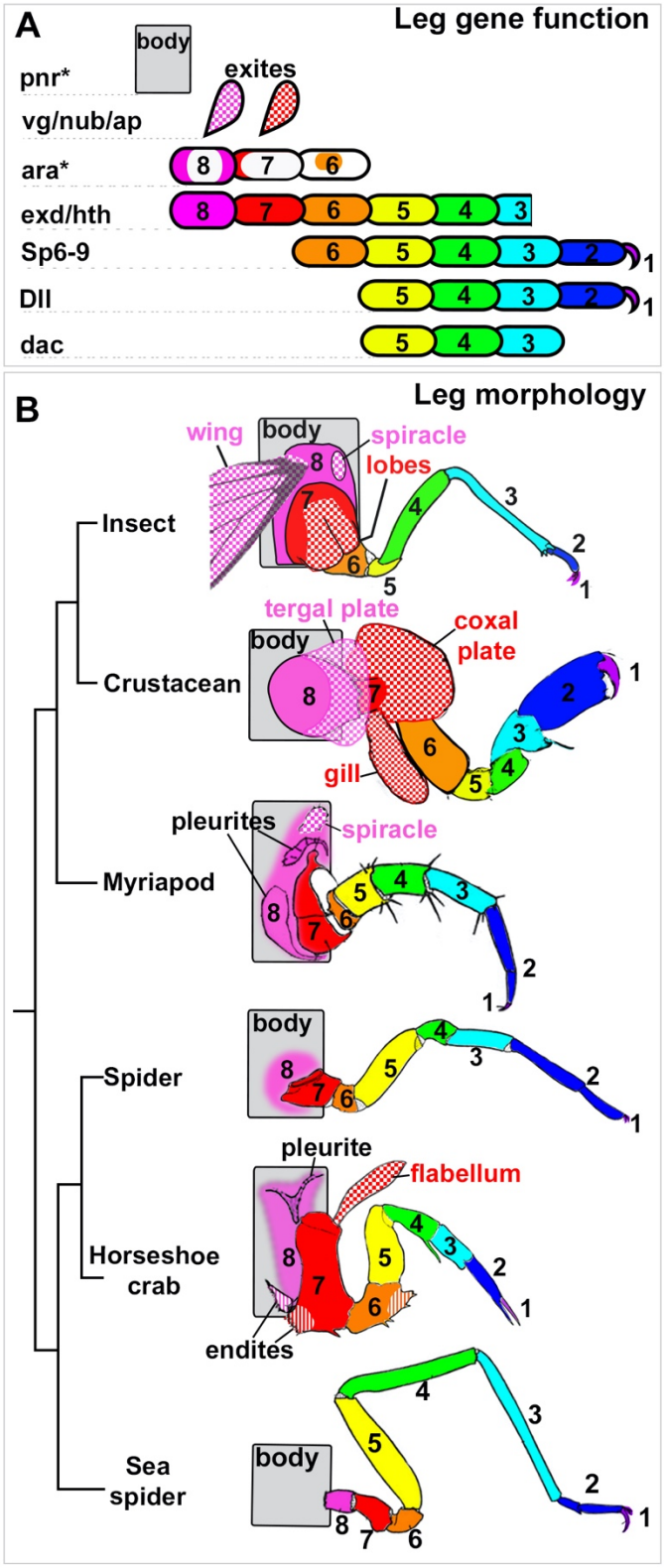


Fig. 6. Expression of *odd-r3* in *Acanthoscurria*. *odd-r3* is expressed in the distal region of each leg segment where the joint will later form. a, b Stage 12.5 *Acanthoscurria* embryos dissected away from yolk mass. Leg segment 7 is readily identified by the presence of a medial bulge, the coxal endite (ce). Proximal to leg segment 7, there is a leg-segment-like bulge (white curly brace), which expresses *odd-r3* in the distal region. *pnr* expression in this late stage embryo is reduced and obscured by other colors. DAPI in b is shown in cyan to better observe morphology. c, d dissected walking leg 1 from slightly earlier embryo, Stage 11.5, where leg segment divisions haven't yet bulged out. *odd-r3* encircles the distal region of each leg segment, including the hypothesized proximal 8th leg segment.


A
Leg gene function
body
pnr*
exites
vg/nub/ap
ara*
8
7
6
exd/hth
8
7
6
5
4
3
Sp6-9
6
5
4
3
2
1
Dll
5
4
3
2
1
dac
5
4
3
B
Leg morphology
Insect
wing
body
spiracle
lobes
Crustacean
tergal plate
body
coxal plate
gill
Myriapod
pleurites
spiracle
Spider
body
Horseshoe crab
pleurite
flabellum
endites
Sea spider
body

430

Fig. 7. Model of how to align all arthropod legs. A. Schematic of which genes function is related to (specific) leg segments. B. Morphology and homologies of arthropod leg segments based on leg gene function in insects, *Parhyale*, and chelicerates. Colors and patterns indicate proposed homologies. Exites (checker pattern); endites (stripe pattern). Drawings in B modified from
435 Snodgrass 1952.

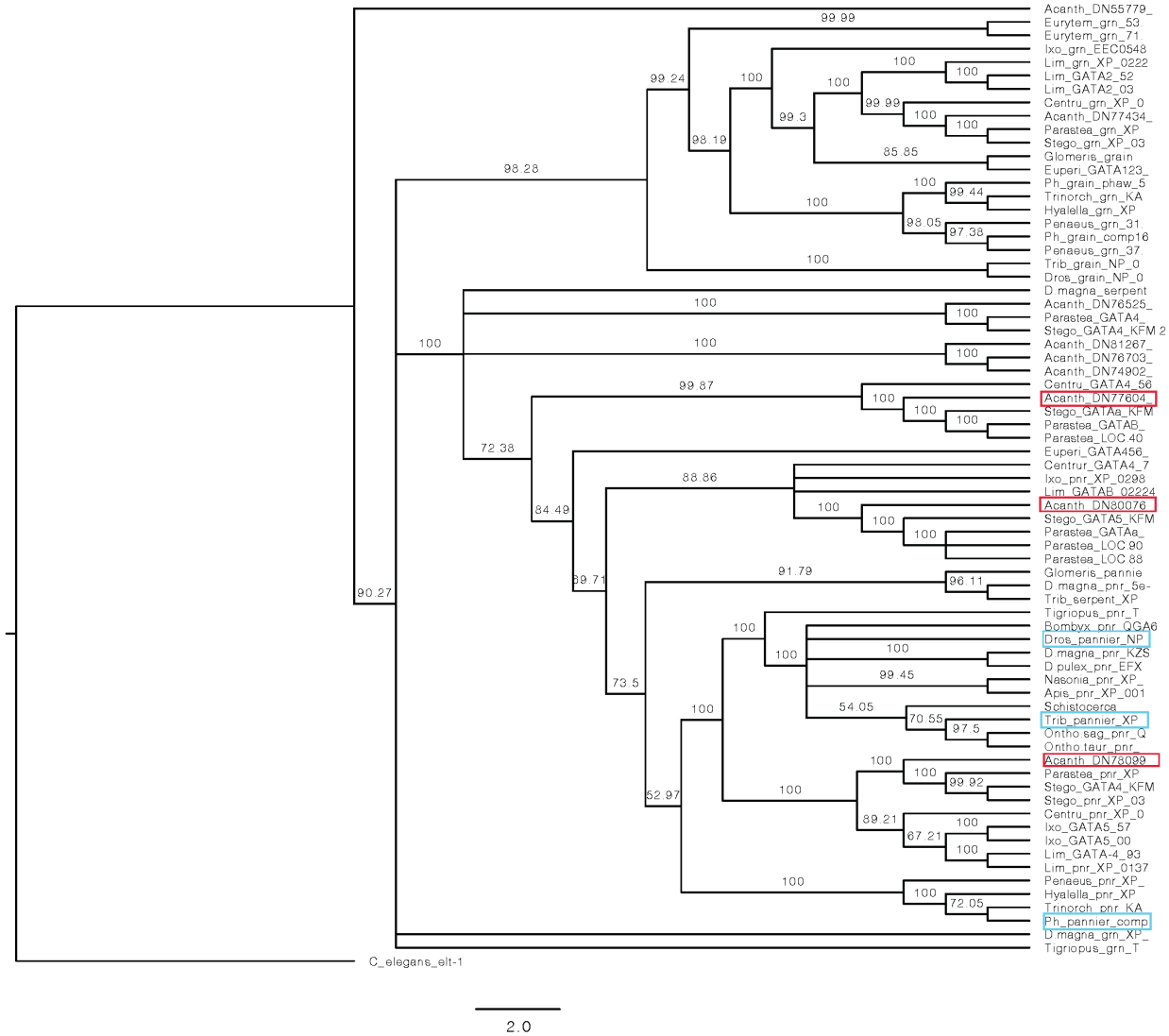


Fig. S1. Three orthologs of *pnr* were identified in *Acanthoscurria* with closest homology to *Drosophila*, *Tribolium*, and *Parhyale pnr* (Fig. S1-2). However, only one of these was expressed at the stages examined, Acanth_DN78099, and was presumed to be *pnr*. Consensus tree generated using Mafft, which gave similar topology to Clustal consensus tree.

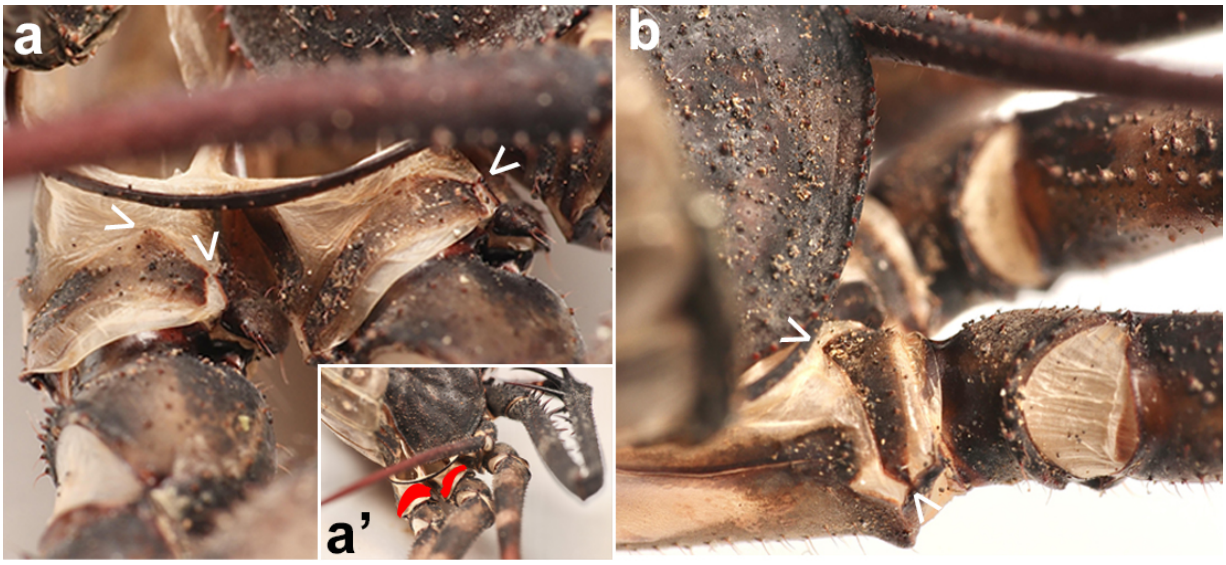


Fig. S2. Proposed precoxa in whip scorpions. Day-old molted exoskeleton. Condyles (points of articulation between adjacent leg segments) of proposed precoxa indicated by >. **a,** dorsal view of molt. **a',** zoomed out view of a. **b.** posterior view.

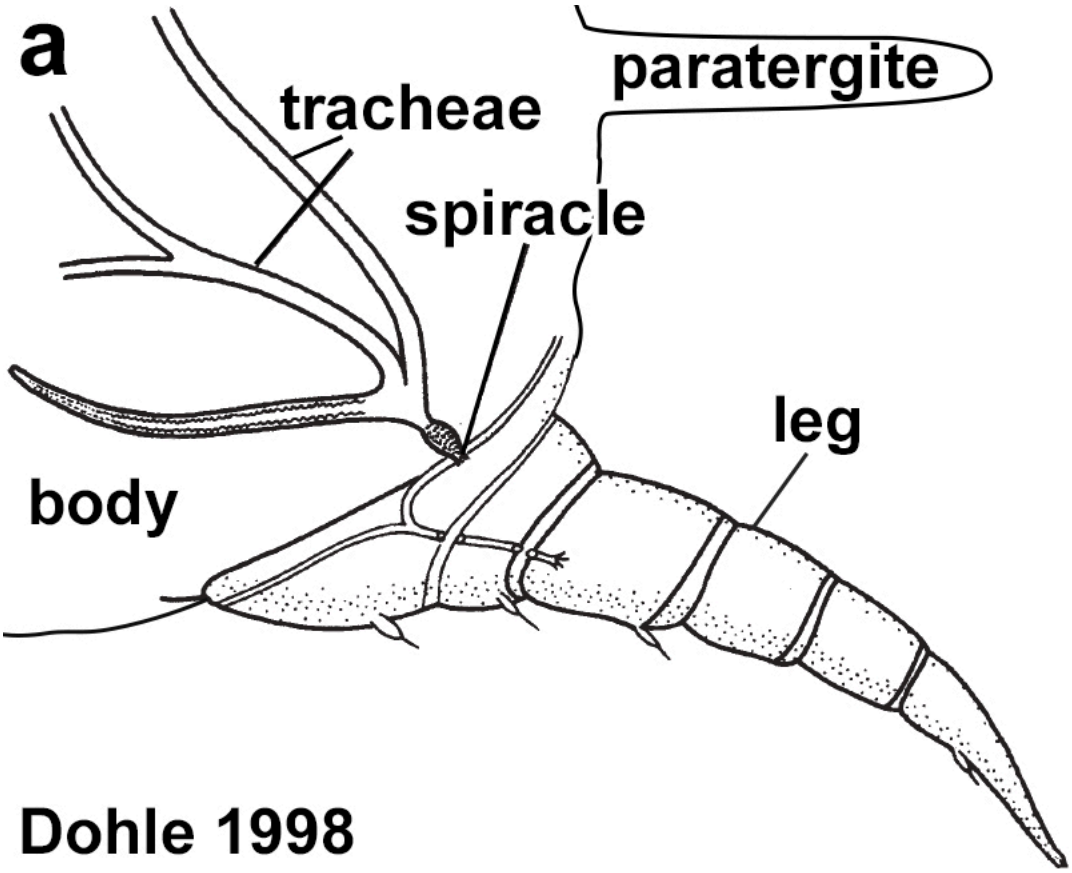


Fig. S3. Respiratory system of myriapod. Modified from Dohle 1998.