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Evolution of the chelicera: a *dachshund* domain is retained in the deutocerebral appendage of Opiliones (Arthropoda, Chelicerata)

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SUMMARY The proximo-distal axis of the arthropod leg is patterned by mutually antagonistic developmental expression domains of the genes *extradenticle*, *homothorax*, *dachshund*, and *Distal-less*. In the deutocerebral appendages (the antennae) of insects and crustaceans, the expression domain of *dachshund* is frequently either absent or, if present, is not required to pattern medial segments. By contrast, the *dachshund* domain is entirely absent in the deutocerebral appendages of spiders, the chelicerae. It is unknown whether absence of *dachshund* expression in the spider chelicera is associated with the two-segmented morphology of this appendage, or whether all chelicerates lack the *dachshund* domain in their chelicerae. We investigated gene expression in the harvestman *Phalangium opilio*, which bears the plesiomorphic three-segmented chelicera observed in "primitive" chelicerate orders. Consistent with patterns reported in spiders, in the harvestman chelicera *homothorax*, *extradenticle*, and *Distal-less* have broadly overlapping developmental domains, in contrast with mutually exclusive domains in the legs and pedipalps. However, unlike in spiders, the harvestman chelicera bears a distinct expression domain of *dachshund* in the proximal segment, the podomere that is putatively lost in derived arachnids. These data suggest that a tripartite proximo-distal domain structure is ancestral to all arthropod appendages, including deutocerebral appendages. As a corollary, these data also provide an intriguing putative genetic mechanism for the diversity of arachnid chelicerae: loss of developmental domains along the proximo-distal axis.

INTRODUCTION

The articulated appendages of arthropods have facilitated the tremendous diversity and evolutionary success of this phylum. Postulated to have evolved from a polyramous ancestral condition, nearly every part of the arthropod leg has undergone extensive evolutionary modifications, enabling adaptations to various ecological niches and environments (Snodgrass 1938; Cisne 1974; Waloszek et al. 2005). Investigation of genetic mechanisms of leg development, principally in the fruit fly *Drosophila melanogaster*, has implicated a suite of four genes that pattern the proximo-distal (PD) axis: *Distal-less* (*Dll*), *dachshund* (*dac*), *extradenticle* (*exd*), and *homothorax* (*hth*). In arthropod walking legs, at least three of these genes (*Dll*, *dac*, and either *exd* or *hth*) are expressed in mutually antagonistic domains. Knockdown of these genes results in loss of the podomeres (leg segments) patterned by that particular gene, engendering the moniker, "leg gap genes" (Dong et al. 2001, 2002; Rauskolb 2001). *Dll* and *dac* pattern distal and medial podomeres respectively; proximal patterning requires the cofactors *exd* and *hth* (Sunkel and Whittle 1987; Cohen and Jürgens 1989; Mardon et al. 1994; González-Crespo and Morata 1996; Lecuit and Cohen 1997; Rieckhof et al. 1997; Casares and Mann 1998; Abu-Shaar et al. 1999; Wu and Cohen 1999; Dong et al. 2001, 2002; Rauskolb 2001; reviewed by Angelini and Kaufman 2005).

An interesting spatial reversal of *exd* and *hth* expression domains has been documented as follows: *exd* is expressed throughout the legs in pancrustaceans (also termed tetraconates), whereas it is restricted to the proximal part in myriapods and chelicerates; *hth* is expressed throughout the legs in myriapods and chelicerates, but is restricted proximally in Pancrustacea (Abu-Shaar and Mann 1998; Abzhanov and Kaufman 2000; Prpic et al. 2001, 2003; Inoue et al. 2002; Prpic and Tautz 2003; Angelini and Kaufman 2004, 2005; Prpic and Damen 2004; Prpic and Telford 2008; Pechmann and Prpic 2009). Because onychophoran leg gap gene domains are comparable to those of pancrustaceans (Janssen et al. 2010), the spatial expression of *exd* and *hth* has been interpreted as a potential synapomorphy for the sister group relationship of chelicerates and myriapods (termed Paradoxopoda or Myriochelata), a relationship recovered in many molecular phylogenetic analyses (e.g., Hwang et al. 2001; Mallatt et al. 2004; Pisani et al. 2004; Mallatt and Giribet 2006; Dunn et al. 2008; von Reumont et al. 2009; Rehm et al. 2011). However, this correlation of leg gap gene domains

remains to be tested in chelicerate and myriapod lineages other than spiders and millipedes.

In contrast with the walking leg, modified appendages are associated with modified leg gap gene patterning. For example, the mandible of pancrustaceans and myriapods, and the maxilla of myriapods are considered gnathobasic (Snodgrass 1938; Popadic et al. 1996, 1998). In these appendages, *Dll* is not expressed in a manner consistent with PD axis formation (Scholtz et al. 1998; Abzhanov and Kaufman 2000; Prpic and Tautz 2003). Similarly, leg gap gene expression in the thoracopods of some crustaceans, and the antennae of insects and millipedes, differs from that in the walking legs in that mutually antagonistic domains are not observed (e.g., Dong et al. 2001; Williams et al. 2002; Prpic and Tautz 2003; Angelini and Kaufman 2004). In the *D. melanogaster* antenna, *hth*, *dac*, and *Dll* have overlapping expression domains and the *dac* medial domain is not functional (Dong et al. 2002; but see Angelini et al. 2009 for a case of a function antennal *dac* domain in *Tribolium castaneum*). Comparable expression domains of leg gap genes occur in the antennae of other insects (Angelini and Kaufman 2004, 2005).

The leg gap genes also play a role in conferring antennal identity. In *D. melanogaster* knockdown of *hth* and *Dll* results in antenna-to-leg transformations, and increasing *dac* expression induces medial leg structures in the antenna (Dong et al. 2001, 2002). A similar effect of *hth* knockdown has been reported in the cricket antenna (Ronco et al., 2008), but in a hemipteran, *hth* knockdown resulted in the loss of the antenna altogether (Angelini and Kaufman 2004). Additionally, *Dll* knockdown does not result in homeotic transformations in the hemipteran antenna (Angelini and Kaufman 2004). Knockdowns or mutations of some other genes downstream of the leg gap genes can also result in homeotic antenna-to-leg transformations (Dong et al. 2002; Toegel et al. 2009; Angelini et al. 2009).

The chelicerate counterpart of the mandibulate antenna is the chelicera, the namesake of this class of arthropods. Chelicerae are the anterior-most pair of prosomal appendages and are generally used for feeding. Homology of the antennae of mandibulates and the chelicerae is based on their deutocerebral innervation and Hox gene boundaries (both are free of Hox expression; Telford and Thomas 1998; Hughes and Kaufman 2002). However, investigation of leg gap gene expression in the appendages of spiders—including both mygalomorphs and araneomorphs—has demonstrated the lack of a *dac* domain altogether in the chelicera, as well as broadly overlapping domains of *hth*, *exd*, and *Dll* (Abzhanov and Kaufman 2000; Prpic et al. 2003; Prpic and Damen 2004; Pechmann and Prpic 2009). The similarity of overlapping expression domains in antennae and chelicerae is remarkable. Given the role of leg gap genes in specifying antennal identity in *D. melanogaster* (Dong et al. 2001, 2002), it has been suggested that leg gap gene domain overlap and activity is requisite for specification of cheliceral morphology in chelicerates (Prpic and Damen 2004; Pechmann et al. 2010).

One limitation of this inference is that cheliceral morphology is quite variable. The chelicerae of spiders are comprised of two segments—the proximal basal segment and the distal fang—and are used for envenomation of prey and/or manipulation of silk. Labidognathous chelicerae (with the appendage perpendicular to the AP axis) do not occur outside of Araneomorphae (the group that includes orb weavers and jumping spiders). Orthognathous (with the appendage parallel to the AP axis) chelicerae occur in Mygalomorphae (tarantula-like spiders) and Mesothelae (spiders with a segmented opisthosoma), as well as three related arachnid orders: Amblypygi, Uropygi, and Schizomida (the four form the clade Tetrapulmonata). The chelicerae of these orders are not chelate (forming a pincer), but rather shaped as a jackknife (Fig. 1). Another four lineages—Solifugae, Ricinulei, Pseudoscorpiones, and acariform Acari—bear two-segmented chelicerae that are chelate, resembling a pair of scissors (acariform mites typically bear two cheliceral articles, but some lineages have a reduced third article, the nature of which is ambiguous; van der Hammen 1989; Evans 1992; Shultz 2007). Finally, the "primitive" orders of Chelicerata—Pycnogonida, Xiphosura, Scorpiones, Opiliones, and the extinct Eurypterida (as well as Palpigradi and the parasitiform Acari)—bear three-segmented chelicerae. In the context of chelicerate phylogeny, the spider chelicera is therefore a derived structure (Fig. 1). Morphological and phylogenetic studies have previously suggested that a three-segmented chelicera is the ancestral condition, and thus the two-segmented morphology would have resulted from the loss of one of the segments, although this hypothesis has not been tested (e.g., Dunlop 1996; Wheeler and Hayashi 1998; Giribet et al. 2002; Shultz 2007).

The occurrence of a cheliceral type with an extra segment is particularly intriguing in the context of leg gap gene domain evolution. However, the expression domains of leg gap genes in chelicerate orders that bear three-segmented chelicerae are not known. As a consequence, it is difficult to generalize patterns reported for leg gap genes in spiders to all chelicerates. For example, the absence of the *dachshund* domain in the spider chelicera could be associated with the two-segmented morphology of this appendage, implying the loss of one segment, rather than with the chelicera itself. In order to test this hypothesis, we examined gene expression of the leg gap genes in the harvestman *Phalangium opilio*, which bear the plesiomorphic three-segmented chelicera, and compared these to data reported for spiders. We also sought similarities in gene expression in the spider and harvestman chelicerae to determine which aspects of PD axis specification are conserved in chelicerates. We show that a *dachshund* domain is present in the three-segmented harvestman chelicera and is

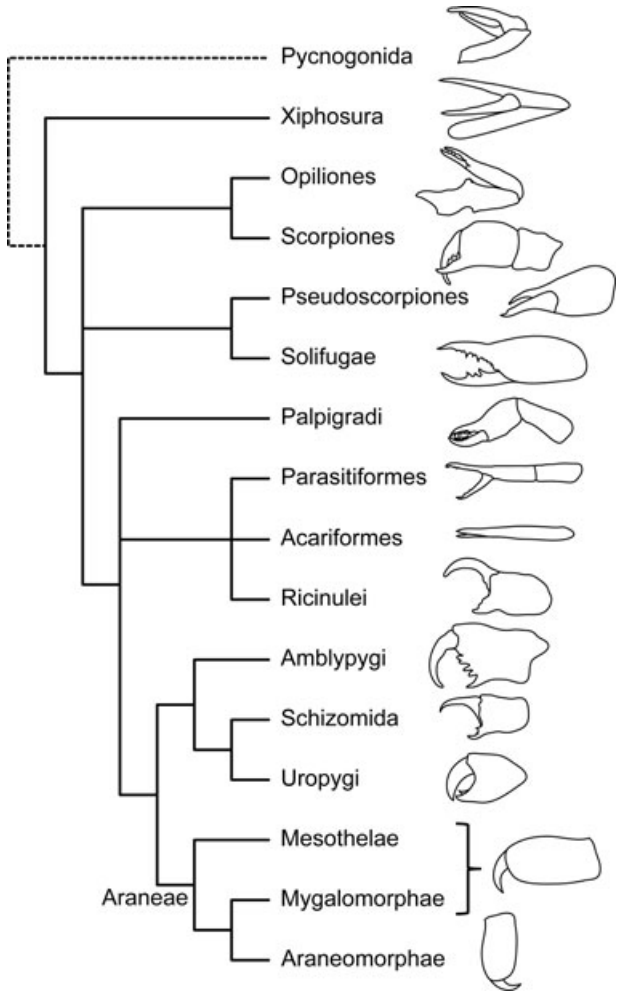

Fig. 1. Phylogeny of Chelicerata indicating relationships among orders and diversity of chelicerae. Constituent lineages of spiders (order Araneae) as indicated. Orientation of the Araneomorphae schematic indicates labidognathous chelicera (perpendicular to body). Topology derived from Giribet et al. (2001), Shultz (2007), and Giribet and Edgecombe (2012).

restricted to the proximal segment of this appendage, which is putatively lost in spiders.

MATERIALS AND METHODS

Embryos

Adults of the synanthropic *P. opilio* (Arachnida, Opiliones, Eupnoi, Phalangiidae) were hand collected between 9 PM and 3 AM from various sites in Weston and Woods Hole (Falmouth), Massachusetts, USA in May through October of 2009–2011. Adults were maintained and embryos collected as previously described (Sharma et al. 2012).

Gene identification and whole mount in situ hybridization

RNA was extracted from a range of embryonic stages using Trizol (Invitrogen) and first strand cDNA synthesis was performed using SuperScriptIII (Invitrogen). A developmental transcriptome of *P. opilio* was generated by sequencing this cDNA in a single flowcell on an Illumina GAII platform, using paired-end 150-bp-long reads. Thinning was performed using 0.0496 as the limit (based on Phred quality scores), and resulting quality of the thinned reads was visualized FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). After thinning, only those terminal bases with a Phred quality score under 30 were trimmed. Assembly was conducted using CLC Genomics Workbench 4.6.1 (CLC bio, Aarhus, Denmark). All four genes were present in single copy and sequences ranged in length from 661 to 1665 bp. Gene identity was confirmed by protein BLAST (NCBI) and visual inspection of amino acid alignments of orthologs across Arthropoda. Sequences of all genes are deposited in GenBank under accession numbers HE805503-HE805507.

Templates for riboprobe synthesis were generated as described by Lynch et al. (2010): genes were amplified by PCR using gene-specific primers (GSP) with an added linker sequence (5′-ggc cgc gg-3′ for the forward primer end and 5′-ccc ggg gc-3′ for the reverse primer). A T7 polymerase binding site for antisense or sense probe synthesis was generated in a second PCR using the forward or reverse GSP and a universal primer binding to the 3′ or 5′ linker sequence with an added T7 binding site, respectively. GSPs were designed from the identified transcriptomic assembly. A list of the primers used for generating sense and antisense probes is provided in Table S1. Probe synthesis and in situ hybridization followed the spider protocols for *Cupiennius salei* (Prpic et al. 2008). The staining reactions for detection of transcripts lasted between 20 min and 6 h at room temperature. Embryos were subsequently rinsed with 1× PBS + Tween-20 0.1% to stop the reaction, counterstained with Hoechst 33342 (Sigma) 10 μg/ml to label nuclei, postfixed in 4% formaldehyde, and stored at 4°C in glycerol. Embryos were mounted in glycerol and images were captured using an HrC AxioCam, a Lumar stereomicroscope driven by AxioVision v 4.8.2, and an AxioImager compound microscope driven by AxioVision v 4.8.2 (Zeiss, Oberkochen, Germany).

RESULTS

Expression of *Po-hth* and *Po-exd*

Po-hth is strongly expressed in the head lobes, the labrum, all of the appendages, and in the ventral ectoderm of all segments (Fig. 2, Supporting information Fig. S1). In the pedipalps and walking legs of early embryos (stage 11), *Po-hth* expression is concentrated in the proximal-most part of

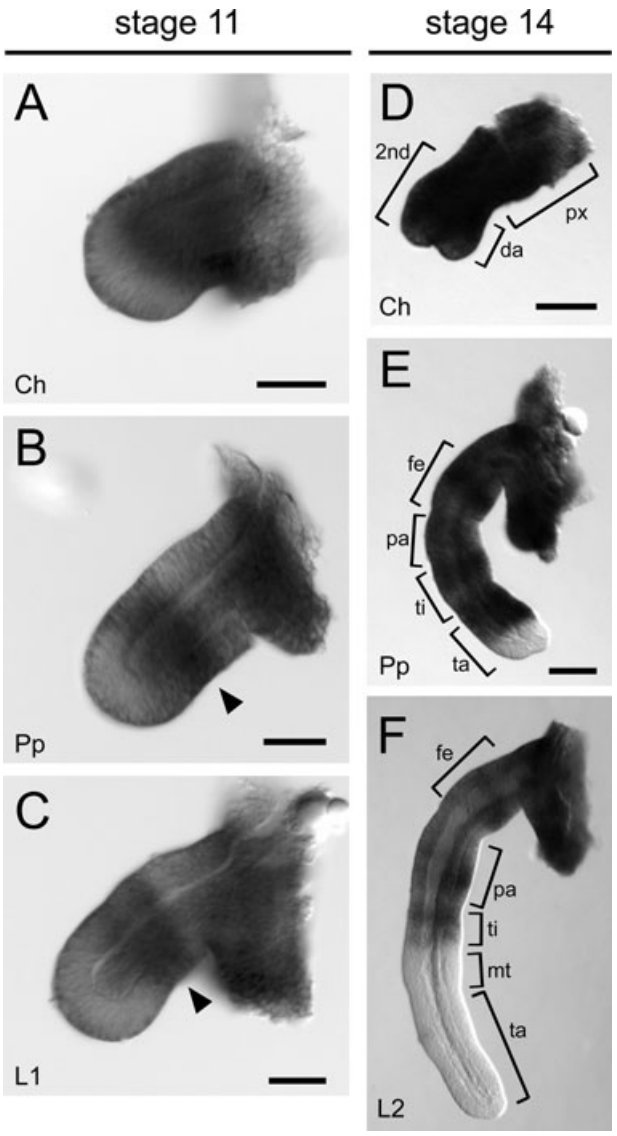

Fig. 2. Expression of the *Phalangium opilio homothorax* gene in the developing appendages. (A–C) Expression in the chelicera, pedipalp, and L1, respectively, of a stage 11 embryo. Arrowheads indicate median ring of expression. (D–F) Expression in the chelicera, pedipalp, and L2, respectively, of a stage 14 embryo. Scale bars for all figures are 50 μm. px: proximal segment of chelicera; 2nd: secondary article of chelicera; da: distal article of chelicera; fe: femur; pa: patella; ti: tibia; mt: metatarsus; ta: tarsus.

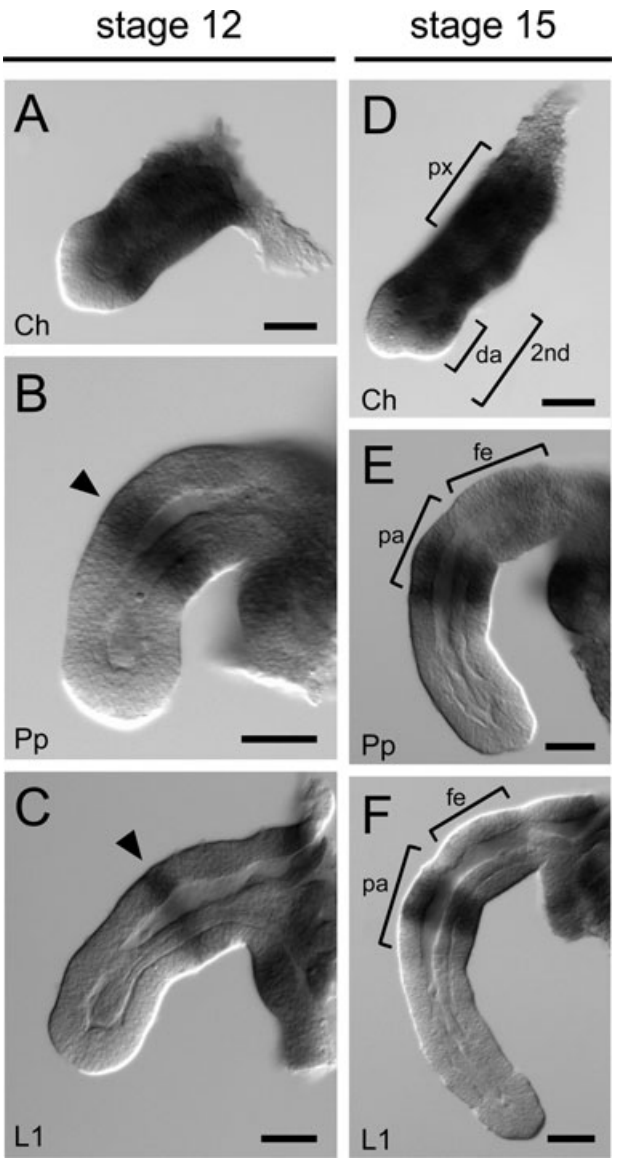

Fig. 3. Expression of the *Phalangium opilio extradenticle* gene in the developing appendages. (A–C) Expression in the chelicera, pedipalp, and L1, respectively, of a stage 12 embryo. Arrowheads indicate distal ring in the patella of the pedipalp and legs. (D–F) Expression in the chelicera, pedipalp, and L1, respectively, of a stage 15 embryo. Scale bars for all figures are 50 μm. Abbreviations as in Fig. 2.

the appendage, and in a separate and medial ring (Fig. 2, B and C). This ring of expression coincides with that of *Po-exd* (see below), although the *Po-hth* medial domain is broader. In the walking legs of older embryos (stage 14), the separate expression domains are less marked; *Po-hth* is strongly expressed throughout the proximal-most part of the leg, including the endites, to the tibia (Fig. 2F). In these older stages, a more distal ring of expression is not observed, in contrast to the spider (the *hth-1* paralog; Prpic et al. 2003). In the pedipalp, *Po-hth* expression is observed throughout the appendage, except for a distal portion of the tarsus (Fig. 2E). In the chelicera, *Po-hth* is expressed throughout the appendage, except for the distal terminus, where expression is slightly weaker (Fig. 2, A and D).

Po-exd is expressed in the labrum, all of the appendages, and in the ventral ectoderm of all prosomal and opisthosomal segments (Fig. 3, Supporting information Fig. S1).

In the appendages, *Po-exd* is strongly expressed in the proximal-most parts of the pedipalps and walking legs, corresponding to the coxa and the endite, and a separate and distinct ring of expression is observed in the patella of the walking legs and pedipalp (Fig. 3, B and C). This ring is retained in older stages, albeit wider and with weaker interconnecting expression in the femur and trochanter (Fig. 3, E and F). In the chelicera, *Po-exd* is expressed throughout the appendage except for the distal terminus; no rings of expression are observed, as in the other appendages (Fig. 3, A and D).

Expression of *Po-dac*

Po-dac is expressed in the central nervous system, in several groups of cells in the head lobes, and in the posterior terminus. In older embryos, *Po-dac* is expressed in the developing pleurites of the opisthosoma. Expression is never detected in the labrum (Supporting information Fig. S2). All six pairs of prosomal appendages express *Po-dac* (Fig. 4, A–F). In early stages (stage 10), expression in all limb buds is similar and occurs in a medial ectodermal ring (Fig. 4, A–C). In older embryos (stage 14), the pedipalps and legs express *Po-dac* in a domain encompassing the podomeres trochanter and femur (Fig. 4, E and F). In the pedipalp and legs, *Po-dac* transcripts are concentrated in the segmental boundaries delimiting the femur, with slightly weaker interconnecting expression in the femur (Fig. 4, E and F). Weak expression is observed more proximally to the coxa; in spiders, this expression is associated with neural structures (Prpic and Damen 2004).

The chelicerae consistently express *Po-dac* as the appendage elongates (Fig. 4, D, Supporting information Fig. S2). In early stages (stage 10), strong expression occurs in the medial part of the cheliceral limb bud ectoderm, in a domain highly comparable to other limb buds (Fig. 4A). This domain does not include any part of the body wall. In older embryos, strong expression of *Po-dac* is retained in the part of the chelicera that corresponds to the proximal segment, and no expression is detected in the secondary or distal articles (Fig. 4D). Contrary to the other leg gap genes, the distal and proximal boundaries of *Po-dac* expression in the chelicera appear sharp rather than diffuse. Herein we consider an expression boundary whose edge is straight and clear to be "sharp" (see, e.g., Fig. 4, A–C) and otherwise to be "diffuse" (see, e.g., Fig. 2, A–C).

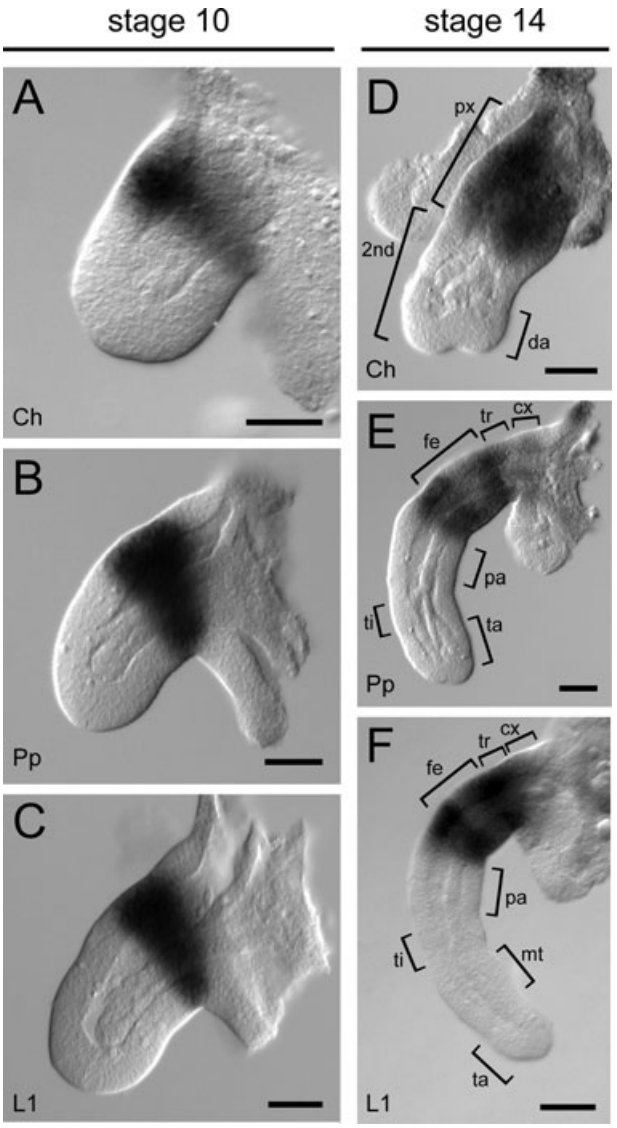

Fig. 4. Expression of the *Phalangium opilio dachshund* gene in the developing appendages. (A–C) Expression in the chelicera, pedipalp, and L1, respectively, of a stage 10 embryo. (D–F) Expression in the chelicera, pedipalp, and L1, respectively, of a stage 14 embryo. Scale bars for all figures are 50 μm. cx: coxa; tr: trochanter. Other abbreviations as in Fig. 2.

Expression of *Po-Dll*

Po-Dll is expressed in all six prosomal appendages, as well as in the developing labrum, the posterior terminus, and the head lobes (Fig. 5, Supporting information Fig. S2). Unlike spiders, harvestmen do not have any opisthosomal appendage-derived organs (e.g., spinnerets) and *Po-Dll* is not expressed in the opisthosoma in a manner suggestive of rudimentary opisthosomal limb buds. In older embryos (stage 14), a pair of strong expression domains is observed in the head lobes, specifically in the part of the eye fields that coalesce toward the midline during development (Supporting information Fig. 2G). An additional and smaller pair of expression domains is observed in the head, slightly posterior and lateral to the first pair (Supporting information Fig. 2G). Expression is also observed in the neuroectoderm along the ventral midline (Supporting information Fig. 2G),

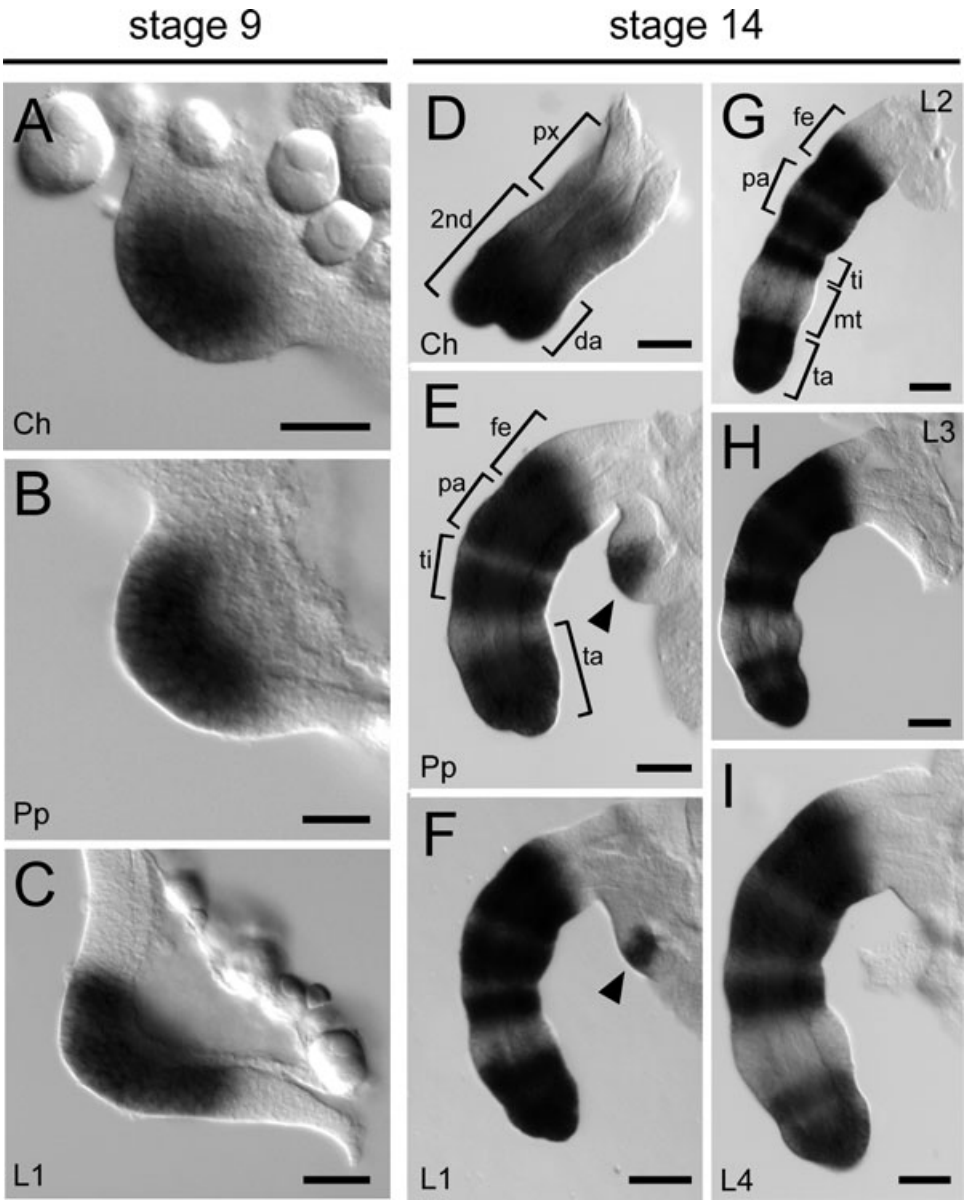

Fig. 5. Expression of the *Phalangium opilio Distal-less* gene in the developing appendages. (A–C) Expression in the chelicera, pedipalp, and L1, respectively, of a stage 9 embryo. (D–I) Expression in the chelicera, pedipalp, and L1–L4, respectively, of a stage 14 embryo. Note the expression in the outgrown endites of the pedipalp and first leg (arrowheads), and the lack of these in the other legs. Scale bars for all figures are 50 μm. Abbreviations as in Fig. 2.

comparable to that in a xiphosuran (Fig. 3J of Mittmann and Scholtz 2001).

In the pedipalps and legs of early embryos (stage 9), *Po-Dll* is strongly expressed in the distal part of the limb bud (Fig. 5, B, C). Subsequently, *Po-Dll* appears throughout the leg as strong rings of expression occurring coincidently with the developing boundaries of the podomeres, while retaining strong expression in the distal-most podomere (the tarsus; Fig. 5, F–I). In older embryos (stage 14), *Po-Dll* is additionally expressed in the outgrowing endites of the pedipalps and first walking legs (denoted L1), but not in L2–L4 (Fig. 5, E–I).

In early stages (stage 9), *Po-Dll* is expressed in the distal part of the cheliceral limb bud, comparably to the pedipalps and walking legs (Fig. 5A). This expression pattern is maintained upon the differentiation of the distal podomere into the secondary and distal articles (stage 14). *Po-Dll* continues to be expressed mostly in the distal part of the appendage, which will form the second and distal articles; and tapering expression is observed extending into the proximal segment

(Fig. 5D). In contrast to the sharp expression boundaries in the other appendages, the proximal expression boundary of *Po-Dll* in the chelicera is diffuse.

DISCUSSION

Here we examined gene expression of the single-copy orthologs of the leg gap genes in the harvestman appendages. We observe that proximal PD axis patterning of the appendages is conserved in Opiliones and Araneae, and resembles the patterning observed in a glomerid millipede. These data are consistent with the Myriochelata hypothesis. Second, we report novel expression domains of *Dll* in apomorphic structures of harvestmen, namely the outgrown endites that form the stomotheca and the portion of the eye fields that form the ocularium. Most significantly, in the harvestman chelicera, the genes *hth*, *exd*, and *Dll* have broadly overlapping expression domains, as in the spider chelicera and the mandibulate antenna, but these are independent of the retention of a *dac* domain, which patterns the proximal segment of the harvestman chelicera.

Proximal patterning in harvestmen legs is consistent with the Myriochelata hypothesis

The expression domains of the leg gap genes *hth* and *exd* in *P. opilio* are comparable, but not identical, to those of spiders. In older stages, *Po-hth* is expressed continuously throughout the appendage, from the coxa and endite to a distal podomere, such as the tibia (*P. opilio* legs) or the tarsus (*P. opilio* pedipalps). This expression domain approximates that of the spider paralog *hth-1*, which is similarly broadly expressed from proximal-most segments to part of the tarsus in the pedipalps and walking legs (Prpic and Damen, 2004; Pechmann and Prpic 2009). A second paralog common to spiders, *hth-2*, is expressed in multiple rings and is believed to be involved in leg segmentation (Prpic et al. 2003; Pechmann et al. 2009), but such rings corresponding to podomere boundaries were not observed in *P. opilio*. Some lineage-specific differences exist between the expression domains of *Po-hth* and spider *hth-1*. For example, a separate and more distal ring of *hth* expression is not observed in *P. opilio*, but has been reported for the *hth-1* paralog of spiders (Prpic et al. 2003; Prpic and Damen 2004). The pedipalps and the walking legs of *P. opilio* also have differing distal boundaries of *hth* expression, unlike spiders. The significance of these differences is not known.

In contrast to *hth*, *Po-exd* is restricted to the proximal segments and a discrete ring of expression in the patella, which closely resembles the expression domain of the *exd-1* paralog in multiple spider species (Abzhanov and Kaufman 2000; Prpic et al. 2003; Prpic and Damen 2004; Pechmann and Prpic 2009). In the millipede *Glomeris marginata*, *exd* is similarly expressed in the legs, albeit without the medial ring domain (Prpic and Tautz 2003).

In spite of these lineage-specific differences, the expression domains of *Po-hth* and *Po-exd* are comparable to those of spiders and a millipede (Abzhanov and Kaufman 2000; Prpic et al. 2003; Prpic and Tautz 2003; Prpic and Damen 2004; Pechmann and Prpic 2009). In general, *hth* is expressed broadly in much of the developing appendage, whereas *exd* is restricted to the proximal podomeres. Taken together with the inverse spatial relationship of *hth* and *exd* in onychophorans and pancrustaceans (Prpic et al. 2003; Prpic and Telford 2008; Janssen et al. 2010), the expression data observed in *P. opilio* are consistent with a sister relationship of chelicerates and myriapods.

The Myriochelata hypothesis is controversial, owing to discordance with morphological and paleontological data, as well as numerous phylogenetic and phylogenomic studies that have recovered chelicerates as sister to the remaining arthropods (e.g., Giribet et al. 2001; Regier et al. 2008, 2010). However, other studies, some with deeper gene sampling, have recovered the monophyly of chelicerates and myriapods (Hwang et al. 2001; Mallatt et al. 2004; Pisani et al. 2004; Mallatt and Giribet 2006; Dunn et al. 2008; Hejnol et al. 2009; von Reumont et al. 2009; Rehm et al. 2011). Consequently, although the spatial relationship of *hth* and *exd* in arthropods constitutes a poorly sampled, one-character system, it is plausible that PD axis patterning in myriapod and chelicerate appendages constitutes a homologous condition. Myriochelata is also supported by detailed similarities in chelicerate and myriapod neurogenesis (Dove and Stollewerk 2003; Kadner and Stollewerk 2004; Mayer and Whitington 2009), which contrasts with the neuroblast-driven system present in insects and crustaceans (Ungerer et al. 2011).

A role for *Dll* in patterning harvestman apomorphies

Consistent with its role in patterning outgrowths, *Dll* is expressed in the distal parts of all appendages. Additional expression domains occur in the labrum and telson, which have been reported in various other arthropod species (e.g., Panganiban et al. 1995; Popadic et al. 1998; Thomas and Telford 1999; Abzhanov and Kaufman 2000). Like the other leg gap genes, *Dll* is known to have additional roles in development beyond the PD axis, such as patterning sensory organs and bristles (Sunkel and Whittle 1987; Cohen and Jürgens 1989; Mittmann and Scholtz 2001; Williams et al. 2002), and even gap gene function in spiders (Pechmann et al. 2011). Here we observed two additional domains of *Dll* function that are unique to the harvestman.

First, *Dll* is expressed in the endites of both the pedipalps and the first walking legs. These domains of expression are similar to the *Dll* expression domains in the endites of

crustaceans (Panganiban et al. 1995). In *P. opilio*, the endites of these two appendages elongate in the adult, forming a preoral cavity called the stomotheca—a structure that occurs only in harvestmen and scorpions, and the putative synapomorphy of this clade (Stomothecata *sensu* Shultz 2007; Fig. 1). The other endites of the harvestman neither elongate nor express *Dll* (Fig. 5, E–I). In the spider, the pedipalpal endite expresses *Dll*, but other endites do not (Schoppmeier and Damen 2001; Prpic and Damen 2004; Pechmann and Prpic 2009). As in the harvestman, the *Dll*-expressing endite of spiders is retained in the adult, forming the spider's "maxilla" (not homologous to the mandibulate maxilla). Taken together, these data suggest that *Dll* is involved in patterning the endites that form gnathobasic mouthparts in chelicerates. Expression data from mouthparts of scorpions, which could further test this hypothesis, are not presently available.

Second, *Dll* is expressed in a pair of domains in the center of the each eye field. *Dll* expression in the head lobes has been observed in other chelicerates, but *Dll* expression in spider and mites is either peripheral or diffuse, in comparison to the harvestman (Thomas and Telford 1999; Abzhanov and Kaufman 2000; Schoppmeier and Damen 2001; Pechmann and Prpic 2009). Moreover, the pair of domains that strongly express *Dll* in *P. opilio* subsequently form a fused outgrowth called the ocularium, a stalk-like structure that bears a single pair of simple ocelli, in the adult (Juberthie 1964). A similar eye mound also occurs in pycnogonids, but as with scorpions, expression data for the pycnogonid eye mound are not presently available. As with the endites, the co-occurrence of the expression domains and subsequent outgrowth in the locality of the expression suggest that *Dll* is involved in ocularium formation.

Functional tests of *Dll* activity by dsRNAi-mediated knockdown have been conducted in a spider and in a mite (Schoppmeier and Damen 2001; Khila and Grbic 2007). In the mite, the knockdown is reported to result in truncation of the pedipalpal endite (Khila and Grbic 2007), whereas in the spider, the effect of the knockdown on the pedipalpal endite (or maxilla) was not specified, but this structure is apparently lost as well (Fig. 4, B and D of Schoppmeier and Damen 2001). Functional methods to test *Dll* activity in the endites and ocularium of harvestmen are not yet developed, but are of significant interest, given other reported cases of *Dll* cooption to form nonappendage structures (e.g., butterfly wing spots, McMillan et al. 2002; beetle horns, Moczek and Rose 2009).

A *dac* domain is present in the three-segmented chelicera

In spiders, *dac* is initially not observed in the two-segmented chelicera, but is expressed proximally and within the appendage in older stages of *C. salei* (Abzhanov and Kaufman 2000; Prpic and Damen 2004; Pechmann and Prpic 2009). This late-stage expression was previously postulated to be of neural nature, as comparable expression also occurred in the coxae of all other appendages of older *C. salei* embryos (Prpic and Damen 2004). In that study, Prpic and Damen (2004) observed that the broadly overlapping domains of *hth*, *exd*, and *Dll* in the spider chelicera resembled the expression of these genes in the antenna of *D. melanogaster*. It was also conjectured that the lack of antagonistic *hth*, *exd*, and *Dll* domains in the spider chelicera was associated with complete loss of the cheliceral *dac* domain.

Unlike the chelicerae of spiders, the plesiomorphic, three-segmented chelicera of the harvestman expresses *dac* in a manner consistent with PD axis patterning during early development. In early embryos, the *dac* domain in the chelicera is topologically indistinguishable from that in the other appendage types. However, as the distal portion of the appendage forms an asymmetrical chela, *dac* is consistently and strongly expressed in the proximal portion of the chelicera. Even after the chelicera has formed the three constituent segments, *dac* is expressed strongly throughout the proximal segment. This may imply that the segment missing in the spider chelicera is the proximal-most one, which is consistent with traditional hypotheses of chelicera evolution based upon morphology (Dunlop 1996; Wheeler and Hayashi 1998; Shultz 2007).

Although we do not currently have the tools to test functionally putative mutual antagonisms of the leg gap genes in the harvestman chelicera, we observe that the expression domains of *Po-hth*, *-exd*, and *-Dll* are broadly overlapping in this appendage in spite of the presence of a *dac* domain, much like their corresponding orthologs in the spider chelicera. These data suggest that the lack of antagonistic domains of *hth*, *exd*, and *Dll* in the spider chelicera is not associated with the absence of the *dac* domain, but rather with the specification of chelicerae and antennae generally. If these broadly overlapping domains are involved in conferring cheliceral identity—as with the *D. melanogaster* antenna—mild knockdown phenotypes of one or more of these three genes could result in chelicera-to-leg transformations. Future work could examine this testable hypothesis, taking advantage of functional genetic tools available in spiders (Hilbrant et al. 2012).

The retention of *dac* in the three-segmented chelicera is remarkable, insofar as *dac* also occurs in homologous appendages (the antenna) of some insects, such as *D. melanogaster* (Dong et al. 2001), *Oncopeltus fasciatus* (Angelini and Kaufman 2004), *T. castaneum* (Prpic et al. 2001), and *Gryllus bimaculatus* (Ronco et al. 2008), but not other panarthropods, such as the isopod *Porcellio scaber* (Abzhanov and Kaufman 2000). *dac* is also not observed in the frontal appendage or jaw of the onychophoran *Euperipatoides kanangrensis* (Janssen et al. 2010). Intriguingly, a large *dac*

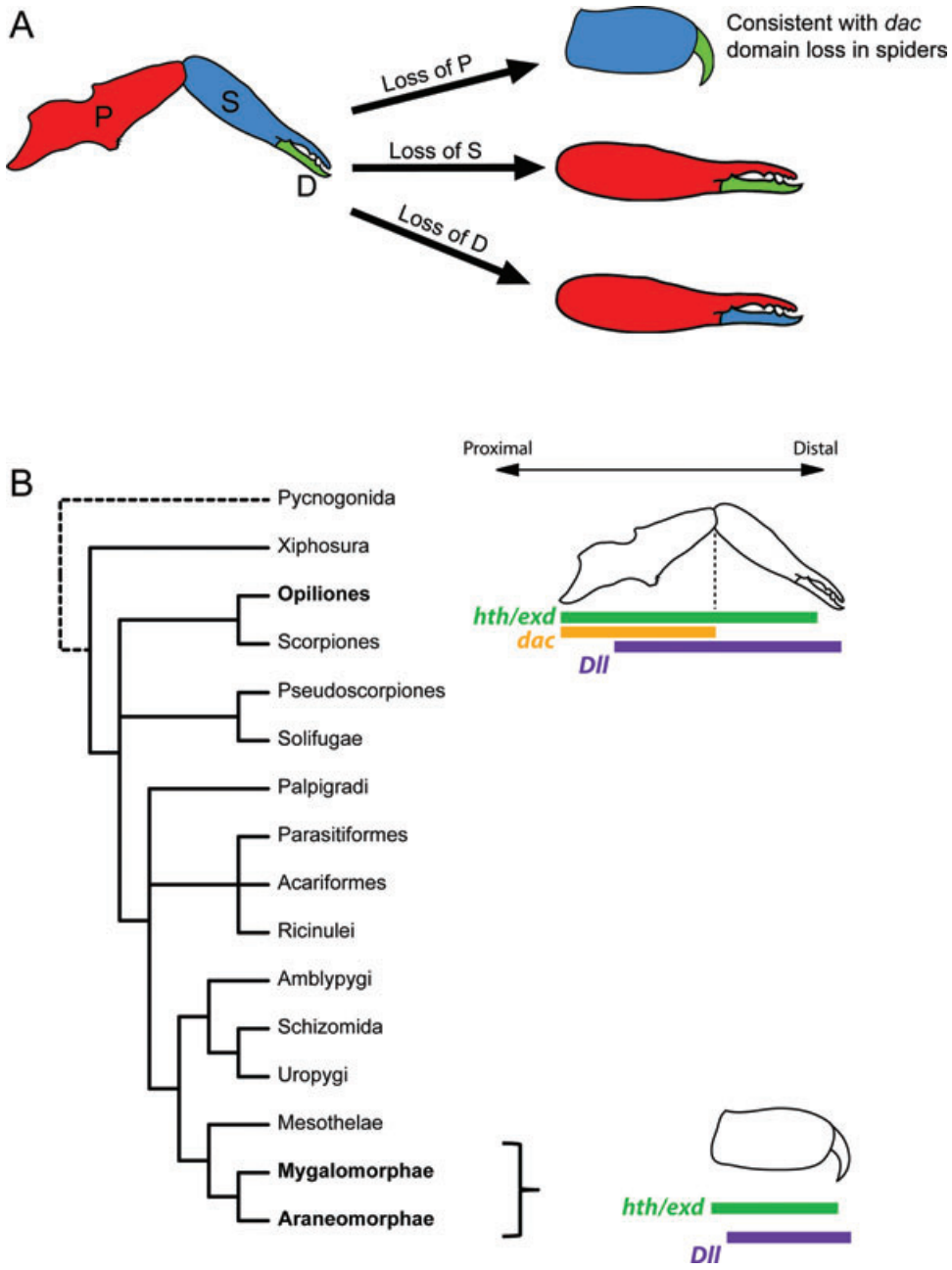

Fig. 6. Alternative hypotheses of chelicera evolution and summary of known leg gap gene expression domains in chelicerae throughout Chelicerata. (A) From a three-segmented ancestral state, a two-segmented chelicera could be obtained by loss of the proximal [P], secondary [S], or the distal [D] segment. If *dac* is considered a marker of the proximal segment, expression data from spiders and the harvestman support a transition of chelicera types by loss of the *dac* domain, and therefore of the proximal segment. (B) Fragmentary leg gap gene expression data are available for the chelicerae of Chelicerata. The complete suite of expression domain is known only for opisthothele spiders and harvestmen. For Xiphosura and Acariformes, only the *Dll* domains have been reported, and are not depicted.

domain comparable that of *P. opilio* has only been observed in one other arthropod: the millipede *G. marginata* (Prpic and Tautz 2003). The relevance of this observation to the Myriochelata hypothesis cannot be assessed given the presently limited data on deutocerebral *dac* domains in myriapods and basal chelicerate orders, but is a matter of interest for future investigation.

The presence of the *dac* domain in some insects has been interpreted as a possible retained rudiment of an ancestral tripartite domain structure (Prpic and Damen 2004).

However, labile deployment of the leg gap genes in modified appendages precludes the assignment of homology of structures on the basis of gene expression domains alone (Williams 1998; Abzhanov and Kaufman 2000). Nevertheless, our observation that a *dac* domain occurs in the medial portion of the deutocerebral appendage in a plesiomorphic order of chelicerates—in addition to a myriapod and some pancrustaceans—lends credibility to the hypothesized tripartite domain structure of this appendage in the common ancestor of arthropods, with subsequent losses of particular domains upon modification (as have occurred in other modified appendage types, such as mandibles and maxillae; Scholtz et al. 1998; Abzhanov and Kaufman 2000; Angelini and Kaufman 2005). However, it is also intriguing that during later developmental stages, both the cofactors *hth* and *exd* are expressed continuously and more distally than the *dac* domain in the harvestman chelicera. To our knowledge, the chelicera of *P. opilio* constitutes the first arthropod appendage wherein this phenomenon occurs, discording with patterns previously observed for appendage regionalization via the leg gap genes (Kojima 2004; Angelini and Kaufman 2005).

Comparative functional data are limited for *dac*, but activity in the deutocerebral appendage appears to vary among species. For example, in *D. melanogaster*, the antennal *dac* domain is small, limited to the third antennal segment (Mardon et al. 1994). Null *dac* mutants bear fusion of the a5-arista joint, but not the loss of any segments, whereas overexpression of the *dac* domain results in medial leg structures in the antenna (Dong et al. 2001, 2002). Similarly, *dac* is weakly expressed in the proximal antenna of *O. fasciatus*, and knockdown of *dac* has no observable effect on the antenna at all (Angelini and Kaufman 2004). By contrast, despite modest antennal expression levels (Prpic et al. 2001), knockdown of *dac* in *T. castaneum* induces truncation of the antenna, owing to the reduction of funicle (medial) segments and fusion of antennal segments in this region, as well as homeotic transformation of the distal funicle articles toward a club-like (distal) identity (Angelini et al. 2009). Thus, in the deutocerebral appendage of at least one arthropod lineage, *dac* acts as a leg gap gene, as well as confers segmental identity along the PD axis.

In the harvestman, *dac* is initially strongly expressed in the median portion of the cheliceral limb bud, and this domain is later constrained to the part of the appendage that forms the proximal segment in *P. opilio*. Definitive determination of the role of *dac* in harvestmen must await the development of functional genetic tools for this system. However, one intriguing possibility is that, if the cheliceral *dac* domain of *P. opilio* functions in a manner similar to that of *T. castaneum*, a knockdown of this gene may result in the loss of the proximal segment, and therefore, in a two-segmented chelicera—the condition that occurs in derived arachnid orders, such as spiders and other tetrapulmonates (Fig. 6A). Such an experimental result, if tested among several major lineages of Chelicerata, would support a clear mechanism for the evolutionary transition from the three-segmented chelicera to the two-segmented types: loss of the *dac* domain along the proximo-distal axis.

However, it is presently unknown whether different lineages of chelicerates with two-segmented chelicerae (e.g., solifuges, pseudoscorpions, amblypygids) pattern this appendage in the same way (Fig. 6B). Phylogenetic approaches have previously coded the two- and three-segmented chelicera as two to three separate character states, presuming homology among these types (Shultz 1990, 2007; Wheeler and Hayashi 1998; Giribet et al. 2002). It remains to be tested whether a two-segmented chelicera can be obtained by alternative modifications of the three-segmented ancestral state, that is by deletions of the second or the distal articles, as alternatives to the proximal article. A survey of leg gap gene expression across Chelicerata, with emphasis on *dac*, may aid in testing the hypothesis of multiple cheliceral *dac* domain losses in derived arachnids as a mechanism for transition to the two-segmented chelicera.

CONCLUSION

The ancient history and plesiomorphic morphology of harvestmen, here represented by *P. opilio*, lend itself to investigation of many aspects of early arthropod evolution. We observed that *dac* is expressed in the proximal segment of the chelicera in the harvestman, whereas neither the *dac* domain nor this segment is retained in spiders. This correlation suggests that cheliceral segment number is determined by the presence of the *dac* domain, providing a putative mechanism for the evolutionary transitions in chelicera morphology.

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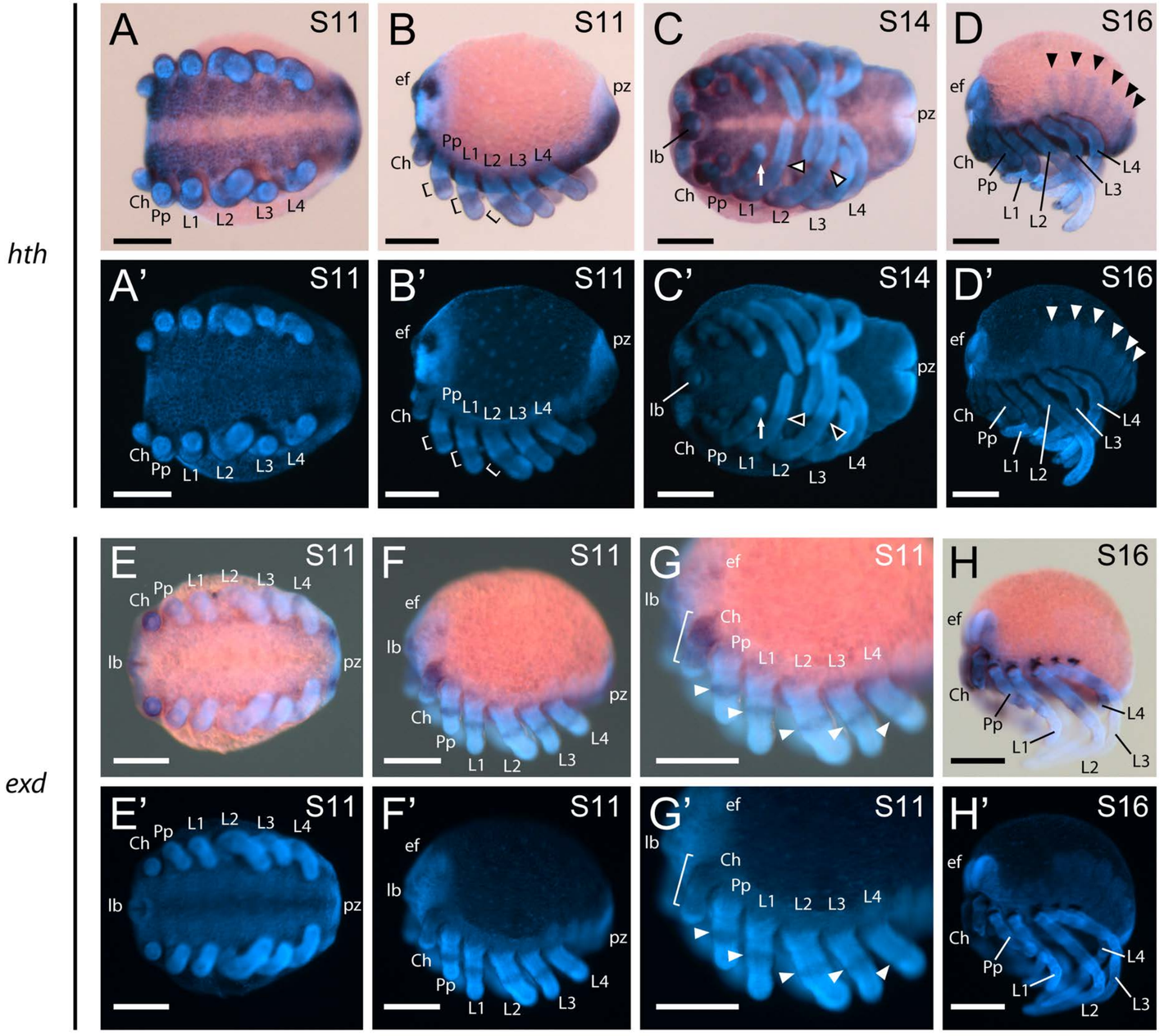
SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Expression of the *Phalangium opilio homothorax* and *extradenticle* genes.

Fig. S2. Expression of the *Phalangium opilio dachshund* and *Distal-less* genes.

Table S1 List of primer sequences used for riboprobe synthesis.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exd
A
S11
B
S11
C
S14
D
S16
A'
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B'
S11
C'
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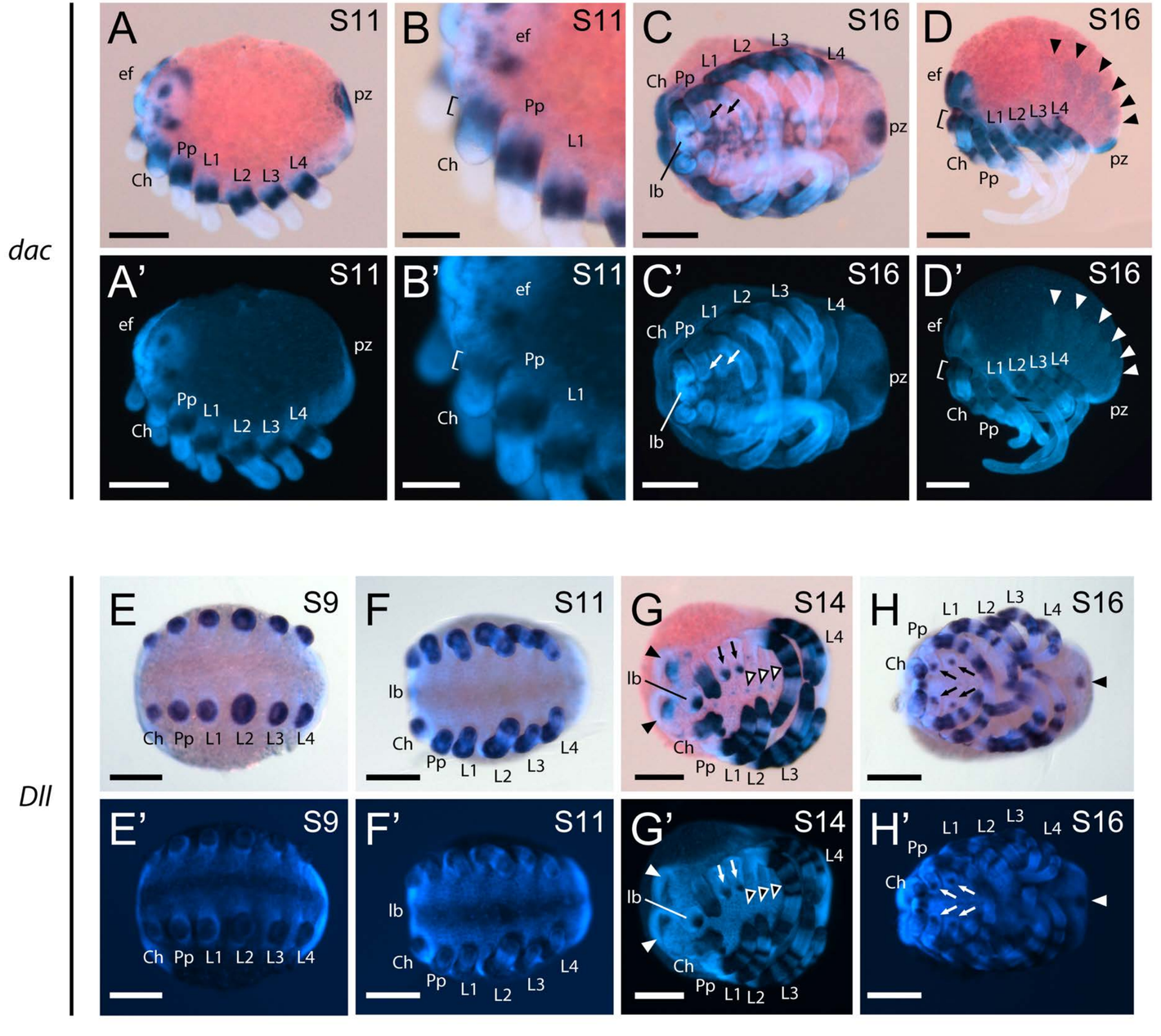
pz
lb
Ch
Pp
L1
L2
L3
L4


dac
A
S11
ef
pz
Pp
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L2
L3
L4
Ch
B
S11
ef
Pp
L1
Ch
C
S16
L1
L2
L3
L4
Ch
Pp
pz
Ib
D
S16
ef
L1
L2
L3
L4
Ch
Pp
pz
A'
S11
ef
pz
Pp
L1
L2
L3
L4
Ch
B'
S11
ef
Pp
L1
Ch
C'
S16
L1
L2
L3
L4
Ch
Pp
pz
Ib
D'
S16
ef
L1
L2
L3
L4
Ch
Pp
pz
Dll
E
S9
Ch
Pp
L1
L2
L3
L4
F
S11
Ib
Ch
Pp
L1
L2
L3
L4
G
S14
L4
Ib
Ch
Pp
L1
L2
L3
H
S16
L1
L2
L3
L4
Pp
Ch
E'
S9
Ch
Pp
L1
L2
L3
L4
F'
S11
Ib
Ch
Pp
L1
L2
L3
L4
G'
S14
L4
Ib
Ch
Pp
L1
L2
L3
H'
S16
L1
L2
L3
L4
Pp
Ch

Table S1 List of primer sequences used for riboprobe synthesis.

Gene	Primer Sequence	Amplicon Length (bp)
homothorax		795
Po_hth_forward	5’ - TTAGCAACGTGTACGCCAAG - 3’	
Po_hth_reverse	5’ - TTAGTCCCGTGTCTTGAGCA - 3’	
extradenticle		591
Po_exd_forward	5’ - GCGCAAGCTAGGAAACACAC - 3’	
Po_exd_reverse	5’ - ATTCCTCCTTGGCTTCTTCG - 3’	
dachshund		786
Po_dac_forward	5’ - AACAACGGAGCCAGAGAGAA - 3’	
Po_dac_reverse	5’ - TTAGAGCCATGGAAGCGACT - 3’	
Distal-less		950
Po_Dll_forward	5’ - GAGCAACTGCCACACAAGAA - 3’	
Po_Dll_reverse	5’ - TTTGCCCTTTCCATTGACTC - 3’	