Report

A Paleozoic Stem Group to Mite Harvestmen Revealed through Integration of Phylogenetics and Development

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Summary

Successfully placing fossils in phylogenies is integral to understanding the tree of life. Crown-group Paleozoic members of the arachnid order Opiliones are indicative of ancient origins and one of the earliest arthropod terrestrialization events [1, 2]. Opiliones epitomize morphological stasis, and all known fossils have been placed within the four extant suborders [3–5]. Here we report a Carboniferous harvestman species, *Hastocularis argus* gen. nov., sp. nov., reconstructed with microtomography (microCT). Phylogenetic analysis recovers this species, and the Devonian *Eophalangium sheari*, as members of an extinct harvestman clade. We establish the suborder Tetrophthalmi subordo nov., which bore four eyes, to accommodate *H. argus* and *E. sheari*, the latter previously considered to be a phalangid [6–9]. Furthermore, embryonic gene expression in the extant species *Phalangium opilio* demonstrates vestiges of lateral eye tubercles. These lateral eyes are lost in all crown-group Phalangida, but are observed in both our fossil and outgroup chelicerate orders. These data independently corroborate the diagnosis of two eye pairs in the fossil and demonstrate retention of eyes of separate evolutionary origins in modern harvestmen [10–12]. The discovery of Tetrophthalmi alters molecular divergence time estimates, supporting Carboniferous rather than Devonian diversification for extant suborders and directly impacting inferences of terrestrialization history and biogeography. Multidisciplinary approaches integrating fossil and neontological data increase confidence in phylogenies and elucidate evolutionary history.

Results and Discussion

Terrestrial arthropods have a sparse fossil record due to low preservation potential; the environments in which they live

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favor rapid decay over burial, and many—such as Opiliones—possess a poorly mineralized exoskeleton. As a result, fundamental questions in arthropod evolutionary history remain unresolved. Examples include the number and timing of terrestrialization events [1], the diagnosis of ancestral and derived characters [13], and the evolutionary relationships of many iconic lineages [14, 15]. The exceptional preservation of Scotland's Rhynie Cherts provided the earliest currently recognized Opiliones (harvestman). This Devonian species, *Eophalangium sheari*, was placed within the suborder Eupnoi on the basis of genitalic morphology [4, 5]. Such clarity of phylogenetic placement is unusual for Opiliones; as an example, a Lower Carboniferous fossil (*Brigantibunum listoni*) was too poorly preserved to permit accurate placement [16]. Furthermore, cladistic analyses are infrequently applied in placing such fossils; two Carboniferous species from the Montceau-les-Mines Lagerstätte are the only nonamber fossil harvestmen to be included in a cladistic analysis to date, being recovered as members of the suborders Eupnoi and Dyspnoi [3].

Phylogenetic Analyses Reveal a Distinct Fossil Harvestman Suborder

Hastocularis argus gen. nov., sp. nov. is a new Carboniferous harvestman, resolved using microtomography (microCT) (see "Systematic Palaeontology" in the Supplemental Results and Discussion available online; Figures 1H–1M and 2; http://dx.doi.org/10.5061/dryad.r32p3). It possesses median eyes, as expected in a modern phalangid (a member of one of the three noncyphophthalmid orders), on an anteriorly projecting ocularium (Figures 1I–1K, black arrowheads) and a pair of raised lateral prosomal tubercles on the carapace (Figure 1I), with associated eyes (Figures 1J and 1K, white arrowheads). Its oval body has a broad prosoma-opisthosoma boundary and dorsal opisthosomal segmentation in the form of seven transverse ridges (http://dx.doi.org/10.5061/dryad.r32p3). Three-segmented chelicerae are tucked between the pedipalps, which have median coxapophyses at the anterior margin of a preoral chamber (http://dx.doi.org/10.5061/dryad.r32p3). Tuberculated legs possess a tarsus divided into seven tarsomeres, and leg II bears coxapophyses. Between the coxae of leg IV is an open gonostome with a protruding penis, whose smooth shaft and dorsal terminal process are visible (Figures 1L and 1M).

But the morphology of *H. argus* includes characters previously considered synapomorphic for both mite harvestmen (Cyphophthalmi) and Phalangida (Figure 1A). The open gonostome and ozophores are exclusive to Cyphophthalmi (Figures 1C and 1D), while a scutum completum (contiguous dorsal prosomal-opisthosomal carapace; Figure 1B) is only found in Cyphophthalmi and one family of Phalangida, Sandokanidae [9, 17]. Intromittent male genitalia, elongate legs with numerous tarsomeres, and the ocularium are characteristic of Phalangida (Figures 1E–1G).

To determine the phylogenetic placement of this group, we included the new species in a matrix comprising 158 morphological characters and five molecular loci for extant species, coding the oldest harvestman fossil, *E. sheari*, in a
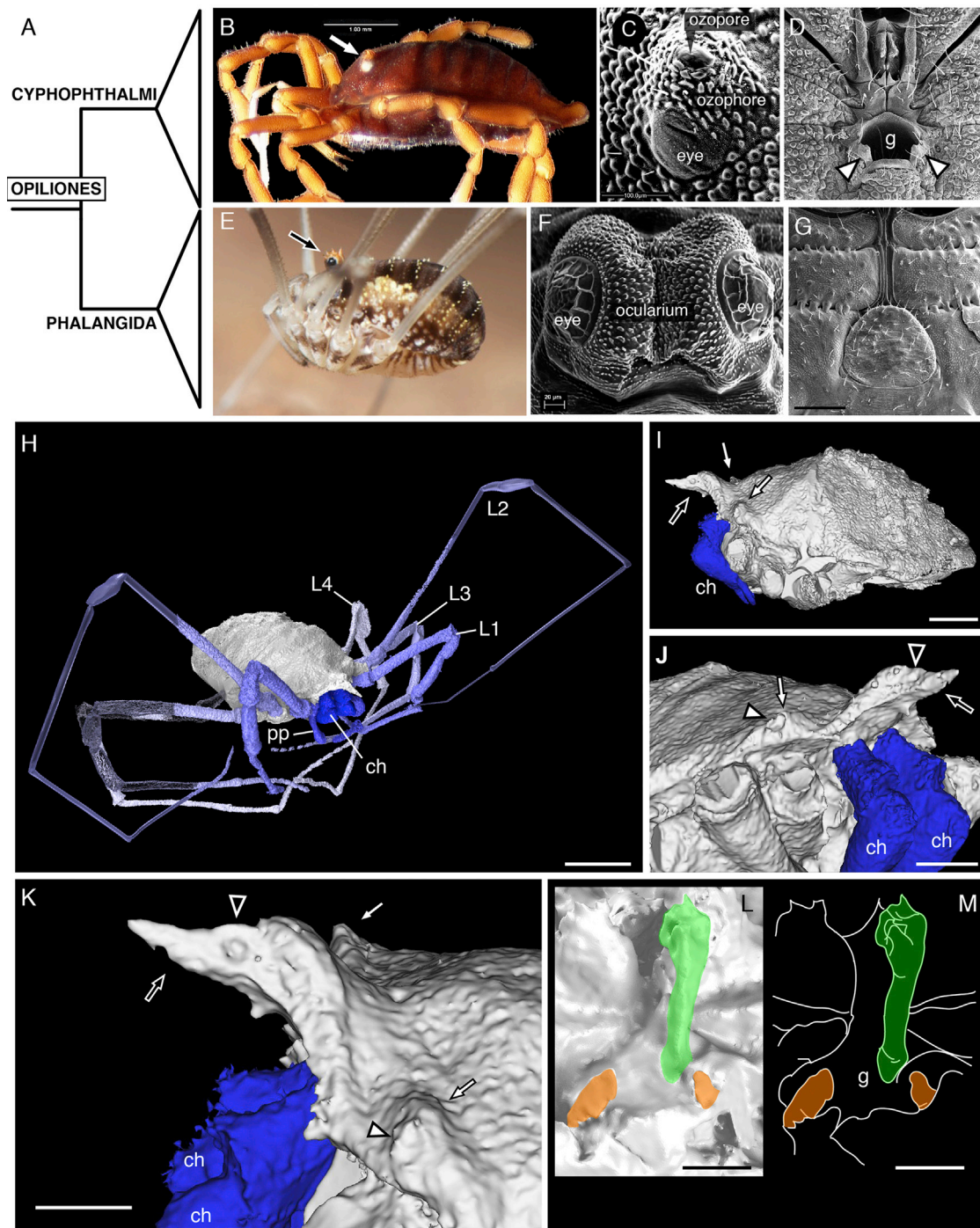
Figure 1. Extant Opiliones and the Identification of a Fossil Stem Group

(A) Basal split between Cyphophthalmi (mite harvestmen) and Phalangida (all remaining harvestmen).

(B–G) Lateral view of adult *Pettalus* (B) with eye mounted on ozophore (arrow) and SEM magnification of the ozophore (C) showing the lateral eye (bottom) and dorsal ozopore (top). The open gonostome of the cyphophthalmid *Metasiro* with lateral flanges (arrowheads) is shown in ventral view (D). Eyes of Phalangida (E) are typically mounted on a centralized ocularium (F), and the gonostome is sealed with a hinged operculum (G).

(H–L) 3D reconstructions of *Hastocularis argus* **gen. nov., sp. nov.**

(H) Whole body and appendages.

(I) Lateral view of body and chelicerae showing ocularium (black arrow) and ozophores (white arrows).

(J) Right anterior region shows the ocularium (black arrow), ozophores (white arrows), and corresponding eyes (arrowheads).

(K) Left anterior region shows bilateral symmetry of ozophores and eyes.

(L) Intromittent genitalia (green) and open gonostome, with lateral flanges (orange).

(M) Schematic interpretation of intromittent genitalia.

ch, chelicera; g, gonostome; ig, intromittent genitalia; L1, first leg; pp, pedipalp. Scale bars represent 250 μm (G), 500 μm (L and M), 1 mm (J and K), 2 mm (I), and 4 mm (H). See also http://dx.doi.org/10.5061/dryad.r32p3.
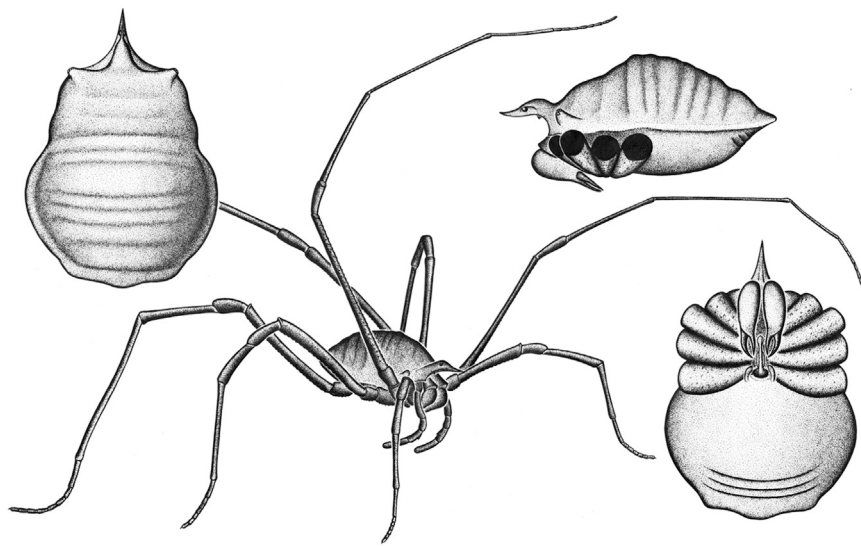
Figure 2. An Idealized Reconstruction of *Hastocularis argus* gen. nov., sp. nov. in Life on the Basis of the MicroCT Models

Shown in dorsal, lateral, ventral, and anterolateral aspect.

species) eye pairs [19]. Harvestmen have at most a single pair of eyes: those of Cyphophthalmi occur laterally, whereas eyes generally occur medially in Phalangida (Figures 1A–1C, 1E, and 1F) [10, 18, 20]. Neither type is definitively homologous to lateral eyes, as would be evidenced by an unambiguous tapetum or facets [10–12]. Thus, the homology of Opiliones eyes remains contentious. A harvestman with both eye pairs—as seen in *Hastocularis argus* **gen. nov., sp. nov.**—would establish the separate evolutionary origins of the cyphophthalmid and phalangid eyes, providing a direct link to the plesiomorphic state observed in other arachnid orders and falsifying the homology of the single eye pair of Cyphophthalmi and Phalangida.

The presence of two sets of eyes in *Hastocularis argus* is supported by structures associated with a specific eye type in modern opilionids (Figures 1C and 1F). Median eyes (Figures 1I–1K, black arrowheads) are located on an anteriorly projecting ocularium (a condensed region of cuticle that bears the median eyes of Phalangida). The raised lateral prosomal tubercles on the carapace are interpreted as ozophores, which bear the laterally occurring eyes in some extant Cyphophthalmi (Figure 1I). Consistent with this interpretation, *H. argus* has a single socket-like depression at the base of the ozophore interpreted as an eye (Figures 1J and 1K, white arrowheads). Given that phylogenetic analyses recover Tetraophthalmi **subordo nov.** as a stem-Cyphophthalmi (or a competing alternative, stem-Phalangida), optimization of this character state suggests that the ancestor of Opiliones also bore both sets of eyes, a plesiomorphic condition that occurs in a grade of chelicerates that includes horseshoe crabs, scorpions, and spiders.

phylogenetic matrix for the first time (http://dx.doi.org/10.5061/dryad.r32p3). Bayesian inference analysis of this total evidence data set recovers *H. argus* as sister species to the Devonian *E. sheari* (posterior probability [PP] = 0.64) in Tetrophthalmi and this clade as sister group to Cyphophthalmi (PP = 0.73; Figure 3A). Lack of nodal support stems from considerable missing data in the fossil taxa and resultant topological instability. We therefore mapped branch attachment frequency (BAF) of Tetrophthalmi for post-burn-in a posteriori trees. The three most frequent placements of this clade are as stem-Cyphophthalmi (BAF = 39.5%), stem-Phalangida (BAF = 20.2%), or stem-Palpatores (BAF = 14.0%), the last being one of the two main divisions of Phalangida (Figure 3A). Ordinal placement of other fossil taxa (e.g., *Ameticos scolos* and *Macrogyion cronus*) is consistent with the total evidence Opiliones analysis previously published (although *E. sheari* was not included in that study [3]).

One of the earliest terrestrial animals, *E. sheari* is of key phylogenetic importance [1, 3, 7]. Previously considered a member of Eupnoi, a suborder that includes many typical modern long-legged harvestmen, this species shares an anteriorly projecting ocularium and a previously overlooked open gonostome with *H. argus* [4]. Due to the previous sectioning of *E. sheari*, ozophores are equivocal in this fossil. But the combination of an ocularium, open gonostome, and intromittent genitalia is unique to (*Eophalangium* + *Hastocularis*). This character combination, coupled with co-occurrence in *H. argus* of median eyes on an ocularium and ozophores, precludes placement in extant Opiliones suborders, thereby supporting the validity of Tetrophthalmi **subordo nov.**

Re-evaluation of Homology Statements of Eyes in Opiliones

Arthropod eyes are labile in nature, and independent, multiple eye losses are common in Chelicerata, the division of arthropods that includes arachnids and horseshoe crabs [2]. The ancestral chelicerate eye state comprises median (simple ocelli) and lateral eyes (e.g., faceted eyes in horseshoe crabs; those with a reflective layer [the tapetum] in spiders) [18]. Various chelicerate lineages have lost the median (e.g., pseudoscorpions), the lateral (e.g., solifuges), or both (e.g., extant ricinuleids, palpigrades, in addition to many troglomorphic

Embryonic Gene Expression in a Phalangida Reveals Vestiges of Ozophores

We reasoned that the presence of multiple eye types in Tetrophthalmi (median and lateral) may have resulted in transient retention of this morphology during embryogenesis of modern species. We therefore investigated the developmental genetics of eye-associated outgrowths (ocularium and ozophores) in a member of Phalangida, *Phalangium opilio*. The appendage-patterning gene *Distal-less* (*Dll*) is commonly co-opted to pattern nonappendage outgrowths. In the model harvestman *Phalangium opilio*, recent work has demonstrated that *Po-Dll* is expressed in domains that give rise to the ocularium. *Po-Dll* knockdown results in the loss of the ocularium [21]. We reinvestigated embryonic *Po-Dll* expression during several consecutive stages of development. In addition to the ocularial domains (Figures 4A–4C, black arrowheads), we observed multiple transient, paired expression domains of *Po-Dll* in the lateral fields ventral to the semilunar grooves (Figures 4A–4C, white arrowheads). These domains peak in
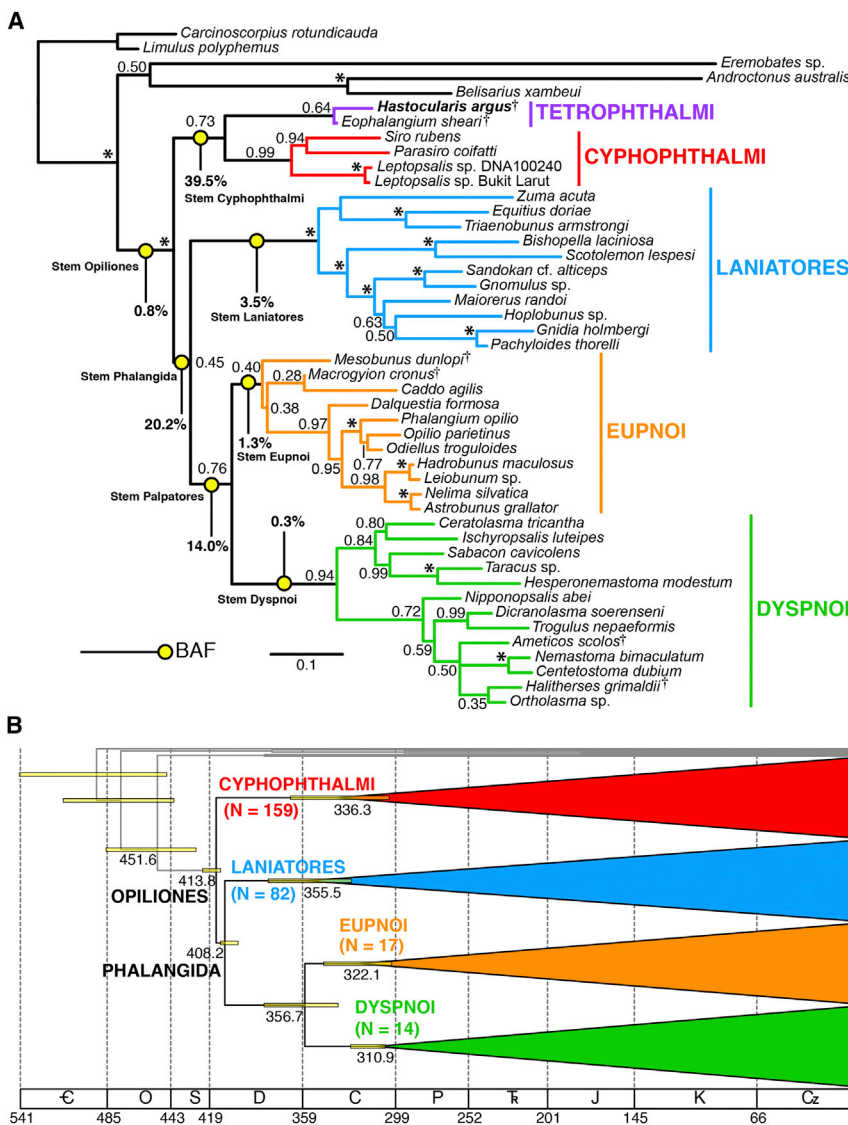
Figure 3. Phylogenetic Analyses of Opiliones

(A) Total evidence phylogeny incorporating morphology and molecules for extinct and extant species. Colored branches correspond to suborders. Numbers on nodes are posterior probabilities. Yellow icons indicate branch attachment frequencies for Tetraophthalmi.

(B) Molecular divergence time estimates treating Tetraophthalmi as stem-Cyphophthalmi. Ingroup clade sizes are not to scale. Numbers on nodes are in Ma.

See also http://dx.doi.org/10.5061/dryad.r32p3.

expression of neurogenetic markers *orthodenticle* (*otd*), *Paired box-6* (*Pax6*), *sine oculis* (*so*), and *empty spiracles* (*ems*) does not contradict the hypothesis of a four-eyed harvestman ancestor, demonstrating plesiomorphic expression patterns (Figure S1 and the Supplemental Results and Discussion, "Embryonic gene expression of neurogenetic markers in *Phalangium opilio*"). These data thus reinforce that ozophores and ocularia are two nonhomologous pairs of eye-bearing outgrowths and suggest that the cyphophthalmid eye is a true arachnid lateral eye.

Increased Accuracy of Harvestmen Molecular Dating Reflects Congruence of Arthropod Evolutionary Dynamics in the Paleozoic

The new phylogenetic placement of *E. sheari* is inconsistent with previous treatments in Opiliones divergence dating that have treated this as a stem- or crown-group Eupnoi [6–9]. Consequently, ages of divergences within Opiliones have been historically overestimated, which has immediate implications for downstream analyses. For example, some groups of harvestmen serve as textbook cases of vicariant biogeography, a discipline contingent upon accurate molecular dating [8, 25–27].

Overestimation of divergence time estimates may also suggest implausible evolutionary scenarios that are inconsistent with biogeochemistry (e.g., availability of atmospheric oxygen) or availability of suitable habitat [28–30]. Previously implied eupnoid diversification in the Devonian, compared to Carboniferous diversifications in a large number of terrestrial crown-group arthropods [1, 19, 31], has made Opiliones a suspiciously ancient outlier.

To reassess divergence times, we constructed a matrix of 272 extant harvestman species and seven chelicerate outgroups for five genes. Using bona fide crown-group Cyphophthalmi and Phalangida fossils and treating Tetrophthalmi as stem-Cyphophthalmi or stem-Phalangida, we estimated the diversification of Opiliones at 414 million years ago and of all four suborders in the Carboniferous (Figure 3B), much younger than the previously proposed Devonian ages for Eupnoi and Dyspnoi [6, 7]. Estimated ages within Cyphophthalmi and Laniatores were unaffected by the new calibration [8, 9]. We

expression strength in stage 14 embryos and wane thereafter. In all Opiliones, these tissues ultimately form the secretory ozopore (the opening of the repugnatorial glands), which is situated at the tip of an outgrowth, the ozophore, in Cyphophthalmi (Figure 1C). In *P. opilio*, an outgrowth does not form in this region in wild-type embryos, and *Po-Dll* knockdown demonstrates no associated loss-of-function phenotype. These data suggest that the domains in the lateral eye fields represent vestiges of ozophores. Similar transient *Dll* domains are reported to signify vestigial outgrowths in other arthropods. For example, they are observed in the intercalary segment of millipedes, which putatively bore true appendages in the ancestor of mandibulate arthropods [22], and in the vestigial exopods of extant horseshoe crabs [23, 24].

These developmental genetic data independently corroborate the hypothesis of a four-eyed harvestman ancestor. Limitations in the tractability of Cyphophthalmi species for developmental genetic techniques have precluded the generation of comparable data for this group. In Cyphophthalmi embryos, we predict conversely strong *Dll* domains in the lateral eye fields and vestigial *Dll* expression in the dorsal anterior of the eye fields, where the ocularium would occur. The
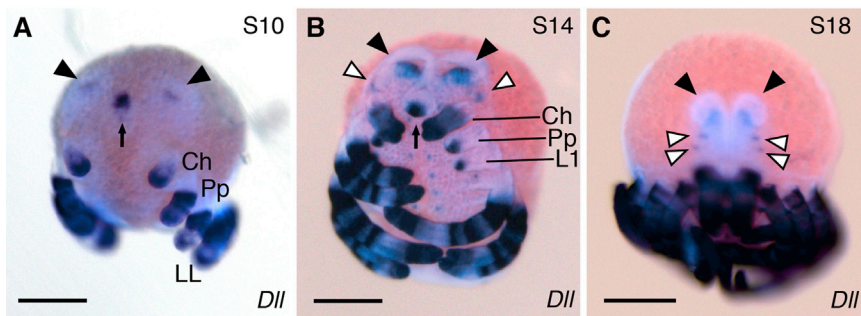
Figure 4. Dorsal Outgrowth and Neurogenesis in *Phalangium opilio*

Expression of *Po-Dll* in intermittent developmental stages shows coalescence of ocularial domains (black arrowheads). Transient appearance of ventrolateral domains (white arrowheads) develops after stage 10 (A), being first observed in stage 14 embryos (B) and waning by stage 18 (C). Abbreviations are as in Figure 1. In (A) and (B), arrows indicate the labrum. Scale bars represent 200 μm. See also Figure S1.

estimated a late Cambrian (491 million years ago) Xiphosura-Arachnida split and the radiation of crown group arachnids during the early Ordovician (475 million years ago). As an external check, these dates are in accord with the earliest known xiphosuran, which is Lower Ordovician in age [32], and the earliest arachnid, which is Silurian [33].

These reassessments offer more-consistent dates of diversification for extant harvestman suborders, which coincide with evolutionary radiations of numerous terrestrial arthropod groups. For example, Carboniferous origins have been reported for such groups as orthosternous scorpions [34], endopterygote insects [35, 36], spiders [37], and numerous paraneopteran [38, 39] and polyneopteran [40, 41] insects. These radiations may have been linked to high atmospheric oxygen concentration in the Carboniferous [42], but could also partially reflect sampling bias [43]. Nevertheless, a wide range of studies across the tree of life [43] and the sedimentary rock record [44] are starting to establish a clear chronology of the development of terrestrial ecosystems. Our estimate of arachnid origins (and thus likely terrestrialization) is in accordance with other recent estimates [30] in suggesting Cambro-Ordivician terrestrialization. Devonian Opiliones origins match well the pattern of basal radiations within major clades throughout the Silurian and Devonian. Stem groups of many terrestrial arthropod lineages are first found in the Devonian, suggesting similar times of origin [1, 31]. Increasingly familiar terrestrial arthropod (and, indeed, invertebrate and vertebrate) fauna then develops in the Euamerican coal forests, as with harvestmen. Our findings thus both provide a novel analysis in reinforcing this chronology and emend a previous outlier in the evolutionary history of Opiliones.

Conclusions

Deciphering the placement of fossil lineages has been a recalcitrant puzzle in many rami of the tree of life, most markedly when a given lineage has a poor fossil record, is not prone to fossilization, or has undergone an ancient rapid radiation. However, placing fossils is integral to recognizing key macroevolutionary transitions, demonstrating the order of character acquisition during evolution, and inferring the ages of ancient divergences. Here we reveal an extinct harvestman suborder through microtomographic investigation of *Hastocularis argus*, coupled with total evidence phylogenetic analysis. This species is a singular discovery, as it demonstrates that the condition of both median and lateral eye types is a lost plesiomorphic state of Opiliones. Independent corroboration of this hypothesis is provided by developmental genetics; gene expression data in *Phalangium opilio* reveal putative vestiges of ozophores during embryogenesis as a pair of transient *Dll* expression domains with no associated phenotype upon

gene silencing of *Dll*. We thus show that fossils can be successfully placed in phylogenies through multiple, consilient approaches to analysis of morphological characters. Our interpretation of eye homology in Opiliones is anticipated to inform future efforts aimed at deciphering the genetic mechanisms differentiating eye types in chelicerates, as well as panarthropods more broadly. In this manner, complementary approaches to evolutionary biology can ground paleontological inference in experimental results and open new avenues of scientific inquiry. Fossils help to explain otherwise enigmatic observations in extant taxa, and the integration of palaeontology, phylogenetics, and development has great potential as a means of understanding morphological evolution within a group.

Experimental Procedures

Materials

A single fossil from Montceau Les Mines, MNHN-SOT 43943, was scanned (Collection Sotty 2, deposited in the Muséum d'histoire naturelle d'Autun belonging to the Muséum national d'Histoire naturelle, Paris). This was a void within a siderite nodule, which has split into four parts; one part could not be located.

MicroCT Reconstruction

Two scans were performed at the Natural History Museum, London on a Nikon HMX-ST 225 with a tungsten reflection target. That of the whole fossil (three parts) was conducted at a current/voltage of 185 μA/180 kV, with a 2 mm copper filter and 3,142 projections, providing an 18.4 μm voxel size. A local scan of the prosoma was performed at a current/voltage of 195 μA/195 kV, with a 0.5 mm copper filter and 6,284 projections, providing a resolution of 6.6 μm. Both were reconstructed with CTPro V2.1, and the resulting tomographic data sets were used to create two 3D, virtual models of the organism (both provided at http://dx.doi.org/10.5061/dryad.r32p3) using the custom SPIERS software suite [45] following the methods of Garwood et al. [46]. Isosurfaces were ray-traced in Blender [47]; missing elements of the limbs were created using the software's mesh creation tools and rendered partially transparent.

Phylogenetic Placement of Fossils

The morphological data matrix compiled by Giribet et al. [20] and modified by Garwood et al. [3] was modified to code the new specimen and additional fossils (*Eophalangium sheari* and *Mesobunus dunlopi*), containing 158 characters and 46 taxa. The morphological data matrix is available in the public database Morphobank (Project 793). The morphological data matrix was analyzed in combination with the five-gene molecular data set of Giribet et al. [6], modified by treatment with GBlocks v.0.91b [48] to cull positions of ambiguous homology. The combined matrix of 4,043 characters is available at http://dx.doi.org/10.5061/dryad.r32p3. These data were analyzed under Bayesian inference in MrBayes v.3.1.2 [49], with the morphological partition assigned a discrete equal rates model [50] and each gene assigned a unique GTR + I + Γ model, as selected in jModeltest v.0.1.1 [51, 52]. Four runs each with four chains, and a default distribution of hot and cold chains, were conducted for 10^7 generations. Convergence diagnostics were assessed in Tracer v.1.5 [53], and 25% of all 16 runs were discarded as

burn-in. Branch attachment frequencies were obtained from a posteriori trees using Phyutility v.2.2 [54].

Estimation of Divergence Times

The five-gene phylogenetic matrix sampling 272 extant harvestman species and seven chelicerate outgroups is available at http://dx.doi.org/10.5061/dryad.r32p3. Ages of clades were inferred using BEAST v. 1.7.4 [55, 56]. Each data partition was assigned a unique GTR + I + Γ model as previously outlined [8, 9], and a birth-death speciation tree prior was implemented. Fossil taxa were used to calibrate divergence times: Arachnida with an exponential prior from 430 to 525, with dates closer to the younger bound upweighted, based on the presence of the earliest fossil scorpions [1, 2]; Opiliones with an exponential prior from 411 to 500 (dates close to 411 upweighted) on the basis of *E. sheari* from the Rhynie chert [4, 5]; Eupnoi, Dyspnoi, and Phalangida with a uniform prior from 411 to 305 and no upweighting on the basis of the fossils reported by Garwood et al. [3]; and an exponential prior for Stylocellidae (105 to 310, dates close to 105 are upweighted, as discussed by Giribet et al. [8]). Analysis is otherwise as reported by Sharma et al. [9], with two Markov chains run for 10^8 generations.

Developmental Gene Expression

Fragments of the genes *Po-ems*, *Po-otd*, *Po-Pax6*, and *Po-so* were isolated from a developmental transcriptome of *Phalangium opilio*, and gene identities were confirmed by reciprocal BLAST as in Sharma et al. [57]. Identification and probe synthesis for *Po-Dll* are described in Sharma et al. [21]. Embryo fixation, riboprobe synthesis, whole-mount in situ hybridization, and imaging were conducted following methods detailed by Sharma et al. [57]. Expression of sense probes revealed only background staining incurred by cuticle deposition. The list of gene-specific primers for sense and antisense riboprobe synthesis is provided in Table S1.

Accession Numbers

The GenBank accession numbers for the neurogenetic marker sequences reported in this paper are as follows: KJ623612 (BankIt1710404 P_opilio_ems), KJ623613 (BankIt1710404 P_opilio_Pax6), KJ623614 (BankIt1710404 P_opilio_so), and KJ623615 (BankIt1710404 P_opilio_otd). The Dryad DOI for the data reported in this paper is http://dx.doi.org/10.5061/dryad.r32p3.

Supplemental Information

Supplemental Information includes Supplemental Results and Discussion, one figure, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.03.039.

Author Contributions

R.G. conducted tomography and digital visualization of *H. argus*, P.S. conducted phylogenetics and evo-devo. All authors contributed intellectually to the study and to completion of the manuscript.

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