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Diverse Adaptations of an Ancestral Gill: A Common Evolutionary Origin for Wings, Breathing Organs, and Spinnerets

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Summary

Changing conditions of life impose new requirements on the morphology and physiology of an organism. One of these changes is the evolutionary transition from aquatic to terrestrial life, leading to adaptations in locomotion, breathing, reproduction, and mechanisms for food capture. We have shown previously that insects' wings most likely originated from one of the gills of ancestral aquatic arthropods during their transition to life on land [1]. Here we investigate the fate of these ancestral gills during the evolution of another major arthropod group, the chelicerates. We examine the expression of two developmental genes, *pdm/nubbin* and *apterous*, that participate in the specification of insects' wings and are expressed in particular crustacean epipods/gills. In the horseshoe crab, a primitively aquatic chelicerate, *pdm/nubbin* is specifically expressed in opisthosomal appendages that give rise to respiratory organs called book gills. In spiders (terrestrial chelicerates), *pdm/nubbin* and *apterous* are expressed in successive segmental primordia that give rise to book lungs, lateral tubular tracheae, and spinnerets, novel structures that are used by spiders to breathe on land and to spin their webs [2]. Combined with morphological and palaeontological evidence [3–9], these observations suggest that fundamentally different new organs (wings, air-breathing organs, and spinnerets) evolved from the same ancestral structure (gills) in parallel instances of terrestrialization.

Results and Discussion

Understanding morphological changes that occurred in the distant past poses a major challenge for evolutionary biology. For example, morphological innovations that took place around 350–450 million years ago are a key to understanding the origin of major terrestrial groups such as insects, arachnids, and land plants, but these early events are obscured by secondary changes that took place during the long intervening periods. Regulatory developmental genes often have well-conserved functions in the development of particular structures or cell types, so comparative analysis of their expression patterns can provide information about such distant evolutionary events. In a previous study, we showed that two developmental genes, *pdm/nubbin (pdm/nub)* and *apterous (ap)*, are similarly expressed in particular epipods/gills in crustaceans and in insect wings. These findings supported an old hypothesis that insect wings originated from ancestral arthropod gills [1, 3, 4]. Here we extend this work to examine the fate of these ancestral gills in the chelicerates, another major arthropod group that includes the horseshoe crabs, mites, scorpions, and spiders (Figure 1).

The expression patterns of *pdm/nub* and *apterous* are good markers for such deep evolutionary comparisons because they appear to be conserved among insects, branchiopod crustaceans, and malacostracan crustaceans for at least 400 to 500 million years of divergent evolution [1, 4, 10]. *pdm/nub* also has a conserved expression pattern in endopods/legs, in a set of rings that correspond to some of the leg joints [1, 11, 12], but this pattern is clearly distinguishable from the gene's expression in insect wings and crustacean gills (Figure 2A). *pdm/nub* is not expressed in endopods/legs that do not have joints, in exopods, or in proximal epipods that are present in some branchiopod and malacostracan crustaceans [1]. Thus, uniform expression of *pdm/nub*, combined with the expression of *apterous*, are diagnostic of structures that derive from a particular type of epipod/gill in diverse species.

Generation of Antibodies for Pdm/Nub

To be able to study the expression of Pdm/Nub in diverse arthropods, we generated antibodies that recognize a conserved epitope in the Pdm/Nub protein. We raised antibodies by immunizing mice with a fragment of Pdm/Nub from *Artemia* [1] and testing the sera for their ability to recognize *Drosophila* Pdm/Nub. We then generated monoclonal antibodies from one of these mice and screened them for their ability to recognize *Drosophila* Pdm/Nub; we recovered a monoclonal, called Mab 2D4, that recognizes a conserved epitope in the homeodomain of this protein (our unpublished data).

These antibodies were tested by immunochemical stainings in diverse arthropods, including insects (*Drosophila*), branchiopod crustaceans (*Artemia*, *Daphnia*), malacostracan crustaceans (crayfish, amphipod), and chelicerates (horseshoe crab and spider; see below). In all of these species, we detected specific expression patterns that were consistent with those previously described for Pdm/Nub [1, 11, 12].

Pdm/Nub Expression in Horseshoe Crab Embryos

First, we examined the expression of Pdm/Nub in embryos of the horseshoe crab *Limulus polyphemus*. Horseshoe crabs are members of the Xiphosurans, the

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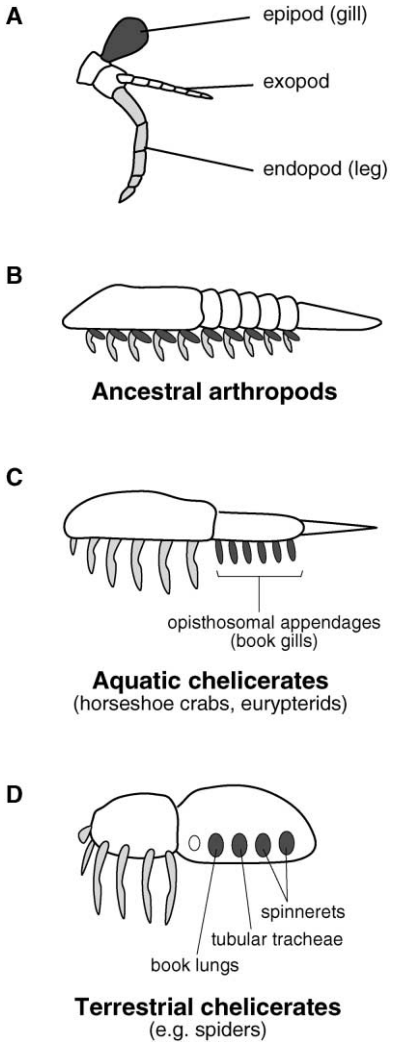

Figure 1. Branched Appendages and the Chelicerate Body Plan

(A) Typical arthropod appendages have a branched structure, including endopods/legs, exopods, and epipods/gills [13].

(B) Ancestral arthropods had branched appendages with distinct ventral (light gray) and dorsal (dark gray) limb branches in most of their trunk segments.

(C–D) The body of chelicerates is typically subdivided in two regions: the prosoma (anterior part) and the opisthosoma (posterior part).

(C) Primitively aquatic chelicerates (horseshoe crabs and extinct eurypterids) have leg-like appendages in the anterior part of their body (prosoma) and book gills in the posterior part (opisthosoma). The prosomal appendages are thought to be homologous to the endopods/legs of other arthropods (in light gray), whereas the opisthosomal appendages may derive from one of the dorsal limb branches. (D) In terrestrial chelicerates, as in spiders, opisthosomal appendages may have been modified to give rise to book lungs, lateral tubular tracheae, and spinnerets, in successive opisthosomal segments. Book lungs and tubular tracheae are internal air breathing organs, whereas the spinnerets are small external protrusions that are used by spiders to spin their webs [2].

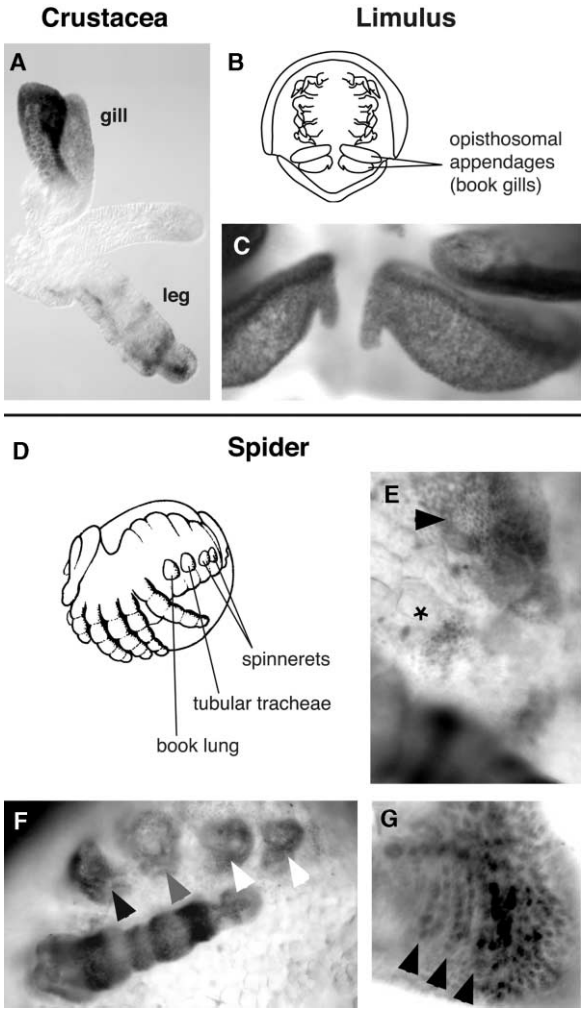

Figure 2. Pdm/Nub Expression in Crustacean Epipods/Gills and in Chelicerate Book Gills, Book Lungs, Tubular Tracheae, and Spinnerets

(A) Pdm/Nub expression in a limb of the crustacean *Pacifastacus leniusculus* (crayfish), showing rings of expression in the leg and strong expression throughout the epipod/gill.

(B and C) Late embryos of the horseshoe crab *Limulus polyphemus* have two pairs of opisthosomal appendages, which will give rise to the genital operculum and the first pair of book gills. Pdm/Nub expression is seen clearly in these opisthosomal appendages (expression is uniform; some parts appear darker because of folding of the tissue).

(D) Schematic representation of a spider embryo, showing the primordia of opisthosomal segments 2–5 that will give rise to book lungs, lateral tubular tracheae, and spinnerets.

(E) Pdm/Nub expression in a book lung primordium (black arrowhead), first opisthosomal primordium (asterisk), and leg (out of focus) in a mid-stage embryo of the spider *Cupiennius salei*. Expression in the first opisthosomal primordium is transient.

(F) Later expression in the leg and in the primordia of book lung (black arrowhead), tubular tracheae (gray arrowhead), and spinnerets (white arrowheads) in the posterior segments of *Cupiennius*. Expression is seen in a set of ring-like domains along the proximodistal axis of the fourth walking leg and in relatively uniform levels throughout the primordia of book lungs, tubular tracheae, and spinnerets.

(G) High magnification of the expression in a book lung of *Cupiennius*. Arrowheads mark developing leaflets of the book lung. In all cases staining is nuclear.

only surviving group of primitively aquatic chelicerates [13], which bear a series of leaf-like appendages in the opisthosomal (posterior) part of their body (Figures 1C and 2B). We were able to obtain immunochemical stainings (as described in [14]) in a few *Limulus* embryos at

mid–late embryonic stages. These show that Pdm/Nub is strongly and specifically expressed in the two pairs of opisthosomal appendages that form during embryonic stages (Figures 2B and 2C); the anterior pair gives rise to the genital operculum, and the posterior one gives rise to the first pair of book gills. Staining is nuclear, as expected for Pdm/Nub, and is uniform throughout these appendages, with a sharp boundary at the point where these appendages connect to the body wall. This distribution is very similar to the expression seen in crustacean gills and in *Drosophila* wings [1, 11], suggesting that the opisthosomal appendages of the horseshoe crab—including the genital operculum and book gills—are likely to be related to the particular epipod/gill that expresses Pdm/Nub in other arthropods. The endopods in these opisthosomal appendages appear to have been lost.

Pdm/Nub Expression in Spider Embryos

The book gills of *Limulus* are external structures consisting of parallel lamellae (leaflets) exposed to the external aquatic medium. The internalization of such structures into the body is envisaged to have given rise to the book lungs of terrestrial arachnids (Figure 1D; [5, 8]), so next we examined whether Pdm/Nub is also expressed in the book lungs of spiders.

Immunochemical stainings in embryos of the spider *Cupiennius salei* show that Pdm/Nub is expressed in a series of paired, segmentally repeated primordia in successive opisthosomal segments (Figures 2D–2F). The strongest and most persistent expression is seen in the primordia that will give rise to the book lungs (second opisthosomal segment), lateral tubular tracheae (third opisthosomal segment), and two pairs of spinnerets (fourth and fifth opisthosomal segments) (Figures 2E and 2F). A small group of cells can also be seen expressing Pdm/Nub weakly and transiently in the first opisthosomal segment (Figure 2E); this appears to be serially homologous to the other Pdm/Nub-expressing primordia, but smaller, and gives rise to no known structure in the adult. During late stages, staining can also be seen specifically in the folded epithelia that form the leaflets of the developing book lungs (Figure 2G). All of these stainings show nuclear localization, as expected for a transcription factor such as Pdm/Nub.

Expression of Pdm/Nub is also seen in the central nervous system (not shown) and in a set of ring-like domains along the proximo-distal axis of the developing legs in the prosomal (anterior) region of spiders (Figure 2F). These expression patterns are clearly distinguishable from expression in the primordia of book lungs, lateral tracheae, and spinnerets and are comparable to the expression seen in the developing central nervous system and endopods/legs of crustaceans and insects [1, 11, 12, 15, 16].

In order to verify the expression pattern described by immunochemical staining, we cloned a fragment of *pdm/nub* from *Cupiennius salei* by PCR with degenerate primers (as described in [1]); we subsequently recovered a cDNA clone using this PCR fragment as a probe to screen a cDNA library (sequence accession number AJ420131). Sequence comparisons suggest that we have cloned the *pdm/nub* ortholog of *Cupiennius* (see the Supplementary Material available with this article online). In situ hybridization with this cDNA as a probe confirms the Pdm/Nub expression patterns described above (our unpublished data).

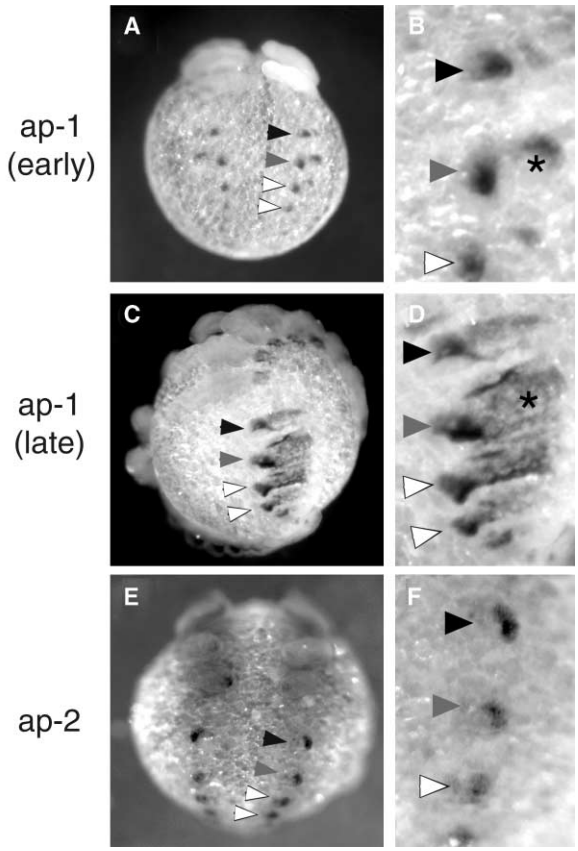

Figure 3. Expression of *apterous* Homologs in the Spider's Book Lungs, Tubular Tracheae, and Spinnerets

(A) Expression of *apterous-1 (ap-1)* in early primordia of the book lungs (black arrowhead), tubular tracheae (gray arrowhead), and spinnerets (white arrowheads) on the opisthosomal segments 2–5 of the spider *Cupiennius salei*.

(B) Higher magnification view of the same embryo, showing additional expression dorsally in relation to these primordia (asterisk).

(C) Later expression of *apterous-1* in the same opisthosomal primordia. The dorsal expression is now stronger, and it is also visible in prosomal segments (dorsally to the walking legs).

(D) Higher magnification view of the same embryo.

(E) Expression of *apterous-2 (ap-2)* in early primordia of the book lungs, tubular tracheae, and spinnerets of *Cupiennius salei*.

(F) Higher magnification view of the same embryo.

apterous Expression in Spider Embryos

To examine the expression of *apterous*, we have also cloned homologs of the *apterous* gene from embryonic cDNA of the spider *Cupiennius salei* by using PCR with degenerate primers (as described in [1], except that nested PCR was carried out with a second forward primer: 5′-GGNAAYAYHAAYTGYAARRANGAYTAYYA-3′). We found two *apterous* genes, *apterous-1* and *apterous-2* (sequence accession numbers AJ420132 and AJ420133), in the spider. Sequence comparisons indi-

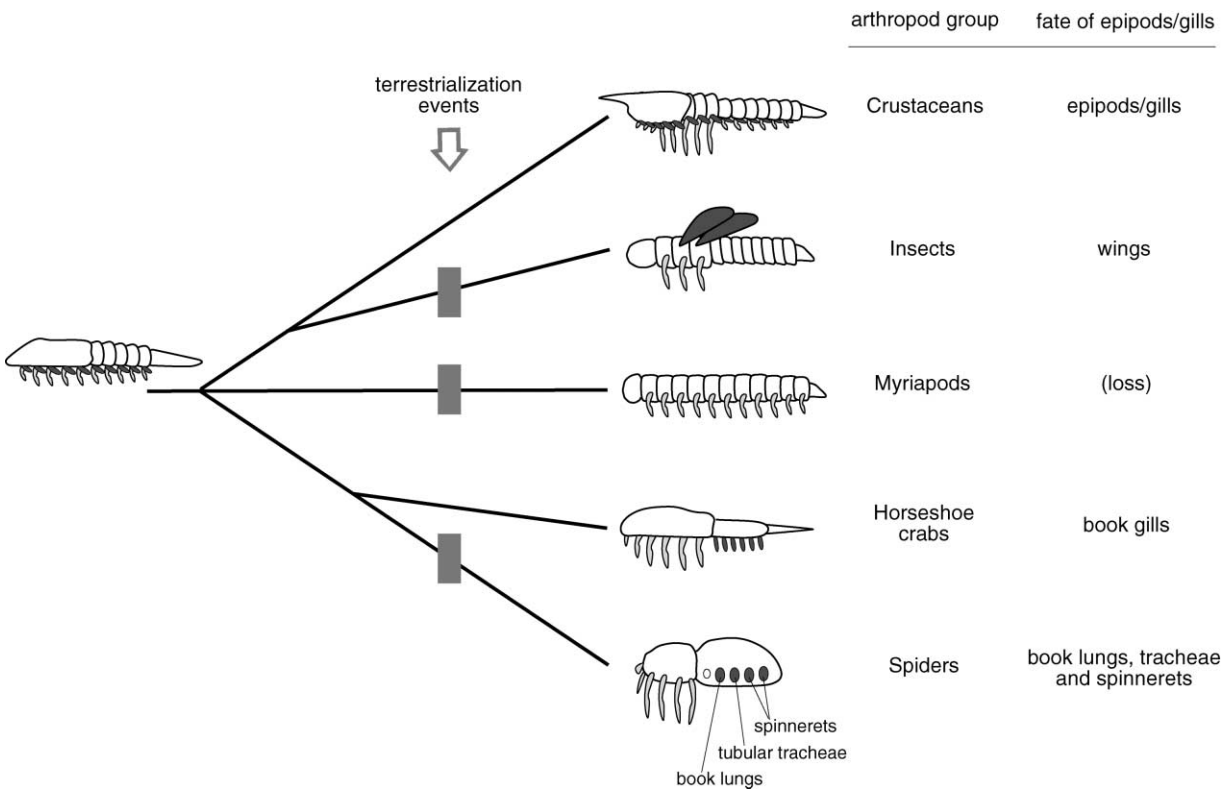

Figure 4. The Evolutionary Fate of Gills in Terrestrial Arthropods

The last common ancestors of all arthropods were aquatic creatures with branched appendages [13, 24]. The ventral branches (in light gray) of these appendages were used mostly for locomotion (e.g., legs), while the dorsal branches, called epipods (in dark gray), were used mostly for respiration and osmoregulation (gills). Endopods/legs are preserved in most arthropods. Epipods/gills are preserved in aquatic arthropods but modified or lost in terrestrial groups, as indicated in the right of the figure. In terrestrial arachnids (spiders and scorpions), a series of related primordia arise in posterior segments of the body. In spiders, the first primordium fails to develop further, the second gives rise to book lungs, the third gives rise to book lungs or to the lateral tubes of the tubular tracheae (depending on the group of spiders), and the more posterior ones give rise to the spinnerets. For simplicity, some appendages or appendage parts are not shown (e.g., antennae, exopods).

cate that both genes are orthologs of the insect and crustacean *apterous* genes (see Supplementary Material); they have probably arisen by a duplication of *apterous* in the lineage leading to spiders.

In situ hybridization in spider embryos (carried out as described in [17]) reveals that both genes are expressed strongly and specifically in the series of opisthosomal primordia that give rise to the book lungs, the lateral tubular tracheae, and the spinnerets (Figure 3) and that their expression overlaps with that of *pdm/nub*. This expression is first seen as a series of specific spots in opisthosomal segments 2–5, during mid-embryonic stages, and persists until late embryonic stages. *apterous-2* expression appears to be restricted to these primordia, whereas *apterous-1* is also expressed in an expanding patch of cells on the lateral body wall, in both prosomal and opisthosomal segments (asterisks in Figures 3B and 3D).

Origin of the Spiders' Book Lungs, Tracheae, and Spinnerets

Our study of Pdm/Nub expression in chelicerates shows that this gene has very specific expression patterns in the developing opisthosomal appendages in *Limulus* and in the primordia that give rise to book lungs, lateral tubular tracheae, and spinnerets in spiders. Furthermore, *pdm/nub* and *apterous* are coexpressed in the primordia of book lungs, tubular tracheae, and spinnerets, as they are only in particular epipods/gills in crustaceans and in insects' wings [1], suggesting that these structures could be related. Book lungs, tubular tracheae, and spinnerets arise from serially homologous primordia in different opisthosomal segments and show almost indistinguishable patterns of gene expression (Figures 2F and 3). When one bears in mind that most opisthosomal segments carried book gills in the ancestors of arachnids (as in the horseshoe crabs [5, 13]), it seems likely that all these structures derive from gills and have preserved similar mechanisms by which they are specified during development. Differences in their morphology are presumably regulated by the action of different Hox genes in each of these segments [17, 18].

In addition to expressing *pdm/nub* and *apterous*, developing book gills, book lungs, and spinnerets are also known to express *Distal-less*, a regulatory gene expressed in most arthropod appendages (including legs, antennae, most gnathal appendages, gills, and wings), consistent with the idea that these structures derive from some type of appendage [14, 18–20]. Furthermore, the primordia of these structures form at the same intra-

segmental position as other appendages, straddling the boundary that separates *engrailed*-expressing from *engrailed*-nonexpressing cells [17, 21]. These observations strongly support the idea that book lungs, lateral tubular tracheae, and spinnerets derive from modified epipods/gills of successive opisthosomal segments.

The hypothesis that these structures derive from the opisthosomal book gills of aquatic chelicerates dates back to comparative anatomical studies of the late 19th century [5–8], but until now many questions have remained open concerning the nature of these appendages and their relationship to other arthropod limbs. Our current work suggests that these organs ultimately derive from a dorsal limb branch (epipod) that was used as a gill in ancestral arthropods and was modified in different ways during terrestrialization in different arthropod groups.

On land, gills cannot function efficiently and become prone to desiccation. This explains why these structures have been modified or lost independently on a number of occasions, during the terrestrialization of different arthropod groups (Figure 4; [22]). For example, many terrestrial crustaceans have their gills in specialized chambers to protect them from desiccation, myriapods and apterygote insects appear to have lost them completely (while evolving novel tracheal systems to carry out the function of respiration on land), and pterygote insects have modified one of these gills to form wings [1, 3, 4, 13, 22]. Thus, it appears that in parallel instances of terrestrialization, the same ancestral structure has given rise to fundamentally different new organs, such as wings, book lungs, tracheal tubes, and spinnerets. Conversely, similar adaptations can sometimes have very different evolutionary histories, as in the evolution of different respiratory systems among different terrestrial groups (e.g., the de novo evolution of tracheal systems in insects and myriapods, several times independently, versus the evolution of spiders' book lungs and tracheae from gills) [13, 22].

As with any type of character (morphological or molecular), similarities in expression patterns can sometimes be misleading for determining homologies [23]. Looking for congruence among independent types of data (morphological, palaeontological, or molecular) and phylogeny is the only way to overcome this problem and trace the origin of morphological innovations such as wings, book lungs, and spinnerets after hundreds of million years of evolution. Our observations are congruent with data from comparative anatomy, palaeontology, and phylogeny [3–9], and this gives us confidence in arguing for the common origin of these structures.

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Accession Numbers

Sequences for *Cs-pdm/nub*, *Cs-ap1*, and *Cs-ap2* may be found under accession numbers AJ420131, AJ420132, and AJ420133, respectively.

Supplementary Material

Diverse Adaptations of an Ancestral Gill: A Common Evolutionary Origin for Wings, Breathing Organs, and Spinnerets

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Supplementary References

S1. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. *22*, 4673–4680.

S2. Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. *4*, 406–425.

Figure S1. Sequence Comparisons and Relationships of the Pdm/Nub and Apterous Homologs of the Spider

(A) Sequence alignment of the POU domain and homeodomain sequences of Pdm/Nub orthologs from arthropods, sea urchins, and vertebrates (Oct proteins). Dots indicate amino acid identity to *Drosophila* Pdm1.

(B) Tree depicting the sequence relationships of the spider's Pdm/Nub to other Pdm/Nub/Oct family members (class II POU-homeodomain proteins) and members of the related class III POU-homeodomains. The tree shows a clear assignment of the spider's Pdm/Nub to the Pdm/Nub/Oct family. Internal nodes in the Pdm/Nub/Oct family are not significantly resolved.

(C) Sequence alignment of conserved regions of Apterous orthologs from arthropods and vertebrates (LH2), including parts of the LIM domains, the homeodomain, and a short stretch of conserved intervening sequence. Dots indicate amino acid identity to *Drosophila* Apterous.

(D) Tree depicting the sequence relationships of the spider's Apterous-1 and Apterous-2 to other Ap/LH2 family members and members of the related ISL family of LIM-homeodomains. The tree shows a clear assignment of the spider's Apterous-1 and Apterous-2 to the Ap/LH2 family. The internal nodes within each family are not significantly resolved. Protein sequence alignments and trees were prepared with the ClustalW program [S1]. Trees were constructed by the neighbor-joining method [S2], based on unambiguously aligned positions for which sequences were available for all proteins. Accession numbers of sequences are as follows: M81957 (*Dm-pdm1*), M81958 (*Dm-pdm2*), Y09913 (*Af-pdm*), AJ420131 (*Cs-pdm/nub*), L04646 (*Sp-Oct*), X13403 (*Hs-Oct1*), M36653 (*Hs-Oct2*), X58435 (*Dm-Cf1a*), Y15070 (*Af-APH1*), X59055 (*Xl-POU2*), X65158 (*Dm-ap*), Y09914 (*Af-ap*), AJ420132 (*Cs-ap1*), AJ420133 (*Cs-ap2*), U11701 (*Hs-LH2*), U89385 (*Dm-isl*), X64884 (*Ot-isl1*), and U07559 (*Hs-isl1*).

A

POU domain

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Drosophila PDM1     PEETTDLEELEQFAKTFKQRRIKLGFTQGDVGLAMGKLYGNDFSQTTISRFEALNLSFKNMCKLKPLLQKWLDDAD
Drosophila PDM2     ..............................................................................E...
Artemia PDM                 ..................................................................E...
Spider PDM          ...M...........................................................................E...
Sea urchin OCT      V..QT.........R.....................................................................
Human OCT1          L..PS.....................................................................E...N..E
Human OCT2          ...PS.........R.....................................................E...N..E
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homeodomain

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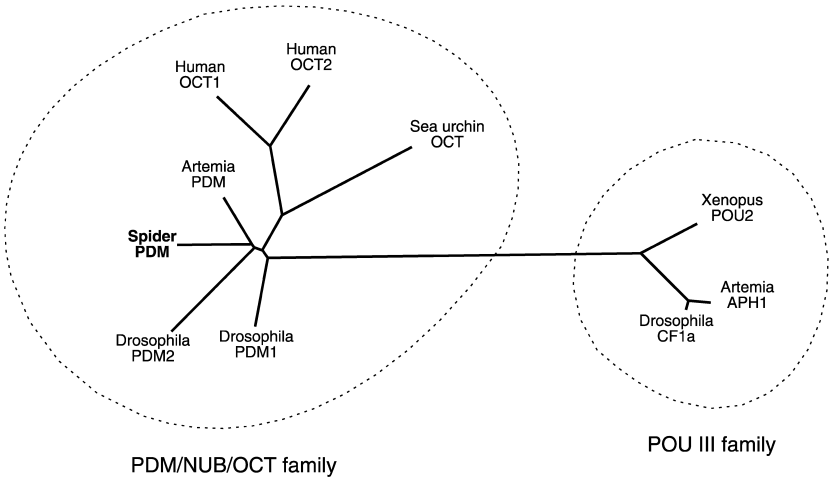
Drosophila PDM1         GRRRKKRTSIETTIRGALEKAFLANQKPTSEEITQLADRLSMEKEVVRVWFCNRRQKEKRINPS
Drosophila PDM2         .............V.TT......M.C.......S..SE..N.D...I..................
Artemia PDM             .............V.IS......G.P.......SM...G.G..............
Spider PDM              .............V.L.......Q.P.......AM..ES.A.....................P
Sea urchin OCT          N...........N..IS...S..MQP.......VL.GEQMG.....................P
Human OCT1              S...........N..V....S..E.........MI..Q.N.....I...............P
Human OCT2              .............NV.F....S............LLI.EQ.H.....I...............C
```

B



C

LIM domains

```
Drosophila AP       SFFGTRRCSRCLASISSNELVMRARNLVFHVNCFCCTVCHTPLTKGDQYGIIDALIYCRHY     QKGRPRKRK
Artemia AP          RLH.I...G..GLVLGP.DL.....DFIY.LS..T.AA.NQS.....IF.MR.GVV....F     .........
Spider AP1          RM.SVK..T..HL...PS.......EH.Y.LH..T.AT.NK.....EYF.LK.DV......     .......Q.
Spider AP2          RL.AVK..A..QRG.FA........D..Y.LH..T.AW.N.A..Q..YF.LR.N.V.....     R........
Human LH2           RR.SVQ..A..HLG..AS.M.....D..Y.L...T..T.NKM..T..HF.MK.S.V....F     .........
```

homeodomain

```
Drosophila AP       RTKRMRTSFKHHQLRTMKSYFAINHNPDAKDLKQLSQKTGLPKRVLQVW
Artemia AP          ........................Q..........A.....S.......
Spider AP1          .P.............A.........................S.......
Spider AP2          ........................Q..........AS....S.......
Human LH2           K..............................A.....T.......
```

D