Evolution of the entire arthropod *Hox* gene set predated the origin and radiation of the onychophoran/arthropod clade

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Background: Dramatic changes in body size and pattern occurred during the radiation of many taxa in the Cambrian, and these changes are best documented for the arthropods. The sudden appearance of such diverse body plans raises the fundamental question of when the genes and the developmental control systems that regulate these designs evolved. As *Hox* genes regulate arthropod body patterns, the evolution of these genes may have played a role in the origin and diversification of the arthropod body plan from a homonomous ancestor. To trace the origin of arthropod *Hox* genes, we examined their distribution in a myriapod and in the Onychophora, a sister group to the arthropods.

Results: Despite the limited segmental diversity within myriapods and Onychophora, all insect *Hox* genes are present in both taxa, including the trunk *Hox* genes *Ultrabithorax* and *abdominal-A* as well as an ortholog of the *fushi tarazu* gene. Comparative analysis of *Hox* gene deployment revealed that the anterior boundary of expression of trunk *Hox* genes has shifted dramatically along the anteroposterior axis between Onychophora and different arthropod classes. Furthermore, we found that repression of expression of the *Hox* target gene *Distal-less* is unique to the insect lineage.

Conclusions: A complete arthropod *Hox* gene family existed in the ancestor of the onychophoran/arthropod clade. No new *Hox* genes were therefore required to catalyze the arthropod radiation; instead, arthropod body-plan diversity arose through changes in the regulation of *Hox* genes and their downstream targets.

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Background

The sudden appearance and remarkable diversity of complex animals in the Cambrian has prompted extensive paleontological [1,2], comparative [3,4] and molecular systematic [5,6] studies of animal relationships and the origin and evolution of metazoan body plans. Current debate is focused on three central issues: the origin of taxa (cladogenesis), the variety of basic designs (morphological disparity) and the evolution of the developmental control systems that regulate these designs [7–9]. Comparisons of evidence from fossils and molecular studies may help to reveal whether the Cambrian marks the true origin of many higher taxa or whether major lineages diverged well before the Cambrian [7–10]. In either of these scenarios, it appears that dramatic changes in body size, pattern and diversity occurred during the radiation of many taxa in the Cambrian, raising a fundamental question: which new developmental mechanisms [11] and genetic information were responsible for the evolution and diversification of larger and more complex animals?

The analysis of arthropods and their relatives may present the best opportunity for an integrated approach to the problem of the evolution of body plans: the fossil record of arthropods is relatively abundant; arthropods are the most speciose and morphologically diverse taxa; and many recent advances in developmental genetics have emerged from the study of one arthropod, *Drosophila melanogaster*. Paleontological, comparative and molecular systematic evidence suggests that arthropods are monophyletic [3,12,13] and descended from a lobopodian ancestor [14]. The most striking trend in the evolution of the lobopodian/arthropod clade has been the diversification of segment types, from the simplicity of some Cambrian lobopodians, with four types of unjointed appendages and a uniformly patterned (homonomous) trunk [14–16], to the complexity of some extant crustaceans and insects, with as many as ten distinguishable appendage types and a highly diversified trunk.

As segmental diversity in highly derived insects such as *Drosophila* is regulated by eight *Hox* genes, it has been postulated that primitive arthropods possessed a more limited set of *Hox* genes which expanded during the course of arthropod and insect evolution [17,18]. Comparative studies of *Hox* genes in various metazoans indicate that many *Hox* genes predate the origin of the insects

[19,20]. The common ancestor of arthropods and vertebrates possessed five or six *Hox* genes [21–23], and most of the insect *Hox* genes were present in the annelid/arthropod ancestor [24–29]. Importantly, arthropods have two unique *Hox* genes, *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*), which are not found in vertebrates [21] or annelids [24,26,27]. The *Ubx* and *abd-A* genes are present in both crustaceans [30] and insects [19,20], the two arthropod classes that have the greatest segmental diversity. It is possible that the evolution of these two *Hox* genes facilitated the diversification of trunk segments during the evolution of diverse arthropods from an ancestor with homonomous trunk segments, a process that included the subdivision of the insect body plan into thorax and abdomen (tagmosis). In this study, we ask the following questions: when did all of the arthropod *Hox* genes arise, and was this before or during the arthropod radiation? And, if *Ubx* and *abd-A* are present in animals with homonomous trunks, how are these genes deployed?

Results and discussion

Myriapod and onychophoran *Hox* genes

To address these questions, we first examined the *Hox* genes from a myriapod, the scolopendromorph centipede *Ethmostigmus rubripes*. In order to identify *Hox* orthologs unambiguously, gene sequences obtained from an initial PCR survey were further extended using vector ligation and degenerate PCR. Characteristic residues within and flanking the homeodomain enabled the identification of centipede orthologs of the *labial (lab)*, *Deformed (Dfd)*, *Sex combs reduced (Scr)*, *Antennapedia (Antp)*, *Ubx* and *abd-A* genes (Figure 1a). The presence of the trunk genes *Ubx* and *abd-A* in the homonomously organized centipede demonstrates that these genes are not unique to tagmatized arthropods, and suggests that they might have been present in even more primitive arthropods.

In order to trace the origin of the *Ubx* and *abd-A* genes further back through evolution, we cloned the *Hox* genes from the Australian onychophoran *Acanthokara kaputensis*. As the Onychophora are considered to be a sister group to the arthropods [2,3] with a fossil record extending back to the late Early Cambrian, the complement of *Hox* genes shared between Onychophora and arthropods would reflect the condition prior to the origin and radiation of the arthropods and perhaps the entire lobopodian/arthropod clade. A PCR survey and the analysis of larger genomic clones revealed that this onychophoran possesses candidate orthologs of all of the *Hox* genes found in *Drosophila*. Sequence alignments have allowed the identification of onychophoran *lab*, *proboscipedia* (*pb*), *Dfd*, *Scr*, *Ubx*, *abd-A*, *Abd-B*, and an additional *Hox* gene which, by virtue of its sequence and by process of elimination, is presumably the *Antp* gene (Figure 1a). The onychophoran *Hox* genes are more similar (in deduced amino-acid sequence) to their arthropod orthologs than to annelid *Hox* genes (Figure 1a), a finding that is consistent with a closer affinity between Onychophora and arthropods than between either group and annelids [2,3]. In particular, the conserved regions of Onychophora *Ubx* (*O-Ubx)* and Onychophora *abd-A* (*O-abd-A)* extend into the sequence downstream of the homeodomain, thus identifying these genes to be orthologs of the arthropod *Ubx* and *abd-A* genes.

Onychophoran *Ubx* gene structure

Drosophila Hox gene structure is unusually complex when compared with vertebrates and annelids. Because the analysis of onychophoran *Hox* genes could shed light on the nature of *Hox* gene structure before the arthropod radiation and the evolution of insects, we cloned and characterized the entire reading frame of *O-Ubx* (Figure 1b). The O-Ubx protein is encoded by two exons; the first exon contains three short regions of amino-acid similarity with the first exon of *Drosophila Ubx*, but is about 120 amino acid residues shorter than the *Drosophila* equivalent (Figure 1b). A single small intron is located in a conserved position just upstream of the homeodomain-containing exon (Figure 1b). Thus, the *O-Ubx* gene is much more compact (~2.5 kb) than the equivalent region of the *Ubx* gene in *Drosophila* (73 kb) [31], and is more similar in size and organization to chordate *Hox* genes.

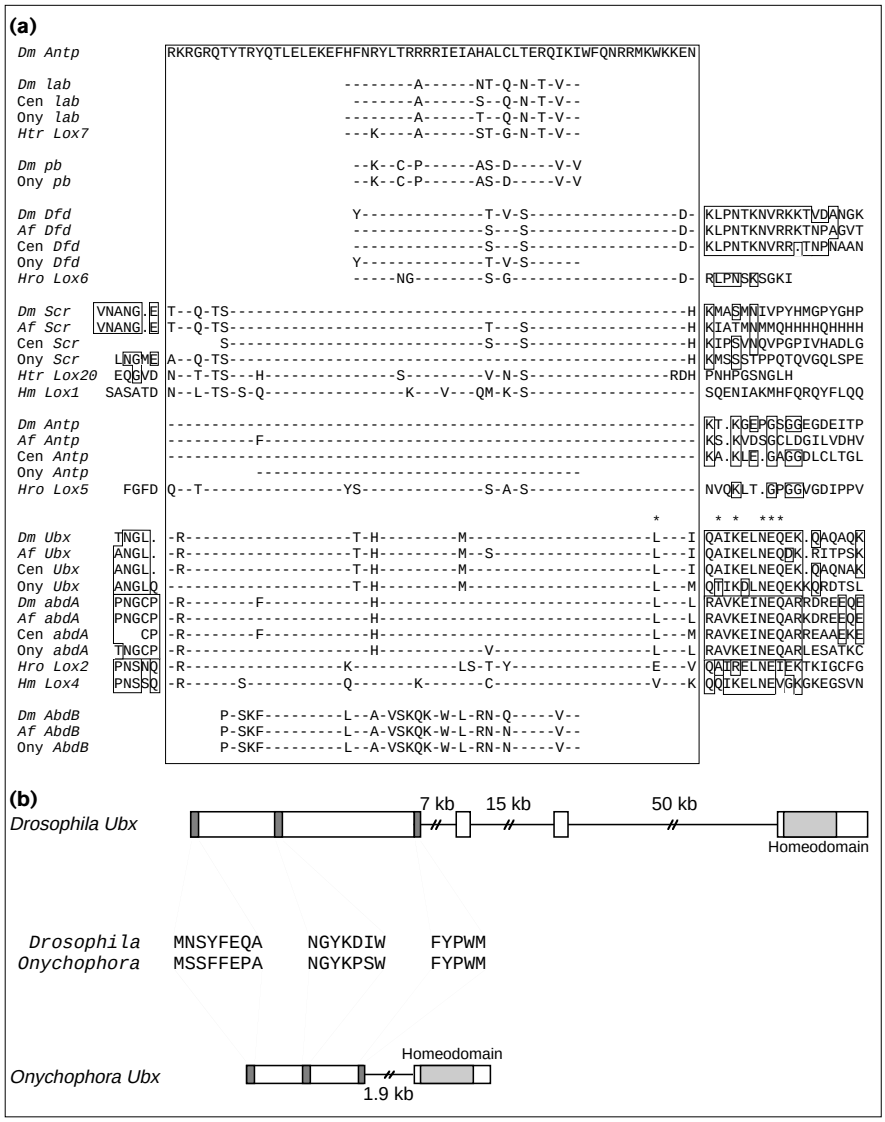
Independent *Hox* gene duplication events in the annelid and onychophoran/arthropod lineages

The presence of *Ubx* and *abd-A* orthologs in Onychophora and their absence in annelids suggests that these two genes have arisen more recently than other *Hox* genes. To analyze the origins of *Ubx* and *abd-A*, we aligned the amino-acid sequences of the homeodomain and flanking regions of onychophoran and arthropod Ubx and abd-A proteins and the annelid Lox2 [28,29] and Lox4 proteins [26] to construct a gene tree. Both parsimony and distance analyses suggest that a single *Hox* gene precursor existed in the common ancestor of both annelids and the onychophoran/arthropod clade and that this gene duplicated and diverged independently in each lineage to create the gene pairs *Ubx/abd-A* and *Lox2/Lox4* ([26,27]; Figure 2a). The presence of *Ubx* and *abd-A* in the onychophoran shows that the duplication event that created these genes occurred before the separation of the onychophoran and arthropod lineages and the diversification of arthropod body plans.

The *ftz* gene predates the arthropods

The centipede and the onychophoran each possess an additional *Antp*-class gene that is not obviously orthologous to any *Drosophila Hox* gene. Alignment of these centipede and onychophoran genes with the *Drosophila fushi tarazu* (*ftz*) gene [32], other insect *ftz* orthologs [33,34], and the crustacean *Hx1* gene [30] along with gene-tree analysis suggests that the centipede and onychophoran genes are most closely related to each other and to the *ftz*-related genes and

Figure 1



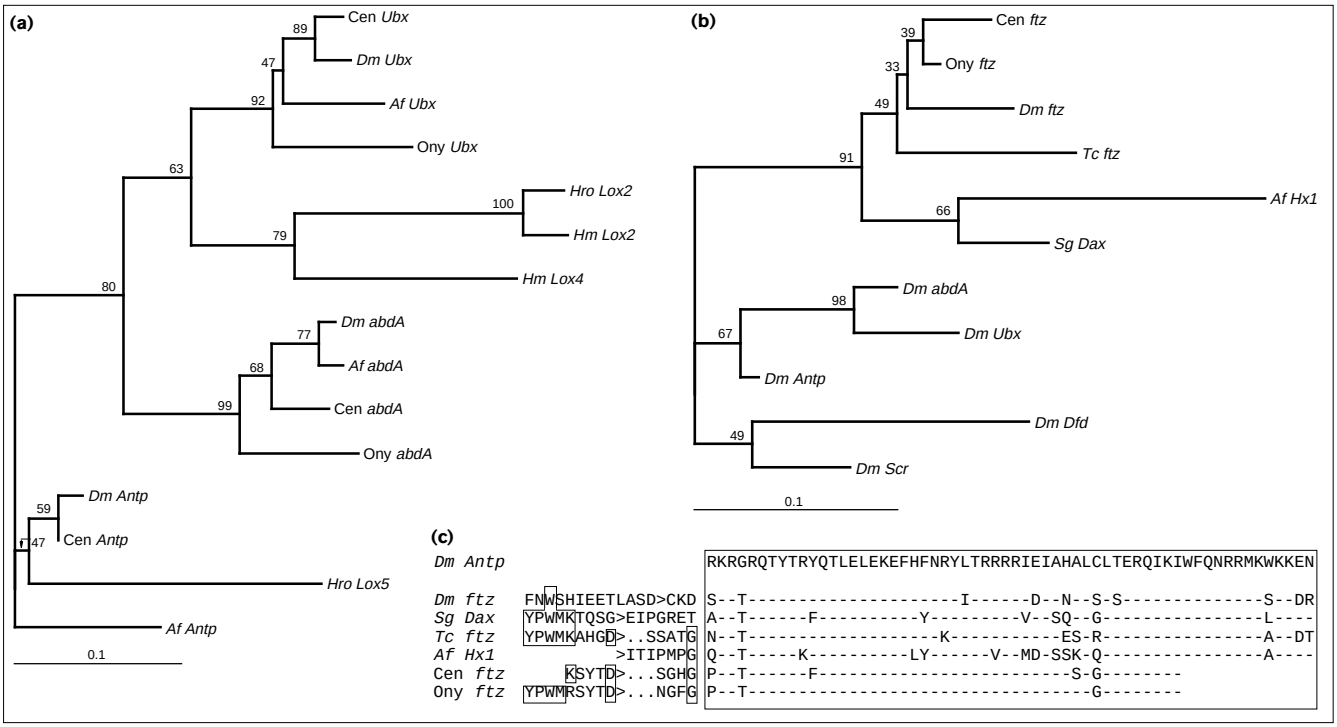
Identification of centipede and onychophoran *Hox* gene orthologs. **(a)** Alignment of the homeodomain and flanking sequences of arthropod [30], onychophoran and annelid [26–29,44] *Hox* genes. Orthologous genes are grouped except for the *Ubx/abd-A/Lox2/Lox4* genes which are shown together to facilitate direct comparison. Sequences that extend beyond the homeodomain and flanking regions of homology are truncated. Within the homeodomain, residues identical to the *Drosophila Antp* gene are indicated with dashes. The onychophoran *Antp* gene was identified on the basis of its sequence homology to insect *Antp* genes and is ruled out as an *Scr* candidate because a large unambiguous *Scr* clone was obtained. In the regions flanking the homeodomain, boxed residues indicate identity among a majority of arthropod sequences (*Lox2/Lox4* residues are boxed when they match either the arthropod *Ubx* or *abd-A* majority). The epitope recognized by the FP6.87 monoclonal antibody is marked by asterisks. Gaps in the alignment are indicated by a full stop. Intron/exon boundaries are inferred from consensus splice sequences. *Af* = *Artemia franciscana*; Cen = centipede; *Dm* = *Drosophila melanogaster*; *Hm* = *Hirudo medicinalis*; *Hro* = *Helobdella robusta*; *Htr* = *Helobdella triserialis*; Ony = Onychophora. **(b)** Comparison of the genomic structure and conserved motifs of the *Drosophila* and onychophoran *Ubx* genes. The coding regions of the *Drosophila* and onychophoran *Ubx* exons are shown as open boxes separated by introns of the indicated sizes. The 3′ exon of each gene contains the homeodomain (light grey region), and the 5′ exon encodes three motifs that are shared between the *Drosophila* and onychophoran genes: MxSxFExA, NGYKxxW, and FYPWM (dark grey boxes; exact sequences for each motif shown in alignment). The onychophoran *Ubx* intron does not appear to contain any microexons similar to those in *Drosophila Ubx*.

thus we suggest that they are *ftz* orthologs (Figure 2b,c). This group of genes shows significantly more amino-acid variation within the homeodomain than typical *Hox* genes. However, all of these genes except *Drosophila ftz* have the YPWM tetrapeptide motif, suggesting that they are related to *Hox* genes. As no *ftz* orthologs have yet been identified outside of Onychophora and arthropods, *ftz* may represent a rapidly evolving *Hox* gene unique to this clade.

Evolution of *Hox* gene regulation in animals with homonomous trunks

The complement of myriapod and onychophoran *Hox* genes demonstrates that the segmental diversity of arthropods evolved without an increase in *Hox* gene number. The evolution of arthropod segmental diversity must therefore have involved regulatory changes in *Hox* genes and/or their targets. More specifically, as some of the major differences between arthropods involve the tagmosis and segmental diversity of the trunk, it is possible that the regulation of *Ubx* and *abd-A* could differ significantly between different arthropod classes. To address this possibility, we examined the expression domains of *Ubx* and *abd-A* in the centipede embryo. Using the monoclonal antibody FP6.87 [35], which recognizes an epitope found in both the centipede Ubx and abd-A proteins (Figure 1a), we observed antigen expression over almost the entire trunk of the embryo (Figure 3a,b). The initial anterior boundary of *Ubx/abd-A* expression in the body wall is in the T2 segment and appears parasegmental (Figure 3a inset), and in later stages of development the boundary shifts

Figure 2



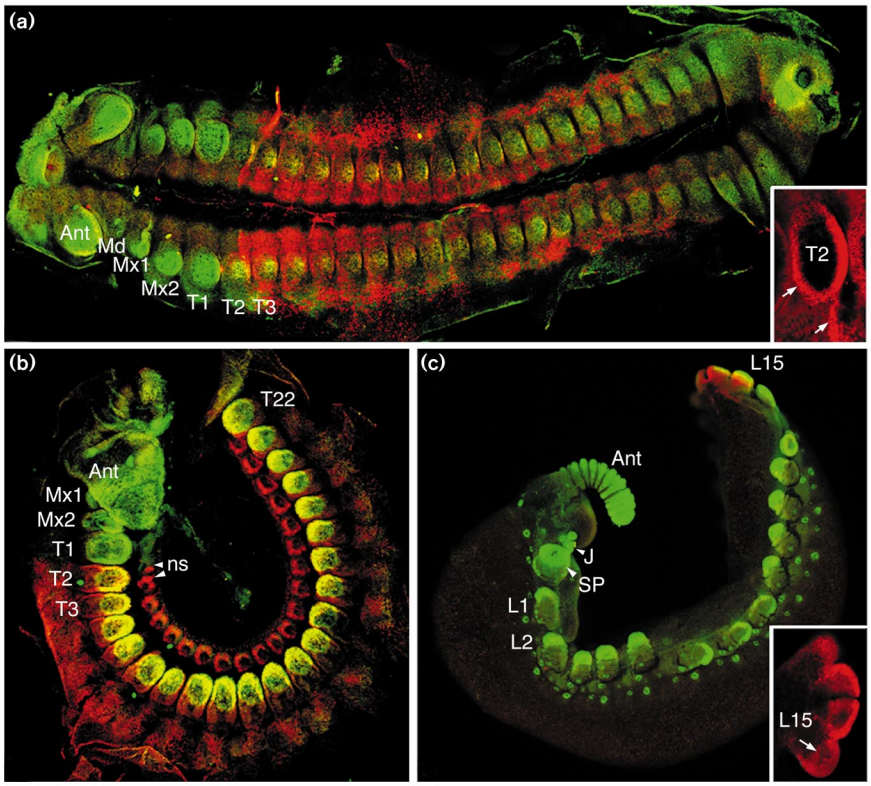
The *Ubx*, *abd-A*, and *ftz* genes are unique to the onychophoran/arthropod clade. **(a)** Phylogenetic gene tree indicating the relationships of *Ubx, abd-A, Lox2* and *Lox4*. The orthology relationships of the onychophoran *Ubx* and *abd-A* genes are highly supported, as indicated by the monophyly of the *Ubx* and *abd-A* genes of onychophora/arthropods. This and other phylogenetic analyses [26,27] suggest that the gene pairs *Ubx/abd-A* and *Lox2/Lox4* arose from independent duplications in the Onychophora/arthropod lineage and the annelid lineage, respectively. *Antp* orthologs from both arthropods and annelids are used as the outgroup. **(b)** Monophyly of *ftz* orthologs. The orthology of onychophoran and arthropod *ftz* genes [30,32–34] is indicated on a phylogenetic gene tree with the *Antp*-class *Drosophila* homeodomain sequences used as an outgroup. For all trees, branch lengths are proportional to the changes per amino-acid position (scale shown) and bootstrap values calculated by least-squares distance methods are shown at each node. *Af* = *Artemia franciscana*; Cen = centipede; *Dm* = *Drosophila melanogaster*; *Hro* = *Helobdella robusta*; *Hm* = *Hirudo medicinalis*; Ony = onychophoran; *Sg* = *Schistocerca gregaria*; *Tc* = *Tribolium castaneda*. **(c)** Alignment of *ftz* orthologs. Amino-acid sequences are aligned with *Drosophila Antp* from the YPWM tetrapeptide motif through the homeodomain. The locations of the introns are indicated by (>) and gaps are indicated by a full stop. The presence of the YPWM motif in the centipede gene is implied by the primer used to clone this sequence (see Materials and methods).

forward to the anterior of the T2 segment (Figure 3b). The *Ubx/abd-A* boundary correlates with the transition in appendage morphology from the poison claw (T1 segment) to the first walking leg (T2 segment; Figure 3b).

The onychophoran body plan and appendage organization are simpler than that of arthropods, with only three distinct head segments followed by a series of homonomous trunk segments, and may reflect a more primitive condition. Given the expression of *Ubx/abd-A* across the trunk of myriapods and crustaceans [36,37], one might expect that these *Hox* genes would be expressed across the lobopod-bearing trunk. We again used the monoclonal antibody FP6.87 to determine the deployment of *Ubx/abd-A* in onychophoran embryos. Although there is a substitution in the presumed epitope recognized by this antibody in O-Ubx (Figure 1a), the antibody recognizes both the O-Ubx and O-abd-A proteins (see Materials and methods). Surprisingly, expression of *Ubx/abd-A* is restricted to the very posterior end of the onychophoran embryo, in the last pair of lobopods and in the terminus (Figure 3c). This *Ubx/abd-A* boundary may correlate with a cryptic transition in segmental identity in this species, as the last lobopod of some fossil lobopodians and extant onychophorans is truncated or vestigial [15,38].

The marked difference in the expression patterns of *Ubx/abd-A* in the centipede and the onychophoran indicates that their homonomous trunks are not regulated by the same *Hox* genes. Although the deployment of *Ubx/abd-A* in the common ancestor of Onychophora and arthropods cannot be determined, it is clear that the anterior boundary of *Ubx/abd-A* expression must have shifted along the antero-posterior axis in one or both lineages. This shift might have been achieved by changes in the regulation of *Hox* genes and/or by the addition or loss of segments. Importantly, the boundary of *Ubx/abd-A* expression consistently correlates

Figure 3



Regulation of *Ubx/abd-A* expression in embryos with homonomous trunks. Double-label antibody stains of *Ubx/abd-A* (red) and *Dll* (green) expression in centipede and onychophoran embryos. **(a)** Stage 4 centipede embryo [45] shown with anterior to the left. *Dll* is expressed in all body outgrowths, including the antenna (Ant), mouthparts (Md, mandible; Mx1, Maxillary segment 1; Mx2, Maxillary segment 2), maxilliped (T1) and walking legs (T2–T22) [45]. The anterior boundary of *Ubx/abd-A* expression lies in T2, where staining appears to be parasegmental in the body wall but the entire T2 limb stains. (Inset: the arrows indicate expression around the base of the limb bud and the anterior boundary in the body wall.) The overlap of *Ubx/abd-A* and *Dll* expression in the trunk limbs is shown in yellow. **(b)** The right side of a slightly older centipede embryo is shown with anterior at the top left. The larger antenna obscures the mandibular and maxillary segments. The anterior boundary of *Ubx/abd-A* has moved to the anterior of the T2 segment in the body wall, but staining in the nervous system (ns) is apparently parasegmental. *Ubx/abd-A* expression continues to the posterior of the embryo at this stage. **(c)** Onychophoran embryo shown with anterior at the top left. The antenna (Ant), jaws (J), slime papillae (SP), lobopods (L1–L15) and some cells in the body wall express *Dll*. *Ubx/abd-A* are expressed in the last pair of lobopods (L15) and in the terminus. A higher magnification view of the posterior segments (inset) shows that the *Ubx/abd-A* boundary lies within the segment bearing the last lobopods (arrow) and that the antigens are localized, as expected, to cell nuclei. *Dll* and *Ubx/abd-A* are co-expressed in the last lobopod and in some cells in the terminus; this overlap of *Ubx/abd-A* and *Dll* expression is shown in yellow.

with a transition in appendage morphology in the trunk of each animal ([36,37] and summarized in Figure 4b). Our data suggest that the expression domains of *Ubx/abd-A* do not demarcate homologous body regions in these taxa, but instead, evolutionary changes in *Ubx/abd-A* deployment underlie the diversification of arthropod body plans that are evident in the Cambrian fossil record.

Hox regulation of specific target genes differs between taxa

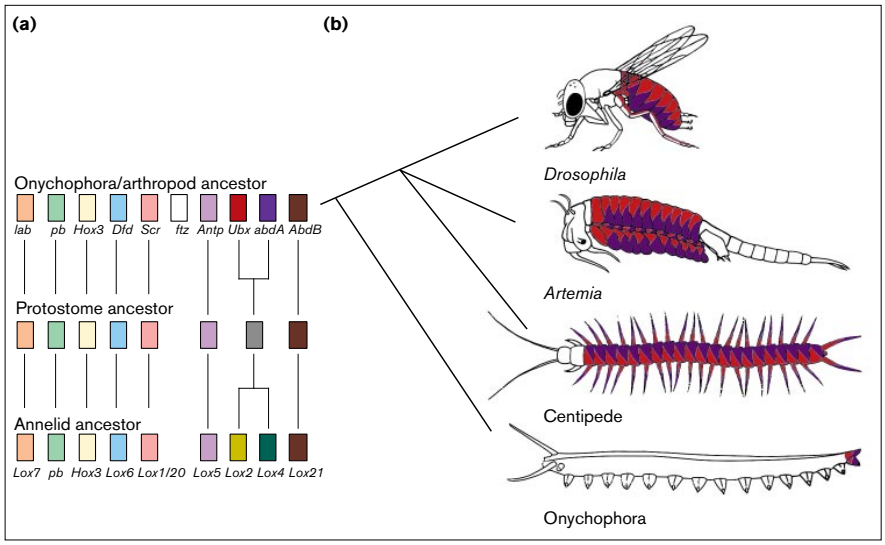
The *Hox* genes control segmental identity by regulating the expression of downstream target genes. In insects, for example, the *Ubx* and *abd-A* gene products suppress limb formation in the abdomen via repression of the *Distal-less (Dll)* gene [39]; however, *Dll* is not repressed in the *Ubx/abd-A* domains in centipede (Figure 3a,b) or onychophoran trunks (Figure 3c) at the stages we surveyed, nor in crustacean trunks [37,39], providing evidence that *Hox*-mediated repression of *Dll* evolved in the insect lineage to sculpt the distinctive limbless insect abdomen. Thus, the evolution of *Hox* regulation of target genes is a second developmental mechanism underlying the diversification of arthropod body plans.

Conclusions

Our results demonstrate that the entire set of arthropod *Hox* genes was present in the onychophoran/arthropod ancestor, and that no new *Hox* genes were required to catalyze the radiation of the arthropods. Instead, changes in the regulation of trunk *Hox* genes along the anteroposterior axis and in *Hox* regulation of major downstream target genes appear to have enabled the morphological diversification of arthropod body patterns. Indeed, if certain phylogenies that depict Onychophora as a primitive lobopodian are correct [14], the diversification of arthropod *Hox* genes would predate the lobopodian radiation as well. The fossil record of Onychophora, other lobopodians and arthropods extends to the Late Early Cambrian (~530 million years ago) [14,15] and the appearance of these taxa is contemporaneous with the 'explosion' of other taxa. *Hox* gene diversification must then have predated this period and could extend to the base of the Cambrian (~543 million years ago) or earlier.

Three new *Hox* genes did arise early in the onychophoran/arthropod clade before the divergence of the Onychophora and the arthropod radiation (Figure 4a). The *Ubx*, *abd-A* and *ftz* genes may represent synapomorphies

Figure 4



Hox gene evolution and the regulation of *Ubx/abd-A* expression in arthropods and onychophorans. **(a)** The *Hox* genes expanded in both the annelids and the onychophoran/arthropod clade by independent duplication events in each lineage. The *Ubx, abd-A* and *ftz* genes are unique to the Onychophora and arthropods and did not exist in the ancient protostome ancestor of annelids and Onychophora/arthropods. Some annelid *Lox* genes are not shown. **(b)** The expression domains of *Ubx* (red) and *abd-A* (purple) are indicated on the body plans of several arthropods and an onychophoran. Where the expression domains of *Ubx* and *abd-A* overlap (*Drosophila, Artemia*) or are not yet distinguishable (centipede and onychophoran), both red and purple are shown. Transient or low level patterns of *Ubx* expression (for example, in *Drosophila* T2 segment) [46] are not shown. The anterior boundary of *Ubx/abd-A* differs between the arthropod classes and the Onychophora, and correlates with transitions in appendage morphology along the anteroposterior axis in each animal: in insects (*Drosophila*), the boundary falls in T3, between the thorax (walking legs) and abdomen (no appendages), and the wing and haltere; in crustacea (*Artemia*), the boundary is between gnathal and thoracic segments that bear distinct limbs; in centipedes, the boundary is located between the poison claw (T1 segment) and the first walking leg (T2 segment); and in Onychophora, the boundary lies between the penultimate and terminal lobopod. The differences in the *Ubx/abd-A* expression domains among arthropods and Onychophora suggest that the evolution of arthropod segmental diversity arose through changes in the regulation of *Hox* genes.

(shared derived characters) for the onychophoran/arthropod clade, as no clear orthologs have yet been identified in other taxa. This raises the exciting possibility that the presence of these genes could be used as phylogenetic tools to resolve the relationship of arthropods to other taxa. The traditional view of annelids and arthropods as sister taxa has been challenged [4] and most protostome relationships are not resolved [2]. Thorough characterization of the *Hox* genes from other protostomes might help to identify the sister taxa of the lobopodian/arthropod clade.

Materials and methods

Cloning of Hox *genes*

Centipede and onychophoran adult DNA was purified from carcasses after the gut contents were removed to prevent potential contamination with prey items. *Hox* gene fragments were amplified from centipede and onychophoran genomic DNA using the degenerate *Hox* primers A, B, E and F [40,41]. Onychophoran *abd-B* was amplified using the *Hox* F primer and two nested *abd-B* specific primers (RNK-3 and RNK-4) [36]. PCR products were ligated into the TA Cloning vector pCR2.1 (Invitrogen). Multiple copies of all PCR products were sequenced, however, only a single clone of *Dfd* was recovered from each species. Two different nucleic acid sequences encoding the same *O-abd-B* amino-acid sequence were obtained. A *caudal* ortholog from Onychophora and an *engrailed* ortholog from the centipede were also identified (data not shown). No orthologs of *Hox3* were identified, perhaps because of substitutions in the *Hox* degenerate primer binding sites. Similarly, no centipede *pb* or *Abd-B* orthologs were cloned, but their presence in Onychophora and chordates demonstrates their existence before the evolution of arthropods. DNA flanking the initial centipede and onychophoran *Hox* clones was isolated by vector-ligation PCR. Genomic DNA was digested with restriction enzymes and ligated and into pBluescript (Stratagene) or pCR2.1. DNA was then amplified using nested gene-specific primers designed from previously isolated PCR products and vector-specific primers. *Hox* gene products were detected by Southern blotting analysis using ^{32}P-labelled DNA encoding the *Drosophila*, centipede or onychophoran homeodomains as probes. The centipede *ftz* gene fragment was amplified using a degenerate primer for the PQIYPWM (single-letter amino-acid code) sequence (dGAGGATC-CCCNCARATHTAYCCNTGGATG) and the F primer. The centipede *Scr* sequence was extended in the 5′ direction using a degenerate primer recognizing ETKRQRT (dGACACNAARMGNCARMGNAC) in nested PCR with the *Hox* B and F primers and a gene-specific primer.Onychophora *Ubx* (2648 bp) and *Scr* (1296 bp) genomic clones were isolated from two subgenomic lambda gt10 libraries after identifying bands of interest on Southern blots of *Eco*RI-digested genomic DNA.

GenBank accession numbers

Centipede gene sequences have accession numbers AFO10172-–AFO10179. Onychophoran gene sequences have accession numbers AFO11272–AFO11282.

Gene trees

Gene trees were constructed with PHYLIP (Phylogeny Inference Package v3.572c). For the *Ubx/abd-A/Lox2/Lox4* tree, up to 83 amino acids containing the homeodomains and flanking conserved sequences were aligned as shown in Figure 1a. The *ftz* alignment consisted of homeodomain sequences only (60 amino acids). The Seqboot program resampled each data set 100 times for bootstrap analysis. The Protdist program calculated distances using a PAM–Dayhoff distance matrix [42] and the Fitch program used the Fitch–Margoliash least-squares model to search for the best trees. Maximum parsimony trees were constructed with the Protpars program. Bootstrap values were calculated with the Consense program.

Immunohistochemistry and analysis of antibody reactivity

Immunohistochemical staining with the FP6.87 and *Dll* antibodies was performed as previously described [37,43]. In order to examine the

reactivity of *O*-Ubx with the FP6.87 antibody, the entire exons encoding the homeodomains of *O*-Ubx and *O*-abd-A were cloned in-frame into the pET28a protein expression vector (Novagen) and expressed in *E. coli* strain BL21 DE3. Western blots of the induced lysates containing onychophoran homeodomain fusions were then assayed with the monoclonal antibody FP6.87 [35]. The putative epitope for this antibody has been mapped to six amino acids that are shared between Ubx and abd-A but are not present in Antp [35,36]. There is a single amino-acid replacement in one of these six residues in *O*-Ubx (Figure 1a). The *O*-Ubx and *O*-abd-A proteins were detected equally well by the FP6.87 antibody by western blotting analysis, indicating that the observed *in situ* immunoreactivity includes both proteins.

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