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ORIGINAL ARTICLE

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Evolution of insect abdominal appendages: are prolegs homologous or convergent traits?

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Abstract Many insects possess abdominal prolegs, raising the question of whether these prolegs are homologous or convergent structures. One way to address this issue is to compare mechanisms controlling the development of prolegs in different insects. Segmental morphologies along the insect body are controlled by the regulatory activities of the Hox proteins, and one well-studied regulatory target is the *Distal-less* (*Dll*) gene, which is required for the development of distal limb structures in arthropods. In *Drosophila* abdominal segments, *Dll* transcription is prevented by Hox proteins of the Bithorax Complex (BX-C). In lepidopteran abdominal segments, circular holes lacking BX-C protein expression allow Dll to be expressed and prolegs to develop. For comparison, we examined protein expression patterns in two species of sawfly from the hymenopteran suborder Symphyta; these insects develop prolegs on all abdominal segments. Interestingly, sawfly prolegs did not express Dll protein at any time, and expressed BX-C proteins throughout development. These results suggest that sawfly prolegs lack distal elements that are present in lepidopteran prolegs. Consistent with this interpretation, the proximal determinant *extradenticle* (*exd*) was present in cell nuclei all of the way to the tip of the sawfly proleg, whereas it was not detectable in the nuclei of cells near the tip of the lepidopteran proleg. Our results support the hypothesis that larval prolegs have evolved independently in the Lepidoptera and Hymenoptera.

Keywords Evolution · Development · Homology · Limb · Arthropod

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Introduction

Larvae of many holometabolous insects possess abdominal appendages, known as prolegs (Snodgrass 1935; Nagy and Grbic 1999). There is considerable diversity in the distribution of prolegs on the insect body, with a wide range of variation in both segmental arrangement and number. Natural selection for function in the larval environment appears to determine whether prolegs are present or absent (Nagy and Grbic 1999). For example, dipteran species that develop prolegs appear to have life history traits that make abdominal appendages useful, such as aquatic habitats or predatory needs. This raises the question of whether prolegs are convergently evolved structures or homologous structures that have been modified and/or lost subsequently in some lineages.

One way of addressing whether prolegs are homologous or convergent is to compare the mechanisms underlying their development. If different taxa develop prolegs using different developmental mechanisms, this would support the hypothesis that prolegs have evolved convergently.

Recent advances in molecular techniques have shed considerable light on the genetic processes that control the development of *Drosophila* limbs. The regulatory activities of the Hox proteins play a key role in determining segmental morphologies along the insect body, including the presence or absence of limbs (Lewis 1978; Kaufman et al. 1980). The development of distal limb structures in arthropods is controlled by a Hox regulatory target, the *Distal-less* (*Dll*) gene (Cohen et al. 1989; Panganiban et al. 1995; Schoppmeier and Damen 2001). In fly abdominal segments, *Dll* transcription is prevented when Hox proteins of the BX-C bind to *cis*-regulatory elements located upstream of the *Dll* transcription start site (Vachon et al. 1992; Castelli-Gair and Akam 1995; Estrada and Sánchez-Herrero 2001).

Previous evolutionary comparisons have suggested that the regulatory interactions suppressing the development of abdominal appendages evolved in two steps within the insects (Palopoli and Patel 1998; Grenier and

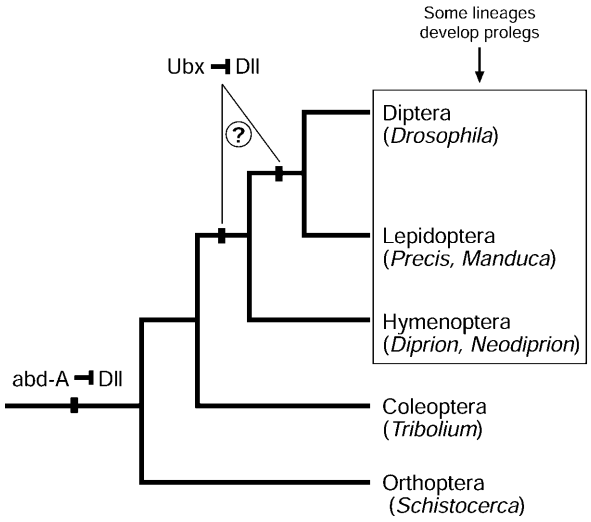

Fig. 1 Evolution of *Distal-less* (*Dll*) repression in the insect abdomen by the Hox proteins of the Bithorax Complex. The repression of *Dll* by *abdominal-A* (*abd-A*) appeared before the evolution of holometabolous insects. The repression of *Dll* by *Ultrabithorax* (*Ubx*) could have evolved either before or after the divergence of the Hymenoptera from the Diptera and Lepidoptera

Carroll 2000; Lewis et al. 2000; Fig. 1). In non-insect arthropods, there appears to be no repression of *Dll* transcription by proteins of the Bithorax Complex (BX-C), and ventral appendages develop on many of the abdominal segments that express BX-C proteins (Averof and Akam 1995; Panganiban et al. 1995; Averof and Patel 1997; Grenier and Carroll 2000). The same appears to be true in one of the most ancient groups of insects, the Collembola (Palopoli and Patel 1998). In the more recently derived Orthoptera and Coleoptera, abdominal-A (abd-A) protein has gained the ability to repress *Dll* transcription, and this prevents limbs from developing in segments posterior to the first abdominal segment (A1). The ability of Ultrabithorax (Ubx) protein to repress *Dll* appears to have evolved in a second step, after the Coleoptera had diverged from the other holometabolous insect orders (Palopoli and Patel 1998; Lewis et al. 2000). In agreement with this model, both Ubx and abd-A appear to repress *Dll* transcription in the Lepidoptera, which are closely related to the Diptera. In the butterfly *Precis coenia*, holes appear in the abdominal Ubx/abd-A expression domains, and this is thought to allow *Dll* to be expressed and prolegs to develop (Warren et al. 1994).

Based on phylogenetic analyses, it has been hypothesized that abdominal appendages are leg homologs that are likely to arise from distinct developmental mechanisms that vary from species to species (Nagy and Grbic 1999). To see if the mechanism underlying the development of prolegs is conserved or not, we used antibodies to examine protein expression patterns in other holometabolous insect species that develop abdominal prolegs: two sawflies of the hymenopteran suborder Symphyta, superfamily Tenthredinoidea. The Hymenoptera are thought to have branched off after the evolution of Coleoptera but before the split between the Antliophora (Diptera, Siphonoptera and Mecoptera) and Amphiesmenoptera (Lepidoptera and Tricoptera) lineages (Whiting et al. 1997).

Materials and methods

Animals used

Eggs of the balsam fir sawfly (*Neodiprion abietis*) that had been laid on balsam fir (*Abies balsamea*) branches in the field in Maine were collected and kept at 15°C until dissection. A colony of introduced pine sawfly (*Diprion similis*) was maintained at 20°C with light (18 h) and dark (6 h) cycles and raised on white pine (*Pinus strobus*) branches. Eggs of the silkworm moth (*Bombyx mori*) were kept at room temperatures until dissection. Tobacco hornworm moths (*Manduca sexta*) were raised at 25°C on artificial food (Carolina Biological Supply), pupae were removed to a large container, and a cardboard tube was placed vertically to allow any emerged adults to crawl up and dry their wings. A deadly nightshade (*Solanum dulcamara)* plant and a common green pepper were placed inside the vessels to stimulate egg laying. The eggs were collected within 5 days of emergence of the adults.

Antibody staining

Embryos were dissected out of the egg in phosphate-buffered saline, and tissues were fixed for approximately 15 min as described by Patel (1994). Once the fixative was washed off, the embryos were dehydrated and stored in methanol at –20°C until staining.

Embryos were stained following the antibody staining procedure described in Patel (1994). The embryos were incubated overnight at 4°C in 10% normal goat serum buffer containing a primary antibody. For *Dll* staining, polyclonal rabbit anti-Dll antibody was used at a concentration of 1:200; for BX-C staining, monoclonal mouse anti-Ubx/abd-A antibody (FP6.87) was used at a concentration of 1:10; and for *extradenticle* (*exd*) staining, polyclonal mouse anti-exd antibody was used at a concentration of 1:5. Embryos were then washed several times and incubated overnight at 4°C in a buffer containing peroxidase-conjugated secondary antibodies at the following concentrations: 1:300 goat anti-mouse for *Dll* staining; 1:200 goat anti-mouse for both *Ubx*/*abd-A* and *exd* staining. Stained embryos were washed thoroughly and then cleared in 70% glycerol.

Protein expression patterns were viewed with Nomarski optics on an Olympus IX70 inverted microscope. A Spot camera (Diagnostic Instruments) connected to a computer was used to capture digital images of embryos under the microscope. Images were cropped and adjusted for contrast with the Adobe Photoshop 5.5 software.

Results and discussion

Lack of Dll expression in sawfly prolegs

As expected, Dll protein appeared in the distal portions of sawfly mouth parts (excluding the mandibles), thoracic limbs, and terminal appendages. Interestingly, however, Dll was completely absent from the abdominal prolegs throughout embryogenesis in both sawfly species examined (Fig. 2). In contrast, Dll is known to be present

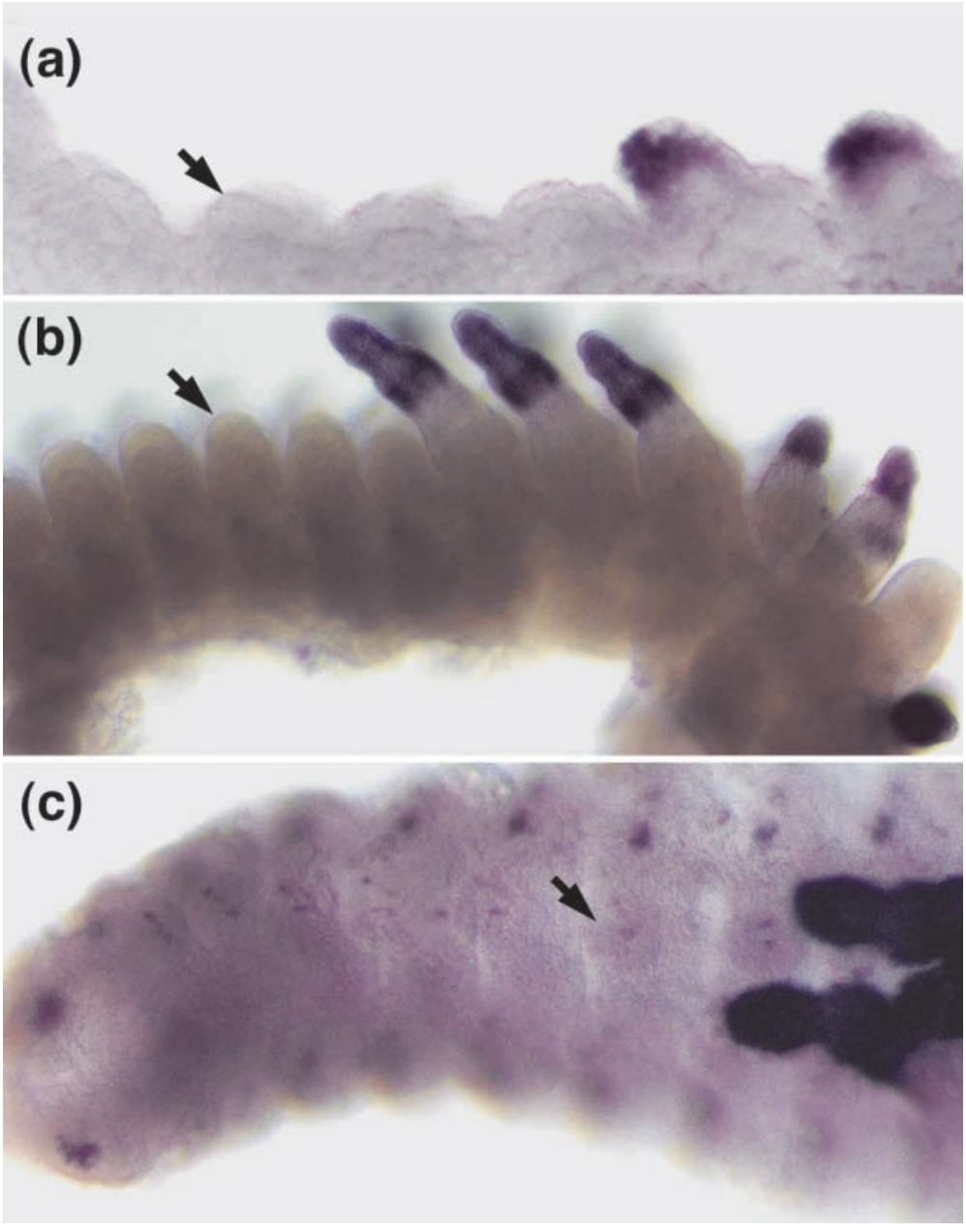

Fig. 2a–c *Dll* expression in sawflies during early (**a**), middle (**b**), and late (**c**) stages of proleg development. Anterior is to the *right*. **a**, **b** Lateral views, whereas **c** is a ventral view. In each panel, the *arrow* indicates a developing proleg on segment A3. **a, b** High levels of Dll expression were evident in the developing thoracic limbs but not in the cells that develop into prolegs or mandibles. **c** Later in embryogenesis, cells in the nervous system and terminal appendages at the end of the abdomen expressed Dll, but there was still no Dll expression in the prolegs. **a** and **c** *Neodiprion abietis*. **b** *Diprion similis*

at high levels in the distal tips of lepidopteran prolegs (Warren et al. 1994).

Expression of BX-C proteins in sawfly prolegs

In both species of sawfly, Ubx/abd-A was present throughout most of the abdomen for most of embryogenesis (Fig. 3). Importantly, each developing proleg contained Ubx/abd-A protein all of the way to the tip of the appendage, with no notable reduction in the levels of these proteins during proleg development. In contrast, circular clearings have been reported to appear in the expression domains of Ubx/abd-A in abdominal segments A3–A6 of lepidopteran embryos, and this is thought to allow these regions to initiate *Dll* expression so that prolegs can develop (Warren et al. 1994). Contrary to a previous report (Zheng et al. 1999), circular clearings of Ubx/abd-A expression were observed in both butterflies and moths (Fig. 4).

Localization of exd protein in sawfly prolegs

The complete lack of Dll protein during the development of sawfly prolegs suggests that they are analogous to the proximal portions of the thoracic limbs, lacking the distal limb segments that are normally patterned by *Dll*. Previous genetic studies have suggested that a typical arthropod thoracic limb can be divided into two distinct regions, which appear to be patterned independently

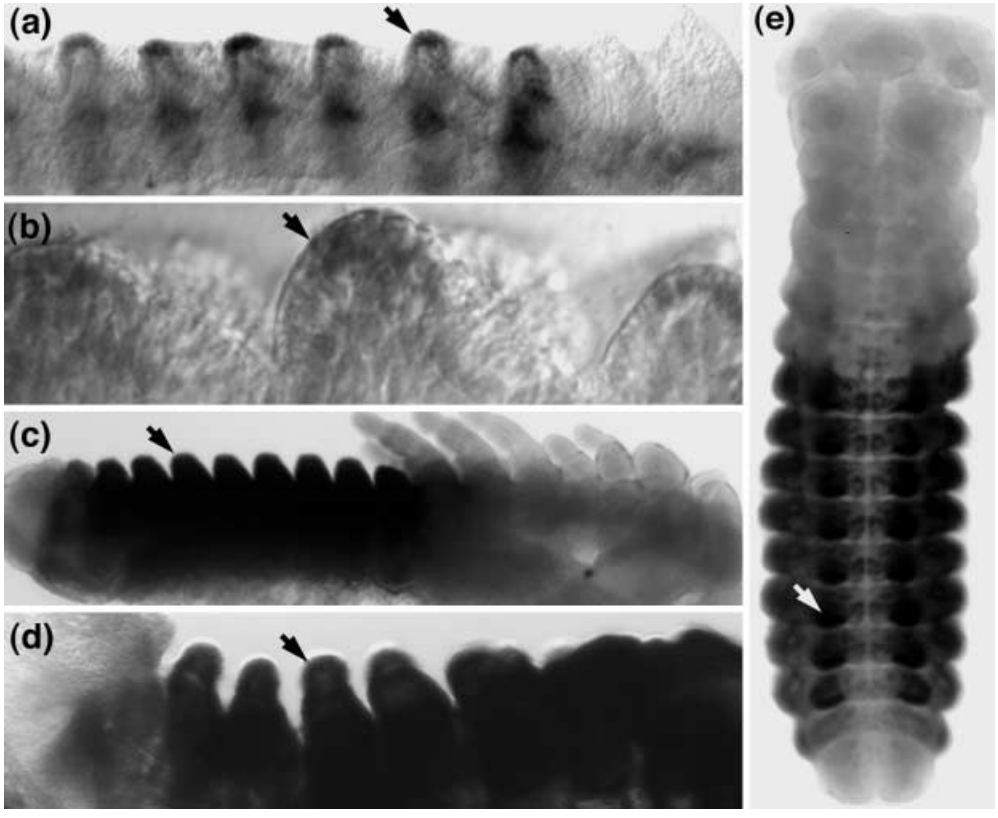

Fig. 3 Ubx/abd-A staining in the abdominal segments of sawflies during early (**a**, **b**), middle (**c**, **e**), and late (**d**) stages of proleg development. Anterior is to the *right* in panels **a**–**d**, and towards the *top* in panel **e**. **a**–**d** Lateral views; **b** is a high-magnification view of the embryo shown in **a**; **e** ventral view. *Arrows* indicate developing prolegs. Ubx/abd-A was expressed to the tips of prolegs throughout the development of the prolegs, and no holes appeared in the domain of Ubx/abd-A expression. **a, b, d** *N. abietis*. **c, e** *D. similis*

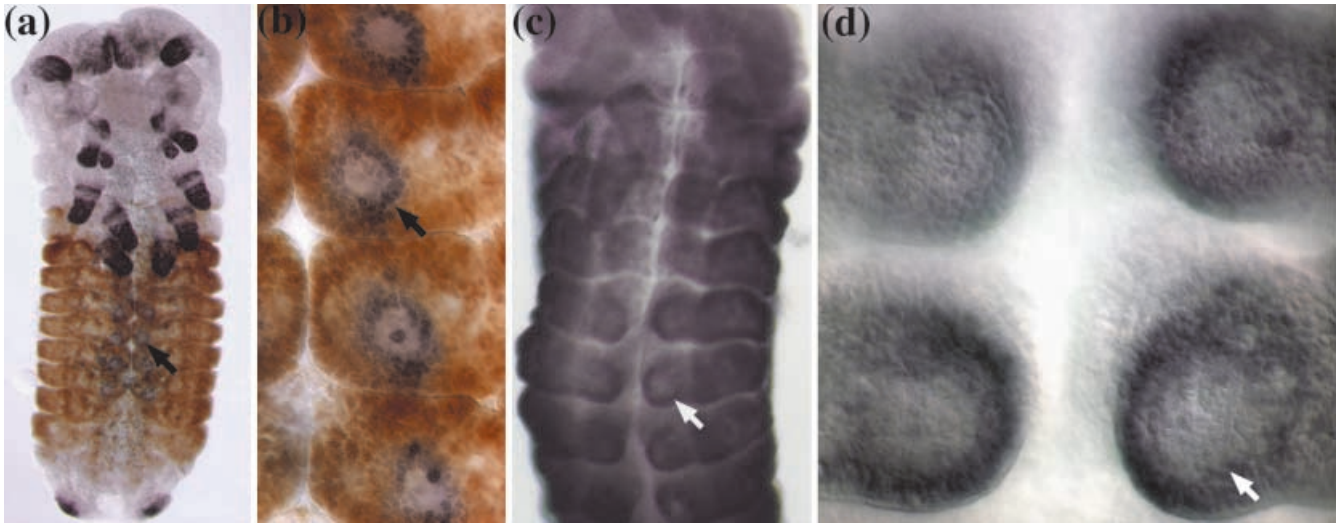

Fig. 4 Circular clearings appeared in the expression domain of Ubx/abd-A in segments A3–A6 of the butterfly *Precis coenia* (**a**, **b**), and the moth *Manduca sexta* (**c**, **d**). Anterior is towards the *top* in all panels. **b** A high-magnification view of the embryo shown in **a**; **e** a high-magnification view of the embryo shown in **d**. In **a** and **b**, the *brown* staining represents Ubx/abd-A expression and *black* staining represents Dll expression. In **c** and **d**, the *black* staining represents Ubx/abd-A expression. In all panels, *arrows* indicate circular clearings in Ubx/abd-A expression

(Gonzàlez-Crespo and Morata 1996; Aspland and White 1997; Abu-Shaar and Mann 1998). In the distal portion of the limb, termed the telopodite, exd protein remains in the cytoplasm, and patterning is controlled by Dll; in the proximal portion of the limb, termed the coxopodite, exd protein is transported into the nucleus and controls patterning in the absence of Dll. If sawfly prolegs are equivalent to coxopodites, then exd protein should be nuclear-localized all of the way to the tip of each proleg. Antibody staining confirmed this prediction (Fig. 5a). This pattern of exd staining can be contrasted to that of all insect thoracic limbs that have been examined (e.g. sawfly

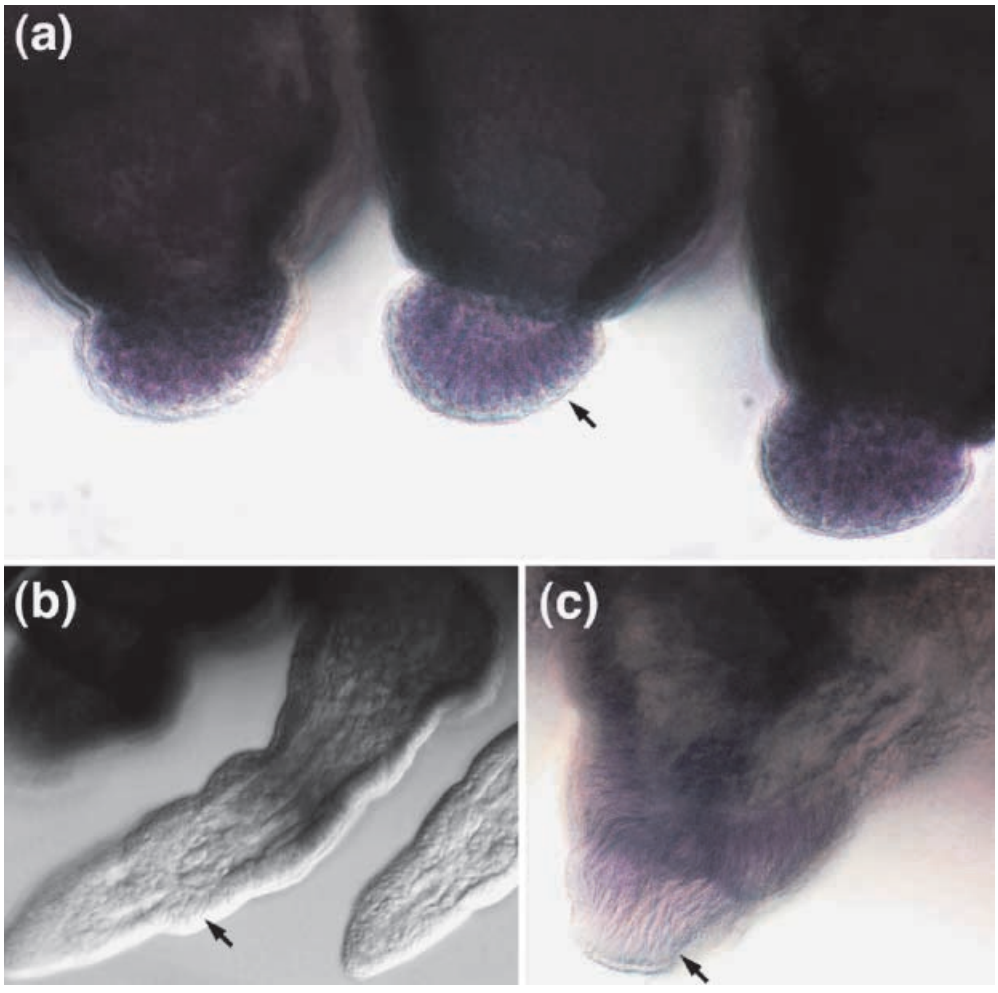

Fig. 5 Extradenticle (exd) staining in the sawfly *D. similis* (**a**, **b**), and the moth *Bombyx mori* (**c**). **a** Exd was nuclear-localized to the tip of the sawfly prolegs (*arrow*). **b** Exd was nuclear-localized in the coxopodite of the sawfly thoracic legs but not in the telopodite (*arrow*). **c** In *B. mori*, exd did not appear to be nuclear-localized in the cells near the distal tip of the prolegs (*arrow*). Morphologically, *B. mori* appeared to have both the telopodite and the coxopodite portions, whereas sawflies appeared to possess only a coxopodite portion

thoracic limbs are shown in Fig. 5b) and to developing prolegs in Lepidoptera (Fig. 5c), where exd protein was not apparent in the nuclei of cells near the distal tips.

Evolution of prolegs

The development of sawfly prolegs without the expression of *Dll*, combined with the nuclear localization of exd protein all of the way to the proleg tips, suggests that sawfly prolegs may be limb bases, like the insect mandibles (Popadic et al. 1998). In *Drosophila*, mutations in the *Dll* gene can result in the development of a stump-like structure that appears to correspond to the coxal segment of a normal leg (Campbell and Tomlinson 1998). Based on our results, we propose that sawfly prolegs are roughly equivalent to the stump-like structure that is developed by these *Dll* mutants, corresponding to the coxal segment of a thoracic leg.

In contrast to sawflies, lepidopteran prolegs appear to have both proximal and distal portions. Morphologically, it is clear that lepidopteran prolegs have cuticular structures at the distal tips that are absent in sawfly prolegs (data not shown). *Dll* expression in lepidopteran prolegs may be required for the formation of these distal structures.

Based upon the phylogenetic distribution of prolegs among the holometabolous insects, Nagy and Grbic (1999) hypothesized that prolegs evolved independently in different lineages. According to this model, although the prolegs in different orders may share homologies at some basic levels (e.g. shared mechanisms of limb development), the particular mechanisms by which the derepression of appendage development occurs in the abdomen are likely to be evolutionary novelties. Our results are consistent with a model of evolutionary convergence, in which derepression of abdominal appendage development has occurred independently in various insect lineages. Future studies on additional holometabolous insect species whose larvae develop prolegs should allow us to model proleg evolution with greater confidence.

Interestingly, sawflies also differ from Lepidoptera in the way the larvae hang onto branches. To grasp onto things, Lepidoptera use both the coxa and the distal cuticular structure of each proleg (Snodgrass 1935). Sawflies hold onto pine needles between each stubby pair of limb bases, but this apparently does not provide adequate

support, so they also tend to curl their abdomen around the branch or needles (unpublished observations). Such behavior was not observed in the lepidopteran larvae examined. Thus the two groups seem to have diverged in their behavior as well as development.

HOX regulatory evolution

Our finding that Dll protein is absent from sawfly prolegs, combined with the observation that Ubx/abd-A proteins are present at high levels in these cells throughout proleg development, is consistent with the current, two-step model of regulatory evolution in the insects (Fig. 1). As in *Drosophila* (Vachon et al. 1992), *P. coenia* (Warren et al. 1994), and *M. sexta* (this study), the expression domains of Ubx/abd-A and Dll proteins did not tend to overlap in the two sawfly species examined. These results suggest that the repression of Dll by BX-C proteins is conserved across the Diptera, Lepidoptera, and Hymenoptera.

The antibody that was used to detect BX-C proteins (FP6.87) recognizes both Ubx and abd-A, so with our results it was not possible to determine the expression domain of each Hox gene separately. This means that we do not have direct evidence that both Ubx and abd-A suppress *Dll* transcription. We consider it likely, however, that both proteins are indeed suppressing *Dll*, as no Dll protein is observed in the sawfly A1 segment. In all insects that have been examined (firebrats, grasshoppers, beetles, and flies), the anterior portion of the A1 segment expresses Ubx but not abd-A, whereas the more posterior segments express abd-A. This anterior boundary of abd-A expression is a highly conserved trait in the insects. Hence, we are assuming that at least the most anterior segment is under the control of Ubx. It would be worthwhile to show experimentally that both proteins suppress *Dll*. A future study using double-stranded RNA interference to block the expression of each gene (e.g. Lewis et al. 2000; Schoppmeier and Damen 2001) would address this question directly.

In the present study, we observed circular clearings in Ubx/abd-A expression in the abdominal segments of the moth *M. sexta*, much like those observed in the butterfly *P. coenia* (Fig. 4). This result contrasts with a previous study reporting the absence of circular clearings in Ubx/abd-A expression in the abdomen of *M. sexta* (Zheng et al. 1999). The contrasting results might be explained by the fact that there tended to be high levels of background staining when this antibody was used in *M. sexta*, which made it more difficult to distinguish the circular clearings in segments A3–A6 (e.g. Fig. 5c).

Given that the repressive function of *Dll* by *Ubx/abd-A* is conserved in the holometabolous insects, the differences in the expression patterns of sawflies and Lepidoptera are likely to be governed by additional genes. Previously, the mandible was the only known example of a ventral appendage in arthropods that develops without the expression of *Dll* (Popadic et al. 1998). Our results indicate that additional ventral appendages are able to develop without *Dll*, and suggest that limb development without *Dll* has evolved independently at least twice in the insects.

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