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Correlation between *Distal-less* Expression Patterns and Structures of Appendages in Development of the Two-Spotted Cricket, *Gryllus bimaculatus*

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ABSTRACT—A million of insect species have been identified so far, displaying a staggering variety of adult morphologies. To elucidate mechanism how such insect morphologies are developed at a molecular level, we investigated developmental process of the two-spotted cricket, *Gryllus bimaculatus*, as a typical hemimetabola, and compared with that of *Drosophila* as an extensively studied typical holometabola. We analyzed expression patterns of homeobox genes of *engrailed* (*en*) and *Distal-less* (*Dll*) during development. In early embryos, *en* is expressed in the posterior compartments of body segments and developing appendages, while *Dll* is expressed in the distal region corresponding to the telopodite of developing appendages. Interestingly, these expression patterns are very similar to those observed in *Drosophila* imaginal discs. In the case of *Dll*, we found that its expression pattern, which is similar to each other in various appendages at early stages, changes in progress with elongation and segmentation, depending on the type of appendages. Late expression patterns of *Dll* are classified into three types: *Dll* expression in the entire region of the antenna, in a distal region of the cercus, and in distal and middle regions of the leg, maxillary and labial palpus, indicating that *Dll* expression patterns are closely related to segmentation patterns of the appendages. Furthermore, since *Dll* is intensely expressed in both sides of the femur-tibia articulation of the leg, we considered that *Dll* is involved in positioning of articulation during the late appendage development. Hence, our results indicated that although common molecules are involved in development of insect appendages, the variety of the morphologies depends on pattern and timing of their expressions.

INTRODUCTION

Insects are vast in number and about a million of species have been identified so far. Their enormous variations in body forms bring ability of adaptation to various environments on the earth. Especially, insects bear several types of appendages such as legs, antenna, mandible, cercus, etc., and their shapes and functions are highly specialized. Recently, mechanisms on development of various appendages of *Drosophila* have been investigated from a molecular biological point of view (Bryant, 1993; Campbell *et al.*, 1993; North and French, 1994; Bonini and Choi, 1995) and various molecules involved in development of appendages have been identified. For instance, it is known that a segment polarity gene of *engrailed* (*en*) is an essential gene for determination of the posterior compartment of *Drosophila* body segments (Karr *et al.*, 1989; Patel, 1994a, b) and the imaginal discs (Kornberg *et al.*, 1985). On the other hand, a homeobox gene, *Distal-less* (*Dll*), is known to one of the essential genes for development of the *Drosophila* appendage. *Dll* is concentrically expressed in appendage imaginal discs (Diaz-Benjumea *et al.*, 1994). Since a *Drosophila* mutant lacking *Dll* activity lost the distal parts of appendages (Cohen and Jürgens, 1989; Cohen *et al.*, 1989), *Dll* is considered to be involved in the proximodistal pattern formation in the appendage.

Although we obtained huge information on developmental mechanism of *Drosophila* appendages, we have still not known about the mechanisms of other insect appendages, for example, butterfly wings, cricket legs, cockroach legs, etc. One of the questions for other insects than *Drosophila* is whether the information obtained on *Drosophila* is applicable to other insects. Recently, Panganiban *et al.* (1994) demonstrated that during early development of larval appendages in *Precis coenia*, *Dll* is expressed in the appendages, as observed in *Drosophila*. Furthermore, Panganiban *et al.* (1995) found that *Dll* is also expressed in the appendages of two crustaceans, *Artemia franciscana* and *Mysidopsis bahia*, myriapods, *Ethmostigmus rubripes*, and chelicerates, *Argiope argentata*. On the other hand, *en* expression has been reported in the

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grasshopper, *Schistocerca americana* (Patel *et al.*, 1989a, b), in the beetle, *Tribolium castaneum* (Patel, 1994a), and its expression pattern closely resembles that in *Drosophila*. These results indicated that common molecules such as *en* and *Dll* are likely to function in appendage formation of other insects similarly as found in *Drosophila*. Thus, the expressions of *en* and *Dll* in developing insect appendages may be used as positional markers: *en* is a posterior compartment marker (Patel *et al.*, 1989a) and *Dll* is a distal marker (Popadić *et al.*, 1996). Furthermore, comparison of the expression patterns of these genes among different appendage types will give us further understanding on structures of insect appendages.

Insects belonging to Pterygota is subdivided into two groups of hemimetabola and holometabola. Since *Drosophila* belongs to the latter, it is interesting to compare expression patterns of various genes found in *Drosophila* with those in hemimetabolan insects. In this study, we focus on the orthoptera as a typical hemimetabola. Their embryos are categorized in the short germ type, while the *Drosophila* embryo is in long germ type. Furthermore, the appendages of orthopterans are initially formed as buds, without forming imaginal discs as observed in *Drosophila*. In the orthopteran insects, we chose a two-spotted cricket, *Gryllus bimaculatus*, to compare the morphological and embryological differences at the molecular level with the fly, because their rearing at a large scale is easy and many eggs are obtained at a time. Since the cricket exhibits fundamental morphologies of appendages like polysegmented antenna, mouth parts of biting type, locomotive leg, and cercus as a sense organ, which are widely found in insects, we can compare developmental processes among those appendages.

Here, focusing on the development of appendages, we first described embryogenesis of *G. bimaculatus* by dividing developmental processes into several stages. Then, the expression patterns of *en* and *Dll* in developing appendages were observed immunohistochemically and compared with those of *Drosophila* imaginal discs. We found that both *en* and *Dll* expression patterns in antenna are similar to those in legs. In addition, we analyzed expression patterns of *en* and *Dll* in the various appendages. We found that *Dll* expression patterns were different among appendage types, although *en* expresses similarly in each posterior compartment. Thus, *Dll* is required for elaboration of the distal part of the appendage, and is involved in determination of the appendage type.

MATERIALS AND METHODS

Animals

Two-spotted cricket, *Gryllus bimaculatus*, was used in this study. To obtain clear results for whole-mount immunostaining, we used a mutant with white eyes (autosomal recessive; *gwhite*) (Fig. 1A), kindly provided by I. Nakatani of Yamagata University. During late embryogenesis and just after molt, their bodies become more transparent than in usual.

Rearing of crickets

All nymphs and adults of *G. bimaculatus* were reared at 27 ± 1°C with humidity of 70 ± 2% under a 10L : 14D photoperiod, and fed on

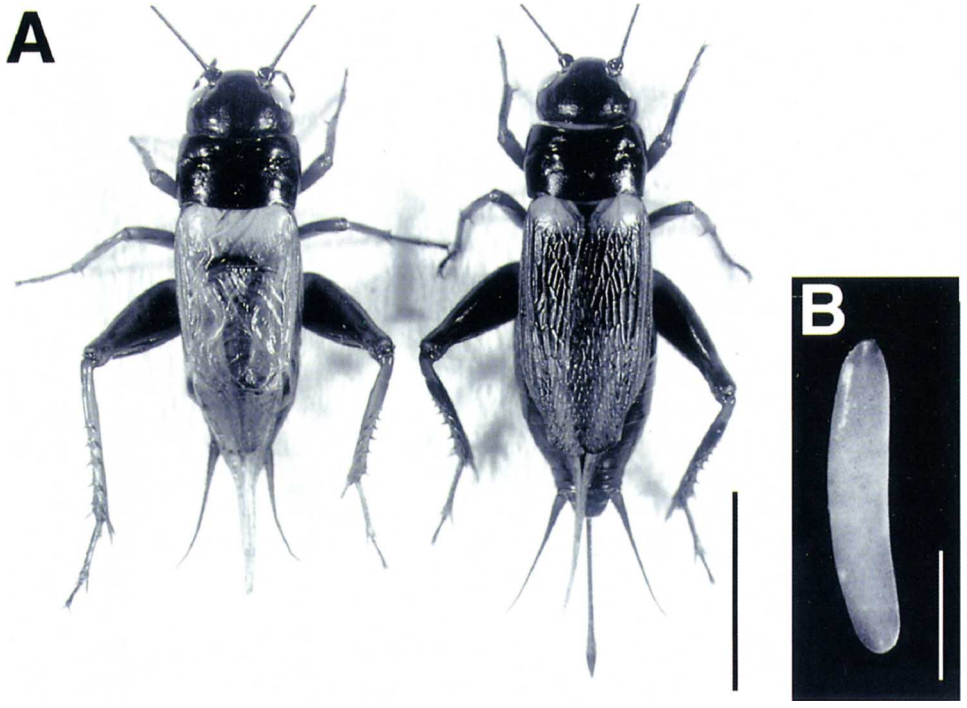

Fig. 1. Adults and an egg of a mutant two-spotted cricket, *Gryllus bimaculatus* (*gwhite*) with white eyes. (**A**) Adult male (left) and female (right). (**B**) Egg. Slightly pointed pole is anterior (upper side). Scale bars = 1 cm (**A**), 1 mm (**B**).

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crashed dog-food and artificial rabbit-food. Under these conditions, nymphs molt every week, and the adults emerge after the 8th molt. Generation time of the cricket is about two months. The 1st-8th instar nymphs were maintained in plastic cases of 10 × 10 × 12 cm, and the adults were in cases with 15 × 15 × 20 cm. Each case contains two cotton wool-plugged plastic tubes for supplying water, and several crumpled papers as a shelter. In the case rearing the adults, a pile of moist and folded tissue papers (about 1 cm in height) was used for females to lay eggs during the dark period. Tissue papers for laid eggs were exchanged with new ones every day, and freshly laid eggs were collected with forceps. The eggs were placed separately on moist tissue papers and incubated under the same conditions as employed for rearing adult crickets (27°C and 70% humidity). Nymphs hatched out from the eggs on 13th day of incubation were transferred to the rearing cases.

Observation of embryogenesis

To observe position and form of cricket embryos within the egg at each stage, eggs were soaked in 30% bleach for 5 min and removed their chorion. Dechorionated eggs were washed thoroughly in phosphate buffered saline (pH 7.4) and then observed in the saline under dark-field illumination, using stereo-microscope. For observation of the embryo morphology, embryos with yolk masses were taken out from the eggs, and then the yolk masses were removed carefully with tungsten needles. The embryos were mounted on microscope slides and observed their detail features under a bright-field microscopy with the Nomarski optics.

Immunohistochemistry

To examine the localization of *engrailed* (*en*) and *Distal-less* (*Dll*) proteins in developing cricket embryos, we used a monoclonal antibody (anti-*en* protein), namely MAb 4D9, purchased from the Developmental Studies Hybridoma Bank (Baltimore, MD, USA) (Patel *et al.*, 1989b), and a polyclonal antibody (anti-*Dll* protein), kindly provided by Dr. Sean Carroll (University of Wisconsin) (Panganiban *et al.*, 1995).

Immunostaining was performed as described previously for grasshopper embryos by Patel *et al.* (1989b). All embryos at stages 5-12 were stained as whole-mount preparation. To detect *en* proteins, the MAb 4D9 supernatant was used at 1:3 dilution and the secondary antibody, peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Lab), was used at a dilution of 1:100. On the other hand, the *Dll* antibody was used at a dilution of 1:200 and the secondary antibody, peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Lab), was used at a dilution of 1:400. To observe the expression domain precisely, the stained embryos were dehydrated in ethanol, placed in xylene, then embedded in paraffin and serially sectioned at 5 μm thickness. Both stained whole embryos and sections were mounted on microscope slides and then observed under a Nomarski differential-interference microscope.

RESULTS

Definition of developmental stages in Gryllus bimaculatus

Although outline of the embryogenesis of *G. bimaculatus* has already reported by Miyamoto and Shimozawa (1983), detailed embryonic structures have not been described. Here, general aspects of morphologies of the developing embryos and metathoracic limbs are briefly illustrated in Fig. 2. Most of the eggs are hatched on the 13th day ± 12 hr under incubation conditions of 27 ± 1°C and 70 ± 2% humidity. We divided total developmental span into 16 stages based on morphological features of developing embryos and appendages, as summarized in Table 1. The correspondence between stages and morphologies are shown in Fig. 2.

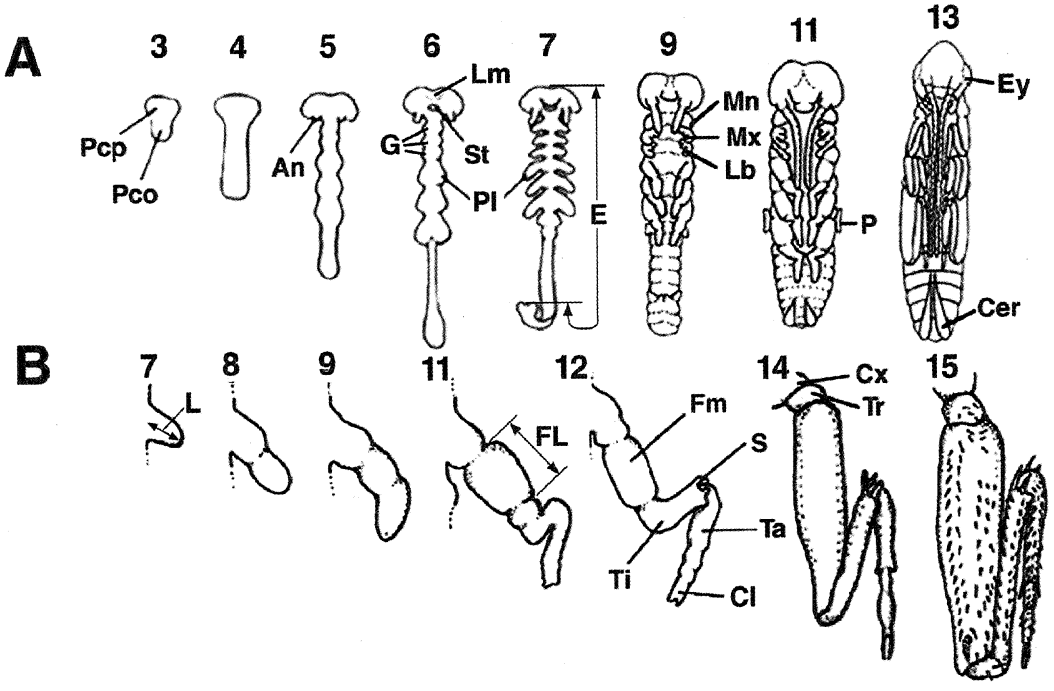

Fig. 2. Illustrations showing embryogenesis in *G. bimaculatus*. Numbers indicate stages. (**A**) Whole embryos (ventral view). (**B**) Metathoracic appendages. Every appendage in *G. bimaculatus* first appears as a bud. An, antenna; Cer, cercus; Cl, claw; Cx, coxa; E, anteroposterior length of embryo; Ey, eye; FL, proximodistal length of femur in metathoracic leg; Fm, femur; G, gnathal segments; L, proximodistal length of metathoracic leg; Lb, labium; Lm, labrum; Mn, mandible; Mx, maxilla; P, pleuropodium Pco, protocorm; Pcp, protocephalon; Pl, prothoracic legs; S, tibial spur; St, stomodium; Ta, tarsus; Ti, tibia; Tr, trochanter.

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Table 1. Embryogenesis in *Gryllus bimaculatus*

stage	day	E/L(or LF)	characteristics
1	~ 1	—	Freshly laid eggs are light yellow in color and 2.88 ± 0.12 mm long (Fig. 1B). Anterior pole of the egg is slightly pointed and the posterior pole blunt. The concave and convex sides are dorsal and ventral, respectively (Fig. 1B). The yolk is finely granular and the embryo is not visible.
2	1 ~ 1.5	—	A heart-shaped germ band appears at the posterior ventral side of the egg.
3	1.5	—	The embryo becomes pear-shaped (Fig. 2A). The wide anterior region of the embryo is protocephalon and the narrow posterior region is protocorm.
4	1.5 ~ 2	—	The protocorm elongates due to proliferation of the caudal region (Fig. 2A).
5	2 ~ 2.5	—	Segmentation begins in the protocormic region. Three thoracic segments are delineated in the middle of the protocormic region. A pair of small antennal rudiments appears at the posterior region of each protocepharic lobe (Fig. 2A).
6	2.5 ~ 3	—	A part of the ventral surface of the protocepharon invaginates medially and becomes the oral opening (stomodeum). A labral rudiment is formed in slightly anterior region of the stomodeum (Fig. 2A). Three gnathal segments are clearly visible. Three pairs of thoracic limb buds develop as small globular swellings (Fig. 2A).
7	3	10.0 ~ 15.0 (E/L)	The abdominal segments are generated gradually from the anterior to the posterior end. The caudal region of the embryo becomes convoluted and ventrally flexed. The gnathal and thoracic limb buds become elongated laterally (Fig. 2A, B). From this stage, to characterize the embryo, we use a ratio (E/L) of the length from head to posterior margin of abdominal last segment (E) to proximodistal length of metathoracic leg (L) as a mesure of the stage (Fig. 2A, B).
8	4	6.5 ~ 7.5	The embryo is fully segmented. The caudal end of the embryo possesses a small invagination (proctodeum). The limb buds (except mandible and cercus) have a medial constriction and divided into two regions (Fig. 2B), which become the telopodite (distal) and the coxopodite (proximal).
9	5 ~ 5.5	3.5 ~ 4.5	The telopodite regions of the thoracic limbs become more elongated and have an additional constriction. The middle compartment becomes the femur, while the distal compartment differentiates in tibia-tarsal regions (Fig. 2B). The maxillary and labial limbs become trilobed (Fig. 2A).
10	5.5 ~ 6	3.0 ~ 3.5	The embryo undergoes katatrepsis; the embryos move around the posterior pole from dorsal to ventral side of the egg.
11	6 ~ 7	8.5 ~ 9.5 (E/LF)	After completion of the katatrepsis, the dorsal closure starts. The labrum shows a transverse groove in the middle (Fig. 2A). Most distal segments of the thoracic limbs become elongated, strongly curved and differentiate into tibial and tarsal regions (Fig. 2B). From this stage, we used the other ratio (E/EL) of E to proximodistal length of femur in metathoracic leg (LF) instead of E/L (Fig. 2B).
12	8	5.0 ~ 8.5	The femoral, tibial, and tarsal segments of the metathoracic leg are clearly distinguishable. At the distal end of tibia, two pairs of spur primordia are generated. The tarsus is divided into three segments and its distal end possesses the claw primordium (Fig. 2B).
13	9	3.5 ~ 5.0	The dorsal closure is completed. The embryo attains its full length and fills the entire egg. Cuticle secreted by the embryo covers over the entire body. Pigmentation in the compound eye is visible. The legs begin to twich (Fig. 2A). Rapid growth of the leg segments starts from this stage.
14	10	3.0 ~ 3.5	Each leg is fully grown. The tibial spurs and tarsal claws become sharp (Fig. 2B).
15	11 ~ 12.5	3.0 ~ 3.5	The embryo is light yellow in color. Regularly arranged black bristles appear on the leg (Fig. 2B) and the posterior ridge of each tergum.
16	12.5 ~ 13	3.0 ~ 3.5	Most of the eggs are hached. The newly hatched nymph is marked by the bright yelow body with transparent legs. Within 30 min from the hatching the nymph becomes black except the eyes, cerci, prothoracic and mesothoracic terga which remain yellow.

Expression patterns of engrailed *in body segments are conserved*

We used a monoclonal antibody 4D9 to observe expression of *engrailed* (*en*) in *G. bimaculatus* embryos at stages 5-12. The *en* expression patterns are shown in Figs. 3 and 4. At stage 5, the *en* expression patterns were segmentally repeated stripes in the gnathal and thoracic segments (Fig. 3A). In addition, a stripe of en also appears in a region becoming the first abdominal segment (Fig. 3A). With the caudal extension of the embryo, the number of abdominal stripes increases and finally sixteen stripes were observed in the fully segmented embryo (Fig. 3C). These stripes are localized in the posterior compartment of every segment. No stripe of en was detected in the most caudal segment, i.e., the eleventh abdominal segment (Fig. 4D), whereas the clear en stripe was found in the posterior compartment of the tenth abdominal segment (Figs. 3C, D and 4D). In progress of development, the gnathal and thoracic stripes become wide. In the thoracic terga, en stripes are much wider than those in abdomens at stage 12 (Fig. 3F). The intensity of expression

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in the segments becomes maximum at stage 11 (Fig. 3D). From this stage, several en-positive cells are regularly arranged at the mid-ventral region of every body segment (Fig. 3H) and in the head (Fig. 3G). Since the *en* expression during neurogenesis has been reported in many organisms including arthropods, annelids, and chordates (Patel *et al.*, 1989b), the en-positive cells may become a subset of neuroblasts, ganglion mother cells and neurons in the central nervous system. The distribution of en-positive cells during neurogenesis in *G. bimaculatus* closely resembles that in the grasshopper, *Schistocerca americana* (Patel *et al.*, 1989b; Condron *et al.*, 1994).

Expression patterns of engrailed *in the posterior compartments of appendages*

The *en* gene is also expressed in developing appendages such as the antenna (Fig. 3F), mandible, maxilla, labium, and thoracic legs (Fig. 4A-C), but not in the developing labrum. During elongation of these appendages, *en* expression is limited to the posterior compartment of each appendage. At

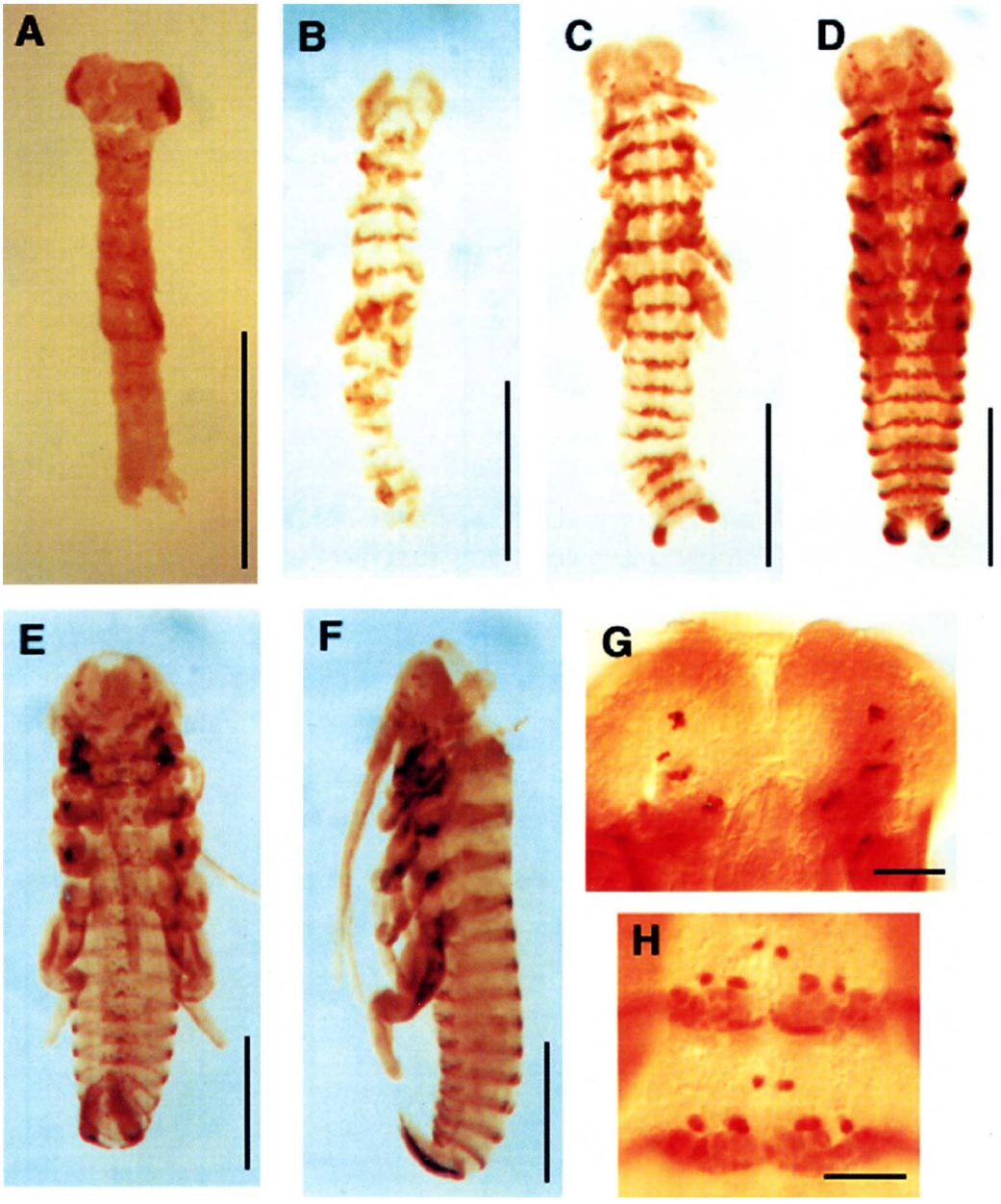

Fig. 3. Expression patterns of *engrailed* in developing embryos. (**A**-**F**) Whole embryos at stage 5 (**A**), stage 7 (**B**), stage 9 (**C**), stage 11 (**D**), and stage 12 (**E**, **F**). en stripes were observed in the posterior margin of each segment. (**G**) The head region at stage 12 where four pairs of patches of en positive cells are arranged at both sides. (**H**) The ventral side of abdomen (the 4th and 5th abdominal segments) at stage 11 where en positive cells are regularly arranged at every segments. Scale bars = 0.5 mm (**A**-**F**), 100 μm (**G**), 50 μm (**H**).

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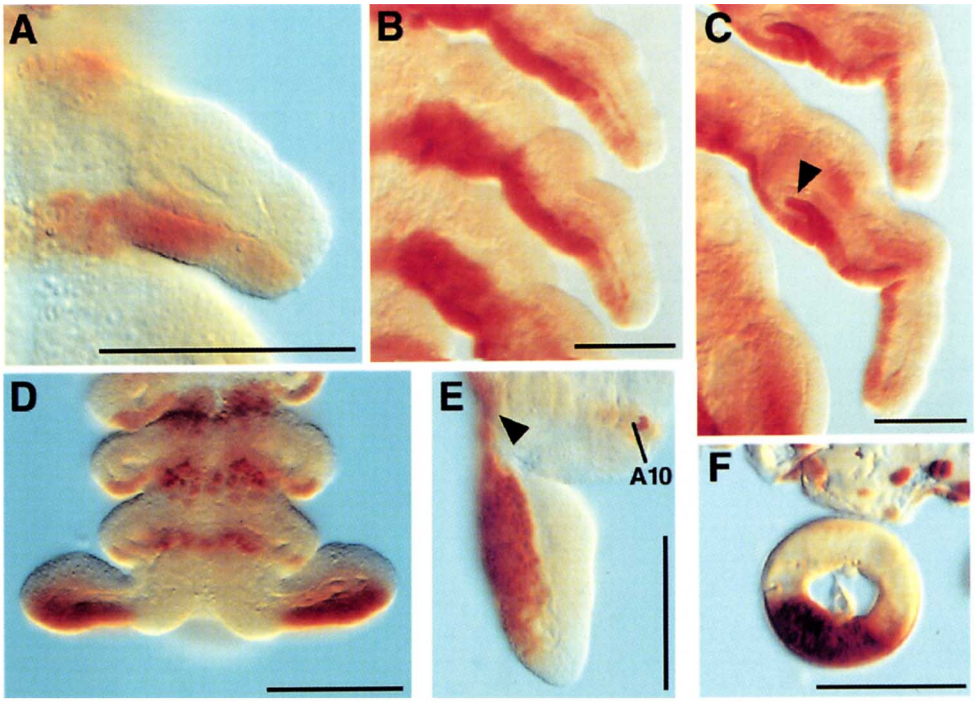

Fig. 4. Expression patterns of *engrailed* in developing appendages. (**A**-**C**) Mesothoracic appendages of embryos at stage 7 (**A**), stage 9 (**B**), and stage 11 (**C**). *en* is expressed at the posterior compartment as in body segments. (**D**-**E**) Cerci of embryos at stage 8 (**D**) and stage 9 (**E**). At stage 11, *en* expression was also observed in the apodeme of the femur (arrowhead) (**C**). At stage 8, expression in cercus appears without connecting any en stripes in the body (**D**). Subsequently, the expression domain is expanded and connected with abdominal 10th stripe (A10) at dorsal (arrowhead) (**E**). (**F**) Cross section of **E**. The expression domain occupies about one third region of circumference. Similar pattern was also observed in other appendages (data not shown). Scale bars = 100 μm.

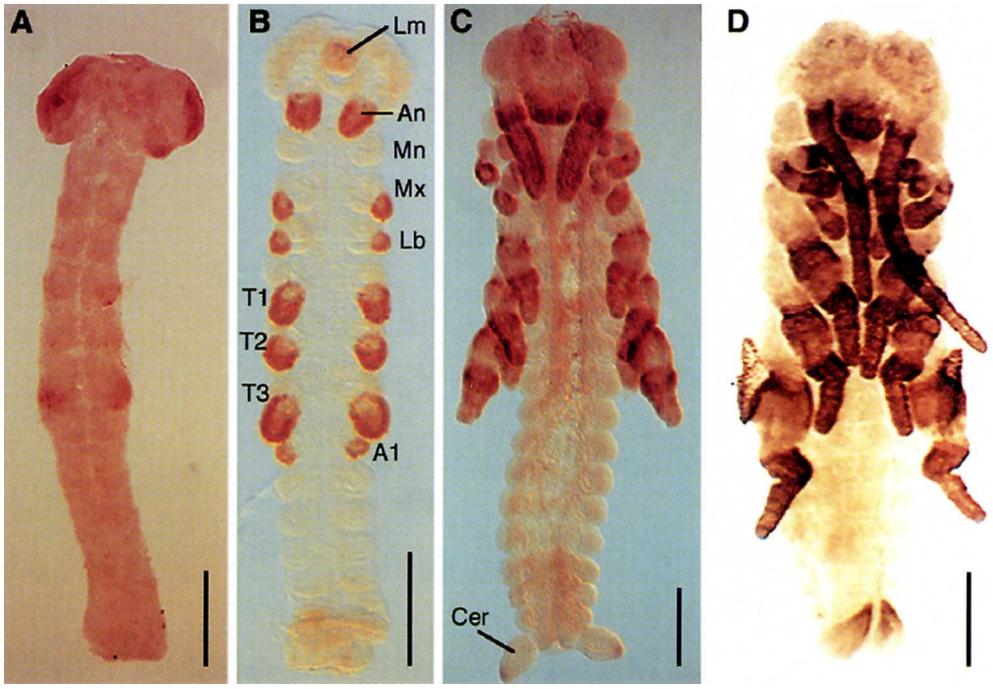

Fig. 5. Expression patterns of *Distal-less* in whole embryos. Each embryo is at stage 5 (**A**), stage 8 (**B**), stage 9 (**C**), and stage 11 (**D**). *Dll* expression was initially observed at primordium of metathoracic appendage (**A**). At stage 8 (**B**), *Dll* is expressed in growing appendages excepting mandible (Mn). The expression was also defined in cercus (Cer) at stage 9 (**C**). With the elongation, each appendage exhibits distinct expression pattern (**C**, **D**). A1, appendage of first abdominal segment; An, antenna; Lb, labium; Lm, labrum; Mx, maxilla; T1-T3, appendages of first -third thoracic segments. Scale bars = 250 μm.

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stage 7 when each appendage bud begins to elongate laterally, the segmental en stripe extends in the posterior portion of each appendage (Figs. 3B and 4A). In thoracic leg buds, the expression in the proximal region is more intense than that in the distal region (Fig. 4A, B). At stage 11, *en* is also expressed in the femoral apodeme in each leg (Fig. 4C). Although no stripe of en is detected in the eleventh abdominal segment of embryo as above mentioned, the *en* expression is observed in developing cercus which is the appendage of the eleventh abdominal segment (Figs. 3C-F and 4D-F). With elongation of the cercus, the expression extends proximally and connects to the en stripe of the tenth abdominal segment (Fig. 4E). In this stage, the orientation of the cercus changes toward the posterior direction along the anteroposterior axis of the body, so that *en* appears expressed in the dorsal region of the cercus (Fig. 4E). A transverse section of the immunostained cercus reveals that en-positive cells are localized in the posterior one third (Fig. 4F). The similar expression pattern was also observed in transverse sections of other immunostained developing appendages (data not shown).

Expression patterns of Distal-less *depend on the type of appendages*

We used a polyclonal antibody to observe expression of *Distal-less* (*Dll*) in *G. bimaculatus* embryos at stages 5~12. Stained embryos are shown in Figs. 5 and 6. *Dll* expression was specifically observed in developing appendages. The initial expression was found in the metathoracic appendage bud at stage 5 (Fig. 5A). By stage 8, *Dll* is expressed in the primordia of labrum, antenna, maxilla, labium, thoracic legs, and preuropodium (Fig. 5B). In addition, *Dll* expression was also observed in the apical region of the cercus at stage 9 (Fig. 5C). As shown in Fig. 6A, initial expressions in the maxillary and labial buds are limited to the distal half regions. These regions become the telopodites defined as the distal shafts of the arthropod limbs (Snodgrass, 1935). The similar expression pattern was also observed in the thoracic appendage buds. However, no *Dll* expression region was found only in mandibular buds (Fig. 6A). These expression patterns are consistent with those reported for *Drosophila melanogaster*, *Precis coenia* (Panganiban *et al*., 1994), and *Thermobia domestica* (Popadić *et al*., 1996).

With further elongation and segmentation of the appendages, the *Dll* expression becomes intense and then the expression pattern changes, depending on the appendage type (Fig. 5C, D). In thoracic legs, intense expressions were observed in the distal and middle regions (Fig. 6C). We found that the distal region becomes tarsus and a part of tibia in future, whereas the middle region corresponds to the entire region of femur. The similar expression pattern was also observed in the labial and maxillary palpus (Fig. 6B, C). *Dll* is also expressed in two endites of the maxilla, the galea and lacinia (Fig. 6B). Interestingly, the expression is restricted to the distal region of each endite, as observed in early gnathal limb buds.

In other types of appendages such as the labrum, antenna, and cercus, no change in *Dll* expression patterns was observed with elongation of the appendages (Fig. 5D), although the expression patterns in the appendages are more or less different from each other. In elongating antenna, *Dll* is expressed in most of the distal region corresponding to future pedicel and flagellum of the antenna, but not in the basal narrow region probably becoming the scape segment (Fig. 6D). The entire region of the growing labrum and the apical region of the cercus also consist of *Dll*-positive cells (Figs. 5D and 6E).

DISCUSSION

Expression patterns of en *and* Dll *are conserved in developing antenna and legs of* Drosophila *and* G. bimaculatus

In the insect appendage formation, two types of developmental process are known: One is seen in holometabolan insects such as *Drosophila*. The appendages are formed as imaginal discs in the larval body. The other type is known in hemimetabolan insects: The appendages are initially formed as buds in the early embryo. In *G. bimaculatus*, the type of the appendage formation belongs to the latter, in which imaginal discs are not formed during appendage development (Fig. 2B). We compared the expression patterns of *en* and *Dll* in the developing antenna and legs of *G. bimaculatus* with those in the corresponding imaginal discs of *Drosophila* as shown in Fig. 7. In the *Drosophila* embryo, *Dll* expression patterns are different between antenna and leg discs, whereas the *en* expression in the posterior compartment is similar between them. The differences between the antenna and leg buds were also observed in *G. bimaculatus* (Fig. 7). Therefore, the expression patterns of *en* and *Dll* in the developing antenna and legs closely resemble those in the *Drosophila* imaginal discs. The similarities of *en* and *Dll* expression patterns between the two organisms imply that common molecules may be involved in development of insect antenna and legs and that their functions may be common in various insects in spite of different mode of appendage development. Since the *en* and *Dll* expression patterns in the crustaceans have been reported to resemble closely those in insects (Patel *et al*., 1989a; Panganiban *et al*., 1995), these genes may function commonly in arthropod appendage formation.

Dll *is expressed in the telopodite of each appendage*

In the early development in *G. bimaculatus*, the similarities in *Dll* gene expression patterns were found among different appendage types except the mandible: *Dll* is expressed in the distal region corresponding to the telopodite of each appendage. Since the morphological analysis of the *Drosophila* mutant lacking the *Dll* activity suggested that formation of the telopodite is regulated by *Dll* (Cohen and Jürgens, 1989), the similarities in *Dll* expression patterns suggest that the initial formation of all appendages except the mandible may occur by determination of the telopodite of the appendage by *Dll*. In addition, *en* expression in the posterior compartment is

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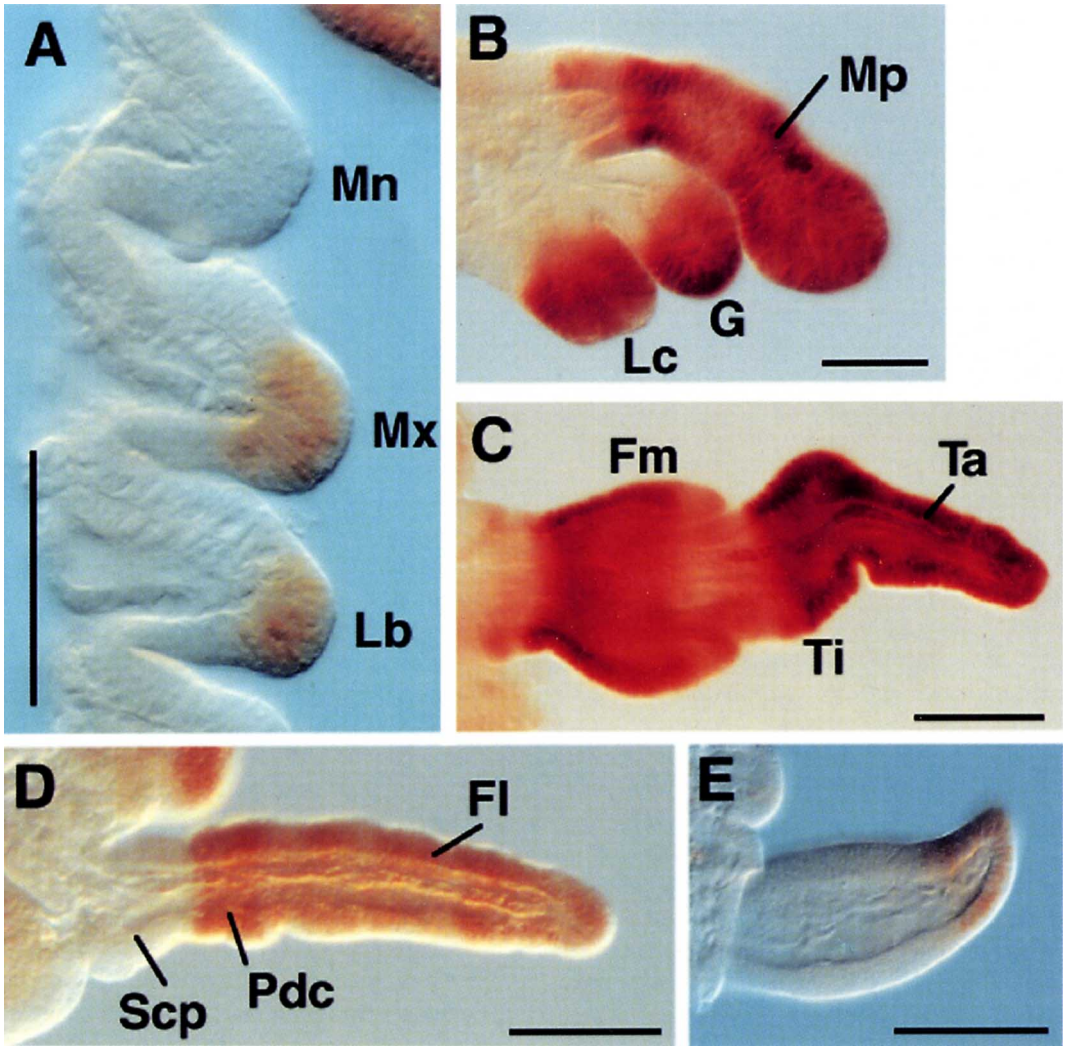

Fig. 6. Expression patterns of *Distal-less* in different appendage types. (**A**) Gnathal appendages at stage 8. *Dll* expressions were observed in distal regions of the maxilla (Mx) and the labium (Lb). No expression domain was observed only in the mandible (Mn). (**B**) Maxilla at stage 11. The expression were observed at the maxillary palpus (Mp), galea (G) and lacinia (Lc). The expression pattern in the maxillary palpus closely resembles that in leg, showing in **C**. (**C**) A mesothoracic leg at stage 11. Intense expression domains were observed in middle and distal regions. (**D**) An antenna at stage 11. The expression domain corresponds to the telopodite of the antenna. (**E**) A cercus at stage 11. The expression is restricted only in the distal region. Fl, flagellum; Fm, femur; Ta, tarsus; Ti, tibia; Pdc, pedicel; Scp, scape. Scale bars = 50 μm (**A**), 100 μm (**B**-**E**).

conserved pattern among different appendage types. Therefore, common expression patterns of the *Dll* and *en* genes in the early appendage formation in spite of different appendage type may support a hypothesis that every appendage type was evolved from a type of limb of a myriapod-like ancestor, which was originally proposed by Snodgrass (1935). He also assumed that in the insect mandible, the telopodite region is absent and consists of only the coxopodite. It is interesting to note that expression of *Dll* was not observed in the cricket mandible. Furthermore, no expression of *Dll* gene in the mandible has been also observed in the lepidopteran insect, *Precis coenia* (Carroll, 1994; Panganiban *et al.*, 1994). These facts support his assumption and suggest that mandible may evolve differently from other appendages in the insect. On the other hand, we found that expression of *en* was not detected in the labrum, although *Dll* is expressed in it. Thus, we assume that although the labrum is originally derived from an appendage, its posterior compartment may be somehow degenerated.

Late expression patterns of Dll *depend on the structure of the appendage*

We found that expression domains of the *Dll* genes become defined finely in each appendage type with the progress of morphogenesis in the appendages. For instance, since the expression domain of the *Dll* gene was found to correspond to the telopodite region in each appendage, we found that the boundary between coxopodite and telopodite corresponds to the articulation region of coxa-trochanter of *G. bimaculatus* in the leg (Fig. 6C) or to the scape-pedicel articulation region in the antenna (Figs. 6D and 8). The *Dll* expression patterns in the maxillary palpus (Fig. 6B) also

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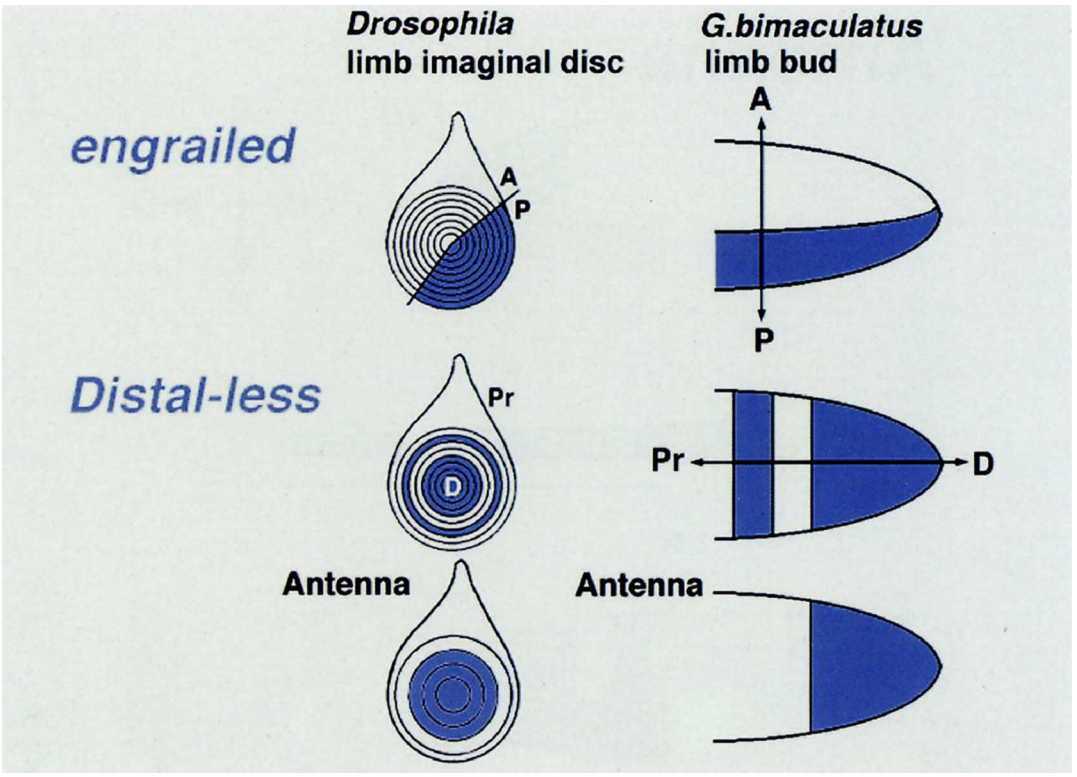

Fig. 7. Schematic illustration of *engrailed* and *Distal-less* expression patterns in *Drosophila* imaginal discs and cricket limb buds. Each gene expression domain found in cricket is similar to that in *Drosophila* in spite of different developmental mode. A, anterior; D, distal; P, posterior; Pr, proximal.

suggest that the maxillary palpus corresponds to the telopodite, as speculated previously (Snodgrass, 1935; Machida, 1981; Bitsch, 1994). In addition, *Dll* expression was also found in the distal regions of the galea and lacinia, which are two endites in the maxilla, showing that they also bear the telopodite elements. Thus, we concluded that the galea and lacinia may be homologous with other appendages.

In cricket embryos, from stage 9 the *Dll* expression patterns begin to change with the elongation and segmentation, depending on the type of appendages (Fig. 5C, D). Final expression patterns of *Dll* are classified into three types. First, the expression was expanded entire region of the appendage as seen in the antenna (Fig. 6D). Second, the expression was observed in a distal region of the appendage as in the cercus (Fig. 6E). Third, as in the leg, intense expression was observed in distal and middle regions (Fig. 6C). The space between the intensely expressed regions corresponds to the position of the prospective femur-tibia articulation (Fig. 8). The maxillary and labial palpus, whose expression pattern of *Dll* belongs to the third type, have an articulation corresponding to the femur-tibia, indicating high homology with the leg. In the maxillary palpus of *G. bimaculatus*, the articulation between the 2nd and 3rd segment from the distal corresponds to the femur-tibia articulation in the leg. On the other hand, in the first and second types, since the space observed in the third type was not found, an articulation corresponding to the femur-tibia may be absent. Thus, we considered that the *Dll* expression patterns in the late appendage development are specific for the appendage type and that these expression patterns may be closely related to the segmentation patterns, especially participated in the positioning of the articulation between the femur and the tibia or between corresponding segments of the appendages.

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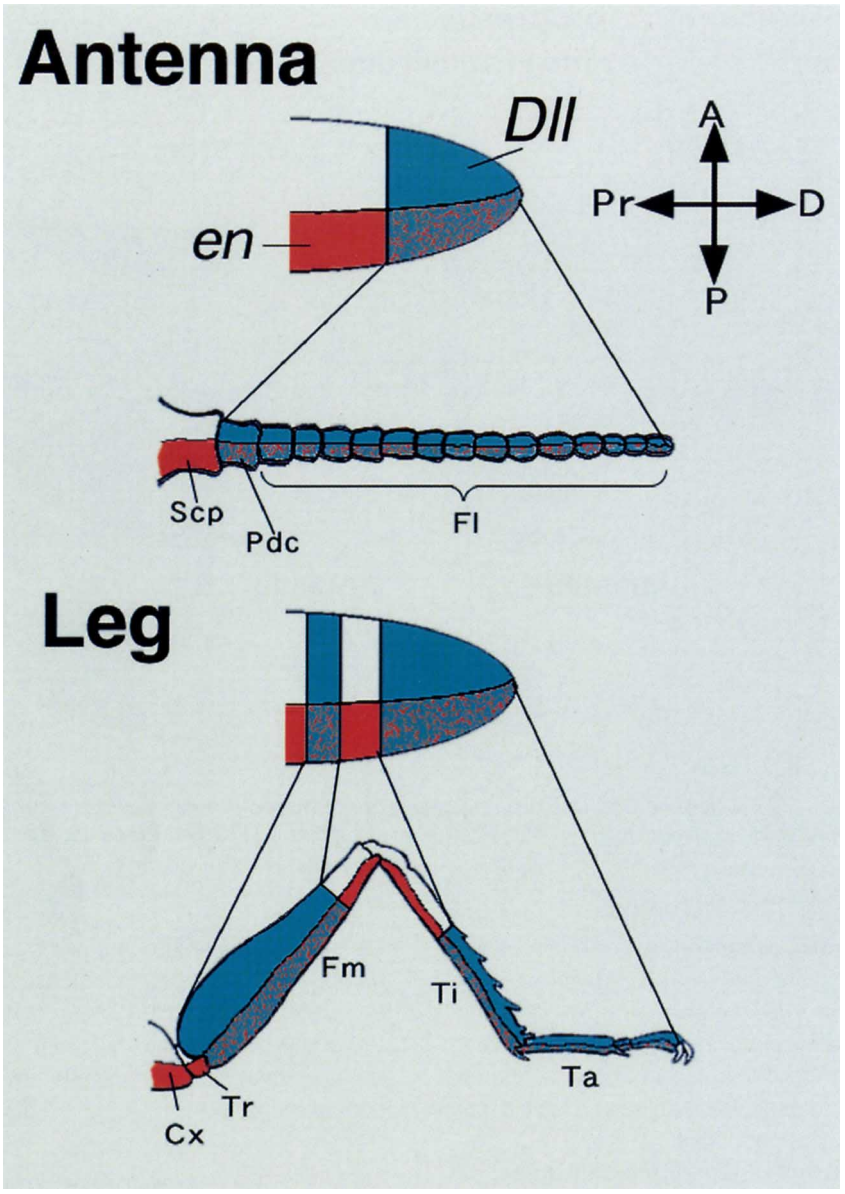

Fig. 8. Illustrations showing *engrailed* (*en*; red) and *Distal-less* (*Dll*; green) expression domains both in cricket antenna and leg. *en* is expressed in the corresponding posterior compartment of the antenna and leg buds. *Dll* expression domains correspond to pedicel (Pdc) and flagellum (Fl) in antenna. Space between two intense expression domains of *Dll* in leg correspond to the region including the femur (Fm) -tibia (Ti) articulation. Cx, coxa; Scp, scape; Ta, tarsus; Tr, trochanter.

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