Correlation of diversity of leg morphology in *Gryllus bimaculatus* (cricket) with divergence in *dpp* expression pattern during leg development

Nao Niwa¹,†, Yoshiko Inoue¹,‡, Akiyoshi Nozawa¹, Mariko Saito¹, Yoshio Misumi², Hideyo Ohuchi¹, Hidefumi Yoshioka¹,§ and Sumihare Noji**

¹Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, Tokushima 770-8506, Japan
²Department of Biochemistry, Fukuoka University School of Medicine, Jonan-ku Fukuoka 814-0180, Japan
*Present address: Department of Developmental Genetics, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan
§Present address: Hyogo University of Teacher Education, 942-1 Shimokume, Yashiro-cho, Kato-gun, Hyogo 673-1419, Japan
†These authors contributed equally to this work.
**Author for correspondence (e-mail: noji@bio.tokushima-u.ac.jp)

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SUMMARY

Insects can be grouped into mainly two categories, holometabolous and hemimetabolous, according to the extent of their morphological change during metamorphosis. The three thoracic legs, for example, are known to develop through two overtly different pathways: holometabolous insects make legs through their imaginal discs, while hemimetabolous legs develop from their leg buds. Thus, how the molecular mechanisms of leg development differ from each other is an intriguing question. In the holometabolous long-germ insect, these mechanisms have been extensively studied using *Drosophila melanogaster*. However, little is known about the mechanism in the hemimetabolous insect. Thus, we studied leg development of the hemimetabolous short-germ insect, *Gryllus bimaculatus* (cricket), focusing on expression patterns of the three key signaling molecules, *hedgehog* (*hh*), *wingless* (*wg*) and *decapentaplegic* (*dpp*), which are essential during leg development in *Drosophila*. In *Gryllus* embryos, expression of *hh* is restricted in the posterior half of each leg bud, while *dpp* and *wg* are expressed in the dorsal and ventral sides of its anteroposterior (A/P) boundary, respectively. Their expression patterns are essentially comparable with those of the three genes in *Drosophila* leg imaginal discs, suggesting the existence of the common mechanism for leg pattern formation. However, we found that expression pattern of *dpp* was significantly divergent among *Gryllus*, *Schistocerca* (grasshopper) and *Drosophila* embryos, while expression patterns of *hh* and *wg* are conserved. Furthermore, the divergence was found between the pro/mesothoracic and metathoracic *Gryllus* leg buds. These observations imply that the divergence in the *dpp* expression pattern may correlate with diversity of leg morphology.

Key words: *Gryllus bimaculatus*, Cricket, Leg development, *hedgehog*, *wingless*, *decapentaplegic*

INTRODUCTION

More than a million insect species have been identified so far, displaying a staggering variety of adult morphologies. One of fundamental questions in biology is how such various morphologies evolved on the earth. One of the ways in which to answer this question is to determine the molecular mechanisms of development in various insects (Akam and Dawes, 1992; Patel, 1993, 1994; Akam, 1994, 1998). The insects can be grouped mainly in two categories, holometabolous and hemimetabolous, according to the extent of their morphological change during metamorphosis. In holometabolous insects, the larvae are usually quite unlike the adult, and the pupal stage is present between the last larval stage and the adult. However, in hemimetabolous insects, the larva hatches in a form that generally resembles the adult, except for its small size and lack of wings and genitalia. How these molecular mechanisms of development differ from each other is thus an intriguing question. Although the mechanism in *Drosophila melanogaster*, which belongs to holometabolous, has been extensively studied, little is known about the mechanism in the hemimetabolous insect.

In order to study development of the hemimetabolous short-germ insect, we have employed the two-spotted cricket, *Gryllus bimaculatus*, instead of the grasshopper, because we can easily rear an inbred line of the cricket and use their eggs at any time (Niwa et al., 1997), and because the cricket is widely used to study insect physiology and neurobiology (Engel and Hoy, 1999; Paydar et al., 1999; Wenzel and Hedwig, 1999, other references are cited therein). There are two major differences in the developmental process between the fly (Fig. 1A-C) and cricket (Fig. 1D,E):

1) Long and short germ – the development of *Drosophila* is typical of the long-germ mode of insect development, in which the pattern of segments is established by the end of the blastoderm stage. Short-germ insects, by contrast, generate all
or most of their metameric pattern after the blastoderm stage by the sequential addition of segments during caudal elongation (Fig. 1D).

(2) Imaginal disc and bud formation – the fly becomes adult through the pupal stage, in which new adult organs develop from the imaginal discs (e.g. Fig. 1B,C), while the cricket becomes adult without any profound discontinuity. The rudiments of the legs, eyes and other most of adult structures are formed by budding formation and already present at hatching (e.g. Fig. 1D,E).

As a first step to answer the question, we focused on expression patterns of the three genes, hedgehog (hh), wingless (wg) and decapentaplegic (dpp), because these genes code key signaling molecules during development that are involved in pattern formation of various tissues including segments, legs, guts, eyes, etc. in Drosophila (Lawrence and Struhl, 1996) as well as in vertebrates (Hogan, 1999). In this study, we cloned cDNAs for the Gryllus hh, wg and dpp genes, and then observed their expression patterns during development. The three genes are expressed in segments, leg buds, the fore- and hindgut, and eyes of the Gryllus embryo. Here, we report results related to leg development as a typical case.

We found that expression patterns of Gryllus hh and wg in the leg bud were exactly the same in the three thoracic legs and principally resembled those observed in the leg imaginal discs of Drosophila. These results suggest the existence of a common mechanism for leg pattern formation. However, it was found that expression pattern of Gryllus dpp in the metamorphic leg bud became distinct in later stages from that in the pro- or mesothoracic leg buds, which become morphologically different legs from the metamorphic leg. Furthermore, we found that Gryllus dpp was expressed in the leg bud in a different way from Drosophila dpp in the leg imaginal disc. Recently, Jockusch et al. (2000) reported that in the grasshopper Schistocerca americana, the expression pattern of dpp differs radically from that of Drosophila dpp. Interestingly, we found that the pattern of Schistocerca dpp is also distinct from the Gryllus pattern. These observations imply that divergence in the expression pattern of dpp may be related to diversity of the leg morphology, rather than difference in the developmental mode.

MATERIALS AND METHODS

Animals
All nymphs and adults of Gryllus bimaculatus (Gb, two-spotted cricket) were reared as described previously (Niwa et al., 1997). Staging of the embryos was carried out according to the Niwa et al., 1997 (see the number in Fig. 1D,E).

Cloning of Gbhh, Gbwg and Gbdpp
A cDNA fragment was cloned by PCR with degenerate primers designed as follows:

5'-MGNTGYAARGARAARTNAA-3' (outside 5' primer), 5'-ACCCARTCRTGCCCGNGG-3' (outside 3' primer), 5'-GT-NATGAAAYCARTGCNGGCGG-3' (inside 5' primer) and 5'-TCAACNCNGCTCNGNC-3' (inside 3' primer) for Gbhh; 5'-GARTGYYAARTGCGAYGNATG-3' (5' primer) and 5'-ANG-TRACRTKRCANCYTYCT-3' (3' primer) for Gbwg; and 5'-GGATTTCCGYTGAGHTNGNCNC-3' (5' primer) and 5'-CCATCGATGAGCRTACRCANCYTYTGGG-3' (3' primer) for Gbdpp.

To isolate longer cDNAs, a cDNA library constructed with mRNA isolated from three-day-old Gb embryos was screened using the corresponding cDNA labeled with [32P]-dCTP. The DNA sequences are available from GenBank/DDBJ under the Accession number AB044709 for Gbhh, AB044713 for Gbwg, and AB044710 for Gbdpp.

Whole-mount in situ hybridization and immunostaining
Standard protocols for whole-mount in situ hybridization using a digoxigenin (DIG)-labeled antisense RNA probe (Wilkinson, 1992) or double probes (one DIG-labeled and one fluorescein isothiocyanate (FITC)-labeled) (Dietrich et al., 1997) for chick embryos and immunohistochemistry for grasshopper embryos (Patel et al., 1989) were used for cricket embryos. A monoclonal antibody against the engrailed protein, Mab 4D9, was purchased from the Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, USA). A polyclonal antibody against the Distal-less protein which is a marker protein for the distal identity of the leg bud (Cohen et al., 1989) was kindly provided by Dr Sean Carroll. Stained embryos were sectioned as described previously (Sasaki and Hogan, 1993).

RESULTS

Cloning of Gryllus cDNAs for hedgehog, wingless and decapentaplegic
We cloned Gryllus cDNA homologs of hedgehog (Gbhh), wingless (Gbwg) and decapentaplegic (Gbdpp) by screening a cDNA library constructed with mRNAs isolated from three-day-old Gryllus embryos (see Methods). Homologies of the deduced amino acid sequences for HH and WG between Gryllus and Drosophila melanogaster (Dm) are 68% (N-terminal half, Tashiro et al., 1993) and 69% (Rijsewijk et al., 1987), respectively. A partial deduced amino acid sequence of GbDPP exhibits higher homology to the corresponding fragment of DmDPP (54%, Padgett et al., 1987), Schistocerca americana DPP (64%, Newfeld et al., 1995) and Tribolium castaneum (Tc) DPP (48%, Sanchez-Salazar et al., 1996) than to that of Dm60A (30%, Wharton et al., 1991; Doctor et al., 1992) and DmSCW (27%, Arora et al., 1994), which belong to the TGFB family. We also cloned a cDNA fragment encoding a partial amino acid sequence of Gb60A that may be the Drosophila ortholog of 60A, because of a high homology of 79% (Wharton et al., 1991; Doctor et al., 1992). Judging from the high homology of the amino acid sequences of the three proteins between Gryllus and Drosophila, and from their expression patterns in embryos (see below), we concluded that Gbhh, Gbwg and Gbdpp were the orthologs of Dmhh, Dmwg and Dmdpp, respectively.

Expression patterns during germ-band elongation (stages 4-5) and segmentation (7-10)
We performed whole-mount in situ hybridization with Gryllus embryos to observe expression patterns of the three genes. In stages 4-5, during which the embryonic rudiment lengthens as posterior segments are added (Fig. 1D), Gbhh and Gbwg were expressed as stripes in either side of each segment boundary, as observed in Drosophila, and in the most posterior regions (Fig. 2), as revealed by double stain (Fig. 2D). With increase in the number of the segmental stripes posteriorly from 6 (Fig. 2A,B) to 7 (Fig. 2C,D), Gbdpp was expressed along the lateral edges of the embryo parallel to the A/P axis (Fig. 3A, see also
At stage 12 of embryogenesis, hh (green) and wg (red) are expressed as stripes in the posterior and the anteroposterior (A/P) compartment boundary, respectively, while dpp (blue) is expressed in two longitudinal stripes in the lateral trunk and dorsalmost region. The primordia of the thoracic imaginal discs of the Drosophila embryo are allocated in response to signals from the wg gene product (see text for details). (B) The relationship between the third instar disc and the adult leg (C) is shown schematically. Distal-less (Dll) (light green) is expressed in the central domain where wg and dpp meet. (D) Illustrations showing Gryllus embryogenesis (ventral view). Numbers indicate developmental stages. In a hemimetabolous insect like a cricket, the adult leg develops from the limb bud formed in the embryo, as schematically illustrated. Gryllus is a short-germ insect: the protocerebrum (Pcp) and protocereum (Pco) of presumptive anterior segments comprise most of the embryonic rudiment which lengthens as posterior segments are added during development. (E) Metathoracic leg formation. An, antenna; Cer, cercus; Cl, claw; Cx, coxa; Ey, eye; Fm, femur; G, gnathal segments; Lb, labrum; Lm, labium; Mn, mandible; Mx, maxilla; P, pleuropodium; Pl, prothoracic leg; S, tibial spur; St, stomodium; Ta, tarsus; Ti, tibia; Tr, trochanter.

Fig. 2. Expression patterns of Gbh h and Gbwg in Gryllus embryos at stages 4-5. All photos represent ventral views of the embryo with anterior to the left. In all panels, arrowheads marks the stripe 5 (the second thoracic segment and new stripe is indicated by a diamond). (A) Expression pattern of Gbh h: 5 segmental stripes and a new stripe are observed. (B) Expression pattern of Gbwg at stage 4. wg stripe initiates from the midline of the germ band and spreads dorsally. (C) Expression patterns of Gbh h and Gbwg at stage 4+: 6 segmental stripes and a new stripe are observed. (D) Double labeling reveals that the Gbwg-expressing cells (dark blue) reside directly anterior to the Gbh h-expressing cells (red). Scale bars, 100 μm.

**Expression patterns during leg-bud formation (stages 6-10)**

The limb buds are formed in the three thoracic segments at stage 6 (Fig. 1D). These grow longer at stages 7-8 (3-4 day) (Fig. 1E) and become folded and grooved. The limb bud will become segmented by stages 9-10 (5-6 day) (Fig. 1E). In stages 7-10, Gbh h was expressed in the posterior region along the entire proximodistal (P/D) axis (Fig. 5A-D). This expression pattern coincided with the EN-expressing region (Niwa et al., 1997). Gbh h was expressed in posterior two third of the leg circumference, as shown in a transverse section of the leg bud (Fig. 5D). However, Gbwg was expressed as a stripe on the ventral side of the A/P boundary along the entire P/D axis of the leg bud, as shown in Fig. 5A (double stain with Gbh h) and in Fig. 5E-H, which is confirmed with transverse sections of the leg bud (Fig. 5H).

Expression of Gbdpp in the limb bud was first detected at stage 6.5 as a stripe, extending along the dorsal side of the limb bud from the longitudinal expression of the embryo to the distal tip of the limb bud continuously (data not shown). By stage 7, a faint Gbdpp stripe was observed along the dorsal side of the limb bud (Fig. 3B), which is then restricted to the dorsal side of the most distal tip of the limb bud (Fig. 3C). In stage 7, Gryllus Distal-less protein (GbdDLL) was detected with the specific antibody in the distal tip of the limb bud (Fig. 5M). At stage 9, Gbdpp was expressed as four spots expression domains became spots in each abdominal segment (Fig. 4G). Expression of Gbdpp was still detected in the cells of the putative dorsal ectoderm (Fig. 4K).
along the P/D axis in the dorsal side of the A/P boundary, although the most distal expression is not detected (Fig. 5J).

At stage 10, the four spots of Gbdpp expression transformed into a nearly circumferential ring near the distal limb tips (data not shown). This ring is initially incomplete ventrally, but eventually closes and clearly extends through both anterior and posterior compartments of the leg. Other domains of dpp expression in more proximal segments still remained in patches at this stage.

**Expression patterns during leg-bud elongation after katatrepsis (stages 11-12)**

In stage 11 (6-7 day) (Fig. 1D), in which the embryo moves around the posterior pole from dorsal to ventral side of the egg (katatrepsis), although signals of Gbhh in the posterior compartment of the abdominal segments became narrow and weak, those in the lateral-dorsal regions of the thoracic segments became intense in addition to the signals in fore- and hind-guts (Fig. 4D). Expression of Gbwg in each segment is localized in the regions where trachea cells differentiate (Fig. 4H). The expression of Gbdpp also became intense as spots in the lateral regions of the thoracic segments (Fig. 4L).

The major leg segments are established at stages 11-12 (6-8 day) (Fig. 1E). In the mesothoracic limb bud, expression patterns of Gbdpp became five circumferential rings and two ventral spots (Fig. 5K), as schematically illustrated in Fig. 5N. The five dpp expression rings were located roughly in the middle of the primary leg segments of the tarsus, tibia and femur (Fig. 5K). In addition, three expression rings appeared in the presumptive three tarsus segments, prior to morphological change (Fig. 5K).
In later stages than stage 11, dpp expression patterns in the prothoracic /mesothoracic (T1/T2 leg buds) became different from those in the metathoracic leg bud (T3 leg bud) (Fig. 6). In the femur and tibia of the T1/T2 leg buds, dorsal and ventral stripes of Gbdpp expression became intense, while the expression remained circumferential in more distal segments. In transverse sections of the T2 tibia, expression of Gbdpp is also found in both dorsal and ventral side intensely (Fig. 5L). However, in the T3 leg bud, circumferential expressions of Gbdpp remained intense in the middle of each segment (Fig. 6).

**dpp expression in Drosophila imaginal discs**

Since expression patterns of Gbdpp were considerably different from those of Dmdpp in the larval leg imaginal disc, we observed expression patterns of Dmdpp in the imaginal disc of the pupa by means of whole-mount in situ hybridization. The expression patterns were similar to the patterns observed when stained with an enhancer trapped line of dpp-lacZ BS3.0 (Fig. 5P-R) (Blackman et al, 1991). These results indicate that Dmdpp is expressed in both dorsal and ventral sides of the A/P boundary. Dmhh (green) is expressed in the posterior compartment, while Dmdpp (blue) is expressed in both dorsal and ventral sides of the A/P boundary. Dmwg (red) expression overlaps Dmdpp expression (blue) in the ventral side of the A/P boundary, as indicated by purple. Expression patterns of Dmdpp in the leg imaginal disc (P,Q) and pupa (R). Schematic representation of expression patterns in the cross section of the pupal leg imaginal disc (S).

**Fig. 5.** Expression patterns of Gbhh, Gbwg and Gbdpp in *Gryllus* leg buds at stage 7, 9 and 11. Expressions of Gbhh was observed in the posterior region of the mesothoracic leg bud at stages 7 (A) (a double stain for Gbhh (red) and Gbwg (dark blue) is indicated by an open arrow), 9 (B) and 11 (C). The anterior side is towards the top. A transverse section at stage 11 reveals that Gbhh is expressed in the posterior region (D). Expression of Gbwg was observed in the ventral side of the limb bud along the anteroposterior boundary at stages 7 (E), 9 (F) and 11 (G). The dorsal side is towards the top. A transverse section at stage 11 reveals that Gbwg is expressed in the ventral region (H). A spot expression of Gbwg appears at the dorsal side of the coxa rudiment. Expression of Gbdpp was observed at the dorsal tip of the limb bud at stage 7 (I), as four spots at the dorsal side of the limb bud along the anterior-posterior boundary at stage 9 (J), and then as seven transverse strips at the regions corresponding to the leg segment structures at stage 11 (K). The dorsal side is to the top. A transverse section of mesothoracic tibia at stage 11 reveals that Gbdpp is expressed in both dorsal and ventral region (L). DLL protein was detected at the distal region of the leg bud (M). Schematic representation of expression patterns of Gbdpp in the leg bud at stage 11 (N). Segmental expression patterns correspond to the leg structures. In a cross section of the middle of the tibia (O), Gbhh (green) is expressed in the posterior compartment, while Gbdpp (blue) is expressed in both dorsal and ventral regions. Gbwg (red) expression overlaps Gbdpp expression (blue) in the ventral side of the A/P boundary, as indicated by purple. Expression patterns of Dmdpp in the leg imaginal disc (P,Q) and pupa (R). Schematic representation of expression patterns in the cross section of the pupal leg imaginal disc (S). Dmhh (green) is expressed in the posterior compartment, while Dmdpp (blue) is expressed in both dorsal and ventral sides of the A/P boundary. Dmwg (red) expression overlaps Dmdpp expression (blue) in the ventral side of the A/P boundary, as indicated by purple. Scale bars, 20 μm in A, E,I,M; 50 μm in B,F,J; 100 μm in C,G,K; 40 μm in D,H,L. Cx, coxa; Fm, femur; Ta, tarsus; Ti, tibia; Tr, trochanter.

**Fig. 6.** Expression patterns of Gbdpp in *Gryllus* thoracic leg buds at stage 11. The dorsal side is towards the top. In the prothoracic (T1) and mesothoracic (T2) leg buds, Gbdpp is expressed as five and seven spots in dorsal and ventral sides, respectively. Location of the spots may correspond to each leg segment of the tarsus (Ta), tibia (Ti), femur (Fm), trochanter (Tr) and coxa (Cx). In the metathoracic (T3) leg bud, Gbdpp is expressed as five circumferential bands (three in presumptive Ta, one in each of Ti and Fm) and two ventral spots (one in each of Tr and Cx). Asterisks indicates a nonspecific staining. Scale bars, 100 μm.
DISCUSSION

In order to examine whether the molecular mechanism of leg development is conserved in the insect, expression patterns of the three signaling molecules of hh, wg and dpp were compared as a first step. The expression profiles of the three genes are found to be essentially conserved in the hemimetabolous short-germ Orthopteran Gryllus and Schistocerca (Jockusch et al., 2000), holometabolous long-germ Dipteran Drosophila and Coleopteran Tribolium (Tcwg, Nagy and Carroll, 1994; Tcddp, Sanchez-Salazar et al., 1996), suggesting the developmental mechanism is common in principle in the insect. However, we found that expression pattern of dpp during leg development was divergent, although expression patterns of hh and wg were essentially conserved. For comparison, the expression patterns of dpp in Gryllus, Drosophila and Schistocerca are schematically illustrated in Fig. 7, in which the processes of insect leg development are divided into two phases: Phase 1 – Allocation of the leg primordium; Phase 2 – leg patterning. In the following, the divergence of dpp expression patterns and its correlation with diversity of leg morphology are discussed for each phase.

Leg allocation in Phase 1

In the allocation phase of Drosophila 5h embryos, Dmwg and Dmhh are expressed in a stripe along the anteroposterior (A/P) compartment boundary (Fig. 7A) and in the posterior region of each segment, respectively (French and Daniels, 1994; Campbell and Tomlinson, 1995 as reviews). However, Dmdpp is expressed throughout the dorsal region (Fig. 7A) and then in the dorsal side of the wg stripe (Fig. 7B) (St Johnston and Gelbart, 1987). Later, the expression changes to give two thin stripes running anteroposteriorly along the length of the embryo (Fig. 1A) (St Johnston and Gelbart, 1987). Recently, Goto and Hayashi (1997) demonstrated that WG, but not DPP, was responsible for initial specification of the limb primordia with a distal identity and for induction ofDll. They proposed a model for the allocation of the limb primordium (the G-H...
model; Goto and Hayashi, 1997) (Fig. 7A,B). A stripe of WG induces the limb primordium expressingDll. Repression ofDll by DPP from the dorsal side and by Spits (Drosophila EGF) from the ventral side limits the limb formation only in the lateral position. Then, DPP specifies proximal cell identity in the primordium in a concentration-dependent manner.

In Gryllus and Schistocerca embryos, expression ofwg was detected in a stripe along the A/P compartment boundary of the body segment (Fig. 7A). In Gryllus embryos, expression ofdpp was first detected along the periphery of the germ band (Fig. 7A). Similar expression patterns were observed also in Tribolium (Sanchez-Salazar et al., 1996). Although the cricket and grasshopper belong to the same Orthoptera, the expression patterns ofSadpp (Jockusch et al., 2000) are more complicated than those ofGbDpp (Fig. 7). In Schistocerca embryos at early stages, Sadpp is expressed in two partial stripes in each hemisegment, intrasegmentally and intersegmentally, paralleling the D/V axis (Jockusch et al., 2000) (Fig. 7A). The intrasegmental stripes extend along both dorsal and ventral sides of the presumptive leg field (Fig. 7B). Early expression patterns ofGbDpp resemble those ofDmdpp or TcDpp more closely than those ofSadpp (Fig. 7A,B). Thus, thewg expression pattern appears conserved in the allocation phase, while early expression patterns ofdpp seem divergent even in the Orthoptera. Thus, more data are necessary to judge whether the G-H model is also applicable as a model for initiation of limb formation in other insects.

**Patterning along the proximodistal axis in Phase 2**

In the Phase 2, in the Drosophila leg imaginal disc, Dmhh is expressed in the posterior compartment of the disc, determining the A/P pattern, and induces Dmdpp and Dmwg expression in the dorsal and ventral side of the A/P boundary, respectively (Figs 1B and 7C) (Campbell et al., 1993; Basler and Struhl, 1994; Diaz-Benjumea et al., 1994). They act cooperatively in a concentration-dependent manner to organize the P/D axis and induce expression ofDll at the center of the disc (Lecuit and Cohen, 1997). In Gryllus and Schistocerca limb buds, since hh and wg are expressed in the posterior and the ventral side of the A/P boundary, respectively, their functions during limb development should be conserved among the fly, cricket, beetle and grasshopper.

However, expression patterns ofGbDpp are considerably different from those ofDmdpp; GbDpp expression is limited to a dorsal stripe transiently around the time of limb bud emerging at stage 6–7 (Figs 3B and 7B), and at this time, expression ofDll was found in the distal tip of the limb bud (Fig. 5M). This transient expression pattern was also reported in Schistocerca embryos (Fig. 7B). In Drosophila, removal ofDpp signaling prior to the second larval instar results in loss ofDll expression, while later removal ofDpp does not affectDll expression, indicating thatDpp is required for the initiation but not maintenance ofDll transcription (Lecuit and Cohen, 1997). Thus, it is reasonable to consider that transientdpp expression is enough to induce expression ofDll, which is required for the P/D leg pattern formation.

In the Drosophila leg imaginal disc, Dmdpp expression is restricted as a stripe in the dorsal side of the anterior compartment (Fig. 1B) (Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Brook and Cohen, 1996). HH was demonstrated to induce expression ofDmdpp in the dorsal side along the A/P boundary (Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Brook and Cohen, 1996), and is repressed in the posterior leg compartment by the EN protein (Sanicola et al., 1995). In the Gryllus leg buds, however, GbDpp is expressed as four spots in the dorsal side of the A/P boundary at stage 9 (Figs 5J and 7D), then expression domains transform into a nearly circumferential ring at stages 10–11 (Figs 5K, and 7E,F), which is located roughly in the middle of the primary leg segments, as observed in Schistocerca. Since GbEN (Niwa et al., 1997) and Gbhh are always expressed throughout the posterior compartment, the relation between the expression domain ofDmdpp with that ofDmhh/Dmen does not hold true in the Gryllus leg bud, indicating divergent roles ofdpp in leg patterning. Jockusch et al. (2000) proposed an idea thatSadpp may play roles in establishment of boundaries between the leg segments, because the appearance of segmentally reiterated rings ofSadpp precedes morphological segmentation of leg buds (Fig. 7E). This idea may be applicable to Gryllus leg buds.

For the patterning along the proximodistal (P/D) axis in the Drosophila limb disc, two models have been proposed: the gradient model (Lecuit and Cohen, 1997; Abu-Shaar and Mann, 1998; Wu and Cohen, 1999) and the intercalation model (Goto and Hayashi, 1999). In the gradient model, it is proposed that since WG and DPP are secreted from the respective ventral and dorsal sides of the cells at the A/P compartment boundary, forming a gradient along the P/D axis, differential response to this gradient leads to circular patterns corresponding to the leg segments. However, in the intercalation model, they proposed that after formation of extreme proximal and distal structures by DPP and WG, the intermediate leg segments are intercalated by a signal from the proximal domain (Goto and Hayashi, 1999). In the case ofGryllus limb buds, the intercalation model appears favorable, because the discrete expression ofGbDpp cannot maintain the DPP gradient in the limb bud necessary for circular patterns corresponding to the leg segments. Furthermore, intercalation of the leg segments occurs during development ofGryllus leg buds (Fig. 1E): the most distal part is formed at first, then limb segmentation occurs at the coxopodite/telopodite boundary, followed by the femur/tibia, trochanter/femur and tibia/tarsus boundaries, judged from the expression patterns of DLL (Niwa et al., 1997), *Gryllus homothorax, dachshund* and aristaless (data not shown). These results are consistent with those observed in the Schistocerca leg buds, using an Annulin antibody (Singer et al., 1992) for detection of the limb segment boundary. The intercalation model may be supported also by the fact that regeneration of amputated cricket legs occurs through intercalation of the missing segments (data not shown) as observed in the cockroach leg (French et al., 1976).

**Patterning along the dorsoventral axis in Phase 2**

For establishment of the dorsoventral pattern of the Drosophila leg disc, it has been suggested that the two signals ofWG and DPP act antagonistically to repress each other’s expression and to specify dorsal and ventral expression patterns (Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Brook and Cohen, 1996). However, in later stages, in early third instar to early pupal leg discs ofDrosophila, Dmdpp is expressed in both dorsal and ventral sides (Figs 5P–Q, and 7E,F), as reported by Masucci et al. (1990) and Jockusch et al. (2000). This faint ventral expression in theDmwg expression domain may
We speculated that the similar difference in expression patterns correlate with leg morphology, implying that temporal and speculation. Thus, the expression patterns of little difference in morphology among the cannot verify our speculation. In Sadpp development is likely to be conserved, judged from expression correlated with morphological diversity.

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