ORIGINAL ARTICLE

Ultrabithorax and abdominal-A specify the abdominal appendage in a dosage-dependent manner in silkworm, Bombyx mori

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In insects, there is a considerable diversity in leg distribution on the body, including number, segmental arrangement, morphological identity and consequent function, but the genetic basis for these differences is not well understood. Here by positional cloning, we showed that a ~ 355 kb region, including *Bombyx mori Ultrabithorax* (*BmUbx*) and *abdominal-A* (*Bmabd-A*), was responsible for the silkworm mutant *Kh-extra-crescents-like* (*E^{Kh}-l*) that displayed additional thoracic limb-like legs on the first abdominal segment (A1) and occasionally on the second abdominal segment (A2). We found that *BmUbx* gene was downregulated at both messenger RNA level and protein level in *E^{Kh}-l* embryo, while its expression domain in the *E^{Kh}-l* embryo was almost the same as that in the wild type. Whereas *Bmabd-A* was upregulated at both levels and was ectopically overexpressed on the supernumerary leg-bearing segments in *E^{Kh}-l*. Compared with the previously reported *E^{cs}-l* mutant in which increased expression of both *BmUbx* and *Bmabd-A* gave rise to ectopic proleg-like appendages on the same segments, we propose that overexpressed *Bmabd-A* gene is capable to promote the outgrowth of extra leg appendages on A1 and A2 segments, whereas *BmUbx* gene is required to specify accurate morphologies of the ectopic legs in a dosage-dependent manner in silkworm. These results provide insights into how these hox genes regulate the leg morphologic diversity on the same segments.
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INTRODUCTION

In insects, there are several sets of serially homologous appendages distributed on the repeated segments, showing a considerable diversity in number, position and morphology. For example, each thoracic segment carries a pair of appendages called thoracic limbs (Jockusch *et al.*, 2004), whereas legs have been suppressed in the abdomen of most insects. However, still some insects possess abdominal appendages called prolegs in several groups, such as the basal hexapod Collembola and larval Lepidoptera (Angelini and Kaufman, 2005). The type of appendage depends on the identity of the segment where they appear, indicating that the Hox genes contribute to appendage specification (Lawrence and Morata, 1994; Morata and Sanchez-Herrero, 1999), through acting on targets downstream of the original proximodistal axis genes (Jockusch *et al.*, 2004).

Hox genes *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) are expressed along the anterior–posterior body axis to determine whether the leg develop on these segments or not and to specify the leg identity in insects. The *Antp* is expressed in the first three thoracic segments and regulates limbs developing on the thorax (Abbott and Kaufman, 1986). A particularly marked example is provided by the *Antp* gain-of-function mutations in *Drosophila melanogaster*, which transform the antennae into second thoracic legs (Kaufman *et al.*, 1990). *Antp* loss-of-function mutation in

silkworm produced the fused thorax and defects in limb development (Chen *et al.*, 2013a). The Hox gene *Ubx* is normally expressed in a complex pattern in the posterior thoracic and anterior abdominal segments of insects, where it has multiple roles in modifying specific morphological differences between the mid-legs (T2) and meta-legs (T3) on the thorax, including appendage size, shape and function (Averof and Patel, 1997; Akam, 1998; Roch and Akam, 2000; Tomoyasu *et al.*, 2005), and inhibit the development of abdominal prolegs via repressing the *Distal-less* (*Dll*) expression (Gebelein *et al.*, 2002; Ronshaugen *et al.*, 2002). Although insects expressed *abd-A* in a similar pattern from the posterior compartment of the first abdominal segment to the remaining abdominal segments (Kelsh *et al.*, 1994; Shippy *et al.*, 1998), there is considerable diversity in the prolegs distribution on the *abd-A* expressed segments among various insects, with a wide range of variation in both segmental arrangement and number. In Diptera *Drosophila* (Vachon *et al.*, 1992) and Coleoptera *Tribolium* (Lewis *et al.*, 2000), *abd-A* suppressed leg development by inhibiting expression of the gene *Dll* leading to the deficiency of abdominal limbs in larvae, however, *abd-A* promotes the development of prolegs in silkworm (Chen *et al.*, 2013a). The *Abd-B* gene is expressed in the last few segments of the abdomen and is required for proleg suppression in these segments (Tomita and Kikuchi, 2009; Xiang *et al.*, 2011). It is therefore of great interest from an evolutionary

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point view to understand how the Hox genes affect the leg morphologic variation, including the identity, number and segmental arrangement, among variety insects, or even between the limbs and proleg within the same species.

In silkworm, *Bombyx mori*, > 30 homeotic mutants belong to pseudoallele group E exhibiting various anomalies in legs development, including number, position and identity (Ueno *et al.*, 1992; Banno *et al.*, 2005). The silkworm E loci were located on the chromosome 6 and were presumed to be analogous to the *D. melanogaster* bithorax complex (BX-C; Ueno *et al.*, 1992; Yasukochi *et al.*, 2004). Over 20 mutants of them present extra legs on A1 or A2 or on both of the two abdominal segments in the larval stage. Furthermore, the identity of these supernumerary legs were thoracic limb-like in some mutants but were abdominal proleg-like in others. Thus, it raises an intriguing question of how these Hox genes regulate the leg morphologic variation on the same segments. To address these questions, here we reported the molecular identification and characterization of the gene responsible for the spontaneous *Kh-extra-crescents-like* (E^{Kh}-*l*) mutant that presents extra thoracic limb-like legs on the first abdominal (A1) segment. Our results indicated that E^{Kh}-*l* locus was restricted within a ~ 355 kb region including the *BmUbx* and *Bmabd-A* gene. Furthermore, we analyzed the expression pattern of *BmUbx* and *Bmabd-A* in the wild type (WT; Dazao) and the E^{Kh}-*l* mutant to determine their role in generating the extra limb-like legs on abdomen.

MATERIALS AND METHODS
Silkworm strains
The E^{Kh}-*l* and Dazao (WT) stains were obtained from the Silkworm Gene Bank at Southwest University (Chongqing, China). E^{Kh}-*l* and Dazao were used as parent strains to produce F_1 offspring. For no recombination in female silkworms, 22 progeny from a single-pair backcross between an F_1 female and a Dazao male (BC_1F) were used for the linkage analysis, whereas 1724 progeny from the cross between Dazao female and F_1 male (BC_1M) were used for the recombination analysis. The developing embryos were incubated at 25 °C with adequate humidity and larvae were reared with fresh mulberry leaves under a photoperiod of 12 h light and 12 h dark at 25 °C.

DNA extraction
Total genomic DNA of parental strains and BC_1 individuals described above were extracted from adults and larvae, respectively. All the samples for DNA extraction were the whole body of the individuals. The samples were disrupted in liquid nitrogen and suspended in DNA extraction buffer (pH 8.0, 10 mM Tris-HCl, 0.1 M EDTA, 0.5% SDS) with 100 μg ml^{-1} proteinase K. Phenol–chloroform extraction was performed after digestion at 55 °C for 5 h. The DNA was precipitated with freezing dehydrated alcohol and dissolved in TE buffer (pH 8.0, 10 mM Tris-HCl, 1 mM EDTA).

Mapping and linkage map construction
Based on the previous studies, the E loci are located at the 21.1 cM on the sixth linkage group (Banno *et al.*, 2005), including the homeotic genes *BmUbx*, *Bmabd-A* and *BmAbd-B* (Ueno *et al.*, 1992), which were located scaffold nscaf2853. We searched the PCR-based amplicon length polymorphisms on nscaf2853 between the Dazao strain and the E^{Kh} mutant as markers for recombination analysis. The primer sequences and position information of the DNA markers for genetic analysis in this study were listed in Table 1.

The markers were first confirmed to be linkage with the E^{Kh} locus by using 20 BC_1F progeny (10 WT and 10 mutant), and subsequent were used for recombination analysis with a large BC_1M mapping populations (1724 individuals). The segregation patterns were analyzed using JoinMap 4.0 software (Van Ooijen, 2006), with a logarithm of the odds threshold of 4.0 and the Kosambi mapping function (Kosambi, 1944).

Quantitative real-time PCR
The relative expression levels of genes, *BmUbx* and *Bmabd-A*, were examined using Quantitative real-time (qRT-PCR). The embryos of E^{Kh}-*l*, E^{cs}-*l* and Dazao were dissected at the 7th day of embryonic developmental stage when the phenotype could be easily distinguished. Each sample contained eight individual embryos and was disrupted with a mini homogenizer (Polytron PT1600E, Kinematica, Lucerne, Switzerland), for RNA extraction using MicroElute Total RNA kit (mega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The complementary DNA was synthesized from total RNA sample using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions. qRT-PCR experiments were performed using the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) with a SYBR Premix EX Taq_kit (Takara), according to the manufacturer's recommended procedure. Referring to the full-length complementary DNA sequence of *Bmabd-A* and *BmUbx* (GenBank Accession number: AB461860 and AB505052), we designed gene-specific primers for qRT-PCR. The eukaryotic translation initiation factor 4A (silkworm microarray probe ID: sw22934) was used as an internal control. The primers used for qRT-PCR were listed in Table 1. The qRT-PCR condition was denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 31 s, 95 °C for 15 s, 60 °C for 20 s and 95 °C for 15 s. All experiments were performed in three independent biological sets and results were presented as mean ± s.d. Statistical analyses were performed using Student's *t*-test ($n = 3$), and *P*-values < 0.05 were considered statistically significant.

Protein isolation and western blotting
Proteins were isolated from embryos at the 7th day of embryonic developmental stage. Embryos were grinded in liquid nitrogen and boiled in 0.02 M phosphate-buffered saline, pH7.4, containing 2% SDS for 30 min. After centrifuging twice at 12 000 *g* for 15 min at 4 °C, we collected the supernatants. The concentration of proteins was determined using a BCA Protein Assay kit (Beyotime, Jiangsu, China) followed the manufacturer's instructions.

Table 1 The primer sets used in this study

Object	Marker	Forward primer	Reverse primer	Position
Linkage analysis	L1035	ACACGGGACTAACGGACTG	AAATTAAGAGGGAACGTATGCA	nscaf2853: 3101369–3102404
	L1094	GGTTTCTCAACGGAACACTACT	CAAAACTTCTACTGCCTGCTTACT	nscaf2853: 3103080–3104173
	L1048	CCGTTGCCTTCCAATCAG	CTGATAACAAAAGGGTTCAAAGAC	nscaf2853: 3112765–3113812
	L1779	CCAGCCTTAGGACGCTCATC	GCAGAGATCCGCCAACTAATAT	nscaf2853: 3205698–3207477
	L1011	TTATTGCTTCGTCCTAAGTATGTC	TAAAACCCTATCGTTCTGCTACTA	nscaf2853: 3225342–3226353
	L1194	ACCTATGTGCTCAGAAAATCGC	CAATCGTTGCCGTGTGAGA	nscaf2853: 3226755–3227948
	L1153	GCGTCTGCGGAGTTACCAT	TTTGTGAAAGTAGAATGTGGGTAAT	nscaf2853: 3356911–3358064
	L1391	TACGACTAAGACGCTGGTGAA	TCCTTGAAAGACTTCCCACC	nscaf2853: 3469287–3470678
Quantitative RT-PCR	sw22934	TTCGTACTGCTCTTCTCGT	CAAAGTTGATAGCAATTCCCT	
	Bmabd-A	GCATTCCACTTCAATCACTATCTG	TCTTCATTCTGTCCTGTTCCTCTC	
	BmUbx	CCACCAGCCTCGTCCTCA	CTTTCCCACCATAGCCATTCT	

For western blotting, proteins were separated by electrophoresis on SDS-polyacrylamide gel electrophoresis. The proteins were then transferred onto polyvinylidene fluoride membranes and probed with rabbit anti-Bmabd-A antibody (Chen *et al.*, 2013b) or rabbit anti-Ubx antibody (Tong *et al.*, 2014). After incubation with horseradish peroxidase-conjugated secondary antibodies (Beyotime) the signals were visualized with Lumi-Light PLUS western blotting substrate (Roche, Mannheim, Germany). Specific protein expression levels were normalized to tubulin for total protein measurements.

Immunofluorescence

Expression pattern of BmUbx and Bmabd-A was assessed using immunostainings in embryos of embryos of E^{Kh-l} and Dazao following the protocol as previously described (Chen *et al.*, 2013b; Tong *et al.*, 2014). The embryos were dissected at 2 days old. We used a rabbit anti-Ubx antibody (at 1:500; a gift from L Shashidhara), and rabbit polyclonal anti-Bmabd-A (at 1:200) antibody. The images were captured on a fluorescence microscope BX51TRF (Olympus, Tokyo, Japan).

RESULTS

Phenotypic characteristics of the E^{Kh}-*l* mutant

Compared with the WT strain (Figure 1a), the larva of E^{Kh}-*l* mutant exhibited a pair of extra rudimentary legs on the first abdominal (A1) segment and ~12% (13 out of 106) presented a pair of smaller ones on the second abdominal (A2) segment (Figure 1b). Advanced magnified observation of the supernumerary legs indicated that they were varied in morphology and length, and showed some bristles and dark pigmentation but typed to neither limb nor proleg based on their morphology at this stage (Figures 1c–e). To further explore the identity of the extra legs, we dissected the embryos in the late developmental stage (7–8 days after oviposition) and found that the E^{Kh}-*l* individuals presented additional legs similar to thoracic limb on the A1 segment according to the similar proximal/distal patterning module (Figures 1f–g). Furthermore, ~5% (5 out of 106) of the E^{Kh}-*l* moths had four pairs of legs compared with three pairs in the WT. The extra legs on the first abdominal segment were much smaller than
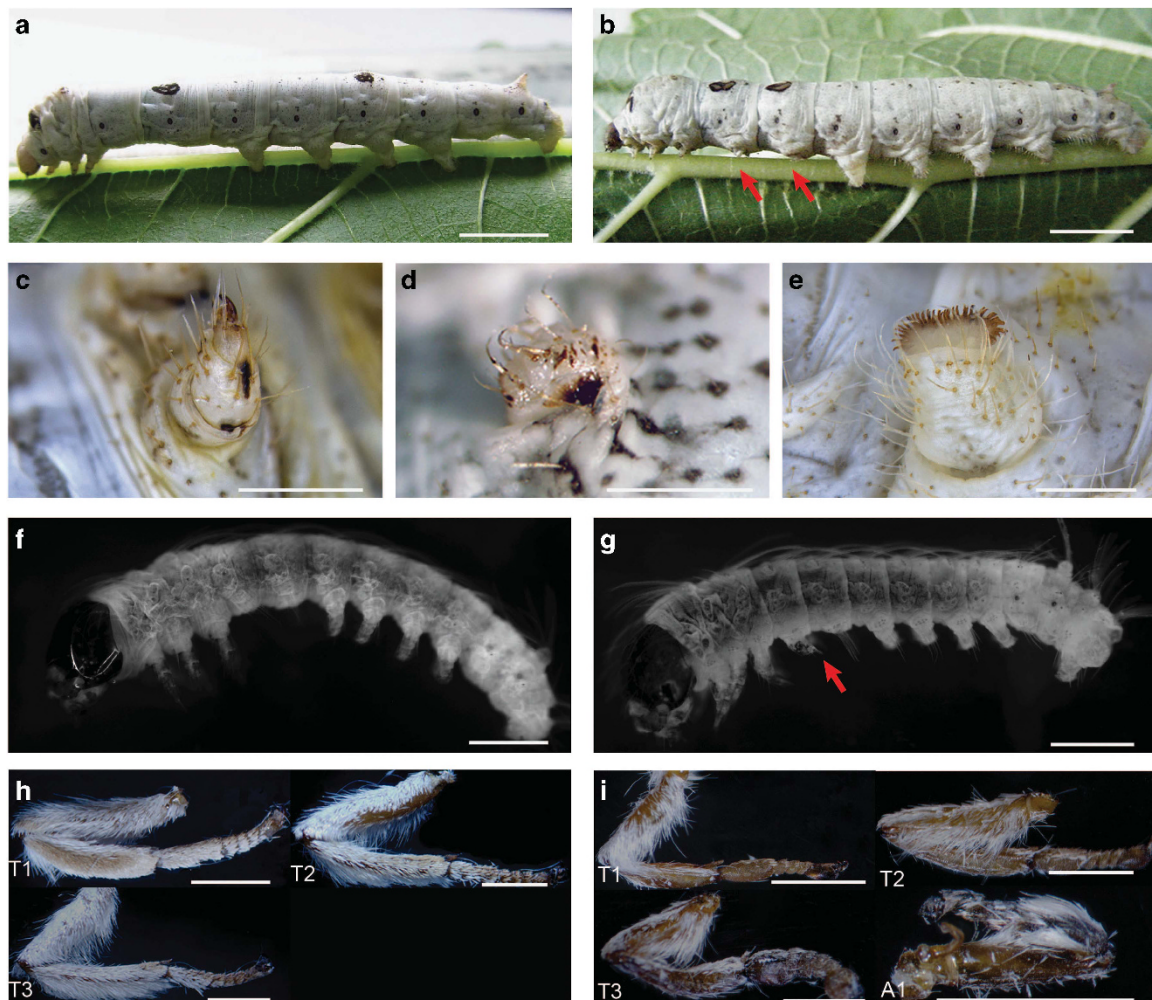
Figure 1 Phenotypes of the Dazao (DZ) wild type and E^{Kh}-*l* mutant. (**a**) Wild-type (Dazao) larvae have one pair of thoracic legs on each thoracic segment and have four pairs of prolegs on A3–A6. (**b**) The E^{Kh}-*l* larvae display supernumerary legs on A1 and A2 segment (red arrow). (**c**) The thoracic limb with bristles and distal claw on the T3 segment in E^{Kh}-*l*. (**d**) The morphology of the additional legs on A1 segment in E^{Kh}-*l*, showing some bristles and dark pigmentation but without distal claw or crochets. (**e**) The abdominal proleg with uniordinal crochets arranged on a lateral penellipse on the A3 segment in E^{Kh}-*l*. (**g**)The E^{Kh}-*l* embryo exhibits a supernumerary pair of thoracic limb-like legs on the A1 segment (red arrows) compared with wild type (**f**). The adults display three pairs of thoracic legs in wild type (**h**). Occasionally, E^{Kh}-*l* mutants display four pairs of legs in the adult stage (**i**). The extra pair of legs on A1 segment are much smaller and almost completely immovable. Scale bar, 1 cm (**a**, **b**); 500 μm (**c–e**); 200 μm (**f**, **g**); 1 mm (**h**, **i**).

the normal legs and were almost completely immovable (Figures 1h–i). Considering the adult legs of *B. mori* develops from the larval thoracic legs (Singh *et al.*, 2007), therefore, the supernumerary legs of E^{Kh-l} homozygotes were completely thoracic typed legs on the first abdominal (A1) segment.

Positional cloning of E^{Kh-l} locus in *Bombyx mori*

Previous linkage analysis efforts mapped the E complex in *B. mori* to BX-C complex on the sixth linkage group (LG6, nascaf2853; Ueno *et al.*, 1992; Xiang *et al.*, 2008; Chen *et al.*, 2013b). To identify the candidate region responsible for the E^{Kh-l} locus, we searched for the genomic polymorphism by PCR between the recurrent parents, Dazao strain and the E^{Kh-l} mutant in the scaffold nscaf2853 on chromosome 6. We found that eight pairs of newly designed markers exhibited polymorphism between them. Then, we performed genetic analysis using 20 BC_1F progeny (10 WT and 10 mutant) that were generated from cross (BC_1F) between the F_1 females and the males of WT strain Dazao, and subsequent fine mapping with a large BC_1M mapping populations (1724 individuals) from cross between F_1 heterozygote males and the females of Dazao strain. The genotyping showed that the E^{Kh-l} locus was delimited between markers L1048 and L1391, and three markers, L1779, L1011 and L1153 were completely linked to the E^{Kh-l} locus (Figure 2a); therefore, the E^{Kh-l} locus was localized within a 355 kb on the nscaf2853 scaffold according to the SilkDB database (Duan *et al.*, 2010). Five predicted genes (*BGIBMGA006389*, *BGIBMGA006487*, *BGIBMGA006388*, *BGIBMGA006387* and *BGIBMGA006386*) were located within this region (Figure 2b). Further bioinformatics analysis revealed that *BGIBMGA006386*, *BGIBMGA006387* and *BGIBMGA006388* were assembled to be *Bmabd-A* gene, and *BGIBMGA006389* was the first exon of *BmUbx*, whereas *BGIBMGA006487* was a wrongly predicted gene in SilkDB. Thus, we concluded that the interval of 355 kb containing the first exon of *BmUbx* and *Bmabd-A* was responsible for the E^{Kh-l} phenotype (Figure 2c).

To determine whether sequence variation exists in the transcripts of *BmUbx* and *Bmabd-A* genes in E^{Kh-l} mutant, we cloned the two genes in both E^{Kh-l} mutant and Dazao strains. The result showed that no sequence difference was observed between E^{Kh-l} and Dazao in the full-length complementary DNA of both genes. Considering the location of the E^{Kh-l} candidate region and the essential function of *BmUbx* and *Bmabd-A* genes in body planning, we next investigated the expression profiles of the two candidate genes in E^{Kh-l} mutant and Dazao strains.
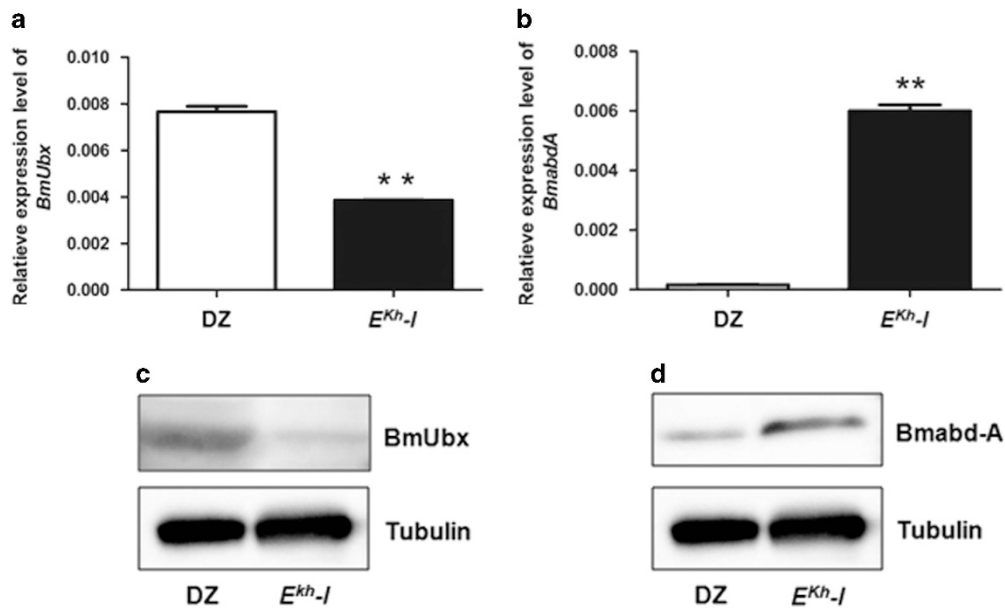
Expression patterns of *BmUbx* and *Bmabd-A* gene

To determine the expression profiles of the *BmUbx* and *Bmabd-A* genes in the E^{Kh-l} mutant, we detected the expression levels of *Bmabd-A* and *BmUbx* at both messenger RNA and protein levels in the embryos (7 days old). The qRT-PCR analysis data showed that the E^{Kh-l} mutant embryos exhibited a decreased level of *BmUbx* expression (Figure 3a), but a significant increased level of *Bmabd-A* expression compared with the WT (Figure 3b). Then, we examined the protein level of BmUbx and Bmabd-A. The western blotting results revealed that BmUbx protein was markedly decreased (Figure 3c), whereas Bmabd-A protein was obviously augmented (Figure 3d) in the E^{Kh-l} mutant, which was consistent with their transcriptional level. These results strongly indicated that the *BmUbx* was downregulated and *Bmabd-A* gene was upregulated at both transcriptional and translational levels in the E^{Kh-l} embryos.

Previous reports have showed that the specific regional functions of individual Hox genes were largely restricted to their expression patterns, the disruption of which can lead to developmental defects (Masumoto *et al.*, 2009; Xiang *et al.*, 2011). To assess whether the expression domains of BmUbx and Bmabd-A were diverged between the E^{Kh-l} mutant and Dazao, we performed the antibody staining in those embryos (2 days old). In the control strain Dazao, the main expression domain of the BmUbx was in the A1 and A2 segments where no leg appendage appeared ($N = 7$; Figure 4a; Supplementary Figure 2A), whereas it was expressed in the same region but in a
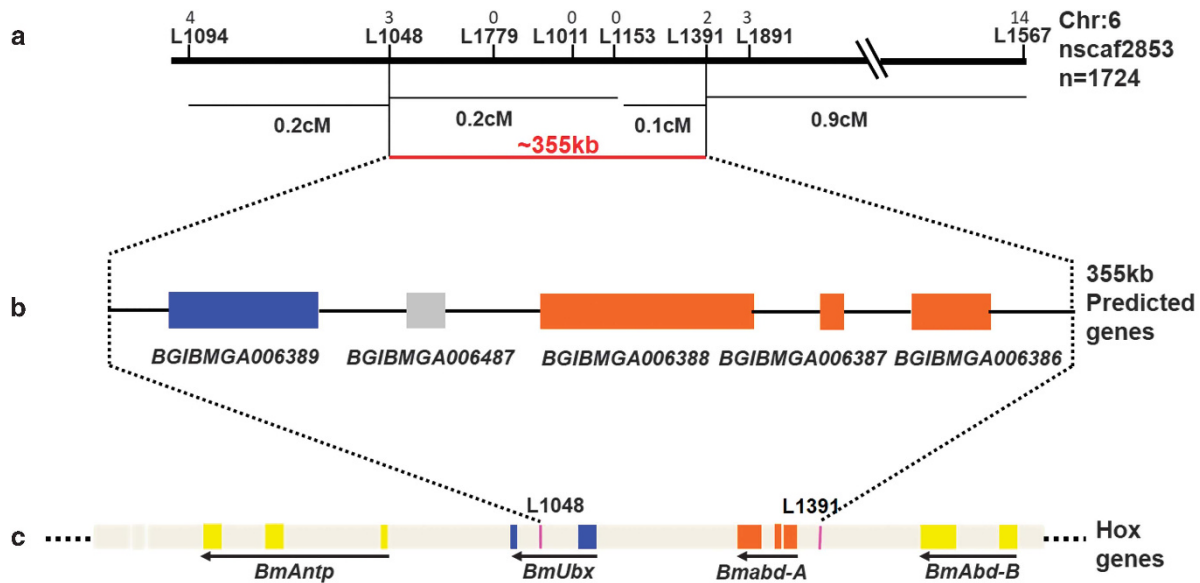
Figure 2 Fine mapping of the silkworm mutant E^{Kh-l} locus on the linkage group 6. (**a**) Physical map resulting from newly developed markers. The E^{Kh-l} locus was narrowed to a ~ 355 kb region between the two markers, L1048 and L1391. The numerals indicate the number of recombinants identified in 1724 BC_1M progeny. (**b**) There are five predicted genes between L1048 and L1391 including *BGIBMGA006389*, *BGIBMGA006487*, *BGIBMGA006388*, *BGIBMGA006387* and *BGIBMGA006386* based on the SilkDB. (**c**) In fact, only the first exon of *BmUbx* and *Bmabd-A* gene was included in the mapped region.

Figure 3 Expression profile of *BmUbx* and *Bmabd-A*. Quantitative RT-PCR analysis of *BmUbx* and *Bmabd-A* in wild type and E^{Kh}-l at embryonic stage (**a**, **b**). Eukaryotic translation initiation factor 4A was used as an internal control. Western blotting analysis of BmUbx and Bmabd-A in wild type and E^{Kh}-l (**c**, **d**). Tubulin was used as internal reference. DZ, Dazao (wild type). (**a**, **c**) The *BmUbx* was remarkably reduced in the E^{Kh}-l embryos, compared with Dazao wild type. (**b**, **d**) To the contrary, *Bmabd-A* was obviously increased in the E^{Kh} embryos. **$P < 0.01$, Student's *t*-test. The data show the mean +s.d. ($n = 3$).
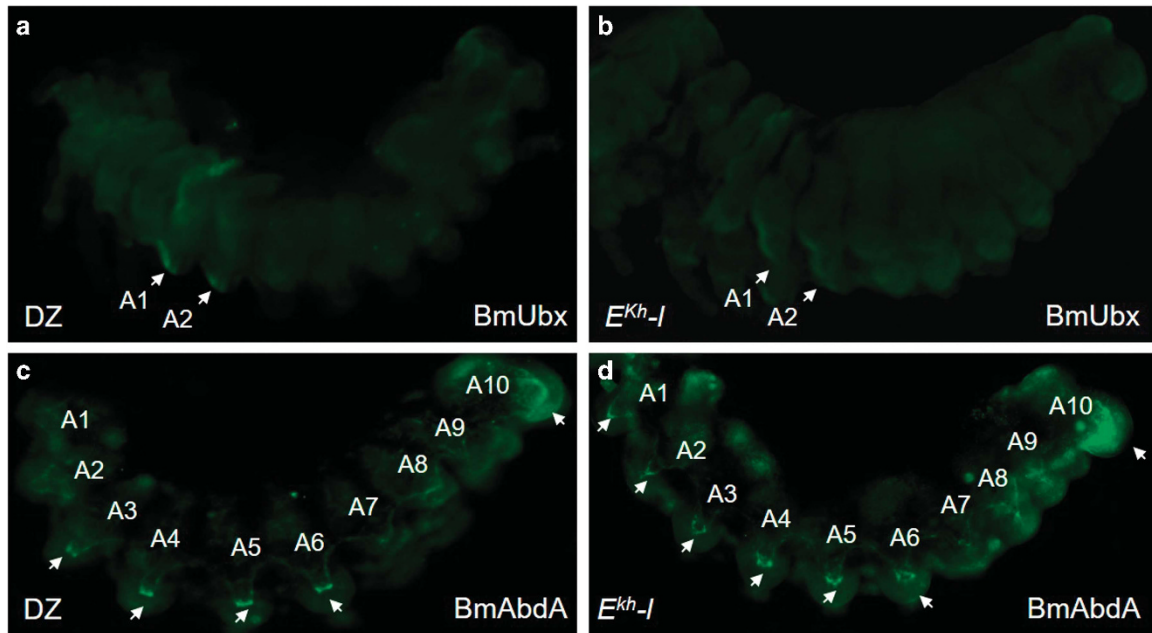
Figure 4 BmUbx and Bmabd-A protein expression patterns in the wild type (Dazao) and E^{Kh}-l mutant. (**a**) In 2-day-old embryo of Dazao, the expression domain of BmUbx was mainly on the ventral side of the A2 segment and both the dorsal and ventral sides of the A1 segment. (**b**) At the same stage, in the E^{Kh}-l embryo, BmUbx was almost expressed at the same domain as wild type but in a lower level. (**c**) In 2-day-old embryo of Dazao, Bmabd-A expression was detectable in the proleg primordias on A3–A6 and A10 segments. (**d**) In the E^{Kh}-l embryos (2 days old), Bmabd-A was not only localized to the A3–A6 and A10 segments, but also was observed at the leg primordias on A1 and A2 segments, where display the supernumerary legs.

seemingly reduced level in the E^{Kh}-l mutant ($N = 12$; Figure 4b; Supplementary Figure 2B). Bmabd-A was detected strongly in the leg primordias of the A3–A6 and A10 segments in WT ($N = 9$; Figure 4c; Supplementary Figure 2C). However, the E^{Kh}-l mutant ectopically overexpressed Bmabd-A in the leg primordias of A1 and A2 segments where extra legs growth out besides the normal expression domain ($N = 6$; Figure 4d; Supplementary Figure 2D). These results suggested that the reduction of BmUbx and the ectopic expression of Bmabd-A on the A1 and A2 segments were responsible for the appearance of the supernumerary limb-like legs in the E^{Kh}-l mutant.

DISCUSSION

E^{Kh}-l, one of the silkworm E loci mutants, exhibits additional thoracic limb-like legs on the A1 segment and occasionally on the A2. In this study, we performed positional cloning and narrowed the E^{Kh}-l candidate region to a ~ 355 kb including the first exon of BmUbx and Bmabd-A gene using a large mapping population (1724 individuals) in the B. mori. By cloning the BmUbx and Bmabd-A in E^{Kh}-l, we did not find any mutation in their transcriptional sequences. The E^{Kh}-l mutant embryos expressed lower level of BmUbx but higher level of Bmabd-A compared with the WT. The previous evidences have reported that many noncoding RNAs, such as intergenic regulatory transcripts and evolutionarily conserved microRNAs, were mapped to the intergenic regions of Hox gene clusters to coordinate their expression through a variety of cis- and trans-regulatory mechanisms in various organisms (Lemons and McGinnis, 2006; Gummalla et al., 2012). However, we did not found any other potential functional transcript within the mapped region by search against the SilkTransDB (http://124.17.27.136/gbrowse2/; Li et al., 2012). The misexpression of BmUbx and Bmabd-A in E^{Kh}-l is probably regulated by some unidentified cis-regulatory elements or transcripts in the mapped region, respectively. Another possibility is that the overexpressed Bmabd-A induced the downregulated BmUbx by the posterior dominance rule in which a more posterior homeotic gene suppresses the expression of the more anterior Hox gene in general. And a third possibility is that the downregulation of BmUbx is causal and leads to the expansion of Bmabd-A, as it has been shown that downregulation of BmUbx through RNAi leads to the development of thoracic-like appendages on A1 (Masumoto et al., 2009). As only two genes were involved in the mapped region, we proposed that the abnormal expressed BmUbx and Bmabd-A were associated with the E^{Kh}-l mutant, but we could not exclude the possibility that other Hox genes, particularly BmAntp, which influences leg development in the thorax, might also be altered in expression level or pattern by indirect regulation of BmUbx misexpression or by regulatory changes in the mapped region.

Previous studies have reported that above 30 homeotic mutants located on chromosome 6 are related to B. mori Hox genes, dozens of them were displayed diversity of supernumerary legs on the first two abdominal segments, including number and morphologic identity (Ueno et al., 1992; Xiang et al., 2008; Chen et al., 2013b). Among them, only two mutants E^{kp}-1 and E^{Cs}-l that present supernumerary abdominal prolegs in the A2 segment have been identified to date (Xiang et al., 2008; Chen et al., 2013b). E^{kp}-1 locus was delimited to a region that included the Bmabd-A and a noncoding RNA, miR-iab-4. Whereas E^{Cs}-l was restricted to a ~ 68 kb sequence in the upstream intergenic region of Bmabd-A containing the miR-iab-4 as well and displayed remarkable ectopically expressed Bmabd-A in the supernumerary prolegs in the A2. In the present study, the Bmabd-A expression was also prominently increased in the E^{Kh}-l mutant, not only the messenger RNA but also the protein. In particular, the immunofluorescence experiment revealed that the Bmabd-A protein was also ectopically overexpressed in the supernumerary legs on A1 and A2 in the E^{Kh}-l mutant, indicating that the Bmabd-A promote the development of extra legs on these two segments. However, the extra legs were limb-like in the E^{Kh}-l instead of proleg-like in E^{kp}-1 and E^{Cs}-l. These results suggested that the overexpressed Bmabd-A was sufficient to promote the outgrowth of additional appendages on A1 and A2, but insufficient to determine their morphological identity. In addition, it is noteworthy that the BmUbx expression level was changed in opposite directions in E^{Kh}-l and E^{Cs}-l even they both overexpressed Bmabd-A on the same segments. The BmUbx

expression was significantly increased in the E^{Cs}-l but was decreased in the E^{Kh}-l mutant (Supplementary Figure 1). We speculate that the reduced BmUbx level specified the additional legs to limb-like in the E^{Kh}-l, whereas the improved BmUbx level specified them to proleg-like identity in E^{Cs}-l. Thus, we proposed that the BmUbx was required to specify accurate morphologies of the extra legs in A1 and A2 in a level-dependent manner (Figure 5). In contrast to the case in D. melanogaster, Ubx and abd-A inhibit proleg development in the abdomen by suppressing Dll expression (Vachon et al., 1992), in silkworm, the two genes determine the number, position and identity of legs in a dosage-sensitive manner.

In addition, even the additional legs displayed morphological similarities with the normal thoracic limb in E^{Kh}-l, they were expressed different Hox genes combinations. The promotion of normal thoracic limbs was carried out primarily by Antp, and in T3 either BmAntp or BmUbx can apparently execute this function, whereas the additional legs in E^{Kh}-l mutant were due to the increased Bmabd-A and decreased BmUbx, suggesting that morphological similarities was not required similar Hox gene expression.

Many insects possess both thoracic limbs and abdominal prolegs, but the molecular origins of the morphological diversity are not well understood. In Panorpidae (Insecta: Mecoptera), the histological and morphological evidence of the embryonic development suggested that the abdominal prolegs of Panorpidae were not serially homologous with the thoracic limbs (Yue and Hua, 2010). The morphological and gene expression analyses of decapentaplegic and Distal-less genes, two genes required for the limb development, indicated that the abdominal prolegs were evolved independently with the limb in the sawfly as well (Oka et al., 2010). So far, it is still unclear the relationship between the two types of legs. In the study, the same segments of silkworm have potential to develop either limbs or prolegs, which was determined by the different combination of Hox genes expression patterns, including expression level and domain. Thus, our findings offer a probable approach to answer the above question by using genetic techniques to
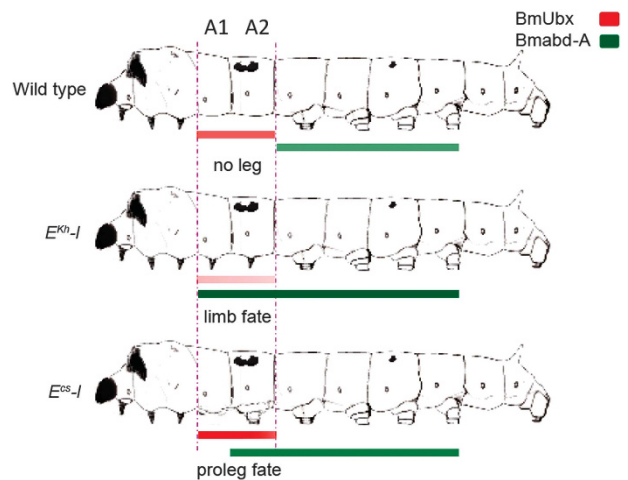
Figure 5 Comparison of the phenotype and the expression of BmUbx and Bmabd-A between E^{Kh}-l and E^{Cs}-l. (a) In wild type, there is no leg on A1 and A2 segments that expressed normal level of BmUbx and Bmabd-A. (b) The downregulated BmUbx and ectopically expressed Bmabd-A caused two extra pairs of limbs on A1 and A2 segments in E^{Kh}-l. (c) E^{Cs}-l mutant that overexpressed BmUbx and ectopically misexpressed Bmabd-A specified the additional legs on the same segments to proleg-like identity. The levels of expression are illustrated by lighter or darker shades. The position of the bars marks the segmental distribution of each gene.

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modify the expression patterns of Hox genes. Further comparative analysis of the morphological changes in appendages and different expressed genes will contribute a lot to reveal the molecular origins of limbs and prolegs. In conclusion, our results show that *BmUbx* and *Bmabd-A* were the candidate genes of the E^{Kh}-*l* mutant. The outgrowth of extra limb-like legs in the A1 and A2 was attributed to the altered expression patterns of the two Hox genes. To be specific, the overexpression of Bmabd-A on the A1 and A2 segment caused the outgrowth of the extra legs, and the decreased expression of the BmUbx on the A1 segment gave rise to the thoracic limb-like leg phenotype. These results provide insights into how these Hox genes regulate the leg morphologic variation on the same segment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

There were no data to deposit.

Abbott MK, Kaufman TC (1986). The relationship between the functional complexity and the molecular organization of the Antennapedia locus of *Drosophila melanogaster*. *Genetics* **114**: 919–942.

Akam M (1998). Hox genes, homeosis and the evolution of segment identity: no need for hopeless monsters. *Int J Dev Biol* **42**: 445–451.

Angelini DR, Kaufman TC (2005). Insect appendages and comparative ontogenetics. *Dev Biol* **286**: 57–77.

Averof M, Patel NH (1997). Crustacean appendage evolution associated with changes in Hox gene expression. *Nature* **388**: 682–686.

Banno Y, Fujii H, Kawaguchi Y, Yamamoto K, Nishikawa K (2005). *A Guide to the Silkworm Mutants: 2005 Gene Name and Gene Symbol*. Kyushu University: Fukuoka, Japan.

Chen P, Tong XL, Li DD, Fu MY, He SZ, Hu H *et al*. (2013a). Antennapedia is involved in the development of thoracic legs and segmentation in the silkworm, Bombyx mori. *Heredity* **111**: 182–188.

Chen P, Tong XL, Li DD, Liang PF, Fu MY, Li CF *et al*. (2013b). Fine mapping of a supernumerary proleg mutant (E(Cs) -l) and comparative expression analysis of the abdominal-A gene in silkworm, Bombyx mori. *Insect Mol Biol* **22**: 497–504.

Duan J, Li R, Cheng D, Fan W, Zha X, Cheng T *et al*. (2010). SilkDB v2.0: a platform for silkworm (Bombyx mori) genome biology. *Nucleic Acids Res* **38** (Database issue): D453–D456.

Gebelein B, Culi J, Ryoo HD, Zhang W, Mann RS (2002). Specificity of Distalless repression and limb primordia development by abdominal Hox proteins. *Dev Cell* **3**: 487–498.

Gummalla M, Maeda RK, Castro Alvarez JJ, Gyurkovics H, Singari S, Edwards KA *et al*. (2012). abd-A regulation by the iab-8 noncoding RNA. *PLoS Genet* **8**: e1002720.

Jockusch EL, Williams TA, Nagy LM (2004). The evolution of patterning of serially homologous appendages in insects. *Dev Genes Evol* **214**: 324–338.

Kaufman TC, Seeger MA, Olsen G (1990). Molecular and genetic organization of the antennapedia gene complex of *Drosophila melanogaster*. *Adv Genet* **27**: 309–362.

Kelsh R, Weinzierl RO, White RA, Akam M (1994). Homeotic gene expression in the locust Schistocerca: an antibody that detects conserved epitopes in Ultrabithorax and abdominal-A proteins. *Dev Genet* **15**: 19–31.

Kosambi DD (1944). The estimation of map distances from recombination values. *Ann Eugenics* **12**: 172–175.

Lawrence PA, Morata G (1994). Homeobox genes: their function in Drosophila segmentation and pattern formation. *Cell* **78**: 181–189.

Lemons D, McGinnis W (2006). Genomic evolution of Hox gene clusters. *Science* **313**: 1918–1922.

Lewis DL, DeCamillis M, Bennett RL (2000). Distinct roles of the homeotic genes Ubx and abd-A in beetle embryonic abdominal appendage development. *Proc Natl Acad Sci USA* **97**: 4504–4509.

Li Y, Wang G, Tian J, Liu H, Yang H, Yi Y *et al*. (2012). Transcriptome analysis of the silkworm (Bombyx mori) by high-throughput RNA sequencing. *PLoS One* **7**: e43713.

Masumoto M, Yaginuma T, Niimi T (2009). Functional analysis of Ultrabithorax in the silkworm, Bombyx mori, using RNAi. *Dev Genes Evol* **219**: 437–444.

Morata G, Sanchez-Herrero E (1999). Patterning mechanisms in the body trunk and the appendages of Drosophila. *Development* **126**: 2823–2828.

Oka K, Yoshiyama N, Tojo K, Machida R, Hatakeyama M (2010). Characterization of abdominal appendages in the sawfly, Athalia rosae (Hymenoptera), by morphological and gene expression analyses. *Dev Genes Evol* **220**: 53–59.

Roch F, Akam M (2000). Ultrabithorax and the control of cell morphology in Drosophila halteres. *Development* **127**: 97–107.

Ronshaugen M, McGinnis N, McGinnis W (2002). Hox protein mutation and macroevolution of the insect body plan. *Nature* **415**: 914–917.

Shippy TD, Brown SJ, Denell RE (1998). Molecular characterization of the Tribolium abdominal-A ortholog and implications for the products of the Drosophila gene. *Dev Genes Evol* **207**: 446–452.

Singh A, Kango-Singh M, Parthasarathy R, Gopinathan KP (2007). Larval legs of mulberry silkworm Bombyx mori are prototypes for the adult legs. *Genesis* **45**: 169–176.

Tomita S, Kikuchi A (2009). Abd-B suppresses lepidopteran proleg development in posterior abdomen. *Dev Biol* **328**: 403–409.

Tomoyasu Y, Wheeler SR, Denell RE (2005). Ultrabithorax is required for membranous wing identity in the beetle Tribolium castaneum. *Nature* **433**: 643–647.

Tong X, Hrycaj S, Podlaha O, Popadic A, Monteiro A (2014). Over-expression of Ultrabithorax alters embryonic body plan and wing patterns in the butterfly Bicyclus anynana. *Dev Biol* **394**: 357–366.

Ueno K, Hui CC, Fukuta M, Suzuki Y (1992). Molecular analysis of the deletion mutants in the E homeotic complex of the silkworm Bombyx mori. *Development* **114**: 555–563.

Vachon G, Cohen B, Pfeifle C, McGuffin ME, Botas J, Cohen SM (1992). Homeotic genes of the Bithorax complex repress limb development in the abdomen of the Drosophila embryo through the target gene Distal-less. *Cell* **71**: 437–450.

Van Ooijen JW (2006). *Joinmap 4.0, Software for the Calculation of Genetic Maps in Experimental Populations*. Kyazma BV: Wageningen, Netherlands.

Xiang H, Li M, Yang F, Guo Q, Zhan S, Lin H *et al*. (2008). Fine mapping of E(kp)-1, a locus associated with silkworm (Bombyx mori) proleg development. *Heredity* **100**: 533–540.

Xiang H, Li MW, Guo JH, Jiang JH, Huang YP (2011). Influence of RNAi knockdown for E-complex genes on the silkworm proleg development. *Arc Insect Biochem Physiol* **76**: 1–11.

Yasukochi Y, Ashakumary LA, Wu C, Yoshido A, Nohata J, Mita K *et al*. (2004). Organization of the Hox gene cluster of the silkworm, Bombyx mori: a split of the Hox cluster in a non-Drosophila insect. *Dev Genes Evol* **214**: 606–614.

Yue C, Hua B (2010). Are abdominal prolegs serially homologous with the thoracic legs in Panorpidae (Insecta: Mecoptera)? Embryological evidence. *J Morphol* **271**: 1366–1373.

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