

Correspondence

A strategy for mapping *bicoid* on the phylogenetic tree

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In *Drosophila*, a gradient of Bicoid protein (BCD) originates from prelocalized mRNA at the anterior pole of the egg and establishes developmental programs including those for the larval head and thorax in a concentration-dependent manner [1,2]. Orthologous proteins have been reported only from cyclorrhaphan flies [3,4]. However, a BCD-like determinant has been postulated for a large variety of insects including leafhopper, beetles and midges [5–7]. It therefore remains to be determined whether diverged *bicoid* (*bcd*) orthologs exist in other insect orders.

bcd encodes a homeodomain-containing transcription factor, and is located immediately upstream of *zerknüllt* (*zen*) in the Hox gene complex (Hox-C) of various drosophilids [8,9]. Based on these findings M. Akam (in [10]) suggested that *bcd* originated as a result of a gene duplication involving *zen*. However, it has been difficult to test this hypothesis due to rapid sequence evolution of *bcd* and *zen* [3]. Only recently, sequence analysis of a *bcd* homolog from the basal cyclorrhaphan fly *Megaselia* (Phoridae) provided direct support for a sister-gene relationship of *bcd* and *zen*, and implicated the position of *bcd* upstream of *zen* in the Hox-C as ancestral [11].

We tested for linkage of *bcd* and *zen* in the blowflies *Calliphora erythrocephala* and *Lucilia sericata* (Calliphoridae, Diptera) where *bcd* homologs have been identified

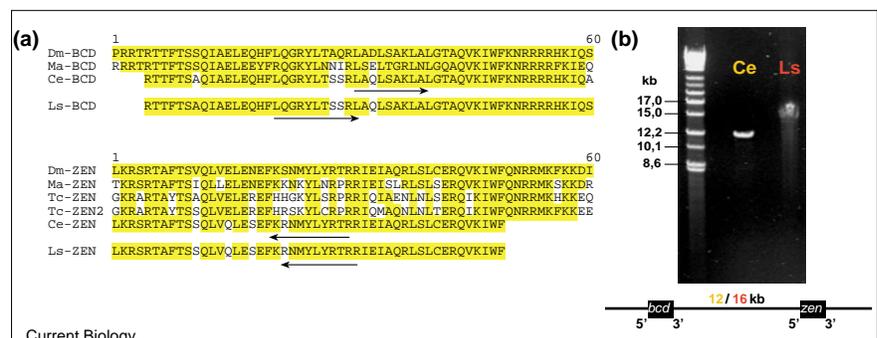
previously [4]. Using degenerate PCR primers we isolated *zen* homeoboxes from both species (Figure 1a). Specific nested primer pairs, when used in long range PCR, amplify single genomic DNA fragments of 12 kb (*Calliphora*) and 16 kb (*Lucilia*), linking the homeoboxes of the *bcd* and *zen* homologs (Figure 1b). The identity of each fragment was verified by Southern-blot hybridization and terminal sequencing. Thus, *bcd* is linked to a Hox class 3 gene in blowflies (Calypttratae) as it is in drosophilids (Acalypttratae), and the 5' to 3' orientation of *bcd* and *zen* with respect to one another is conserved. This linkage was probably inherited from the common ancestor of the monophyletic Schizophora (Acalypttratae and Calypttratae) [12], which comprise the majority of family-level diversity of Diptera [13]. Our observations strongly support the hypothesis that *bcd* arose as a tandem duplication of *zen* within the Hox-C (Figure 2). We conclude that analysis of the relevant Hox-C portion of selected species provides a means to map the origin of *bcd* on the phylogenetic tree.

In the red flour beetle *Tribolium castaneum*, a holometabolous insect distantly related to flies, orthologs of

the eight arthropod homeotic genes [14], as well as *ftz* [15] and *zen* [16] are arrayed in the same order as their *Drosophila* counterparts (Figure 2), an order that has been maintained for over 300 million years. To determine whether a highly diverged *bcd* ortholog is located in its predicted position in the *Tribolium* Hox-C we sequenced a BAC clone spanning the region from the 5' exon of *mxp/Hox2* to *Tftz* (Genbank accession AF321227). We analyzed this sequence using BLAST (NCBI), and the BCM genefinder program (Baylor College of Medicine Web site) to predict open reading frames and putative transcription units. We found a second *zen* gene (see the homeodomain comparisons in Figure 1a) immediately downstream of the one previously identified. These genes are most likely the result of an independent duplication in the lineage leading to *Tribolium*. Although known transcription units were faithfully predicted, no other homeodomain-encoding sequences were found. The *Tribolium* Hox-C does not, therefore, contain a *bcd* ortholog in the interval between *mxp/Hox2* and *TcDfd/Hox4*.

Conservation of the relative positions of *zen* and *bcd* in blowflies

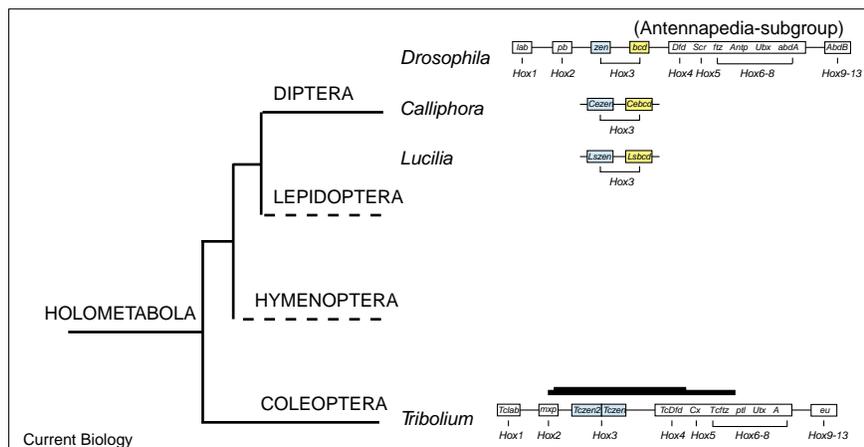
Figure 1



bcd-zen linkage in blowflies. (a) Homeodomain sequence alignments of BCD [4, 11, 17] and ZEN from *Drosophila melanogaster* (Dm) [18], *Megaselia abdita* (Ma) [11], *Tribolium castaneum* (Tc), *Calliphora erythrocephala* (Ce) and *Lucilia sericata* (Ls) with internal primer positions (arrows) used for long range DNA

amplification experiments and sequencing. The partial *zen* homeoboxes of *Calliphora* and *Lucilia* were amplified with degenerate primers as described previously [11]. (b) Amplified DNA fragments from *Calliphora* (12 kb) and *Lucilia* (16 kb) that were used as template for terminal sequencing.

Figure 2



Simplified phylogenetic tree of holometabolous insects, adapted from [19]. Linkage of Hox genes in *Drosophila* [8,9,20,21], *Calliphora*, *Lucilia* and *Tribolium* [14–16,22] is indicated to the right (not to scale). Note that a second *zen* gene (not shown) has been reported from *D. melanogaster* but not from other drosophilids. The extent of the *Tribolium* BAC clone is shown by a line above the *Tribolium* Hox-C; the sequenced region is marked by a

thick line. Abbreviations: *lab*, labial; *pb*, proboscipedia; *zen*, zerknullt; *bcd*, bicoid; *Dfd*, Deformed; *Scr*, Sex-combs reduced; *ftz*, fushi-tarazu; *Antp*, Antennapedia; *Ubx*, Ultrabithorax; *abdA*, abdominal-A; *AbdB*, Abdominal-B; *mxp*, maxillopedia; *Cx*, Cephalothorax; *ptl*, prothoraxless; *Utx*, Ultrathorax; *A*, Abdominal and *eu*, extraurogompfri.

and drosophilid fruitflies, combined with the absence of a *bcd* gene in the corresponding position in the *Tribolium* Hox-C (Figure 2), provide direct support for the hypothesis that *bcd* originated recently [11], presumably after the basal radiation of holometabolous insects. As the homeotic complexes of additional holometabolous insects are analyzed for *bcd*, our understanding of the origin of *bcd* will be refined.

Our conclusion that *bcd* emerged after the basal radiation of holometabolous insects does not necessarily imply that beetles and more primitive insects develop without an anterior determinant. In fact, several observations have been taken as evidence for such a factor in beetle and leafhopper development [5,6]. If these inferences are correct our results suggest that BCD functionally replaced an ancestral anterior determinant.

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