SUPPLEMENTAL MATERIAL

Table S1. Statistical analysis of CNS characteristics between regions for the three pair-wise species comparisons.

Source of variation[(1)]	Species pair	Density		Size distribution		Nucleotide identity (%)		Sequence in CNS (%)	
		d.f.	F	d.f.	G	d.f.	F	d.f.	F
Between the six regions	mel-pse	5	1.74	60	104.34***	5	4.84***	5	7.17***
	mel-buz	5	15.31***	60	58.19	5	2.13	5	16.16***
	pse-buz	5	18.75***	60	64.82	5	3.30*	5	18.76***
Between Hox regions	mel-pse	2	0.23	24	30.63	2	0.13	2	1.06
	mel-buz	2	1.40	24	26.52	2	0.49	2	1.19
	pse-buz	2	1.18	24	38.04*	2	4.55*	2	1.56
Between non-Hox regions	mel-pse	2	3.34*	24	24.90	2	9.71***	2	3.64*
	mel-buz	2	9.44***	24	16.68	2	2.69	2	11.71***
	pse-buz	2	11.02***	24	9.65	2	0.40	2	10.52***
Hox vs non-Hox regions	mel-pse	1	1.58	12	60.36***	1	4.54*	1	26.48***
	mel-buz	1	54.87***	12	17.41	1	4.29*	1	55.00 ***
	pse-buz	1	69.34***	12	19.59	1	6.63*	1	69.65***

* P < = 0.05; ** P < 0.01; *** P < 0.001.

(1) Variation between the six regions (df = 5) can be partitioned into three components: variation between the three Hox gene regions (df = 2), variation between the three non-Hox gene regions (df = 2) and variation between Hox and non-Hox gene regions (df = 1).

Table S2. Statistical analysis of CNS characteristics between species pairs for the different regions.

Source of variation[(1)]	Region	Density		Size distribution		Nucleotide identity (%)		Sequence in CNS (%)	
		d.f.	F	d.f.	G	d.f.	F	d.f.	F
Between the three species pairs	Hox genes	2	15.58***	24	28.23	2	0.70	2	43.97***
	Non-Hox genes	2	49.44***	24	9.59	2	1.89	2	34.10***
mel/buz *versus* pse/buz	Hox gene	1	2.09	12	2.96	1	0.08	1	1.69
	Non-Hox gene	1	0.10	12	3.60	1	0.19	1	0.03
mel/pse *versus* mel/buz or pse/buz	Hox gene	1	29.06***	12	27.69**	1	1.32	1	86.24***
	Non-Hox gene	1	98.78***	12	5.28	1	3.59	1	68.17***

** P < 0.01; *** P < 0.001.

(1) Variation between the three species pairs, mel/pse, mel/buz and pse/buz (df = 2) can be partitioned into variation between the equally distant species pairs mel/buz and pse/buz (df = 1) and variation between the closely related mel/pse and the other two species pairs (df = 1).

Table S3. Statistics of the sequencing process of two BAC clones from a *Drosophila buzzatii* genomic library.

BAC clone	**5H14**	**40C11**
Number of clones in plasmid library (insert size 1.5 kb)	960	1,056
Number of clones sequenced by one end	77	-
Number of clones sequenced by both ends	712	864
Number of reads in shotgun sequencing	1,501	1,728
Coverage	7.7x	6x
Number of contigs (> 2kb) after automatic assembly	7	7
Number of contigs (> 2kb) after manual editing	4	6
Number of contigs (< 2kb) after manual editing	2	0
Number of extra reads from plasmid library (clone ends)	47	43
Number of extra reads from plasmid library (custom primers)	22	31
Number of PCR fragments sequenced (physical gaps)	1	3
Number of reads from PCR fragments	4	11
Number of reads for finishing	73	85
Total number of reads produced	1,574	1,813
Total number of reads in final assembly	1,391	1,396
Average read size (bp)	829	890
Size of BAC clone insert (bp)	124,024	132,938
Error rate (per 10 kb)	< 0.01	0.17

Table S4. Primer pairs used for cDNA amplification.

gene	species	forward	reverse	Size
lab	*D. buzzatii*	CATCCGTGTCAGCTGCTAAC	TTCACGCGCTTCTTCTGCTTCAT	570 bp
	D. repleta	TCCAGCACCGAAACTACCTG		400 bp
	D. virilis			400 bp
	D. melanogaster			433 bp
pb	*D. buzzatii*	TCAATTCGCAGCCGTCGATG	GAGAGCGTTTGCCTCTTGTG	458 bp
	D. melanogaster			475 bp
abdA	*D. buzzatii*	TCGCAGTACTCGTCTCTG	CTGTGTGCAAGCGTTGC	504 bp
	D. virilis			501 bp
	D. repleta		CTGGTAGAACTGCGCAA	424 bp

Correspondence of regulatory sequences shown in Figure 3 with their domains of expression

labial gene (Chouinard and Kaufman 1991)

1	No Enhancer Activity
2	Anterior Midgut
3	Maintenance in Procephalon; Eye-Antennal Disc
4	Initiation in Procephalon
5	Imaginal Disc Repressor
6	No Enhancer Activity
7	Dorsal Ridge
8	Posterior Midgut
9	Late Embryonic Head Expression
10	No Enhancer Activity

proboscipedia gene (Kapoun and Kaufman 1995)

1	Imaginal Antennal and Thoracic Enhancers
2	Basal Promoter
3	Embryonic Mesodermal, Maxillary, and Labial Lobe Enhancers
4	Embryonic Maxillary and Labial Lobe Enhancers and Labial Discs Enhancers
5	Late Embryonic Labial Lobe Enhancers (weak) and Labial Discs Enhancers
6	Redundant Maxillary and Labial Lobe Enhancers
7	“Cryptic” Enhancers

Figure S1. Detail of two CRM. Fragments of the AVID alignments (mel/pse and mel/buz) used to generate the VISTA plots of Figure 3. (**A**) lab550-HOMRE (Homeotic Response Element): The four binding sites of this CRM are conserved between all three species in one CNS. (**B**) iab2(1.7) (fragment): All species show five Hunchback (HB) binding sites (three are conserved between all three species; one is shared by *D. melanogaster* and *D. pseudoobscura*; another one is shared by *D. pseudoobscura* and *D. buzzatii*; and there is one unique in *D. melanogaster* and another one in *D. buzzatii*); a unique Krüppel (KR) binding site, where point mutations (red uppercase) in *D. melanogaster* cause gain of expression mutants (Hab1 and Hab2) (Shimell et al. 1994), is conserved in all three species. This region contains 9 CNS in the *D. melanogaster-D. pseudoobscura* comparison and 6 CNS between *D. melanogaster-D. buzzatii*. PATCH (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi) was used for Hunchback binding site prediction in the iab2(1.7) enhancer.

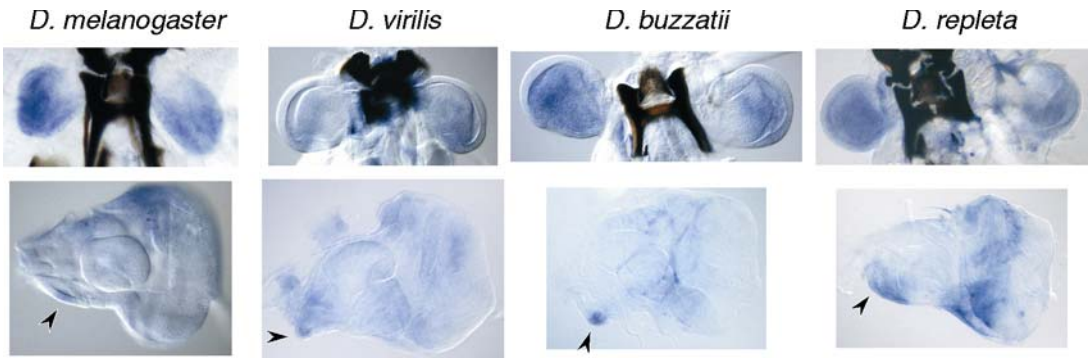

Figure S2. **Expression pattern of *pb* in imaginal discs**. Labial disc: *pb* is expressed in the labial disc of late third instar larvae and prepupae of the four tested species. Expression is detected over the whole disc, which will give rise to the proboscis. Eye-antennal disc: *pb* is also expressed in the presumptive palpus (arrowhead) of the eye-antennal disc of prepupa and white pupa, which will give rise to the maxillary palps. In overstained discs some *pb* expression can be detected in the presumptive ocellus. Some staining has also been observed in the lateral side of the stalk in white pupae eye-antennal discs of *D. repleta*. However, it has not been determined whether this expression domain is shared by the other species, as this region is easily broken.

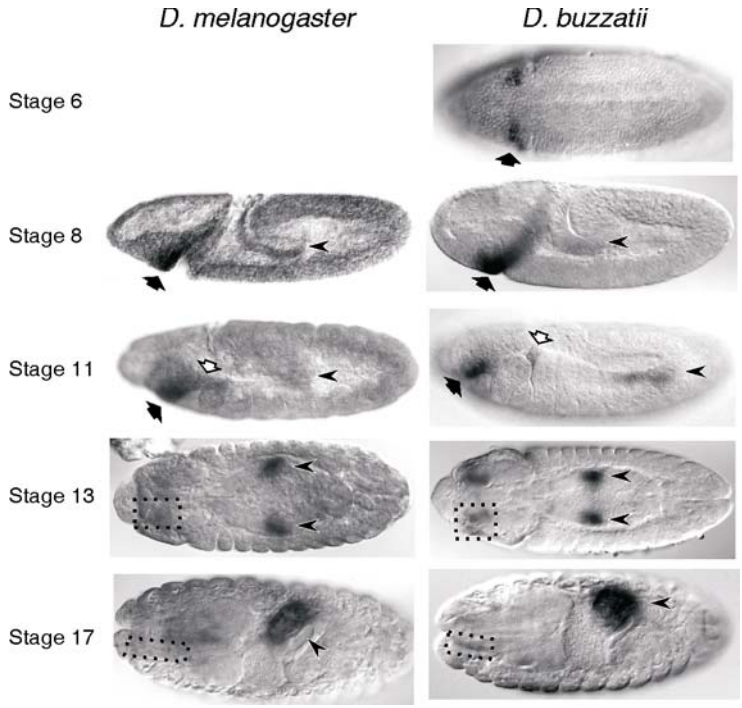

Figure S3. **Expression pattern of *lab* in embryos.** Cephalic region: *lab* is expressed in a ventral stripe just anterior to the cephalic furrow, which corresponds to the intercalary segment (black arrow). This domain of expression was first detected at stage 6 in *D. buzzatii* and at stage 8 in the other species. Later on *lab* is expressed in the dorsal ridge (white arrow) (stage 11-13) and in sensory structures derived from the intercalary segment (boxed area). Midgut: at stage 8 *lab* expression is detected weakly in the anterior and posterior midgut primordial (arrowheads). When the two primordia fuse (stage 12-13) *lab* expression in this region suddenly increases. Expression is kept at high levels in the second midgut constriction and finally in the second midgut chamber (stage 17).

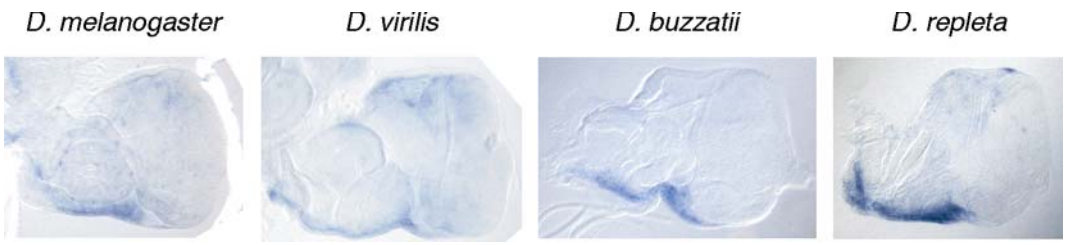

Figure S4. **Expression pattern of *lab* in the eye-antennal disc**. Expression of *lab* was detected in the peripodial membrane of the eye-antennal disc from late third instar larvae and prepupae. This region will give rise to the lateral and posterior head capsule. No expression has been detected in other imaginal discs.

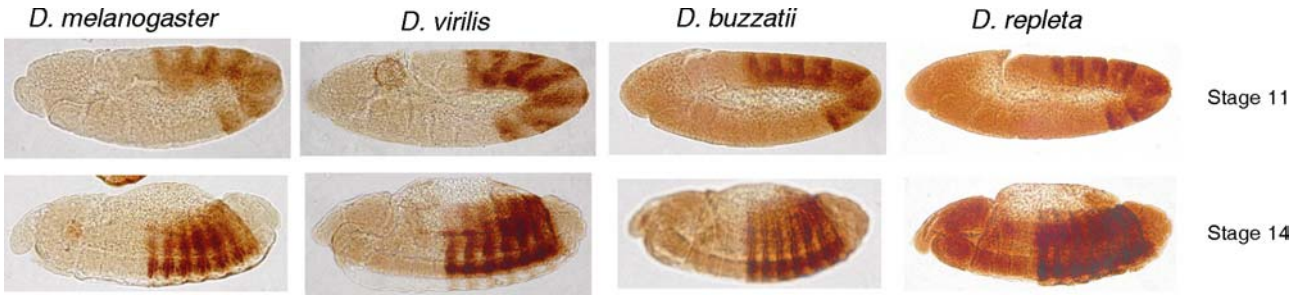

Figure S5. **Expression pattern of *abdA* in embryos**. ABD-A protein is detected in parasegments 7 through 13 (PS7-13). It is first detected in the ectoderm at stage 10 and later on (stage16-17) is detected on the ventral nervous system.

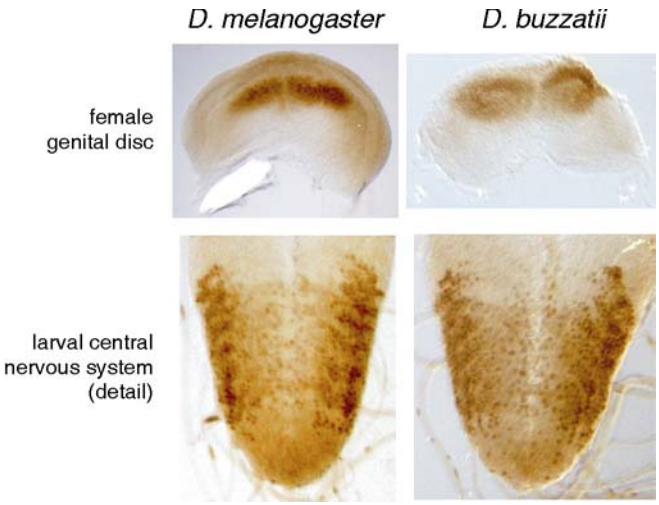

Figure S6. **Expression pattern of *abdA* in imaginal discs**. Female genital disc: ABD-A protein is observed in the presumptive internal genitalia region (derived from the A8 segment) of the female genital disc. Proper staining with anti-ABD-A was only obtained for *D. melanogaster* and *D. buzzatii*. All imaginal discs were tested for *abdA* expression. Central nervous system of the larva: ABD-A protein is always detected in seven stripes in the larval central nervous system of the four species, although the morphology of the larval central nervous system is slightly different.