CHAPTER 1

Analysis of changes in expression of regulatory genes of sex comb development during evolution of sex comb.
1.1 Introduction:

Sex comb is a sexually dimorphic trait—a group of male specific bristles present on the tarsal segments of the prothoracic leg (T1) in Sophophoran species of Drosophila (fig 1.10a). It is absent from the rest of the species of Drosophilidae. Thus the sex comb is an evolutionary innovative trait (Lakovaara and Soura, 1982; Lemeunier et al., 1986; Kopp and True, 2002). The chaetotaxy of the prothoracic leg of females do not show variation from the chaetotaxy of the prothoracic leg of males of the non-Sophophoran group (Kopp and True, 2002; Barmina and Kopp, 2007). As previously mentioned there exist a dramatic variation in the number, position and morphology of sex comb in different species of Sophophora enabling sex comb to enjoy a diverse evolutionary phylogenetic position (Kopp and True, 2002; Kopp and Barmina, 2005). Sex comb has got diverse roles in courtship and mating (Spieth, 1952; Cook, 1977; Ng and Kopp, 2008), indicating that their evolution has been driven by sexual selection. The sex comb develops from a group of bristles called the transverse bristle row (TBR) which is present on the T1 of both the males and females of Drosophilidea (Hannah-Alavah, 1958; Tokunaga, 1962). The TBR which are recruited to develop into sex comb undergo two important events of modifications i) rotation ii) chitinization (Held et al., 2004; Atallah et al., 2009; Tanaka et al., 2009). The transformation of the primitive bristle to the newly evolved sex comb should have been resulted from a genetic shift in the developmental circuit regulating the formation of the primitive bristles on the tarsal segments of the prothoracic leg.
Genetic and mutational analysis in *D. melanogaster* has shown that *Sex combs reduced* (Scr), *dachshund* (dac), *bric-a-brac* (bab) are the crucial regulators of the genetic network underlying the formation of the sex comb (Godt et al., 1993; Docquier 1997; Rogers et al., 1997; Cauderc et al., 2002; Randsholt and Santamaria, 2008). Findings in the *Sophophoran* group reveal that presence/absence of sex comb in the tarsal segments is correlated with the spatial and quantitative plasticity and modulation of Scr expression (Barmina and Kopp 2007; Randsholt and Santamaria, 2008; Kopp, 2011). dac gain of function in ts1 and tarsal segment 2 (ts2) in *D. melanogaster* induces development of ectopic sex combs in these segments. The ectopic sex comb is correlated with ectopic SCR expression (Randsholt and Santamaria, 2008). These data proved that dac is the genetic regulator of Scr. bab loss of function mutants have a similar effect with ectopic sex comb in distal tarsal segments with a correlation with SCR expression in these segments, suggesting bab to be the genetic repressor of Scr. Earlier studies (Barmina and Kopp 2007; Randsholt and Santamaria, 2008), have shown that, divergence in the number of sex combs in tarsal segments of *Sophophoran* species is not associated with variation in expression pattern of DAC and BAB in the prothoracic leg discs among these species. The enigmatic question which remains unanswered is “what are the changes in the genetic circuit, which has given the potential of evolving the primitive bristles to the evolutionary innovation- sex comb in the *Sophophoran* group of *Drosophila*” The present work in this chapter is an attempt to identify the genetic changes in
developmental pathway which has lead to the evolution of sex comb from the primitive bristle pattern. The expression pattern of the key regulators of the sex comb development has been analyzed in representative species of the newly evolved Sophophoran group and the non-Sophophoran group of Drosophila. The expression pattern of the candidate genes namely Scr, dac and bab, were compared between the selected species primitively lacking sex comb and species with sex comb to figure out the possible genetic changes that might have driven the evolution of sex comb.
Chapter 1

1.2 Materials and Methods:

1.2.1 Drosophila media:

All fly stocks used in this study were grown on standard white wheat cream agar medium. In one litre of water 100gm of jaggery was added, it was allowed to boil. 100gm and 10gm of wheat cream flour and agar powder were added. The ingredients were allowed to cook. The flame was reduced and 7.5 ml of propionic acid was added. The cooked media was poured into sterilized media bottles and plugged with cotton. Yeast granules were added after cooling of the media. Flies were grown at 22±2ºC.

1.2.2 Fly Stocks:

D. melanogaster, and D. rajasekari were selected from the melanogaster group, D. n. nasuta, D. kepuluana, D. kohkoa, D. s. neonasuta, are from the immigrans group, D. hydie from the repleta group, and D. virilis from the virilis group. All the fly stocks were collected from Drosophila stock centre, University of Mysore.

1.2.3 Staging and sexing of pupae:

Larvae were cultured at 22º±2º C on white wheat cream agar medium and fed with diluted yeast solution twice a day. The concentration of the yeast solution was increased as the larvae grew older. The larvae were staged till first, second, third instar and white prepupal stage (zero hour pupae). To obtain synchronised
pupae, white pre-pupae were collected from culture bottles. Pupae were sexed depending on the presence and absence of the developing testis which is visible in the form of a bubble like structure in the lateral side of the larva in the posterior part in case of males (Geigy, 1931; Aboim, 1945). The zero hr pupae were then placed on a moist tissue paper in a petridish and aged at $22^\circ\pm2^\circ$ C for 3-4 hrs.

### 1.2.4 Pupal dissection:

3-4 hrs pupae were taken in a cavity block and washed with 1XPBS (Sodium chloride 130mM, Disodium hydrogen phosphate, 70mM, Sodium dihydrogen phosphate 30mM, pH 7.2), to remove the media sticking on them. Half of the pupae were cut off from the posterior end. Gut and fat bodies were carefully removed and the pupae were cut open laterally from both the sides using micro scissor. The dorsal wall of the pupae were removed carefully and fixed in 4% paraformaldehyde (4% Paraformaldehyde was prepared by diluting 16% paraformaldehyde with PBS pH 7.2, prepared freshly before used), for 15 min at room temperature and washed twice with PBT (PBS+0.1% triton- X 100). The everting prothoracic leg discs were removed carefully with micro needle and forceps. 10-20 such discs of males and females were used for immunostaining.

### 1.2.5 Immunostaining:

10-20 males and females of 3-4hrs pupae were dissected for prothoracic discs in cold Phosphate buffered saline (PBS), (Sodium chloride 130mM,
Disodium hydrogen phosphate, 70mM, Sodium dihydrogen phosphate 30mM, pH 7.2). The prothoracic imaginal discs were transferred immediately in cold 4% paraformaldehyde (4% Paraformaldehyde was prepared by diluting 16% paraformaldehyde with PBS pH 7.2, prepared freshly before used) at room temperature. The discs were washed with PBT (PBS pH 7.2, with 0.1% triton X-100) for 10mins X 3. The prothoracic imaginal discs were blocked in PBT with 15% Normal goat serum (NGS) for 2 hrs at room temperature (RT). The samples were incubated in primary antibody in 15% normal goat serum, overnight at 4°C. Samples were washed with PBT for 2 hrs with frequent changes. Washes were conducted at RT. Discs were incubated for 2hrs in Biotin conjugated secondary antibody at RT. The imaginal discs were washed in PBT for 2hrs and treated with Streptavidin-Horse radish peroxidase (Streptavidin-HRP) complex at a concentration of 1:500 (Streptavidin-HRP: 15% NGS in PBT) for 2 hrs at RT and washes were given in PBT for 2hrs. The HRP- reaction was performed in staining solution containing DAB (250µg/ 200µl of PBT) and H₂O₂ (1% hydrogen peroxide in double distilled water). Several washes were given in PBT for 30min with frequent changes.

Mouse anti-SCR 6H4.1 (DSHB), antibody was used at a dilution of 1:60 (anti-SCR: 15% NGS in PBT), mouse anti-DAC Mabdac, (anti-DAC: 15% NGS in PBT) was used at a concentration of 1:90. Rat anti- BAB2 was used at a concentration of 1: 4000, (anti- BAB2: 15% NGS in PBT).

Secondary antibodies used was Goat anti-mouse IgG, 1:1000 (Goat anti-mouse IgG: 15% NGS in PBT) for anti-SCR antibody and anti-DAC antibody.
Goat anti-rat IgG, 1:500 (Goat anti-rat IgG: 15% NGS in PBT) was used as secondary antibody for anti-BAB antibody.

**Mounting and observation:**

The prothoracic imaginal discs were mounted on a clean micro slide in 50% glycerol (PBS pH 7.2 with glycerol, 1:1). Maximum care was taken to avoid air bubble while placing the micro cover slip. The observation was done using a Leica bright field microscope at 10X, 20X and 40X. The staining pattern and the diagrammatic representation of the observation were noted down.

**Imaging and image processing:**

Imaging was performed on a Leica bright field microscope using DMRA2 camera and LAS software. The images were arranged and labeled using adobe photoshop software version-7.

**1.2.6 In-situ hybridization:**

**1.2.6.1 Isolation of plasmid DNA:**

DH5α bacteria containing dac cDNA cloned into pFLC-I vector procured from BDGP were cultured in 3ml of Luria bertani medium containing 50µg/ ml ampicillin (stored at 4°C) by incubating overnight with vigorous shaking. The plasmid DNA was isolated as described by Sambrook and Russell (2001). 1.5 ml of the overnight culture was poured into a micro centrifuge tube and centrifuged at 12,000 rpm for 30 seconds at 4°C. The supernatant was discarded and the bacterial pellet was cleared of the medium completely. The bacterial pellets were
resuspended in 100µl of ice–cold alkaline lysis solution I by vigorous vortexing. 200µl of freshly prepared alkaline lysis solution II was added to the bacterial suspension. The contents were mixed by inverting the tube rapidly five times taking maximum care to close the tube tightly. The tubes were stored on ice for 3-5 min. 150µl of ice–cold alkaline lysis solution III was added, dispersing the alkaline lysis solution III through the bacterial lysate by inverting the tubes several times. The tubes were stored on ice for 3-5 min. The bacterial lysate was centrifuged at 12,000 rpm for 5 mins at 4ºC. The supernatant was transferred to a fresh tube. Equal volume of equilibrated phenol: chloroform: isoamyl alcohol (25:24:1) was added, and vortex for 1 min, the emulsion was centrifuged at 12,000 rpm for 2 min at RT. The aqueous upper layer was transferred to a fresh tube. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the extract, vortexed for 1 min and centrifuged for 5 min at RT. The nucleic acid from the aqueous phase was precipitated by adding two volumes of chilled ethanol kept at -20ºC for 30 min. The precipitated nucleic acid was pelleted by centrifugation at 12,000 rpm for 20 min at RT. The supernatant was removed and the tube was allowed to stand on a paper towel to allow the liquid to drain away. Any drop of fluid adhering to the sides of the tube was removed using an autoclaved micropipette tip. 1ml of 70% ethanol was added and the tube was inverted several times. The DNA was recovered by centrifugation at 12,000 rpm for 15 min at RT. The supernatant was removed and the tubes were allowed to stand on a paper towel to allow the liquid to drain away. Any drops of ethanol formed at the sides of the tubes were removed. The tubes were stored at room
temperature till the ethanol has evaporated and no fluid was visible in the tube (5-10 min). The nucleic acid was dissolved in 50µl of 1XTE containing 20µg/ml DNase free RNase A (pancreatic RNase). The solution was gently mixed for few seconds. The DNA solution was stored at -20°C.

**Reagents for isolation of plasmid DNA:**

a) Luria bertani medium: To 950 ml of deionized H₂O, 10g tryptone, 5g yeast extract, 10g Nacl were added. It was stirred well until the solute was dissolved. The pH was adjusted to 7.0 with 5N NaOH. The volume of the solution was adjusted to 1 liter with deionized water.

b) Alkaline lysis solution I: 50mM glucose, 25mM Tris–cl pH 8.0, 10mM Ethylenediamminetetraacetate (EDTA) pH 8.0.

c) Alkaline lysis solution II: 0.2 N NaOH, freshly diluted from 10N solution, 1% Sodium dodecyl sulfate (SDS).

d) Alkaline lysis solution III: 5M potassium acetate 60.0 ml, Glacial acetic acid 11.5ml, H₂O 28.5 ml. The resulting solution is 3M with respect to potassium and 5M with respect to acetate. The solution is stored at 4ºC and transfered to an ice bucket just before use.

e) 1X Tris-EDTA pH 8: 10X Tris-EDTA pH 8 which consist of 100mM Tris–Cl pH 8.0, 10mM EDTA pH 8.0 was diluted to 1X with autoclaved double deionized water.
1.2.6.2 Linearising the plasmid DNA:

**pFLC-I Polylinker for RE clones:**

<table>
<thead>
<tr>
<th>M13 -21 primer</th>
<th>T7 promoter</th>
</tr>
</thead>
</table>
| `ttgtaaaacgacgccagtgaattgtatatagctccctccggtggccgccccg`
| `aacatTTTgctccggtcacttaaTCTagTgtatatTccgcttaacccgtgagctgactctgaggccgagctccccggtggccgccccg`
| `ataacttcgtatagcatacattatacctggtattcggccgagctcgaattcgagagcgg`
| `tattgaagcatatcntagtataattgttaaacctagctccggtttagccggctcgagcttaagcagcgtctcgc`
| `cDNA polyA ctgcctcagatgccggcctactgtaggggaggggggtaccagctttt`
| `ANDc Tylop gagaggctcaaatcagccggttagcctcctcccccggcgtgtgaaaa`
| `gctccccctttagtgagggttaattccagcttggccgaatacaggtctctgtgtagctccttctgtgttgaaattgttatcc`
| `caagggaaactcactcccaattaaagctcgaaccgcattagtaaccagtgtcgacaagacacacctttacaaatagg`
| `T3 promoter`                  | `M13 Reverse primer`         |

Fig 1.1: pFLC-I vector of BDGP-DGRC, RE clone showing EcoR1 and Apa1 restriction digestion sites (arrows). The vector comprises of 2990 base pair. T7 and T3 promoter of the vector were used for *in-vitro* transcription to synthesised the sense and anti-sense probes respectively. EcoR1 restriction digestion site was used to linearised the vector for the synthesised of anti sense probe. Apa1 restriction digestion sites were used to linearise the vector for the synthesised of sense probe.

Fig 1.2: Polylinker of pFLC-I vector of RE clone showing T7, T3 promoter and restriction enzyme cutting sites of EcoR1 and Apa1 respectively. Blue colored arrow indicates the direction at which transcription initiates for T7 promoter. Red arrow indicates the direction at which the transcription initiates for T3 promoter. T3 promoter and EcoR1 restriction enzyme cutting site was used for the synthesised of anti-sense probe. T7 promoter and Apa1 restriction enzyme cutting site was used for the synthesised of sense probe.
Sequence of dac insert:

```
AATTTCTGAGTGAACATCGTCGATCGTACTCGAACACGACACAGTCCCGTTCTCCAAAA  
TTCCAGTCAAAAAACGGTTATTTGCAACTCGATTAACACAGGGGCAATCGGAAAAAAAACT   
TAGAAATATTCAGAGTATATATTCCAGCAGAATTACAAAAAGGAAATGTCGGATAGGTCCAAAT   
TAGCTCCATTTGGCTTTTTCTAAAACATATTAAAGCAAATGTGCAATTTATCTCTCTGTAAATTT  
TAGAAAAATGCAGAATAATACTAATGAAAAAGAGTGGCTTATTTATACGATAGGTTATTTATAT   
TTCCAGTACGAAAACGGTTATTTGCAGAATTACAAAAAGGAAATGTCGGATAGGTCCAAAT   
TAGCTCCATTTGGCTTTTTCTAAAACATATTAAAGCAAATGTGCAATTTATCTCTCTGTAAATTT  
TAGAAAAATGCAGAATAATACTAATGAAAAAGAGTGGCTTATTTATACGATAGGTTATTTATAT   
TTCCAGTACGAAAACGGTTATTTGCAGAATTACAAAAAGGAAATGTCGGATAGGTCCAAAT   
TAGCTCCATTTGGCTTTTTCTAAAACATATTAAAGCAAATGTGCAATTTATCTCTCTGTAAATTT  
TAGAAAAATGCAGAATAATACTAATGAAAAAGAGTGGCTTATTTATACGATAGGTTATTTATAT
```

Fig 1.3: Sequence of dac insert in pFLC-I vector of RE64054, BDGP-DGRC clone. The insert is 1997 base pair in length which was linearised in the pFLC-I vector by restriction digestion. EcoR1 was used for the synthesis of anti-sense probe using T3 promoter. Apa1 restriction digestion enzyme was used for the synthesis of sense probe using T7 promoter.

2990 base pair pFLC-I vector (fig 1.1) with 1997 base pair dac insert (fig 1.3) of the RE clone was linearised using EcoR1 restriction digestion enzyme to synthesised anti-sense probe. Apa1 restriction digestion enzyme was used to linearised the above vector to synthesized the sense probe. The poly linker of pFLC-I vector of RE clone is given in fig 1.2. The restriction digestion mixture includes 5µg Plasmid DNA, with dac insert, 5µl 10X buffer for restriction
endonuclease, 3µl restriction endonuclease (Apa1 and EcoR1) depending on sense and antisense probe. The 50 µl restriction digestion reaction mixture was incubated for 3hrs at 37ºC.

1.2.6.3 In-vitro transcription and synthesis of digoxiginin-labeled RNA probes:

Anti sense dac probe was synthesised from EcoR1 digested fragment bearing T3 promoter using DIG RNA labeling kit. Sense probe was similarly synthesised from Apa1 digested fragment bearing T7 promoter according to the protocol supplied with DIG/RNA Labelling kit (Roche Diagnostics).

1µg plasmid DNA containing dac insert with double deionised water (sterile made RNase free by DEPC treatment), were added in a sterile RNase free vial to make the sample volume upto 13µl.

The reaction vial was placed on ice and the following reagents were added

10X NTP labeling mixture 2 µl
10X Transcription buffer 2 µl
Protector RNase inhibitor 1 µl
RNA Polymerase
T3 (for antisense probe) 20U/ µl 2 µl
OR
T7 (for sense probe) 20U/ µl 2 µl

The reaction mixture was mixed gently and centrifuged briefly.
It was incubated for 2hrs at 37ºC. 2 µl of RNase free DNase I, was added to remove template DNA. It was incubated for 15 minutes at 37ºC. The reaction was stopped by adding 2 µl 0.2 M EDTA (pH 8.0).

1.2.6.4 Alkaline hydrolysis:

The fragment length of RNA probe was cut down to approximately 850 bases by limited alkaline hydrolysis as described in Cox et al.;(1984). 1µg of the dac probe and equal volume of sterile RNase free, DEPC- treated, double deionised water were taken in a sterile tube. Twice the volume of the above reaction mixture of carbonate buffer (40mM NaHCO3 pH 10, 60mM NaCo3 pH 10) was added. The reaction mixture was incubated for 8 min at 60ºC. Equal volume of neutralizing buffer (3M sodium acetate pH 6, v/v 1% acetic acid) was added. 3 volume of chilled ethanol was added and incubated for 3hrs at -20ºC. Centrifuged at 12,000 rpm for 15 min at 4ºC. Washes were given with 70% alcohol. The pellets were dried and resuspended in 100µl of double deionised DEPC treated water.

1.2.6.5 Hybridisation signal detection:

Hybridisation signal detection of in-situ hybridization was conducted as described by Tautz and Pfeifle (1989), with the slight modifications.

Prothoracic imaginal discs from 3-4 hrs pupae were dissected in cold Phosphate Buffered Saline (Sodium chloride 130mM, Disodium hydrogen phosphate, 70mM, Sodium dihydrogen phosphate 30mM, pH 7.2). They were
fixed in 4% paraformaldehyde in PBS for 20 min at RT. The discs were washed for 10 min X 3 times in 100% ethanol. The samples were incubated with xylene: ethanol (1:1) for 10 min. 2-3 rinses were given in 100% ethanol followed by 5 minutes incubation in methanol. The samples were rehydrated in a graded methanol v/v PBT (0.1% tween-20 in PBS) series (80%, 50%, 25%) and finally to PBT. The samples were fixed with 4% paraformaldehyde for 20 min. Washes with 0.1% PBT were given for 10 min X 3 times. Proteinase K treatment (5µg/ml of PBT) were given for 15 minutes. Washes were given with 0.1% glycine (0.1% glycine in PBS) for 10min X 3 times. The samples were refixed with 4% paraformaldehyde for 20min followed by washes with PBT (0.1%). Samples were incubated with pre-hybridization buffer at 56ºC for *D. melanogaster* and 42ºC for *D. n. nasuta* for 1 hr. Samples were incubated with hybridization buffer overnight at 56ºC and 42ºC for *D. melanogaster and D. n. nasuta* respectively. Washes were given with post hybridization buffer: PBT (3:1), post hybridization: PBT (2:1), post hybridization: PBT (1:1) and with PBT for 30 min each respectively. The samples were incubated with 1% bovine serum albumin (BSA) in PBT for 1 hour followed by incubation with anti-digoxiginine antibody (Roche diagnostics), at a dilution of 1: 1000 (antibody: 1% BSA). Washes with PBT (0.1%) were given for 1 hour (with 4 changes). The samples were equilibrated with Alkaline phosphate buffer (1mM levamisol, 100mM Nacl, 100mM Tris/ Hcl pH 9.5, 0.1% tween-20) for 10 min. The chromogenic reaction was carried out with a mixture of NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) with alkaline phosphate at a dilution of
8:1000. The samples were incubated till a dark purple color is developed. Frequent washes were given with PBT (0.1%).

**Mounting and observation:**

The prothoracic imaginal discs were mounted on a clean micro slide in 50% glycerol. Maximum care was taken to avoid air bubble while placing the micro cover slip. The observation was done using a Leica bright field microscope at 10X, 20X and 40X. The staining pattern and the diagrammatic representation of the observation were noted down.

**Imaging and image processing:**

Imaging was done using Zeiss Axio Cam MRc5 bright field microscope. The images were arranged and labeled using adobe photoshop software version-7.

**Reagents for *in-situ* hybridization:**

a) 4% Paraformaldehyde: as described for immunohistochemistry.

b) 20X saline-sodium citrate (SSC):

Dissolved 175.3 g of NaCl and 88.2 g of sodium citrate in 800ml of water. The pH was adjusted to 7.0 with a few drops of 14N of HCl. The volume was adjusted to 1 litre with water. It was sterilized by autoclaving and stored at 4ºC.

c) Salmon Sperm DNA (SSD): 10mg/ml
10mg of SSD is added in 1ml of nuclease free water in a 1.5 ml of eppendorf tube. It is sonicated upto 20-50base pair fragments, treated with 0.1% DEPC treated water. The concentration was made up to 200µg SSD/300 µl of prehybridisation buffer

d) Prehybridization buffer:

50% formamide, 5% 20X SSC, 200µg/ ml SSD, 0.1% Tween-20, 50 µg/ ml heparin, 10 µg/ ml tRNA, the volume was adjusted to 5 ml with nuclease free water.

e) Hybridization buffer with Anti-Sense dac probe or Sense dac probe:

Prehybridization buffer with 1.8 mg (anti sense probe or sense probe) / 50 ml of hybridisation buffer.

f) Post hybridization buffer:

50% formamide, 5% 20X SSC, 0.1% Tween-20, the volume was adjusted to 5 ml with nuclease free water.

g) NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt) mixture: 8:1000 (NBT+ BCIP: alkaline phosphate).
1.3 Results:

The expression patterns and the results of the comparative analysis study of the important candidate genes regulating the development of sex comb between the representative species of the species primitively lacking sex comb and the species with sex comb are illustrated below.

1.3.1 Sex combs reduced:

Different species of *Drosophila* with and without sex comb enjoying different phylogenetic positions were taken up as representative species for the comparative analysis of the expression pattern for SCR by Anti-SCR antibody staining. The species bearing sex comb comprises *D. melanogaster* and *D. rajasekari* from the *melanogaster* group. The species group primitively lacking sex comb considered for present analysis include *D. n. nasuta*, *D. s. neonasuta*, *D. kohkoa*, *D. kepuluana* from the *immigrans* group, *D. hydei* from the *repleta* group and *D. virilis* from the *virilis* group.

1.3.1.1 SCR expression in species with sex comb:

SCR could be seen in tibia (ti) and first tarsal segment (ts1) of the prothoracic leg discs in members of species with sex comb (Barmina and Kopp, 2007; Randsholt and Santamaria, 2008). Here a strong SCR expression could be detected in the ts1 and ti of male prothoracic leg discs of 3-4 hrs pupa (fig 1.4a). The SCR expression extends till the distal tip of ts1 (fig 1.4e).
Fig 1.4: SCR expression profile in representative species of Drosophila with sex comb (D. melanogaster a, b and D. rajasekari c, d). 3-4 hrs old pupae were dissected for prothoracic leg discs and subjected for anti-SCR antibody staining. SCR could be detected in the tibia (ti) and the 1st tarsal segment (ts1). Strong expression of SCR is seen in the ts1 which is comparable with the SCR expression in the tibial segment. Arrow indicates the distal tip of the ts1. In males of these species with sex comb (a) D. melanogaster and (c) D. rajasekari the SCR
expression extends till the distal tip of ts1 (fig e, enlarged portion of distal tip of ts1 of male \textit{D. melanogaster}). In female of the species with sex comb (b) \textit{D. melanogaster} and (d) \textit{D. rajasekari}, the SCR expression does not extend till the distal tip of the ts1 (fig f, enlarged portion of distal tip of ts1 of female \textit{D. melanogaster}). Scale bar in (a) is 0.1mm and is same for images (b, c, d). Scale bar in (e) is 0.1mm and is same for (f).

In female individuals of \textit{D. melanogaster} a strong SCR expression could be seen (fig 1.4b), but the expression does not extend till the distal tip of ts1 (1.4f). Thus in \textit{D. melanogaster} there exists a dimorphic expression of SCR between male and female individuals. This dimorphism particularly corresponds to the region of sex comb development. In \textit{D. rajasekari}, another species with sex comb taken up for the study, SCR expression shows a similar expression pattern with \textit{D. melanogaster}. In both male (fig 1.4c) and female (fig 1.4d) individuals, SCR expression is strong in ts1 and ti with a distally extended expression specific to only males.

\textbf{1.3.1.2 SCR expression in species primitively lacking sex comb:}

The expression pattern of SCR in prothoracic leg discs of male and female individuals in 6 species primitively lacking sex comb is depicted in fig 1.5a-l. SCR expression was seen in ts1 and ti. The intensity of expression of SCR in ti in these species is comparable to that of the expression in ti in \textit{D. melanogaster} in strength. The expression in ts1 is very weak and hardly detectable in the individuals of these combless species namely \textit{D. n. nasuta}, \textit{D. s. neonasuta}, \textit{D. kokhoa}, \textit{D. kepuluana}, \textit{D. hydei}, \textit{D. virilis} (fig 1.5 a, c, e, g, i, k). SCR expression could be seen in ts1 and ti, with a strikingly reduced expression in ts1 and there exist no dimorphic expression of SCR between male and female individuals of
Fig 1.5: SCR expression profile in some of the selected species of Drosophila without sex comb in male and female (a, b) D. n. nasuta, (c, d) D. s. neonasuta, (e, f) D. kokhoa, (g, h) D. kepuluana, (i, j)
D. hydei, (k, l) D. virilis. 3-4 hrs pupae were dissected for prothoracic leg discs. The prothoracic leg discs were subjected for anti-SCR antibody staining. SCR could be detected in the tibia (ti) and 1st tarsal segment (ts1). The level of SCR expression in the ts1 is remarkably lower than the SCR level in the ti. There is no noticeable difference in the SCR expression between the male and female in the ts1 of the prothoracic leg discs. Scale bar is 0.1mm and is same for all the images displayed.

the species without sex comb in ts1 (fig 1.5b, d, f, h, j, l). Overall there is a strikingly reduced expression of SCR in ts1 and there is no sexual dimorphism.

1.3.2 dachshund expression:

D. melanogaster was taken up as a representative candidate species from the species with sex comb for studying dachshund expression. D. n. nasuta, D. s. neonasuta, D. kepuluana, D. virilis were analyzed from the species group without sex comb. First level of expression analysis was carried out by anti-DAC antibody staining in 3-4hrs pupal prothoracic leg discs and the results are presented in fig (1.6 a-j)

In male and female D. melanogaster (fig 1.6 a-b), strong expression of DAC could be observed in the presumptive ti, ts1 and ts2 of the prothoracic leg discs. The expression in ts2 is comparatively lower to that of ts1. There exists no noticeable expression difference between the male and female prothoracic leg discs. In the four species from the cluster primitively lacking sex comb viz: D. n. nasuta (1.6 c-d), D. virilis (1.6 e-f), D. s. neonasuta (1.6 g-h), D. kepuluana (1.6 i-j), the prothoracic leg discs stained with anti-DAC antibody did not show any detectable signal. Expression pattern was sexually monomorphic. Observation was same in both male and female discs of all the species under this group.
Since the expression pattern of anti-DAC antibody staining in sex comb lacking species seemed to be intriguing, a reconfirmation experiment was performed, to check the binding efficiency of the antibody.

**Fig 1.6:** DAC expression pattern in some of the representative species of *Drosophila*. 3-4 hrs pupae were dissected for prothoracic leg discs and subjected to anti-DAC antibody staining. In species
with sex comb (a, b) male and female *D. melanogaster*, DAC could be detected in the tibia (ti), tarsal segment 1 (ts1), and tarsal segment 2 (ts2). The expression level of DAC is weaker in ts2 than in ts1. Dimorphic expression of DAC could not be observed in the prothoracic leg discs of male and female *D. melanogaster*. In species without sex comb, male and female (c, d) *D. n. nasuta*, (e, f) *D. virilis*, (g, h) *D. s. neonasuta*, (i, j) *D. kepuluana*, negligible amount of DAC expression could be detected. Scale bar is 0.1mm and is identical in all the images.

Immuno staining was conducted on prothoracic leg discs and eye antennal imaginal discs of 3rd instar larvae of *D. melanogaster* and *D. n. nasuta*. Positive signal of DAC was observed in the (fig 1.7a) prothoracic and (fig 1.7b) eye antennal imaginal discs of *D. melanogaster*. Immuno-histochemistry with the same antibody could not detect the presence of DAC in (fig 1.7c) eye antennal imaginal discs of *D. n. nasuta*. 

Fig 1.7: DAC profile in imaginal discs of *D. melanogaster*, and *D. n. nasuta* as reveal by anti-DAC antibody staining. 3rd instar larvae were dissected out for prothoracic and eye-antennal imaginal discs and subjected for anti-DAC antibody staining. DAC could be clearly seen in the prothoracic leg discs (a) and eye-antennal imaginal discs (b) of *D. melanogaster*. Eye-antennal imaginal discs (c) of *D. n. nasuta* show negative signal for DAC. Scale bar is 0.1mm and is same for all the images.
1.3.2.1 Expression of \textit{dac} analysed as transcripts:

As the previous experiment suggest anti-DAC antibody raised from \textit{D. melanogaster} DAC protein, probably cannot detect DAC in the species without sex comb viz \textit{D. n. nasuta}, \textit{D. virilis}, \textit{D. s. neonasuta}, \textit{D. kepuluana} or compels one to the speculation that DAC is absent in the species without sex comb taken up for the study.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1_8}
\caption{\textit{dac} transcripts as revealed by \textit{in-situ} hybridization in 3-4 hrs male prothoracic leg discs of \textit{Drosophila}. \textit{dac} could be seen in (a) \textit{D. melanogaster}, a species with sex comb and (b) \textit{D. n. nasuta} a species without sex comb. Both the species show a positive signal for \textit{dac} transcripts irrespective of the presence and absence of sex comb. \textit{dac} transcript could be detected in the tibia (ti), tarsal segment 1 (ts1), tarsal segment 2 (ts2). Scale bar is 0.1mm in both the images.}
\end{figure}

The speculation needs to be reconsider as DAC has an important function to play in leg development of \textit{Drosophila} (Mardon \textit{et al.}, 1993), and the legs of
the species without sex comb analyzed for DAC staining were properly formed. This indicates that the function of DAC towards leg development is taken care of. The possible reason might be DAC in *D. melanogaster* has undergone a change to an extent that anti-DAC antibody raised from *D. melanogaster* has fail to recognize DAC in the primitively combless species.

In order to check *dac* expression at its transcription level *in-situ* hybridization was conducted. *dac* transcripts localization by *in-situ* hybridization was done using Dig anti-sense probe, in the 2-3hrs pupal prothoracic leg discs of *D. melanogaster*, a species bearing sex comb and *D. n. nasuta* a species primitively lacking sex comb. The results are presented in fig 1.8. *dac* transcripts could be detected in the presumptive ti, ts1 and ts2 (fig 1.8a) in a similar pattern as shown by immuno-staining in *D. melanogaster*. The *in-situ* hybridization result of *D. n. nasuta* (fig 1.8b) as opposed to the negative staining that was seen in DAC immunostaining, depicts a similar profile as that of *D. melanogaster*. The signal intensity in *D. n. nasuta* discs seems to be less intense when compared with the discs of *D. melanogaster*. This is because the Dig anti-sense probe was designed using *D. melanogaster* cDNA as template. Probably due to divergence in the sequence of *dac* between *D. melanogaster* and *D. n. nasuta*, hybridization might not be efficient in *D. n. nasuta* as that in *D. melanogaster* discs. The experiment demonstrates that despite the negative signal generated by antibody staining, *in-situ* hybridization detects *dac* transcripts in *D. n. nasuta* discs and confirms DAC expression in the prothoracic leg discs.
1.3.3 *bric-a-brac* (*bab*)

![Fig 1.9: BAB 2 staining in some of the representative species of *Drosophila* with sex comb and cluster primitively lacking sex comb. 3-4hrs pupae were dissected for prothoracic leg discs and are subjected to anti-BAB antibody staining. BAB 2 could be observed in tarsal segment 1 (ts1) to tarsal segment 4 (ts4) with a gradient expression from ts1 to ts4. Expression is highest at ts4 and ts3 and lowest at ts1. Dimorphic expression of BAB 2 could not be detected between male and female prothoracic leg discs in the representative species studied. No remarkable difference between the species with sex comb and the cluster primitively lacking sex comb could be witnessed. BAB 2 staining in male and female (a, b) *D. melanogaster*, (c, d) *D. kepuluana*, (e, f) *D. s. neonasuta*, (g, h) *D. virilis*, (i, j) *D. n. nasuta*. Scale bar is 0.1mm in all the images displayed.](image-url)
The candidate species taken up to study BAB expression pattern are *D. melanogaster* from the species group with sex comb and *D. n. nasuta, D. s. neonasuta, D. kepuluana, D. virilis* from the species primitively lacking sex comb of *Drosophila*. Expression pattern analysis was done by anti-BAB antibody in 3-4hrs APF of the prothoracic leg discs. In *D. melanogaster* (fig 1.9 a, b) BAB is expressed from the ts1 to tarsal segment 4 (ts4). BAB shows a graded pattern from ts4 to ts1 with highest expression pattern in ts4 and ts3 and lowest in ts1. Males and females do not show remarkable differences in the expression pattern of BAB. The expression in the species primitively lacking sex comb viz, *D. kepuluana, D. s. neonasuta, D. virilis, D. n. nasuta* is shown in the fig (1.9 c-j). It resembles the expression pattern of *D. melanogaster* with a graded expression from ts4 to ts1. Males and females show similar expression pattern.

The emerging picture from immuno-histochemistry of BAB is there exists no remarkable difference in the expression pattern of BAB in representative candidate species taken up, between newly evolved species with sex comb and species group primitively lacking sex comb. There is no sexual dimorphism in its expression even in species with sex comb.
1.4 Discussion:

During evolution new morphological character makes an appearance by possible shifts at cross roads in developmental pathways. An insight into such switches can be obtained by comparative analysis of expression of components of a genetic circuit that regulates different events of development of a trait. Here is an attempt to identify the genetic changes in the genetic regulators of sex comb development, which has lead to the evolution of sex comb from primitive bristle pattern, expression pattern of the genetic regulators of sex comb development viz: Sex combs reduced, dachshund and bric a brac were analyzed. Comparative analysis of the expression pattern of the genetic players were observed between the species bearing the primitive bristles and the species with sex comb.

1.4.1 Marked enhancement in expression of SCR features the species with evolutionary novelty sex comb, as against primitively sex comb lacking species:

*Sex combs reduced* is a homeotic gene which specifies the prothoracic segment along anterior/posterior axis of the body of *Drosophila* (Struhl, 1982). Its function is required for the development of labial derivatives such as the adult proboscis and the larval salivary glands, posterior head, prothoracic leg, sex comb development in prothoracic leg (Lewis *et al.*, 1980; Wakimoto and Kaufman, 1981; Struhl, 1982; Panzar *et al.*, 1992; Percival-Smith *et al.*, 1997; Rogers *et al.*, 1997). Sex comb is lost entirely in homozygous Scr null clones (Struhl, 1982) and in trans heterozygous combinations of strong hypomorphic
mutations (Lewis et al., 1980, Pattatucci et al., 1991). A strong reduction in the number of sex comb teeth is seen in weaker hypomorphic combinations and flies heterozygous for Scr nulls (fig 1.10b) or deficiencies (Lewis et al., 1980; Kaufman et al., 1980; Pattatucci et al., 1991). There is an increase in the number of sex comb teeth when Scr is duplicated (Capdevila et al., 1986; Kennison and Russell 1987; Boube et al., 1997). Gain-of-function mutations that cause ectopic expression of Scr in T2 and T3 thoracic legs are sufficient to induce ectopic sex combs in these legs (Hannah-Alava 1964; Kaufman et al. 1980; Shroff et al., 2007). Analysis of temperature-sensitive Scr alleles reveals that Scr is required for sex comb development during late third larval instar and early pupal development (Pattatucci et al., 1991). Ectopic expression of Scr in ts2 and ts3 induced sex comb development in these segments in prothoracic leg of D. melanogaster (Barmina and Kopp, 2007). These mutational studies have shown that Scr is one of the key regulators of sex comb development in D. melanogaster.

SCR profile was analyzed in Sophophoran group which display a wide range of sex comb diversity by earlier workers (Barmina and Kopp, 2007; Randsholt and Santamaria, 2008). Sophophoran group bearing different number of sex comb e.g. one comb (D. erecta, D. eugracilis), two combs (D. takahashi, D. kikkawai, D. pseudoobscura), three sex combs (D. elegans), or species which has secondarily lost sex comb (D. lucipenis, D. prolongata) were stained for SCR expression.
In all these species, a strict correlation was found between SCR expression level in the tarsal primordia and, the number of tarsal segments that differentiate combs. *D. erecta, D. eugracilis* develop a single comb and showed SCR in ts1. *D. takahashi, D. kikkawai,* and *D. pseudoobscura* differentiate combs on ts1 and ts2, and express SCR in the corresponding tarsal segments. *D. elegans* which carry combs on ts1, ts2 and ts3 show SCR on ts1, ts2 and ts3. Striking lower level of SCR was seen in species which has secondarily lost sex comb (*D. lucipenis, D. prolongata*). This study showed that SCR also has evolutionary role to play within *Sophophoran* group in relation to sex comb diversity.

The question asked here is, whether the emergence of sex comb in *Sophophoran* group from the primitively sex comb lacking *Drosophila* is
underpinned by a change in Scr expression levels/domain in the prothoracic leg discs.

In the present study SCR expression was analyzed in *D. melanogaster* and *D. rajasekari* from the *Sophophoran* group. *D. melanogaster* is a species with a single sex comb whereas *D. rajasekari* a species with two sex combs. Expression in *D. melanogaster* is the same as reported by earlier workers (Barmina and Kopp, 2007; Randsholt and Santamaria, 2008). This is the first report of SCR expression in *D. rajasekari*. The two sex combs are in ts1 and the SCR expression is similar to that of *D. melanogaster*.

Important finding was that SCR expression observed in ts1 of the primitive species lacking sex comb namely *D. n. nasuta, D. s. neonasuta, D. kepuluana, D. kohkoa, D. hydei, D. virilis* were strikingly lower (fig 1.5a-l) to the SCR expression observed in the ts1 of the *Sophophoran* species (*D. melanogaster, D. rajasekari*) (fig 1.4 a-d). The intensity of SCR expression in the ti of the two groups were same which proves equal antibody binding efficiency in the two groups analyzed.

Sivanantharajah and Percival-Smith, (2009) has shown that SCR forms a complex with Probosipedia and Extradendicle. This complex is essential for the development of proboscis and salivary gland. Mutations in the dimer formation domain of SCR which is an octapeptide motif, affects sex comb formation in a highly dose sensitive manner. These findings imply the probable requirement of SCR protein’s function as homodimer in sex comb development
and a resulting dose dependency. The low expression of SCR in primitively sex comb lacking group seems to be unable to reach a threshold to gain the potential of transforming the primitive bristle into the evolutionary novelty – the sex comb.

*D. melanogaster* has been found to exhibit sexually dimorphic expression of SCR as reported by earlier studies (Barmina and Kopp, 2007; Tanaka *et al.*, 2011). The present study shows the sexually dimorphic expression of SCR in males and females in *D. rajasekari* as well (fig 1.4 c, d). The dimorphic expression of SCR is a typical pattern of *Sophophoran* species which have prominent sex comb. In males the expression extends till the distal margin of the ts1 which correlates to the position of sex comb development, whereas in females SCR expression does not extend till the distal margin of ts1 (fig 1.4 a, c males and 1.4 b, d females). Second important finding in the present study is that there is no such sexual dimorphism in SCR expression in the primitively sex comb lacking species.

The low expression of SCR in primitive species without sex comb seems to be insufficient for transforming the primitive bristles into the sex comb. Gain of high expression of SCR in the *Sophophoran* group is one of the key factor and acquiring sexual dimorphism with expression of SCR, so that it is made as a male specific character is the second key step for gaining the evolutionary novelty - sex comb in these species.
The difference in the expression pattern of SCR between the *Sophophoran* group and the non-*Sophophoran* group could be due to the divergence which SCR regulatory sequence has undergone during the course of evolution. Another strong possible reason for the variation in expression pattern might be due to a change in the upstream regulators of Scr. To check the second hypothesis expression pattern of genetic regulators of Scr were studied. Expression pattern of *dac* and *bab*, the genetic activator and repressor of Scr (Randsholt and Santamaria, 2008), were studied and a comparative study was conducted between the species with and without sex comb.

1.4.2 Altered expression of Dachshund is associated with presence/absence of sex comb between the *Sophophoran* and the non-*Sophophoran* group:

The nuclear Dachshund protein specifies the eye, Central nervous system, genital identities and is particularly required for the development of all medial leg territories (Mardon *et al*., 1994; Martini *et al*., 2000; Keisman and Baker, 2001). DAC is necessary and sufficient for eye development. Along with *Eyeless, Sine oculis and Eyes absent*, it forms the core of the retinal determination network. DAC also plays a key role in the development of medial leg territories. DAC domain 1 is the essential domain required for the development of eye and leg in *D. melanogaster* (Mardon *et al*., 1994). Work of Martini *et al*., (2000), have demonstrated that *dac* plays a key role in brain development during pupariation in *Drosophila*. In *dac* mutant there is a marked
reduction in the number of α lobe axons, a disorganization of axons extending into horizontal lobes, and aberrant projections into brain areas normally unoccupied by mushroom body processes. Keisman and Baker (2001), have shown that dac is differentially expressed in the male and female genital discs. It plays sex-specific roles in the development of the genitalia. The sex determination hierarchy mediates this sex-specific deployment of dac by modulating the regulation of dac by the pattern formation genes wingless (wg) and decapentaplegic (dpp). The sex determination pathway acts cell-autonomously to determine whether dac is activated by wg signaling, as in females, or by dpp signaling, as in males.

Earlier studies by Docquier (1997) and Docquier et al., (1999) reveals the role of DAC in activating sex comb formation. This study has been done in Montium-like mutant of D. melanogaster. MtlT5 possessed an ectopic sex comb on ts2 whereas Mtl101 shows ectopic sex combs on ts2 and ts3. MtlT5 is an insertion of a transposon at the 3’ of the dachshund gene, and Mtl101 is an internal deletion of this transposon (Docquier 1997). Anti–DAC antibody staining in the prothoracic leg discs of MtlT5 and Mtl101 reveals ectopic expression of DAC in ts1-ts3 and ts1-ts4 respectively (Randsholt and Santamaria, 2008) as opposed to the strong expression which is restricted to ts1 in wild type D. melanogaster.

Randsholt and Santamaria, (2008), also found out the correlation of dac and Scr in the morphogenesis of sex comb development. SCR was monitored in MtlT5 and Mtl101 mutants and it could be seen in the ts1-ts2 in MtlT5 and
ts1-ts3 in *Mtl101*, ectopic sex comb development and ectopic DAC were correlated in the distal tarsal segment primordial of *Mtl* with ectopic SCR expression. These findings proved that DAC promotes sex comb development through SCR. Thus *dac* functions as genetic activator of SCR.

The present study on comparative analysis of *dac* between group primitively lacking sex comb and group with sex comb revealed an interesting scenario. In *D. melanogaster* DAC can be seen in the ts1 and ts2 with a lower expression pattern in ts2 to ts1. Analysis within *Sophophoran* group between species with variation in sex comb, has been done taking examples of one sex comb (*D. melanogaster*), two sex comb (*D. bocqueti*), respectively. Randsholt and Santamaria (2008), in this study have shown that species within *Sophophoran* group with variations in sex comb number do not show variation in DAC expression. In this context it was asked if DAC has undergone any relevant shift in its expression pattern between *Sophophoran* group and the primitively sex comb lacking group in correspondence with the gain or down regulation of SCR expression.

Expression of DAC was analyzed in the species primitively lacking sex comb of the present study viz: *D. n. nasuta, D. virilis, D. s. neonasuta,* and *D. kepuluana* and DAC was found to be expressed at negligible levels (fig 1.6 c-j). Though the results depicted by the immuno-histochemistry, indicate absence of DAC in the prothoracic leg discs of the species group without sex comb taken up for the study, but the results had to be reconsidered as DAC plays a key role in the development of legs and eyes. *dac* mutant legs are truncated
due to merging of the femur, tibia, and the upper tarsal segments into a small, poorly defined tissue mass (Mardon et al., 1994; Giorgianni and Mann, 2011). Complete loss of dac function results in having little or no eye. There will be a dramatic decrease in the number of ommatidia from 750-800 in wild type to 0-15 in dac mutant (Mardon et al., 1994). The segments in the prothoracic leg (femur, tibia, and the upper tarsal segments), and eyes in the species lacking sex comb are properly developed suggesting that the roles of DAC in context with leg and eye development are fulfilled. The fact that even the other segments of leg discs and the eye antennal discs stained negative in immunostaining experiment prompted to reinvestigate the issue of DAC expression at different levels.

Interestingly, the experiments conducted to assay the expression pattern of DAC in eye antennae and prothoracic leg discs of third instar larvae in D. melanogaster and D. n. nasuta give similar results. A positive signal could be observed in the imaginal discs of D. melanogaster (1.7 a, b) but there was negligible signal in the imaginal discs of D. n. nasuta (1.7c).

Transcriptional activity of dac was analyzed in two species namely D. melanogaster (fig 1.8a) and D. n. nasuta (fig 1.8b) by mRNA in-situ hybridization. dac transcripts could be seen in the ti, ts1 and ts2 of prothoracic leg discs in D. melanogaster as well as in D. n. nasuta. A striking difference could be seen in 3-4 hrs prothoracic leg discs of D. n. nasuta a species without sex comb by in-situ hybridization as against the negative results revealed by immunostaining (fig 1.6a). In-situ hybridization results confirm that there is
indeed expression of dac in the prothoracic leg discs of species primitively lacking sex comb (1.8b).

The immunostaining V/S transcript localization results lead to an important dimension of the dac evolution among the two clusters of species. The antibody used in the experiment was raised against D. melanogaster DAC protein. The fact that the discs from the primitive cluster stain negatively with the antibody despite the transcript localization by in-situ hybridization, strongly imply that the degree of change that the protein has undergone renders DAC from the primitive species lacking sex comb undetectable by anti melanogaster–DAC antibody.

Earlier studies have proved that DAC is a genetic activator of Scr (Randsholt and Santamaria, 2008) and high expression of Scr is necessary for the development of sex comb (Barmina et al., 2007), in this context it can be suggested that the newly evolved DAC protein in the species with sex comb might have acquired the potential of interaction and facilitate activation of Scr and thus by “heterotypy” (Arthur, 2004), have altered the regulatory pathway of sex comb development.

1.4.3 No divergence in BAB profile between the species primitively lacking sex comb and the species with sex comb:

The bric a brac locus consists of two structurally and functionally related genes, bab1 and bab2 which act as a homeotic and morphogenetic regulator in the development of ovaries, appendages and the abdominal pigmentation
(King, 1970; Godt et al., 1993; Godt and Laski, 1995; Sahut- Barnola et al., 1995; Kopp et al., 2000; Cauderc et al., 2002). \(bab1\) and \(bab2\) encode a single nuclear protein each (Cauderc et al., 2002). \(bab2\) plays a predominant role in ovarian and particularly in leg development (Cauderc et al., 2002).

\(bab\) also plays a key role in the development of ovary which is shown by the mutant phenotype. In context to the development of ovary, \(bab\) mediates the formation of the terminal filaments, stacks of cells that are required for the formation of ovarioles (King, 1970; Godt and Laski, 1995; Sahut-Barnola et al., 1995) and contribute to the regulation of germline and follicle stem cell divisions during oogenesis (Lin and Spardling, 1993; Forbes et al., 1996). Earlier studies by Godt et al., (1993), showed that the three pairs of legs in both males and females suffer \(bab\) mutation. The two main characteristic of a \(bab\) mutant leg is i) shortening of tarsal segments resulting due to fusion of tarsal segments and loss of tarsal joints ii) a transformation of the bristle pattern of distal tarsal segments towards the bristle patterns of the ts1. ts5 and ts4 exhibit the maximum sensitivity towards the fusion of tarsal segments. The stronger the \(bab\) mutation, the further proximal the fusion extends. ts5 to ts2 get fused into a single segment in \(bab\) null mutants, sensitivity to a transformation of the bristle pattern of tarsal segments decreases from proximal to distal, involving only ts2 in weak \(bab\) mutants and ts2-ts4 in strong \(bab\) mutants.

Work of Kopp et al., (2000), has shown that abdominal pigmentation which is recently evolved in the \textit{melanogaster} group of \textit{Drosophila} is
controlled by bab gene. bab integrates regulatory inputs from the homeotic and the sex determination pathways. bab expression is modulated segment and sex specifically in sexually dimorphic species, but is uniform in sexually monomorphic species. bab has an important role in regulating sexually dimorphic trait like abdominal pigmentation in D. melanogaster (Kopp et al., 2000; Couderc et al., 2002; Gibert et al., 2007; Williams et al., 2008). In male D. melanogaster the posterior abdomen is covered by fully pigmented cuticle which is absent in females. bab encode dominant repressors of pigmentation. BAB shows dimorphic patterns of expression which are inverse of the melanic pigmentation on the abdomen of each sex, sexually dimorphic bab expression resulted from a composite activity of two separate CREs (Cis regulatory elements) in the bab locus (Williams et al., 2008). One CRE is expressed equivalently in both the sexes (monomorphic) in the anterior abdominal segments, whereas the other CRE directs female specific (dimorphic) bab expression in the posterior segments, direct binding of the region and sex specific transcriptional factors Abdominal B (ABD-B) and DSX dictates the region and sex specific activity of the dimorphic CRE (Williams and Carroll, 2009). In D. melanogaster the female isoform of DSX activates the bab dimorphic CRE by binding to two sites, whereas in males, the male isoform of DSX represses expression by binding to these same sites (Williams and Carroll, 2009).

In context to sex comb development bab mutants exhibit a transformation of the bristle pattern in the tarsal segments, leading to the
development of ectopic sex combs in distal tarsal segments (Godt et al., 1993; Cauderc et al., 2002). SCR expression analysis on the prothoracic leg discs of bab mutant shows the presence of SCR in the position of ectopic sex comb (Randsholt and Santamaria, 2008). These finding confirms that the ability to differentiate distal combs in bab mutant is therefore associated with ectopic SCR in comb bearing primordial, showing that bab acts genetically as a repressor of Scr. BAB profile was observed in Sophophoran species bearing different numbers of sex combs viz, D. melanogaster (one sex comb), D. kikkawai, D. ficusphila (two sex combs), and D. elegans (three sex combs). These Sophophoran species exhibiting divergence in the number of sex combs do not show changed BAB profile from D. melanogaster (Barmina et al., 2007; Randsholt and Santamaria, 2008). A clear picture of BAB in species primitively lacking sex comb help to understand whether BAB expression modulations in these species serve as an important factor for the origin of sex comb from the primitive state particularly since it is a sexually dimorphic trait. BAB was examined in some of the species primitively lacking sex comb namely D. n. nasuta, D. s. neonasuta, D. kepuluana and D. virilis. D. melanogaster was taken as a representative species with sex comb. BAB could be detected from ts1-ts4 in a gradient manner (fig 1.9a). The expression level increases from the proximal tarsal segments to the distal tarsal segments with highest expression seen in ts3-ts4 and lowest expression in the ts1.

BAB profile in the species primitively lacking sex comb taken up for the study does not show any remarkable divergence from that seen in D.
melanogaster (fig 1.9 c-j), irrespective of the change in the SCR expression. As mentioned earlier BAB is genetic repressor of SCR and the development of sex comb requires enhanced expression of SCR. It might also be possible that the Cis-regulatory element (CRE) of Scr has undergone a change as far as the interaction of BAB is concerned in the two groups of species studied. Both in D. melanogaster and the species without sex comb the high expression domains of SCR and BAB do not overlap, therefore the differential response of Scr CRE to BAB, does not seem to have lead the divergence in the expression pattern of SCR between the Sophophoran and the non-Sophophoran groups of Drosophila.

BAB has a crucial role to play in the sexually dimorphic abdominal pigmentation in Drosophila and it shows a dimorphic expression in male and female individual with respect to abdominal pigmentation. To analyze whether BAB has a similar role to play in sex comb evolution, a comparative study of BAB expression in female and male were made. It was found that there was no dimorphic expression of BAB in D. melanogaster (fig 1.9 a, b), as well as species without sex comb (fig 1.9 c-j). Considering the role played by BAB in the evolution of other sexually dimorphic traits like abdominal pigmentation it can be suggested from the present study that BAB is not directly or singly responsible for sexually dimorphic expression of SCR in the prothoracic leg discs.

Based on these data it can be concluded that BAB pattern in species primitively lacking sex comb and species with sex comb is similar and it was
likely present in the common ancestor of flies with and without sex comb. No variation in BAB expression would have played a key role in initiating the evolutionary variation sex comb in species with sex comb from species without sex comb.