Molecular localization of the P-element insertion lines of *Drosophila melanogaster*.

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Abstract

Isolation of *Drosophila* olfactory mutants is important in identifying the genes mediating the sense of smell. The P-element used in mutagenesis, P[lArB] and P[GawB], allow for the rescue of genomic DNA, immediately downstream of its site of insertion, in a plasmid (Plasmid Rescue). Localization of the P-element was carried out using a novel standardized plasmid rescue methodology in one P[lArB] line (1110) and four P[GawB] lines (191, OK294, OK309, and 238Y). Furthermore, points of insertion in these four P[GawB] lines were confirmed by our standardized inverse PCR methodology. Also, P-elements in four new P[GawB] lines (003, OK140, OK284, and OK301) were localized using the same inverse PCR methodology. Thus, in a total of nine lines, P-element insertion point was localized by either plasmid rescue or inverse PCR or both. The localization of P element insertion in these lines indicates about nearby candidate genes, which could be investigated further for any altered phenotype in these lines.

Introduction

The fruit fly, *Drosophila melanogaster*, can smell and discriminate a wide variety of odors with remarkable sensitivity and specificity. First single-gene olfactory mutations were isolated by Rodrigues and Siddiqi (1978, 1981). The P-element has been the workhorse of *Drosophila* genetics since it was developed as a tool for transgenesis in 1982; the subsequent development of a variety of systems based on the transposon have provided a range of powerful and flexible tools for genetics and genomics applications. The P-element used in mutagenesis, like P[lArB] and P[GawB], allow for the rescue of genomic DNA, immediately downstream of its site of insertion, in a plasmid (Plasmid Rescue). This rescued DNA then serves as a handle in the molecular characterization and the subsequent cloning of the gene. Inverse PCR (iPCR) is a method for the rapid *in vitro* amplification of DNA sequences that flank a region of known sequence. The method uses the polymerase chain reaction (PCR), but it has the primers oriented in the reverse direction of the usual orientation. A limitation of standard PCR is that 5' and 3' flanking regions of your DNA fragment of interest must be known. Inverse PCR allows you to conduct PCR when you only have the information of one internal sequence. Thus, in a total of nine lines, P-element insertion point was localized by either plasmid rescue or inverse PCR or both.

Materials and Methods:

Stocks of flies were maintained on standard cornmeal medium (Lewis, 1960) under a 14-hour light and 10-hour dark cycle at 24°C.

Materials for plasmid rescue:

Homogenization Buffer:

Tris HCl, pH 7.5	50 mM
NaCl	60 mM
EDTA, pH 8.0	10 mM
Sucrose	5 %

Lysis Buffer:

Tris HCl, pH 9.0	300 mM
EDTA, pH 8.0	100 mM
SDS	0.625 %
Sucrose	5 %

RNase A:

Stock solution	10 mg/ml

For stock solution, dissolve DNase free RNase A in,

Tris HCl, pH 7.5	10 mM
NaCl	15 mM
Double distilled water	as per required

Heat the solution at 100°C for 15 minutes on heating block. Allow to cool slowly to room temperature and dispense into aliquots and keep at -20 ^{0}C.

Proteinase K:

Stock solution	20 mg/ml
Dissolved in,	
Tris HCl, pH 8.0	50 mM
Calcium acetate	1.5 mM
Double distilled water	as per required

Dispensed in small aliquots and kept at -20°C.

Phenol : Chloroform : Isoamyl alcohol :
25 : 24 : 1

TBE Buffer, pH 8.3:

Stock solution	5 ×
Working solution	0.5 ×

Autoclave and dilute to make working solution and prefer to use freshly diluted.

Ethidium bromide (Etbr):

Stock solution	10 mg/ml

Add 1 g of Etbr to 100 ml of double distilled water. Wrap and the container in aluminum foil or transfer the solution to a dark bottle and store at room temperature.

LB Medium (Luria–Bertani Medium/Broth):
To make 100 ml, add

Tryptone	1 g
Yeast Extract	0.5 g
NaCl	1 g

Shake until the solutes are dissolved, adjust the pH to 7.0 with 5 N NaOH, then make up the volume to 100 ml using distilled water. Sterilize by autoclaving and keep at room temperature.
For LB-Agar plates, just add 1.5 g of Agar powder to 100 ml of LB broth and then autoclave it.

SOB-Agar plate with Antibiotic:
To make 100 ml of SOB medium/broth, add

Tryptone	2 g
Yeast Extract	0.5 g
NaCl	0.05 g
KCl (250 mM)	1 ml (final concentration 2.5 mM)
$MgSO_4$ (0.5 M)	2 ml (final concentration 10 mM)

Shake until the solutes are dissolved, adjust the pH to 7.0 with 5 N NaOH, then make up the volume to 100 ml using distilled water. To make SOB-Agar, add 1.5 g Agar and boil to dissolve it. Then get it autoclaved.

When using, melt SOB-Agar and add
$MgCl_2$ (2 M) 0.5 ml
Swirl mix, and when temperature drops to ~50°C – 60°C, add
Ampicillin (50 mg/ml) 100 µl for 100 ml SOB medium (final concentration 50 µg/ml)

SOC Medium:
After SOB broth is autoclaved and its temperature is dropped down to 60°C or less, add 200 µl of 1 M Glucose (filter sterilized using 0.22 µm filter) into 10 ml of SOB medium.
And before use, add 50 µl of 2 M $MgCl_2$ in 10 ml of SOC medium.

Antibiotics:

Tetracycline –
- Stock solution: 5 mg/ml in Ethanol (light sensitive)
- Working concentration: 50 µg/ml (1:100)
- Store at -20°C.

Chloramphenicol –
- Stock solution: 34 mg/ml in Ethanol (light sensitive)
- Working concentration: 170 µg/ml (1:200); 25 µg/ml (for XL-10 Gold *E. coli*)
- Store at -20°C.

Ampicillin –
- Stock solution: 50 mg/ml in sterile water
- Working solution: 100 µg/ml (1:500)
- Sterilize through 0.22 µm syringe filter unit.
- Store at -20°C.

Plasmid Isolation:

Solution I:

Glucose	50 mM
Tris HCl, pH 8.0	25 mM
EDTA, pH 8.0	10 mM

The solution should be autoclaved and kept at 4°C.

Solution II:

NaOH	0.2 N
SDS	1 %

The solution should be prepared fresh and used at room temperature.

Solution III:
For 100 ml,

Potassium acetate (5 M)	60.0 ml
Glacial acetic acid	11.5 ml
Double distilled water	28.5 ml

The resulting solution is 3 M with respect to Potassium and 5 M with respect to Acetate.
The solution should be stored at 4°C and transferred to ice bucket just before use.

Materials for inverse PCR:

Buffer A:

Tris HCl, pH 7.5	100 mM
EDTA, pH 8.0	100 mM
NaCl	100 mM
SDS	0.5 %

The first three components should be autoclaved and SDS should be added later and kept at room temperature.

LiCl/KAc Solution:

KAc	5 M
LiCl	6 M

The above two components should be autoclaved separately.
Mix 1 part 5 M KAc stock : 2.5 parts 6 M LiCl stock just before the experiment, the mixture should be freshly prepared.

TE Buffer (pH 8.0):

Tris HCl, pH 8.0	10 mM
EDTA, pH 8.0	1 mM

The buffer should be autoclaved and kept at room temperature.

RNase A:

Stock solution	10 mg/ml
Working solution	100 µg/ml with autoclaved double distilled water.

Prepared as described for Plasmid Rescue above.

Methodologies

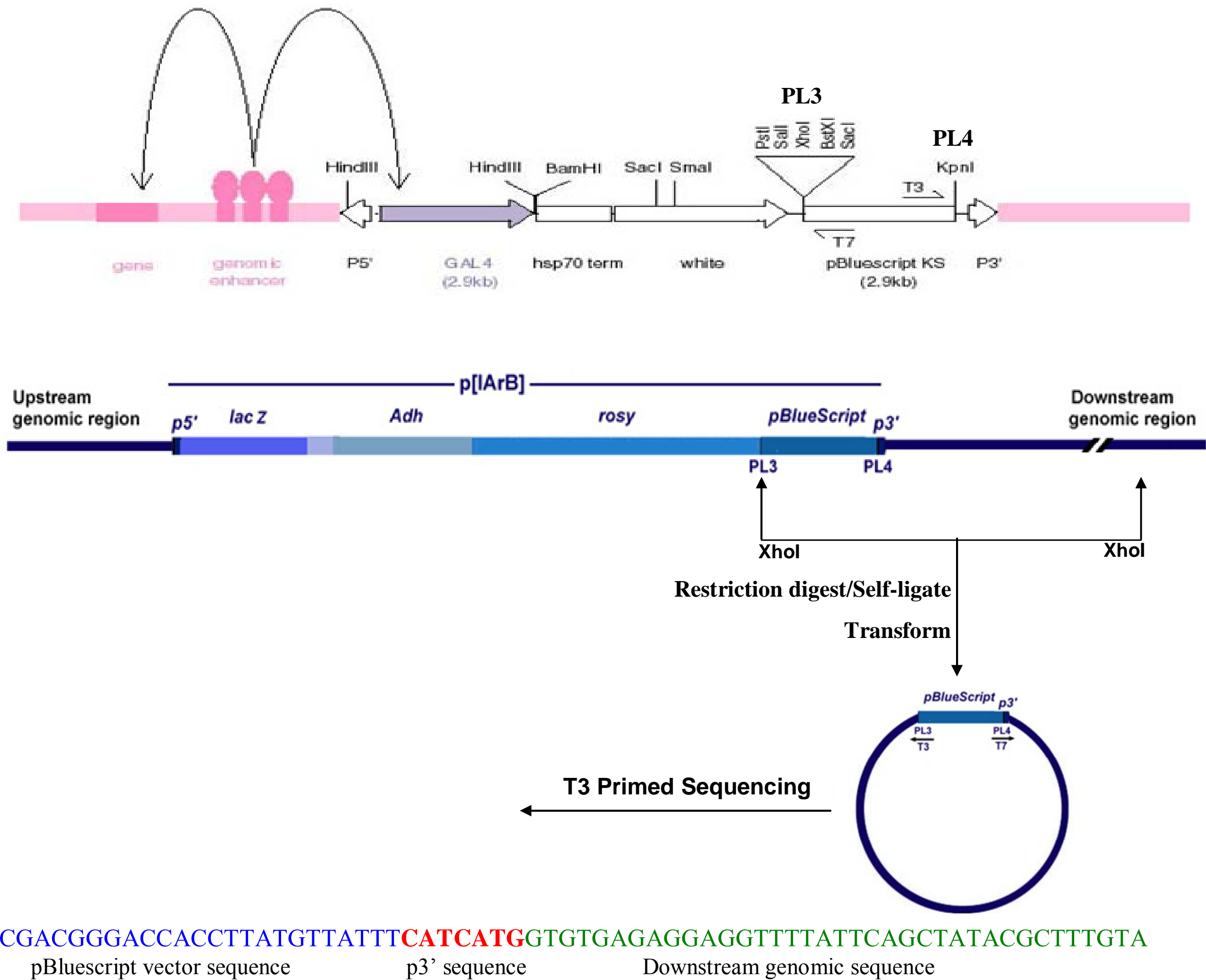


Figure 1. Schematic of the plasmid rescue. Genomic DNA extracted from P[GawB] and P[lArB] insertion lines, digested with XhoI (cutting at the polylinker PL3 site of the P element and at the next restriction site in the downstream genomic region). The DNA was then self-ligated, the ligated DNA was used for transformation, and the cells were plated on the selection medium containing Ampicillin, Tetracyclin, and Chloramphenicol.

Plasmid Rescue:

In plasmid rescue, genomic DNA from the P element insertion lines was extracted, restriction digested with XhoI, a restriction enzyme of the polylinker 3 (PL3) site of the P element, ligated under conditions to favor self-ligations, the ligated DNA was transformed into *E. coli* (XL-10 Gold strain) by electroporation and the transformants were selected for Ampicillin, Tetracyclin and Chloramphenicol resistance (Figure 1). Plasmid DNA was extracted from the transformants, restriction digested using XhoI and NotI (PL4 restriction enzyme) to know the size of insert. This plasmid DNA with insert has T3 and T7 promoter/priming sites, and DNA primers specific to these two sites were used to obtain the 3' and 5' end sequences of the rescued DNA. The T3 primed 3' end sequence includes a part of the pBluescript vector, followed by the seven base pair P element inverted repeat (p3'), CATCATG and the downstream genomic region. The first base following the inverted repeat, therefore, corresponds to the exact site of P element insertion (Wilson *et al.,* 1989).

In order to determine the site of P element insertion on the genome, the 5' and 3' end sequences were matched with the *Drosophila* genome sequence available online at the National Center for Biotechnology Information (NCBI) and the FlyBase websites. This was done by using BLAST (Basic Local Alignment Search Tool) sequence similarity search program (Altschul *et al.,* 1990).

Extraction of genomic DNA from flies:

Homogenization and lysis:

- Take 30 anaesthetized flies in a 1.5 ml eppendorf tube.
- Freeze the tube by dipping in liquid N_2.
- Homogenize in 300 µl of chilled homogenization buffer using a polypropylene pestle homogenizer.
  Crush gently till cuticle of the fly is visibly broken into small pieces.
  Leave on ice.
- Add 300 µl of lysis buffer.
  Mix gently by inversion.

RNase treatment:

- Add 10 µl of RNase A (from 10 mg/ml stock).
  Mix gently.
  Incubate at 37°C for 30 minutes.

Proteinase treatment:

- Add 5 µl of Proteinase K (from 20 mg/ml stock).
  Mix gently.
  Incubate at 37°C for 30 minutes.

Phenol/Chloroform/Isoamyl alcohol extraction:

- Extract at least two times (optimum three times) with equal volume of Phenol : Chloroform : Isoamyl alcohol (25:24:1).
  Add 600 µl of Phenol : Chloroform : Isoamyl alcohol.

Mix by inverting 10-15 times until the aqueous layer turns milky.
Spin at 13000 rpm for 10 minutes at room temperature.
Separate aqueous phase (top layer) and collect ~550 µl of aqueous phase.
Add 550 µl of Phenol : Chloroform : Isoamyl alcohol.
Repeat as above.
Collect ~500 µl of aqueous phase.

Chloroform extraction:

- Add 500 µl of Chloroform.
  Mix by inverting 10-15 times vigorously for two minutes.
  Spin at 13000 rpm for 5 minutes at room temperature.
  Collect top 450 µl of aqueous layer.
  Repeat as above.

Alcohol precipitation:

- Add 900 µl (two volumes) of absolute alcohol.
  Mix gently, DNA precipitates.
  Let stand on ice for 15 minutes.
  Spin at 13000 rpm for 10 minutes.
  Discard ethanol.
  Add 600 µl of 70 % alcohol for washing.
  Rinse briefly.
  Spin at 13000 rpm for 15 minutes at room temperature.
  Discard ethanol.
  Remove excess with pipette.
  Leave at room temperature for ~20 minutes to evaporate.
  Resuspend the pellet in 30 µl of sterile water by leaving stand for 5 minutes and tapping. About 0.5 µg/µl of DNA is obtained. Store at 4°C.

Digestion of genomic DNA (50 µl) :
Set up the following digestion reaction (with a PL3/cloning enzyme):

Genomic DNA (~2 flies)	5.0 µl
10 × buffer	5.0 µl
Sterile water	37.5 µl
BSA	0.5 µl
Restriction enzyme (Xho I)	2.0 µl

- Incubate in water bath at 37°C for 3 hours.
- Heat the tube at 65°C for 20 minutes on heating block to stop the reaction.
- Check 10 µl of the reaction on 0.8 % TBE-agarose gel.

Precipitation of digested genomic DNA :

- Add 60 µl of sterile water to the leftover 40 µl of digested genomic DNA reaction.
- Put on the heating block at 42°C.
- Extract once with Phenol : Chloroform : Isoamyl alcohol (25:24:1)
  Add 100 µl of Phenol : Chloroform : Isoamyl alcohol
  Vortex for 20 seconds.

Spin at 13000 rpm for 10 minutes at room temperature.
Collect 90 µl of supernatant in a fresh tube.

- Extract once with Chloroform
  Add 180 µl of Chloroform.
  Vortex for 20 seconds.
  Spin at 13000 rpm for 5 minutes at room temperature.
  Collect 80 µl of aqueous top layer in a fresh tube.
- Precipitation of the digested DNA
  Add 9 µl of 3 M Sodium acetate (pH 5.0)
  Add 180 µl of chilled absolute alcohol.
  Mix gently.
  Keep on ice bath for 30 minutes.
  Spin at 13000 rpm for 30 minutes at 4°C.
  Discard the supernatant.
  Rinse with 100 µl of 70 % alcohol.
  Spin at 13000 rpm for 15 minutes at 4°C.
  Remove ethanol.
  Spin briefly again.
  Remove traces of ethanol.
  Let air dry for ~20 minutes at room temperature.
  Re-dissolve the pellet in 20 µl of sterile water.

Ligation of the digested genomic DNA (200 µl):
Set up the following ligation reaction :

Digested genomic DNA	10.0 µl
10 × ligation buffer	20.0 µl
Sterile water	169.0 µl
T4 DNA Ligase	1.0 µl

- Incubate at 16°C for 16 hours.

Precipitation of the ligated DNA :

- Add 20 µl of 3 M Sodium acetate (pH 5.0)
- Add 400 µl of chilled absolute alcohol.
- Invert mix gently.
- Leave over night at -20°C.

Pelleting the ligating DNA:

- Spin at 13000 rpm for 30 minutes at 4°C.
- Discard the supernatant.
- Rinse with 100 µl of 70 % alcohol.
- Spin at 13000 rpm for 30 minutes at 4°C.
- Remove ethanol.
- Spin briefly again.
- Remove traces of ethanol.
- Let air dry at room temperature for ~20 minutes.
- Re-dissolve in 5 µl of sterile water.

Transformation and analysis:

- Thaw out XL 10 Gold *E. coli* electrocompetent cells on ice for 15 minutes.
- Add ligated DNA at volume no greater than 4 µl to 40 µl of electrocompetent cells. Mix gently with pipette keeping it cooled.
- Transfer the content to the bottom of sterile Bio-Rad 0.2 cm electroporation cuvette kept on ice, remove any air bubble.
- Wipe out the moisture from the outer surfaces of the cuvette using a tissue paper, and pulse (electroporate) at the following settings -

Voltage	2.5 KV
Resistance	200 Ω
Capacitance	25 µF

  Ideal time constant obtained: 4.5 seconds
- Immediately add 1 ml SOC medium containing 20 mM Glucose kept at room temperature. Mix gently using pipette. Addition of medium at room temperature provides a heat shock that increases the efficiency of transformation.
- Transfer to 1.5 ml sterile eppendorf tube and incubate at 37°C with mild shaking for one hour.
- Plate out required dilution (up to 200 µl per 90 mm plate) on SOB-Agar medium containing 20 mM $MgSO_4$ and the appropriate antibiotics.

Pouring LB-Agar plates:

For XL 10 Gold *E. coli* cells –

Melt the LB-Agar medium and cool it to ~50°C – 60°C, and then add

Tetracycline	2 ml in 200 ml of LB-Agar
Chloramphenicol	148 µl in 200 ml of LB-Agar
Ampicillin	400 µl in 200 ml of LB-Agar

- Pick up at least 6 independent transformants per each line and re-streak on fresh LB-Agar plates with appropriate antibiotics added like above.
- Incubate at 37°C till well separated colonies are seen.

Plasmid miniprep from the transformants:

- Inoculate single colonies from each of the 6 independent transformants into 5 ml of LB-antibiotics medium in a culture tube.
- Grow over night at 37°C in a mild incubator shaker.
- Chill culture on ice for 15 minutes.
- Transfer 1.5 ml of culture to sterile eppendorf tube.
- Harvest cells by spinning at 13000 rpm for 30 seconds at room temperature.
  Decant supernatant.
  Again transfer 1.5 ml of culture to the same tube.
  Spin at 13000 rpm for 30 seconds at room temperature.
  Decant supernatant.
  Re-spin cells for 30 seconds.
  Remove remaining LB medium.
  Leave tubes on ice.
- Add 150 µl of Solution I.

Resuspend the bacterial pellet by vortexing.
Vortex vigorously till cells are mixed properly.
Leave on ice till chilled down (≤ 5 minutes).
Make Solution II in mean time as follows –

For 1 ml, add

10 N NaOH	20 µl
10 % SDS	100 µl
Sterile water	880 µl

- Add 300 µl of freshly prepared Solution II. Pour the Solution II very slowly with the wall of the tube and invert mix very gently for ~5 times.
  Leave on ice for 10 minutes till slight precipitation appears.
- Add 250 µl of Solution III, it leads to clump formation. Invert mix very gently. Leave on ice for 10 minutes.
- Pellet cell lysate by spinning at 13000 rpm for 5 minutes at room temperature. The white crap settles down.
  Carefully collect ~650 µl of supernatant avoiding the white crap completely. If white material is still visible in supernatant, re-spin the supernatant to remove the white material completely.

RNase treatment:

Add 10 µl of RNase A (from 10 mg/ml stock). Vortex for a second to mix it properly.
Incubate at 65°C for 30 minutes on heating block. This step can be done at 37°C as well.

- Add 650 µl of Phenol : Chloroform : Isoamyl alcohol (25:24:1).
  Mix by vortexing for 30 seconds. Also, mix thoroughly with jerks.
  Spin at 13000 rpm for 5 minutes at room temperature.
  Collect 600 µl of aqueous layer in a fresh tube.
  Repeat the above process.
  Collect 550 µl of aqueous layer in a fresh tube.
- Add 550 µl of Chloroform.
  Mix by vortexing for 30 seconds. Also, mix thoroughly with jerks.
  Spin at 13000 rpm for 5 minutes at room temperature.
  Collect 500 µl of aqueous layer in a fresh tube.
  Repeat the above process for two more times.
  After the third processing, collect 400 µl of aqueous layer in a fresh tube.
- Add 2 volumes (800 µl) of chilled absolute alcohol slowly.
  Mix gently by inversion.
  Leave at -20°C for one hour or over night.
  Spin at 13000 rpm for 30 minutes at 4°C. This spin can be done at room temperature as well.
  Decant ethanol.
- Add 400 µl of 70 % alcohol.
  Rinse pellet by tapping and inverting repeatedly; can be vortexed as well.
  Spin at 13000 rpm for 10 minutes at 4°C. This spin can be done at room temperature as well.
  Remove ethanol.
  Re-spin briefly and remove excess ethanol with pipette.
  Air dry the pellet at room temperature for ~20 minutes.
  Resuspend in 20 µl of sterile water. Mix well by pipetting and tapping.
- Check the plasmid DNA obtained by digesting with cloning enzyme and running on a mini gel. A single band of size more than 3 Kb (size of pBlueScript plasmid) should be seen.

Plasmid DNA digestion (20 µl):

Plasmid DNA	2.0 µl
10 × Buffer	2.0 µl
Sterile water	14.3 µl
BSA	0.2 µl
Restriction enzyme (Xho I)	1.5 µl

- Double digest with the cloning enzyme and a PL4 enzyme to release the insert.

Preparation of electrocompetent cells:

- Inoculate a single colony of XL-10 Gold *E. coli* from a fresh Agar plate into a flask containing 50 ml LB media with antibiotics.
- Incubate the culture over night at 37°C in a rotary shaker maintained at 250 rpm.
- Inoculate 25 ml each in two 500 ml pre-warmed LB media with antibiotics in conical flasks.
- Incubate at 37°C in a shaker maintained at 250 rpm till the OD_{600} reaches to 0.4 (~2.5 hours).
- Rapidly transfer the flasks in ice-water bath. Keep for ~15-30 minutes. Swirl occasionally.
- Pellet down the bacterial cells by transferring the culture repeatedly into 250 ml pre-chilled centrifuge bottles, and by spinning at 2500 rpm for 15 minutes at 4°C.
- Re-suspend the pellet in 250 ml of ice-cold sterile double distilled water. Swirl mix properly to wash the bacterial cells, maintaining the low temperature.
- Spin at 2500 rpm for 15 minutes at 4°C. Repeat the above process.
- Re-suspend the pellet in 250 ml of ice-cold sterile 10 % Glycerol. Swirl mix properly maintaining the low temperature.
- Spin at 2500 rpm for 15 minutes at 4°C. Repeat the above process.
- Re-suspend the pellet in 10 ml of ice-cold sterile 10 % Glycerol. Swirl mix properly maintaining the low temperature.
- Spin at 2500 rpm for 20 minutes at 4°C.
- Decant the Glycerol from the bottles and re-suspend the pellet into the remaining Glycerol in the bottle by swirling, maintaining the low temperature.
- Make 40 µl aliquots of the cells in pre-chilled sterile eppendorf tubes and using cooled sterile pipette tips. Keep the tubes on ice.
- 40 µl checked in ice-cold electroporation cuvette for arcing.
- Immerse the tubes in liquid N_2 and transfer to a -80°C freezer till further use for electroporation (transformation).

Inverse PCR:

In inverse PCR, there are initial common steps as that of plasmid rescue. The genomic DNA was digested with DpnII restriction enzyme and then it was ligated in a condition favoring self-ligation. The ligated DNA was directly taken for PCR, using PGaw2 and PGaw3 primers (Figure 2). The PCR product (shown in Figure 3) was gel extracted and given for DNA sequencing using the SP1 (for 5’ end) and SPEP1 primer (for 3’ end). Then the sequence was matched with the *Drosophila* genome sequence databases as in case of plasmid rescue.

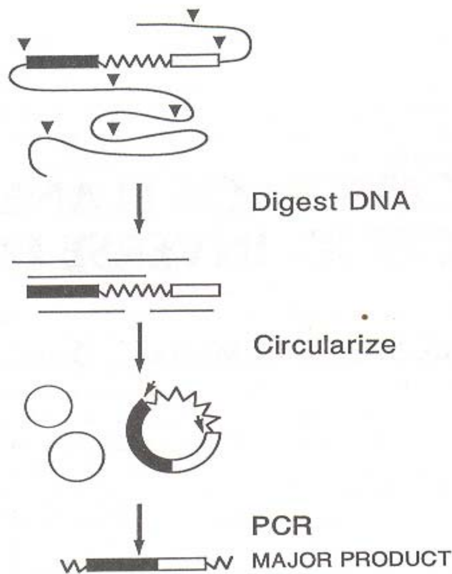


Figure 2. Schematic of the inverse PCR. The core region (the P element P[GawB]) is depicted as a jagged line. Filled and open boxes represent the upstream and downstream flanking regions, respectively, and restriction enzyme (DpnII) recognition sites are denoted by triangles. Oligonucleotide primers (PGaw2 and PGaw3) constructed to anneal to the core region and the direction of DNA synthesis are shown by arrows.

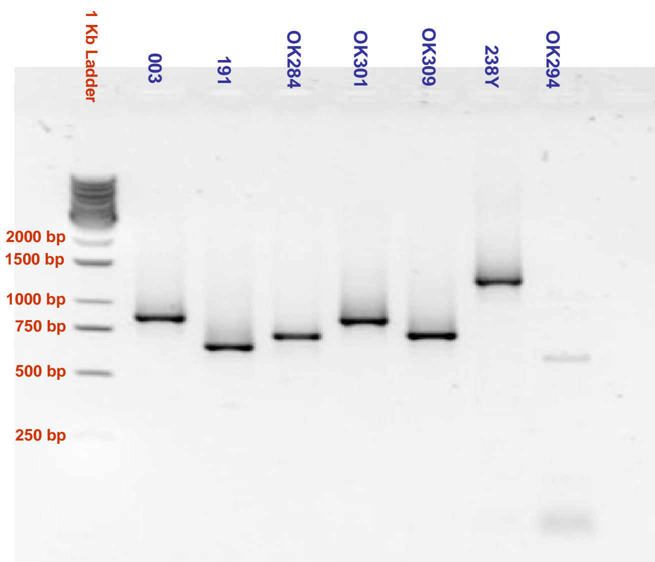


Figure 3. Inverse PCR of P[GawB] insertion lines. The presence of single band in each of the lines indicates that there is only one copy of the P element inserted in them. And the varying sizes of the bands are indicative of different sites of P element insertions and thus recognition sites for DpnII in the genome.

Quick fly genomic DNA preparation from flies:

(modified from the methods described by E. Jay Rehm and Roger Hoskins of BDGP)

- Take 30 anaesthetized flies in a 1.5 ml eppendorf tube.
- Freeze the tube by dipping in liquid N_2.
- Grind flies gently, without shearing genomic DNA, in 200 µl of Buffer A with polypropylene homogenizer on ice.
- Add an additional 200 µl of Buffer A and continue grinding on ice, until only cuticles remain.
- Incubate at 65°C for 30 minutes on dry heating block.

- Add 800 µl of LiCl/KAc solution, invert mix several times slowly.
- Incubate on ice for at least 10 minutes.
- Spin at 12000 rpm for 15 minutes at room temperature.
- Transfer 1 ml of the supernatant into a fresh tube, avoiding floating crud.
  If crud transfers, re-spin to exclude the crud completely.
- Add 600 µl of Isopropanol and invert mix very slowly for several times.
- Spin at 12000 rpm for 20 minutes at room temperature.
- Aspirate away supernatant, leaving the pellet intact.
  Quick spin it again and aspirate using pipette.
- Wash pellet with 500 µl of 70 % alcohol.
- Spin at 12000 rpm for 10 minutes at room temperature.
- Aspirate and discard the supernatant.
  Quick spin again and aspirate using pipette.
- Air dry pellet for ~30 minutes at room temperature.
- Resuspend the pellet in 150 µl of TE buffer (pH 8.0).
  Leave it over night at room temperature to dissolve the pellet completely.

Digestion of genomic DNA (25 µl):
Set up the following digestion reaction (with DpnII restriction enzyme):

Genomic DNA (~2 flies)	10.0 µl
10 × buffer	2.5 µl
Sterile water	9.5 µl
RNase A	2.0 µl (from 100 µg/ml stock)
Dpn II	1.0 µl (5-10 Units)

- Incubate at 37°C in water bath for 3 hours.
- Heat the tube at 65°C for 20 minutes on heating block to stop the reaction.
- Check 5 µl of the reaction on 0.8 % TBE-agarose gel.

Ligation of the digested genomic DNA (400 µl):
Set up the following ligation reaction:

Genomic DNA digestion reaction (~1 fly)	10.0 µl
10 × ligation buffer	40.0 µl
Sterile water	346.0 µl
T4 DNA Ligase (2 Weiss Units)	4.0 µl

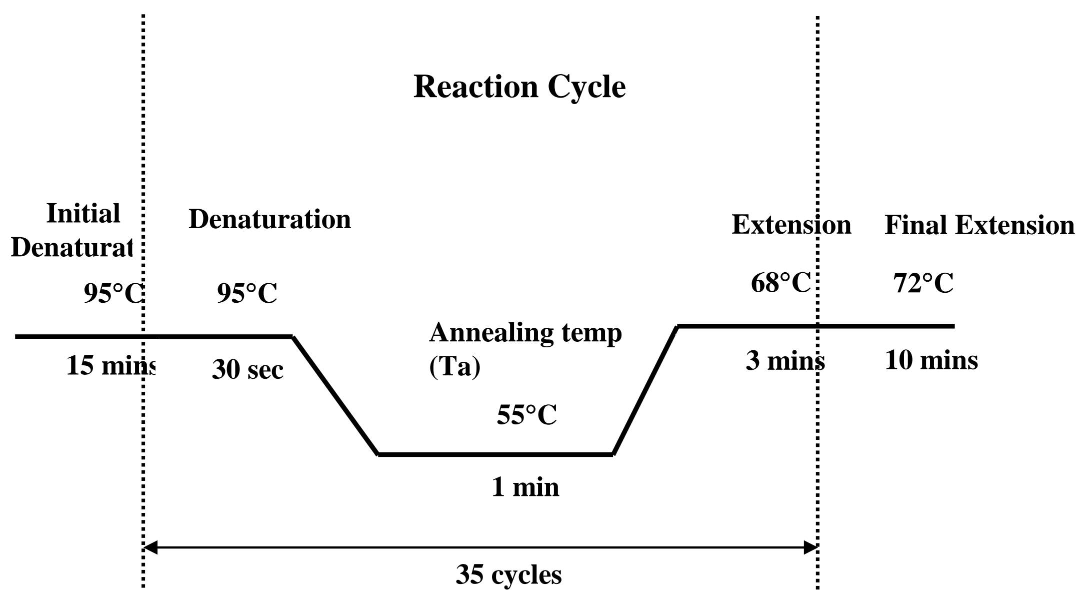
- Incubate at 16°C for 16 hours.
- Add 40 µl of 3 M Sodium acetate (pH 5.0) and 1 ml of absolute alcohol. Invert mix gently and chill for 1 hour at -80°C.
- Spin at 16000 rpm for 30 minutes at 4°C.
- Decant supernatant and aspirate the remaining alcohol using pipette.
- Air dry the pellet at room temperature for ~20 minutes.
- Resuspend in 150 µl of TE buffer (pH 8.0). Keep it for at least one hour at room temperature to dissolve the pellet completely.

Inverse PCR reaction (50 µl):
Set up the following PCR reaction (to amplify the 5' end):

Sterile water	33.0 µl

10 × PCR buffer	5.0 µl (1 × final concentration)
10 mM dNTP mix	2.0 µl (0.4 mM final concentration)
10 µM Primer PGaw2	2.0 µl (0.4 µM final concentration)
10 µM Primer PGaw3	2.0 µl (0.4 µM final concentration)
Taq polymerase	1.0 µl (5 Units)
Ligated genomic DNA	5.0 µl

PCR cycling parameters :



- Check 5 µl of PCR reaction on 1 % TBE-agarose gel.
- Sequence with SP1 primer (for 5' end).
- To amplify the 3' end, use Plw3-2 and PRY4 primers set with Ta as 55°C.
- Sequence with SPEP1 primer (for 3' end).

Primers used for amplification and sequencing:

(A) Amplification of PGawB insertion lines by inverse PCR:
5' end amplification:
PGaw2: 5' – CAGATAGATTGGCTTCAGTGGAGAC – 3'
PGaw3: 5' – CGCATGCTTGTTCGATAGAAGAC – 3'

3' end amplification:
Plw3- : 5' – TAACCCTTAGCATGTCCGTGGGGTTTG – 3'
PRY4: 5' – CAATCATATCGCTGTCTCACTCA – 3'

End sequencing of PCR products in PGawB insertion lines:
SP1 (*for 5' end sequencing*):
5' – ACACAACCTTTCCTCTCAACAA – 3'
SPEP1 (*for 3' end sequencing*):
5' – GACACTCAGAATACTATTC – 3'

(B) Plasmids with insert obtained from Plasmid rescue:
T3 (*for 3' end sequencing*):
5' – AATTAACCCTCACTAAAGGG – 3'
T7 (*for 5' end sequencing*):
5' – TAATACGACTCACTATAGGG – 3'

Table 1.

S. No.	Strain	Cytological location	Site of insertion	Genes in the vicinity of insertion
1	003	X	8788241 bp	*Moesin (Moe)* *CG1885*
2	191	X	10633066 bp	*CG32676* *Raspberry (Ras)*
3	1110	2R	16470254 bp	*l(2)05510* *Nnf1a* *Bancal (bl)*
4	OK140	2R	15614715 bp	*snoRNA:185* *tRNA:E4:56Fb*
5	OK284	3L	18703561 bp	*CG12477* *CG18363*
6	OK294	X	2685775 bp	*white (w)* *CG32795*
7	OK301	X	22232347 bp	*fog* *CG41475*
8	OK309	3L	20396454 bp	*trbl* *CG13248*
9	238Y	3L	14267346 bp	*frizzled (fz)*

Results and Discussion

The localizations of the P-element insertions and the nearby candidate genes are summarized in Table 1.

Until recently, the most popular method for molecular localization was the plasmid rescue. In this, the genomic DNA is extracted, restriction digested, self-ligated and the self-ligated DNA is transformed into the bacteria, and the transformants are selected for antibiotic resistance. There is lack of widely used complete plasmid rescue protocol available. Using some existing literature and talking to experienced people, we have standardized this protocol in our lab, and it works fine. But, this procedure is a bit lengthy and the transformation and selection procedure is a little tricky in terms of efficiency and possibilities of contaminations or false positives. The easier and more reliable method could be the inverse PCR method. This method does not require transformation and selection procedure. The self-ligated DNA can be directly amplified by using specific primers which are oriented in the reverse direction of the usual orientation. And the PCR product can be directly sequenced after cleaning. One more advantage of using inverse PCR is that it tells about the copy number of the P-element insertion in the genome. The presence of single band after PCR indicates that there is only one P-element insertion in the genome. There are two widely used methods for

inverse PCR described in the Berkeley *Drosophila* Genome Project (BDGP) (by E. Jay Rehm) and in Gal4 Enhancer Trap Insertion Database (GETDB). For optimum results in our case, we have employed some modifications in these protocols and used, like the method of grinding flies, centrifugation steps, the incubation temperature and time for ligation and most importantly we standardized the PCR conditions. We found that removing two bases from 3' end of the *PGaw2* primer gives better result, and also we standardized the best annealing temperature to be 55°C for *PGaw2* and *PGaw3* primers pair. These conditions produced distinct quality bands, more suitable for the downstream DNA sequencing. Four lines (*191, OK294, OK309,* and *238Y*) were localized both by plasmid rescue and inverse PCR methods independently and the analysis confirmed that the point of insertion of the P-element is the same in both the methods. That shows both the methods could be used to localize the P-element insertions successfully and the results coincide.

Using these approaches, a total nine lines (*003, 191, 1110, OK140, OK284, OK294, OK301, OK309,* and *238Y*) have been localized at base pair level. Few of these lines show interesting olfactory behavioral phenotypes at larval or adult or both stages. The olfactory behavioral tests have been done in our lab for these lines at both larval and adult stages (unpublished data). The localization of P-element insertions in these lines tell about the nearby candidate genes, which could have a role in the behavioral phenotype observed. There are few lines which have interesting genes in the vicinity which are known to have roles in different sensory organs developmental processes and signaling pathways. And there are few genes, the function of which is still not known. The line *003,* which shows normal response to all the concentrations of the three odors tested at larval stage, adults show decreased response to Ethyl acetate & 1-Hexanol and normal response to only Isoamyl acetate, has P-element insertion in the intron region of the gene *Moesin* (*Moe*). This gene is involved in many biological processes like anatomical structure development; anterior/posterior axis specification; organelle organization and biogenesis; oocyte axis determination; actin filament-based process; sensory organ development; cytoskeleton organization and biogenesis; organ morphogenesis and compound eye photoreceptor development. It would be interesting to study the behavioral phenotype of the alleles of this gene. This gene may have a role to play in the behavioral phenotype we are seeing in the line *003*. But, it needs to be established by various approaches like excision of P-element, complementation analysis and gene expression studies. In the line *191* larvae shows decreased response to Ethyl acetate & Isoamyl acetate and normal response to 1-Hexanol, but adults show decreased response to Ethyl acetate & 1-Hexanol and increased response to Isoamyl acetate, has P-element insertion in the intron region of the gene *CG32676*. Its molecular function is unknown. There is little information about its involvement in some cellular processes and protein modification processes. In the line *1110*, there is a gene *l(2)05510*, just 621 bp from the insertion site towards 5’ side. Its function is not known. In the line *OK140*, there is a gene *snoRNA:185*, 112 bp from the insertion site towards 5’ side and its function is unknown. Also, there is another gene *tRNA:E4:56Fb*, 250 bp from the insertion site towards 3’ side and it is involved in translation. In the line *OK294*, the P-element is inserted in the intron region of a gene *white* (*w*). This protein has ATPase activity, coupled to transmembrane movement of substances; eye pigment precursor transporter activity, and transmembrane receptor activity. There is another gene *CG32795* which is 1800 bp from the insertion site towards 5' side, its function is not known. In the line *OK301*, the insertion is again in the intron region of a gene called *fog*. It is involved in the biological processes like terminal region determination; torso signaling pathway; regulation of development, heterochronic; ventral furrow formation and morphogenesis of embryonic epithelium. In the line *OK309*, there is a gene *trbl*, 1746 bp from the insertion site towards 5’ side. Its molecular function is described as: ATP binding; protein kinase activity; protein serine/threonine kinase activity. In the line *238Y*, there is a gene *frizzled* (*fz*), very near to the P-element insertion, it is present just 101 bp

from the insertion site towards 3' side. So, this P-element insertion could be affecting the *frizzled* gene and so its functions, and it is worth investigating at greater detail. The molecular function of this gene is described as: Wnt receptor activity; Wnt-protein binding; transmembrane receptor activity; non-G-protein coupled 7TM receptor activity; G-protein coupled receptor activity. It is involved in many biological processes like anatomical structure development; cell communication; sensory organ development; signal transduction; macromolecule localization; protein localization; regulation of cellular component organization and biogenesis; Wnt receptor signaling pathway. Thus, this gene is reported to be involved in sensory system development and signal transduction pathways which are important for mediating sense of smell and learning and memory.

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Exploring the mutagenic activity of colchicine in *Drosophila.*

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Introduction

Drosophila melanogaster has been used successfully to evaluate *in vivo* genetic endpoints resulting from DNA damage, and diverse mating systems have been employed to determine the effect of genotoxins on germinal and somatic cells. In the present study, we assayed colchicine (CO) for the induction of somatic mutation and mitotic recombination in the *Drosophila* Somatic Mutation and Recombination Test (SMART).

Material and Methods

Mating System

Standard cross (SC): 3-day-old virgin females from the $flr^3/TM3, Bd^S$ *stock* were mated with *mwh/mwh* males. From this cross, two types of progeny were recovered: inversion-free and inversion-carrier flies. During larval stage they are indistinguishable from one another. As adults, the presence of the Bd^S marker allows the classification of the progeny based on wing phenotype: wild-type wing borders in the inversion-free flies ($+, flr^3 / mwh, +$), and nicks in the wing border of inversion-carrier flies ($TM3, Bd^S / mwh, +$) (Figure 1a).