FLP-mapping: A universal, cost-effective, and automatable method for gene mapping

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Abstract

Genetic mapping with DNA sequence polymorphisms allows for map-based positional cloning of mutations at any required resolution. Numerous methods have been worked out to assay single nucleotide polymorphisms (SNPs), the most common type of molecular polymorphisms. However, SNP genotyping requires customized and often costly secondary assays on primary PCR products. Small insertions and deletions (InDels) are a class of polymorphisms that are ubiquitously dispersed in eukaryotic genomes and only about 4-fold less frequent than SNPs. InDels can be directly and universally detected as fragment length polymorphisms (FLPs) of primary PCR fragments, thus eliminating the need for an expensive secondary genotyping protocol. Genetic mapping with FLPs is suited for both small-scale and automated high-throughput approaches. Two techniques best suited for either strategy and both offering very high to maximal fragment-size resolution are discussed: Analysis on non-denaturing Elchrom gels and on capillary sequencers. Here, we exemplify FLP-mapping for the model organisms *Drosophila melanogaster* and *Caenorhabditis elegans.* FLP-mapping can, however, be easily adapted for any other organism as the molecular biology involved is universal. Furthermore, we introduce *FLP mapper*, a JAVA-based software for graphical visualization of raw mapping data from spreadsheets. *FLP mapper* is publicly available at http://bio.mcsolutions.ch.

Key words: Genetic mapping, sequence polymorphisms, InDels, model organisms, Drosophila melanogaster, Caenorhabditis elegans, mapping software, *FLP mapper*

1. Introduction

Small insertions/deletions (InDels) constitute a ubiquitous and frequent class of biallelic sequence polymorphisms in eukaryotic genomes. In complex genomes such as rice and human, InDels roughly constitute between 20% of the sequence polymorphisms and occur as frequently as once every 1000 bps to about once every 5000 bps [1-3]. Similar frequencies have been observed in lower metazoans. Some studies reach highly divergent conclusions about InDel frequency, e.g. varying between 7 % and 37 % in Arabidopsis. In part, high estimates may be attributed to sequencing errors, as about two third of InDels are 1 bp polymorphisms that have a low confirmation rate [2,4].

Genotyping with InDels offers the main advantage that the measured output is the size of a primary PCR-product rather than its sequence. Fragment length polymorphisms (FLPs) can

be easily measured on standard lab equipment at standardized conditions. The determination of sequence differences (SNPs) on the other hand involves either secondary, oftenly costly and complex enzymatic reactions on the primary product (e.g., primer extension and primer ligation-based techniques, SNPs on chips [5]) or the analysis of different properties of homo- and heteroduplexes of primary PCR-products at partially denaturing conditions. The latter principle generally needs optimization for every single PCR product and in part specialized machinery (e.g., SSCP and DHPLC [6]).

Microsatellites (STRs) constitute a FLP subclass that has been widely used for genetic mapping by the same methodology [7]. They differ from InDels in that they are comprised of repetitive sequence, which may cause PCR problems leading to "stuttering" products. Besides, STRs are far less common than InDels - best illustrated by the current numbers of entries in the dSNP database for human polymorphisms (http://www.ncbi.nlm.nih.gov/SNP/) containing about 25 million SNPs, 1.5 million InDels, but only about 5000 STRs.

Thus, FLP-mapping combines the advantages of genetic mapping with STRs and SNP-mapping, respectively: Allele calling is based on robust methodology on the primary PCR product and InDel density on average may be sufficient to even map down to a gene.

FLP-mapping is both suited to a small-scale approach in labs equipped with basic instrumentation (i.e., gel apparatus and thermocycler) and to high-throughput procedures including automated PCR-setup in 96- or 384-well format, multiplexing strategies, fragment analysis on capillary sequencers, and software-supported allele calling. Here we exemplify both strategies by providing protocols for high-throughput fragment analysis and for low and medium throughput genotyping on high-resolution non-denaturing gels.

Besides, we present *FLP mapper*, a software that is designed to graphically display the genotypes of recombinant chromosomes.

2. Materials

(see note 1)

2.1. Links to SNP and InDel databases

human:	http://snp.cshl.org/
	http://hgvbase.cgb.ki.se/cgi-bin/main.pl?page=data_struct.htm
mouse:	http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=snpQF
C. elegans:	http://genomebiology.com/2005/6/2/R19 (additional data files 12 to 17)
	http://www.wormbase.org/db/searches/strains

http://genomeold.wustl.edu/projects/celegans/index.php?snp=1

Drosophila: http://genomebiology.com/content/supplementary/gb-2005-6-2-r19-s18.pdf

http://www.fruitfly.org/SNP/

http://flysnp.imp.univie.ac.at/

Arabidopsis: http://www.arabidopsis.org/servlets/Search?action=new_search&type=marker

Zebrafish: http://zfin.org/cgi-bin/webdriver?MIval=aa-crossview.apg&OID=ZDB-REFCROSS-010114-1

http://www.sanger.ac.uk/Projects/D_rerio/DAS_conf.shtml

2.2. Worm lysis

1. Worm lysis buffer (10x): 500 mM KCl, 100 mM Tris-HCl pH 8.2, 25 mM $MgCl_2$, 4.5% NP-40 (=IGEPAL CA630, Sigma-Aldrich), 4.5% Tween-20 (Sigma-Aldrich), 0.1% Gelatin (Sigma-Aldrich). Store at room-temperature.
2. Proteinase K (100x, from *Tritirachium album*, Sigma-Aldrich): 10 mg/mL in water. Store at -20°C.

2.3. Fly lysis

1. Squishing buffer (10x): 100 mM Tris-HCl pH 8.2, 10 mM EDTA pH 8.0, 2% Triton X-100 (Sigma-Aldrich), 250 mM NaCl. Store at room-temperature.
2. Proteinase K (100x, from *Tritirachium album*, Sigma-Aldrich): 20 mg/mL in water. Store at -20°C.
3. Vibration mill (Retsch MM30).

2.4. PCR

1. Taq polymerase (5U/µl, Euroclone Life Sciences) and 10x buffer without $MgCl_2$: 50 mM KCl, 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8.8, 0.1 % Tween-20. Add $MgCl_2$ from a 50 mM stock solution.
2. Phusion polymerase (2U/µl, Finnzymes) and 5x buffer: HF buffer containing 7.5 mM $MgCl_2$ is provided with the enzyme.
3. 2 mM dNTPs: Made from 100 mM dATP, dTTP, dGTP, dCTP stock solutions (Roche).
4. Primers: Working concentration 10 pmol/µl, made from 100 pmol/µl stock solution.
5. Thermoycler: Should be equipped with a 96-well thermo-block (e.g., MJR, Applied Biosystems, Biometra).

2.5. Capillary electrophoresis

1. Denaturation buffer: HiDi formamide (Applied Biosystems).
2. Size standard: LIZ500 (Applied Biosystems).
3. Running parameters for fragment analysis on an ABI3730 capillary sequencer (Applied Biosystems): Oven temperature 66°C, buffer temperature 35°C, pre-run voltage 15 kV, pre-run time 180 sec, injection voltage 2 kV, injection time 10 sec, first readout 200 ms, second readout 200 ms, run voltage 15 kV, voltage number of steps 10, voltage step interval 50 sec, voltage tolerance 0.6 kV, current stability 15 uA, ramp delay 500 sec, data delay 1 sec, run time 850 sec.
4. Capillary length: 36 cm.
5. Polymer: POP7 (Applied Biosystems).

2.6. Gel electrophoresis

1. TAE buffer (40x): per liter 145.37 g Tris(hydroxymethyl) aminomethane ("Tris"), 11.16 g $Na_2EDTA \times 2\ H_2O$, 34.4 ml glacial acetic acid. Concentration of 1x TAE is 30 mM.
2. SYBR green I / SYBR gold (Invitrogen) staining solution: 1:10000 dilution in 10 mM TAE. Stock solution shall be stored in 5 μl aliquots at -20°C (note 2).
3. Ethidium bromide (EtBr, Sigma-Aldrich) staining solution: 0.4 μg/ml EtBr in water. Store 10 mg/mL stock solution in light-protected bottle at 4°C (note 3).
4. Loading buffer (6x): 30% (v/v) Glycerol, 0.25% (w/v) xylene cyanol, 0.25% (w/v) bromphenol blue. Store at 4°C.
5. Gels: Elchrom Spreadex pre-cast gels that are non-denaturing, but offer very high to maximum resolution.
6. Submarine Gel system: Elchrom provides the specialized SEA2000 system for its gels featuring a thermostated water-bath. However, Elchrom gels can be run on any gel platform with at least 600 ml buffer volume and a width of at least 15 cm (note 4).
7. Transilluminator: Should be equipped with a 254 nm UV source for achieving maximum sensitivity of SYBR-stained gels.

2.7. Software

1. Allele calling on capillary sequencer-generated electropherograms: GeneMapper v3.7 (Applied Biosystems).
2. Visualization of raw mapping data: *FLP mapper*. Our software is specifically designed to extract and graphically display the genotype of recombinant chromosomes from Excel

spreadsheets. Two algorithms are implemented: A) Deduction of the genotype of the recombinant chromosome in the presence of an unrecombined counter-chromosome (e.g., suited to *Drosophila* and mammalian genetics). B) Visualization of both recombinant chromosomes of self-fertilizing organisms (like e.g. *C. elegans*).

The program can be downloaded freely from http://bio.mcsolutions.ch and runs on all operating systems supporting Java Runtime Environment (JRE).

3. Methods

3.1. Establishing a FLP map

1. From a suitable database (2.1.) choose candidate InDels/FLPs in the region of interest. For chromosomal linkage analysis at least one (central) FLP per chromosome is required, for mapping at highest resolution FLPs have to be spaced appropriately in a subchromosomal region. 1 bp FLPs do not pose a resolution problem on sequencers, but may approach the limits of resolution on Elchrom gels (3.6.). Besides, unverified 1 bp FLPs frequently are due to sequencing artefacts and thus have a lower confirmation rate than larger FLPs.
2. Design primers spanning the polymorphism to amplify fragments in the size range of 50 to 350 bp at an annealing temperature of 56 to 60°C. This task can be performed by the program Primer3, which is available as an online service on the world wide web at: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (note 5). For FLPs being resolved at a sequencer one primer has to be fluorescently labelled. 1-bp FLPs that have to be separated on Elchrom gels shall have an approximate length of 100 bp.
3. Genotype parental chromosomes and verify the fragment length polymorphism on the system of choice. If FLPs shall be resolved on sequencers and primer costs shall be kept low, it is advisable to first confirm the FLP with unlabelled primers on high-resolution Elchrom gels (3.6.).
4. From the verified FLPs choose assays that are evenly spaced on the genetic map at the required resolution. E.g., if a resolution of <=1 cM is demanded, take one FLP every 2 cM.
5. Genotype other chromosomes used for the mapping experiment.

3.2. Worm lysis

1. Collect individual worms in microfuge tubes or in wells of a 96-well microtiter plate each containing 10 µl 1x lysis buffer (1.0 µl 10x buffer, 0.1 µl proteinase K, 8.9 µl water).

2. Shock-freeze in liquid nitrogen.

3. Digest worm suspension by placing tubes/plates at 60°C for 60 min. Heat-inactivate proteinase K for 30 min at 90°C (note 6). Cool down to 4°C. Store at -20°C. Avoid more than 10 freeze-thaw cycles.

4. Dilute suspension 10-fold prior to use for PCR. Use 2 µl diluted extract per PCR reaction (note 7).

3.3. Fly lysis

A. Bulk preparation

1. Place single flies into wells of a 96-well deep-well plate (Eppendorf) filled with each 200 µl squishing buffer (20 µl 10x buffer, 2 µl proteinase K, 178 µl water).

2. Add one Tungsten carbide bead (Qiagen) per well.

3. Seal deep-well plate with a rubber mat (Eppendorf) and clamp it into the vibration mill.

4. Shake for 20 seconds at 20 strokes per second.

5. Transfer suspension into a 96-well PCR-plate (MJR) avoiding debris (note 8).

6. Incubate plate for 30 min at 37°C and subsequently for 20 min at 95°C to heat-inactivate proteinase K (note 6). Cool down to 4°C. Store at -20°C.

7. Dilute suspension 20-fold prior to use for PCR. Use 2 µl diluted extract per PCR reaction (note 7).

B. Single preparation

1. Place a single fly into a microfuge tube and add 50 µl squishing buffer (5 µl 10x buffer, 0.5 µl proteinase K, 44.5 µl water).

2. Squish fly with the pipette tip or, alternatively, use a pestle (Treff-Lab) fitting the microfuge tube to grind the fly.

3. Incubate tube for 30 min at 37°C and subsequently for 20 min at 95°C to heat-inactivate proteinase K (note 6). Cool down to 4°C and add 150 µl water. Store at -20°C.

7. Dilute suspension 20-fold prior to use for PCR. Use 2 µl diluted extract per PCR reaction (note 7).

3.4. PCR

A. Using Taq Polymerase

1. Mix 2 µl DNA (diluted single worm lysate or single fly extract) with 23 µl PCR reaction mix (2.5 µl 10x buffer, 1 µl $MgCl_2$, 0.5 µl dNTPs, 1 µl forward-primer, 1 µl reverse-primer, 16.95 µl water, and 0.05 µl Taq) in an appropriate microfuge tube or PCR-plate (MJR) on

ice. Multiplexing two to three different primer pairs in one reaction is usually possible without further optimization.

2. Cycling parameters: 2 min 95°C, 10 cycles: 20 sec 95°C, 20 sec 61°C (-0.5°C for each cycle), 45sec 72°C, 24 cycles: 20 sec 95°C, 20 sec 56°C, 45sec 72°C, final extension: 10 min 72°C, hold at 12°C.

B. Using Phusion Polymerase

1. Mix 2 µl DNA (diluted single worm lysate or single fly extract) with 23 µl PCR reaction mix (5 µl 5x buffer, 2.5 µl dNTPs, 1 µl forward-primer, 1 µl reverse-primer, 13.25 µl water, and 0.25 µl Phusion) in an appropriate microfuge tube or PCR-plate (MJR) on ice. Multiplexing two to three different primer pairs in one reaction is usually possible without further optimization.

2. Cycling parameters: 30 sec 98°C, 30 cycles: 10 sec 98°C, 20 sec 60°C, 15 sec 72°C, final extension: 10 min 72°C, hold at 12°C.

3.5. Capillary electrophoresis

Taq polymerase tends to catalyze the addition of an adenosine to the 3' end of PCR-products. This activity may yield two products exhibiting a size difference of 1 bp. A-addition could constitute a genotyping problem especially for 1 bp FLPs and for small FLPs causing polymerase "stuttering". Two different strategies are feasible to control A-addition: First, the non-fluorescent primer can be "pig-tailed" on the 5' end, which leads to nearly 100% A-addition on the fluorescently labelled strand [8]. Second, A-addition can be prevented by using Polymerases like Pfu (Promega) or Phusion (2.5., 3.5., note 9). An example of a 1 bp FLP amplified with either Taq or Phusion polymerase is given in figs. 1A and 1B.

1. Add 2 µl PCR-reaction (performed with one labelled primer) to 198 µl water. Dilution is necessary to reduce signal intensity and to reduce protein content that potentially could harm capillaries.
2. Mix 2 µl of the diluted PCR-reaction with 10 µl HiDi formamide containing LIZ500 size standard (25 µl LIZ500 in 10 ml HiDi) in 96-well sequencing-plates (Applied Biosystems).
3. Denature by heating mixtures to 95°C for 2 min.
4. Separate fragments on an ABI3730 capillary sequencer using POP7 polymer according to running parameters given in 2.6.
5. Analyze data and perform allele calling using GeneMapper Software. In "Tools – Options - Anaylsis" choose "Duplicate homozygous alleles" function. Export raw genotyping data in Microsoft Excel-compatible form by the "Export Table" function as "Tab-delimited text

(.txt)". Export the following data: Sample name, marker, allele 1, allele 2.

3.6. Gel electrophoresis

For low to medium throughput genotyping applications, fragment analysis on non-denaturing gels is ideal because of convenience and technical simplicity. However, due to relatively low resolution conventional agarose gels are only suited for the minority of FLPs exhibiting large size differences. Elchrom Spreadex gels are as easy to handle as agarose gels, but offer higher resolution, under ideal conditions down to the limit of 1 bp (see fig. 1C). Allele calling has to be performed manually. Thus, this fragment separation technique is most adequate for small genotyping projects and for FLP assay validations with non-fluorescent primers.

1. Choose Elchrom Spreadex gels (http://elchrom.com/public/index.php?article=144) with appropriate number of slots and offering optimal resolution for the fragments of choice. Take gels with 8 cm length for 1 bp FLPs.
2. Unpack gel and place it into submarine gel unit filled with TAE (note 4). If the gel has been stored at 4°C allow for a 30 min equilibration. The optimal buffer temperature is 55°C, but runs at lower temperatures are possible.
3. Per slot load a mixture of 2 µl PCR-reaction, 1 µl loading buffer, and 2 µl water.
4. Running conditions depend on the gel used and the resolution requirements. A typical run would be for 1 ½ to 2 ½ hrs at 120 V at 55°C.
5. Transfer gel into SYBR green/ SYBR gold or EtBr staining solution, respectively, and float them for 30 or 45 min in an appropriate gel tray. Up to four gels may be stained simultaneously in 50 ml (notes 2 and 3).
6. Destain for 30 min in water. SYBR stained gels may also be destained in destaining solution (Elchrom).
7. Place gel onto a transilluminator equipped with a 254 nm UV source and record gel image.
8. Perform allele calling and chart data in a Microsoft-Excel sheet into four columns: Sample name (i.e., recombinant), marker (i.e., FLP-assay), allele 1, and allele 2. Store the data in the Excel "Text (Tab-delimited) (*.txt)" format.

3.7. *FLP mapper* software

FLP mapper is most useful for complex crossings involving three different genotypes: The mutant genetic background, the wild-type background recombined with the mutant, and a third genetic background into which recombinant chromosomes are crossed. This third genotype may be a mixture of both parental genotypes. For mapping projects involving many markers it is therefore laborious to delineate the genotype of the recombinant chromosome in the presence of the third chromosome **[9].**

FLP mapper firstly accesses the three different genotypes stored in an Excel-compatible database. Next, it extracts the genotype of the recombinant chromosome from a spreadsheet listing both alleles by subtracting the genotype of the third chromosome. Recombinant genotypes are then listed and coloured such that the most informative recombinants can be easily spotted. This algorithm is tailored for the needs of *Drosophila* and mammalian genetics.

A second algorithm is optimized for the genetics of the self-fertilizing organism *C. elegans*. As both chromosomes are recombinant, subtraction is not necessary and both genotypes are graphically displayed.

1. Download software from http://bio.mcsolutions.ch
2. To generate a project click "new" in the upper left panel. Existing projects can be opened, closed, and saved by pressing the respective adjacent buttons.
3. After a program has been created, "import-options" appear in the main panel. This option allows to define the content of the raw mapping data in Excel .txt format. The program will cross-reference to four columns of the Excel sheet containing the sample name ("sample"), the name of the assay ("marker"), and the genotypes of the two loci for a given marker and sample ("allele 1" and "allele 2").
4. In order to import raw data press "files". In the middle upper panel an "import" symbol will appear that allows to choose a file when activated. The file name now appears in the left window section (see fig. 2A).
5. The strain genotype and mutant selection options can be chosen by activating the "compiling-options" in the task-bar. By choosing this option the middle upper panel will change to reveal a "new" option symbol. Selection of a new option reveals four pull-down menus from which the database of strain genotypes (see also 8), the genotype of the mutagenized strain, the genotype of the wild-type strain used for recombination, and the genotype of the strain into which the recombinant chromosome is crossed can be chosen. Finally, there is an option to indicate whether the recombinants have been selected to exhibit or not to exhibit the mutant phenotype ("selection for/against mutation"). These parameters should be saved by clicking onto "save" in the upper panel. Several different parameter permutations can be saved as options.

6. Next, by returning to the "files" task, choose the genotype option defined before on a pull-down menu. Furthermore, the output format (and the model organism) can be determined as well.

7. The data now have to be compiled by pressing the "compile" button in the middle upper panel. Finally, an .html output file is generated after activating the "export" symbol (see figs. 2 B, C).

8. Genotype databases can be imported into *FLP mapper* as Excel .txt files. In the menu bar choose "database", click on "import" and select the file to be imported. To create your own database refer to the format of the *Drosophila* and *C. elegans* databases supplied with the program.

9. Output colours can be changed. Click "export-models" in the task bar, select respective default file, copy central field, click "new", paste data, change hexadecimal codes of colours (http://www.december.com/html/spec/color.html), click "save".

10. Compiling algorithms are provided for *Drosophila* genetics, which may be suited to vertebrate genetics as well, and for *C. elegans* genetics. Algorithms can be imported and exported after selecting the respective button in the task-bar via the icons in middle upper panel. If necessary, algorithms can be self-programmed in JAVA.

4. Notes

1. Water for molecular biology applications should have a resistivity of 18.2 MΩ, a total organic carbon content of less than five parts per billion, and should be pyrogen- and nuclease-free (less than 0.001 EU/mL) (e.g., from a Milli-Q water purification system, Millipore).

2. SYBR green and SYBR gold are extremely light sensitive and thus shall not be frequently thawed and frozen again. Therefore it is advisable to aliquot fully thawed stock solution into 5 μl portions at dim light. One aliquot, diluted 10000 times in 10 mM TAE is sufficient to stain four gels at once. Do not re-use staining solution.

3. EtBr is mutagenic and should be removed from the staining solution with an appropriate removal system (e.g., charcoal filter, Schleicher and Schuell).

4. The gel tanks must be filled with TAE and may never get into contact with TBE. A single TBE filling spoils the submarine gel system for use with Elchrom gels forever.

5. Usually, primers designed by hand according to the formula $T_M=4x(G+C)+2x(A+T)-5$ °C, devoid of repetitive or palindromic sequences, and ideally ending with a G or C work well.

6. Heat-inactivation is absolutely crucial, as one of the most common causes of PCR failure are trace-amounts of proteinase K. Some protocols only include a 10 min heating step,

which may be insufficient. Incubation, heating, and cooling can be performed in a thermocycler.

7. Dilution of crude extracts improves PCR success-rate and also reduces the risk of spoiling capillaries by proteins.

8. Tungsten carbide beads can be re-used after an overnight incubation in 0.1 M HCl and subsequent thorough washing in water.

9. Phusion polymerase is expensive and thus is not recommended for general use. However, it is very robust and yields good results and is preferable to Pfu polymerase for applications where A-addition is unwanted.

5. References

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6. Figure Legends

Figure 1: Resolution of FLPs on sequencers and on non-denaturing gels

ZH3-23, a 1-bp FLP between the *C. elegans* strains Bristol and Hawaii, amplified with Taq polymerase (A) and Phusion polymerase (B), respectively, resolved on an ABI3730 sequencer and displayed by GeneMapper software (Applied Biosystems). Each homozygous and the heterozygous genotypes are shown. In order to perform unambiguous allele-calling when Taq catalyzed A-addition occurs, the peak without A-addition for the smaller and the peak with A-addition for the larger fragment have to be assigned (A). Without A-addition each electropherogram is unambiguous (B).

C) FLPs *3L021* and *2R114* amplified from respective homozygous and heterozygous *Drosophila* strains *yw, EP3L, FRT80, EP2R,* and *FRT42* and resolved on non-denaturing Elchrom Spreadex gels. The 5-, 6-, and 11-bp *3L021* and the 12-bp *2R114* FLPs are clearly separated. Resolution of 1-bp FLPs is possible for smaller, ca. 100 bp fragments. Size standards M3 and M1 are from Elchrom.

Figure 2: Graphical display of raw mapping data by *FLP mapper*

A) Screen-shot of the *FLP mapper* window exhibiting raw mapping data of the file "selection-for" in the central window. Compiling-options, compiling-algorithm, and export-model have been defined and then chosen from the pulldown-menus. The data can now be compiled and exported as .html file by clicking on the icons in the middle upper panel. B) Output in .html format from the example shown in A). Recombinants R101 to R126 have been selected for exhibiting the mutant phenotype. Both the FRT42D/EP0755 recombinant chromosome and the counter-chromosome FRT42Dw^{+}cl have been genotyped for FLP-markers 2R027 to 2R036 that are situated from proximal to distal on the right arm of chromosome 2 [9]. *FLP mapper* only displays the genotype of the recombinant chromosome. Genotypes excluding the candidate locus are in dark grey and genotypes including the locus are in light grey. Empty fields were not genotyped due to PCR-failure. The most informative recombinant R107 determines that the candidate region is proximal to 2R028. C) Output in .html format for recombinants R6 to R20 that have been selected for the wild-type allele (against the mutant allele). Both recombinant and non-informative chromosomes have been genotyped, but only the recombinant chromosomes are listed by *FLP mapper*. Again, genotypes excluding the candidate regions are dark grey. The informative recombinants place the candidate region distal to 2R017. The combination of both mapping data localizes the mutation between 2R017 and 2R028.
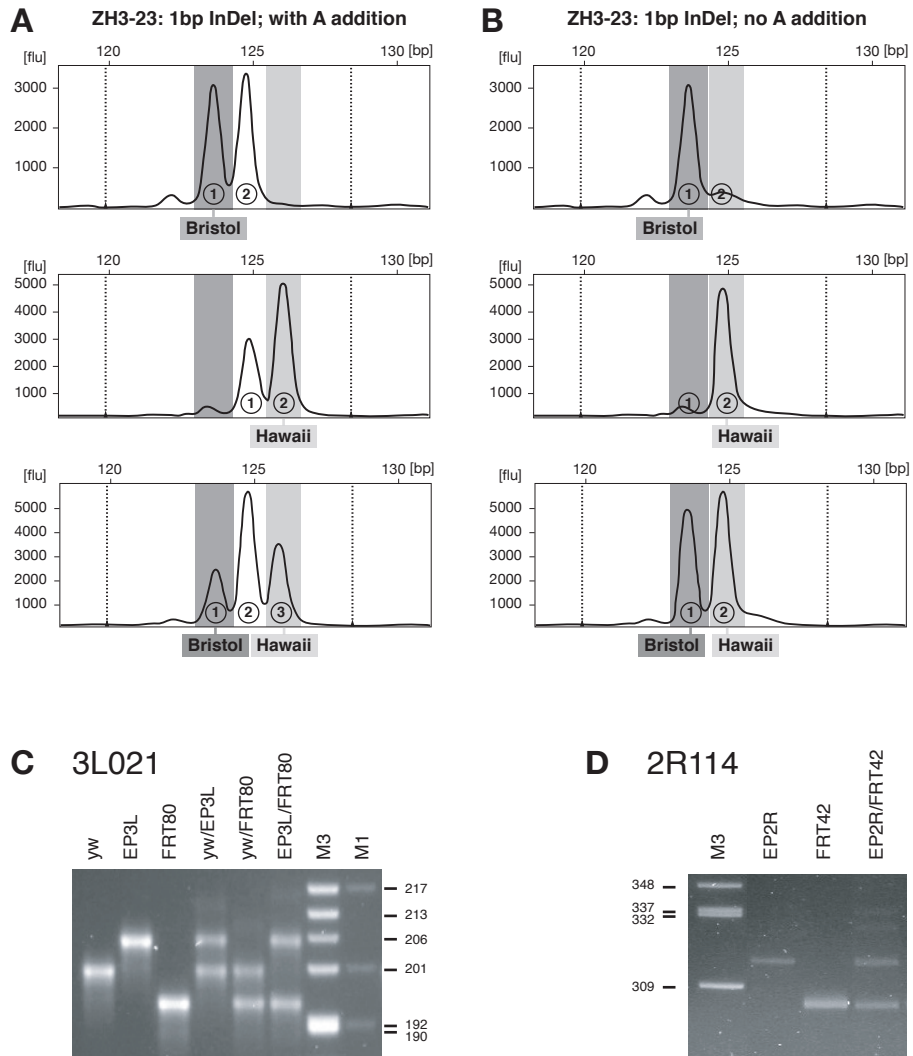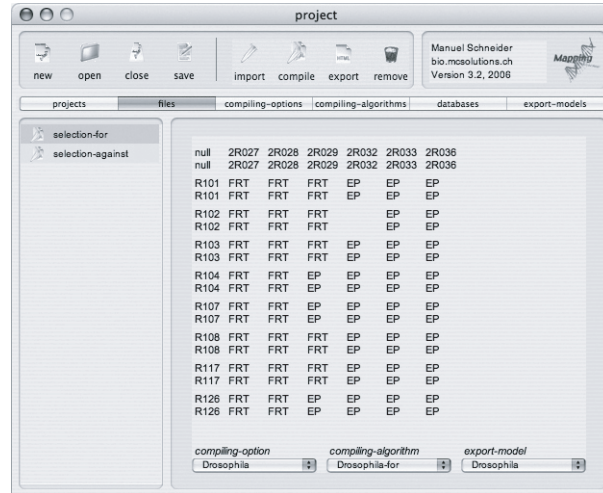
Figure 1

A ZH3-23: 1bp InDel; with A addition

B ZH3-23: 1bp InDel; no A addition

C 3L021

D 2R114

Figure 2

A

B

selection-for

	2R027	2R028	2R029	2R032	2R033	2R036
R101	FRT	FRT	FRT	EP	EP	EP
R102	FRT	FRT	FRT		EP	EP
R103	FRT	FRT	FRT	EP	EP	EP
R104	FRT	FRT	EP	EP	EP	EP
R107	FRT	EP	EP	EP	EP	EP
R108	FRT	FRT	FRT	FRT	FRT	EP
R117	FRT	FRT	FRT	FRT	FRT	FRT
R126	FRT	FRT	EP	EP	EP	EP

Samples [8], Markers [6]; Datenbank - D melanogaster [FRT42D, EP0755, FRT42Dw+cl]; Selection [true]

C

selection-against

	2R017	2R039	2R060	2R068	2R083	2R096	2R118	2R130	2R139
R6	EP	EP	EP	EP	EP	EP	EP	EP	EP
R7	FRT	EP	EP	EP	EP	EP	EP	EP	EP
R8	FRT	EP	EP		EP		EP	EP	EP
R9	EP	EP	EP	EP	EP	EP	EP	EP	EP
R10	FRT	EP	EP	EP	EP	EP	EP	EP	EP
R11	FRT	EP	EP	EP	EP	EP	EP	EP	EP
R12	FRT	EP	EP	EP	EP	EP	EP	EP	EP
R13	FRT	EP	EP	EP	EP	EP	EP	EP	EP
R14	FRT	EP	EP	EP	EP	EP	EP	EP	EP
R15	FRT	EP	EP	EP	EP	EP	EP	EP	EP
R16	FRT	EP	EP	EP	EP	EP	EP	EP	EP
R17	EP	EP	EP	EP	EP	EP	EP	EP	EP
R18	EP	EP	EP	EP	EP	EP	EP	EP	EP
R19	EP	EP	EP	EP	EP	EP	EP	EP	EP
R20	FRT	EP	EP	EP	EP	EP	EP	EP	EP

Samples [15], Markers [9]; Datenbank - D melanogaster [FRT42D, EP0755, FRT42Dw+cl]; Selection [false]