Southern blotting, Determining Loss of DNA Methylation, and Mapping *dim* mutations

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Bi410

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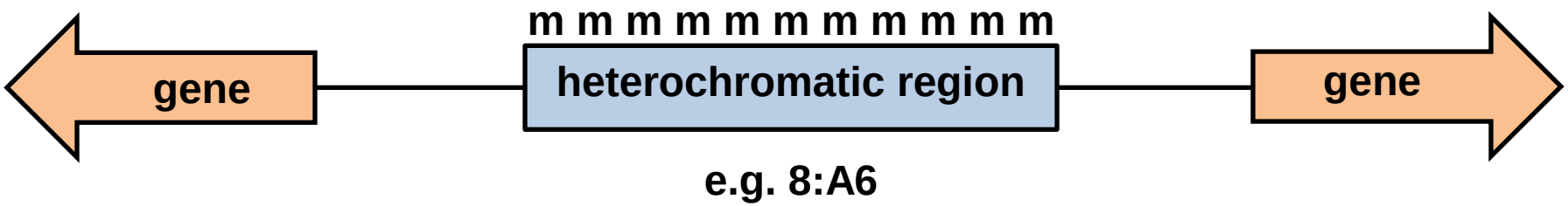
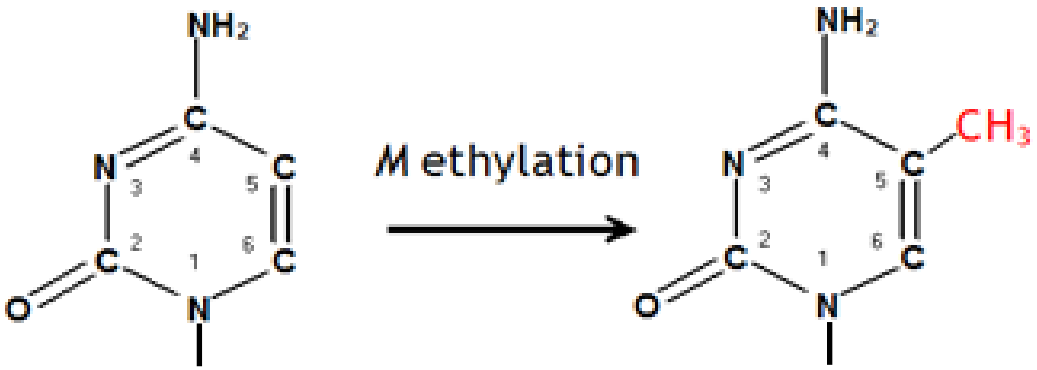
Heterochromatic regions throughout the genome

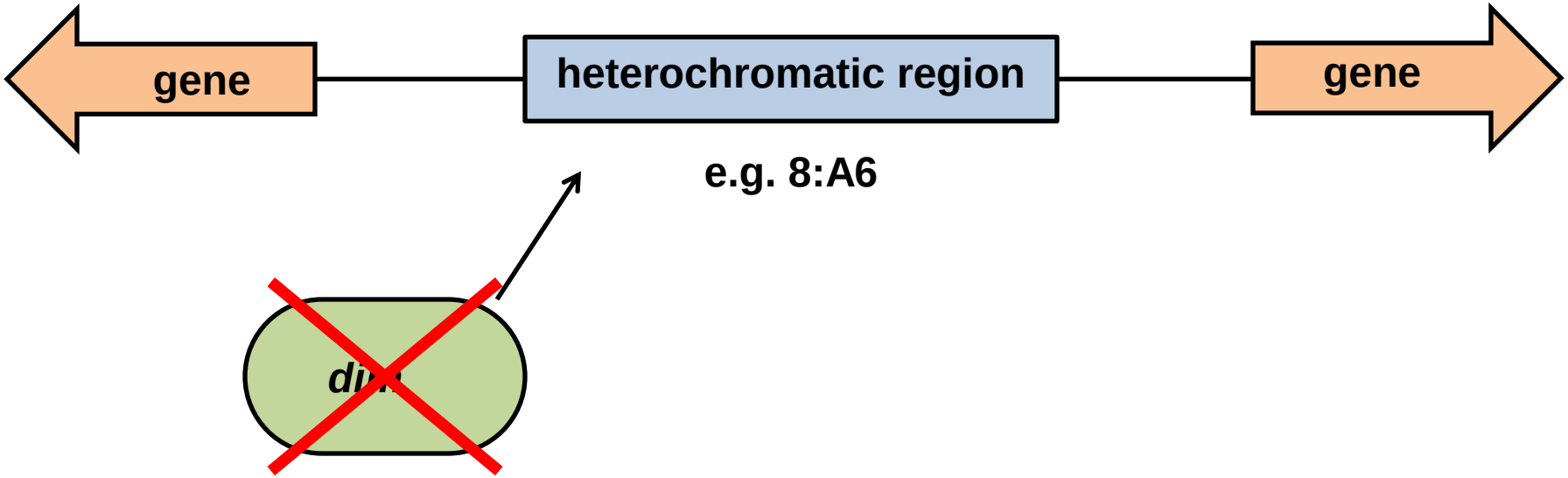


e.g. 8:A6



- heterochromatic regions are found across the Neurospora genome
- A:T rich, often repeated sequences, marked by DNA methylation
- Several proteins are needed to place DNA methylation at heterochromatin

defective ***i***n ***m***ethylation (***dim***)

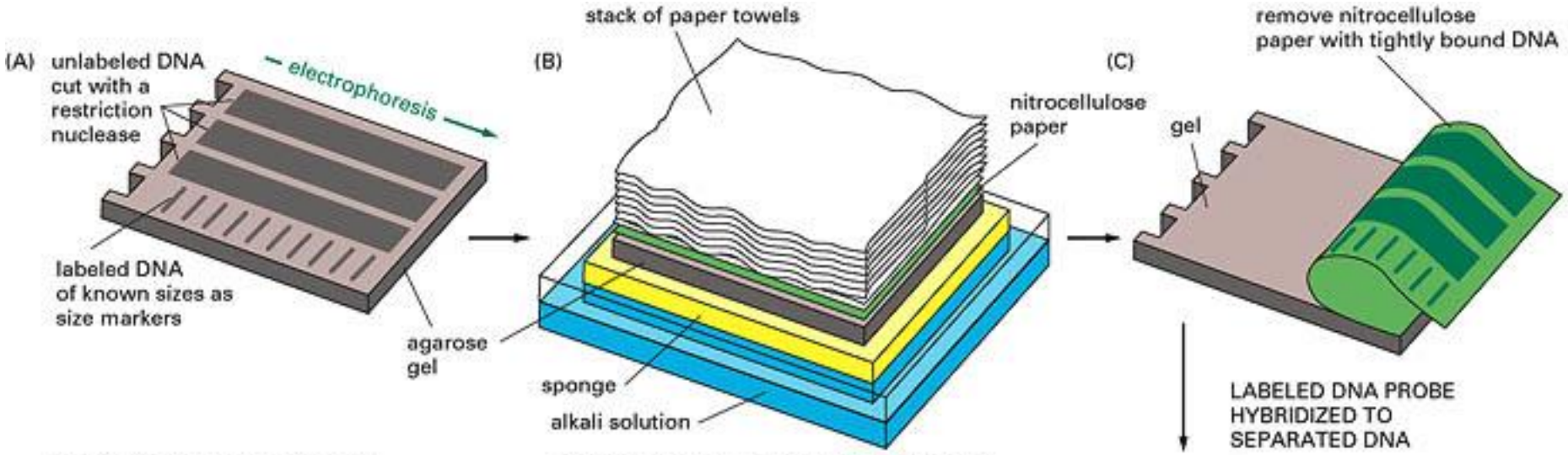


- Mutations in any one of the genes needed to catalyze DNA methylation that render that gene's protein product inactive result in a loss of DNA methylation

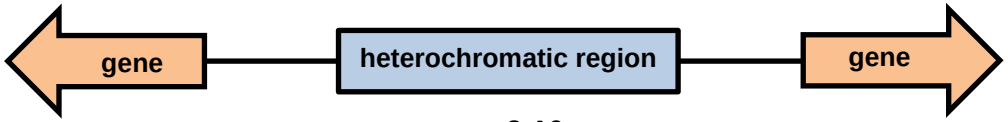
- Genes are termed ***dim*** (***d***efective ***i***n ***m***ethylation)

How do we monitor DNA methylation status?

Southern blotting!



probe with ^{32}P DNA specific to a heterochromatic region



e.g. 8:A6

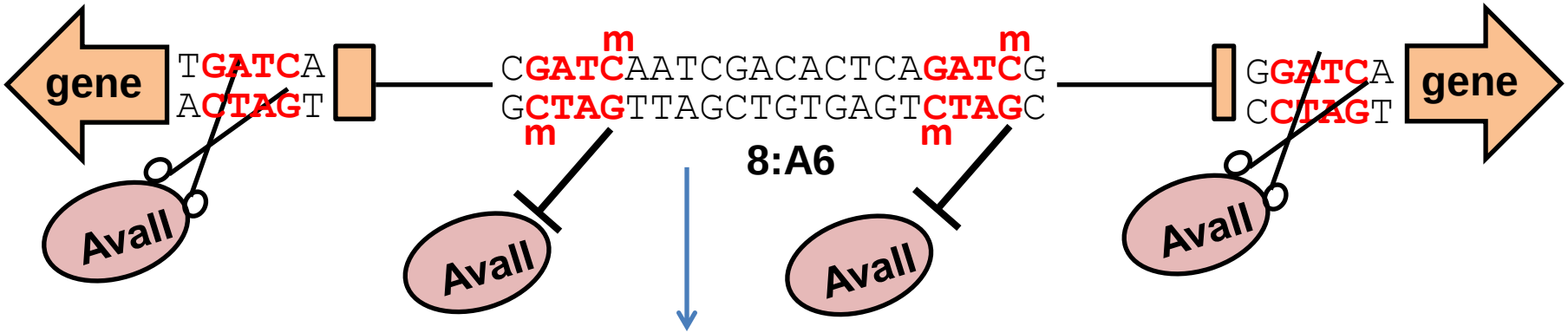
image from: http://jdutchstevens.tripod.com/id9.html

The key to Southern blotting for DNA methylation is the restriction enzyme used!

Unmethylated
DNA

smaller DNA pieces

DNA that has cytosines methylated

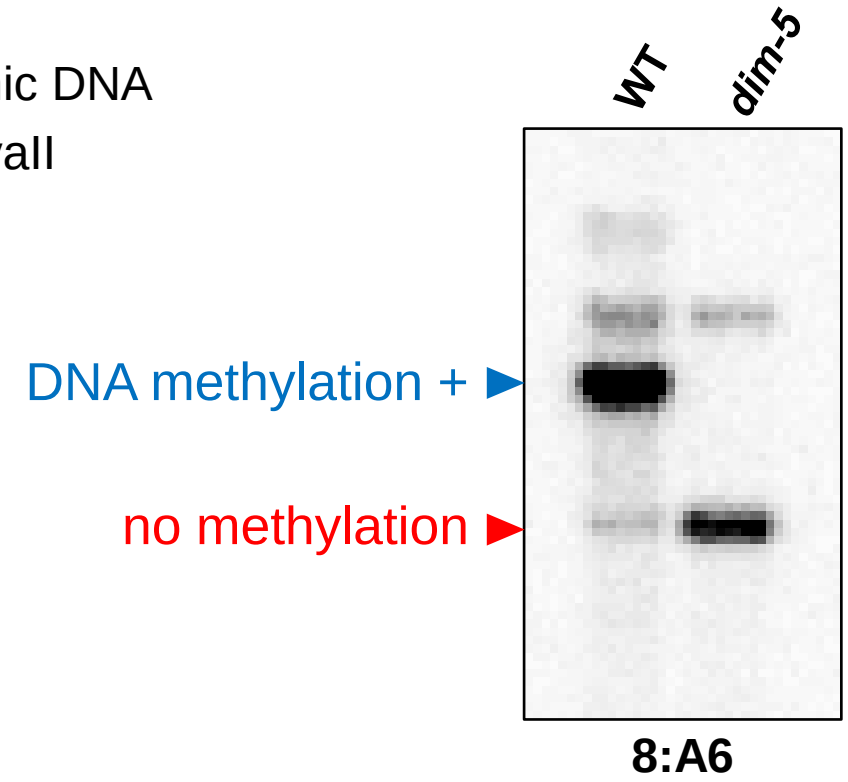


LARGER DNA pieces

Analyzing DNA methylation status of strains

Isolate genomic DNA

Digest with AvaII

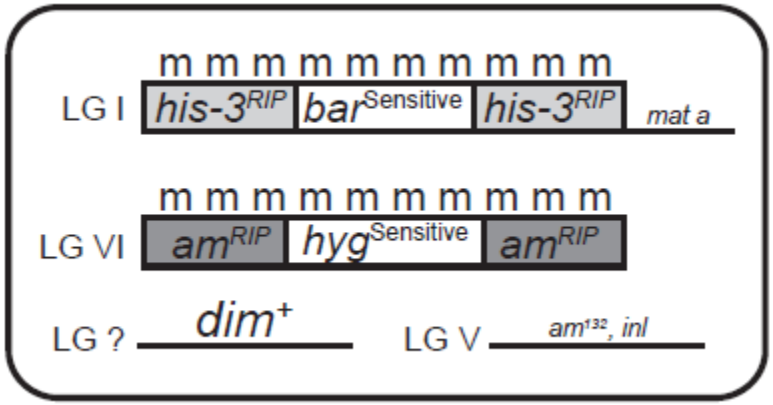


This membrane with AvaII cut genomic DNA was probed with ^{32}P labeled DNA from the 8:A6 region (a heterochromatic region).

Most of the DNA in a WT strain is methylated (bigger products) while DNA from the dim-5 strain does not have DNA methylation, and can be cut by AvaII

What you guys are doing: finding more genes important for DNA methylation

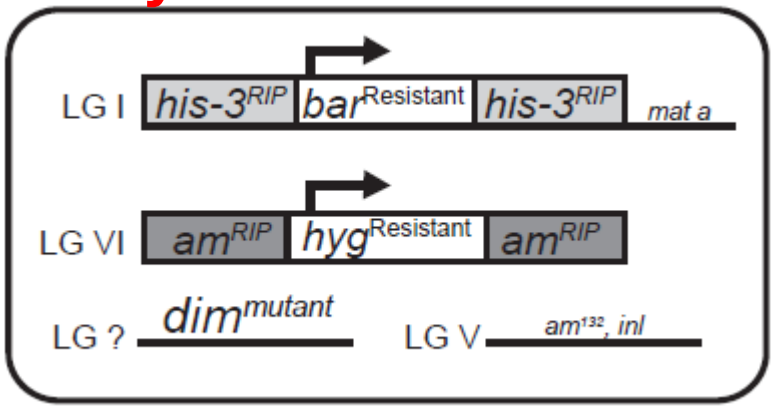
N2977



- Has DNA methylation
- drug resistance cassettes are silenced (strain cannot grow with drugs)

UV

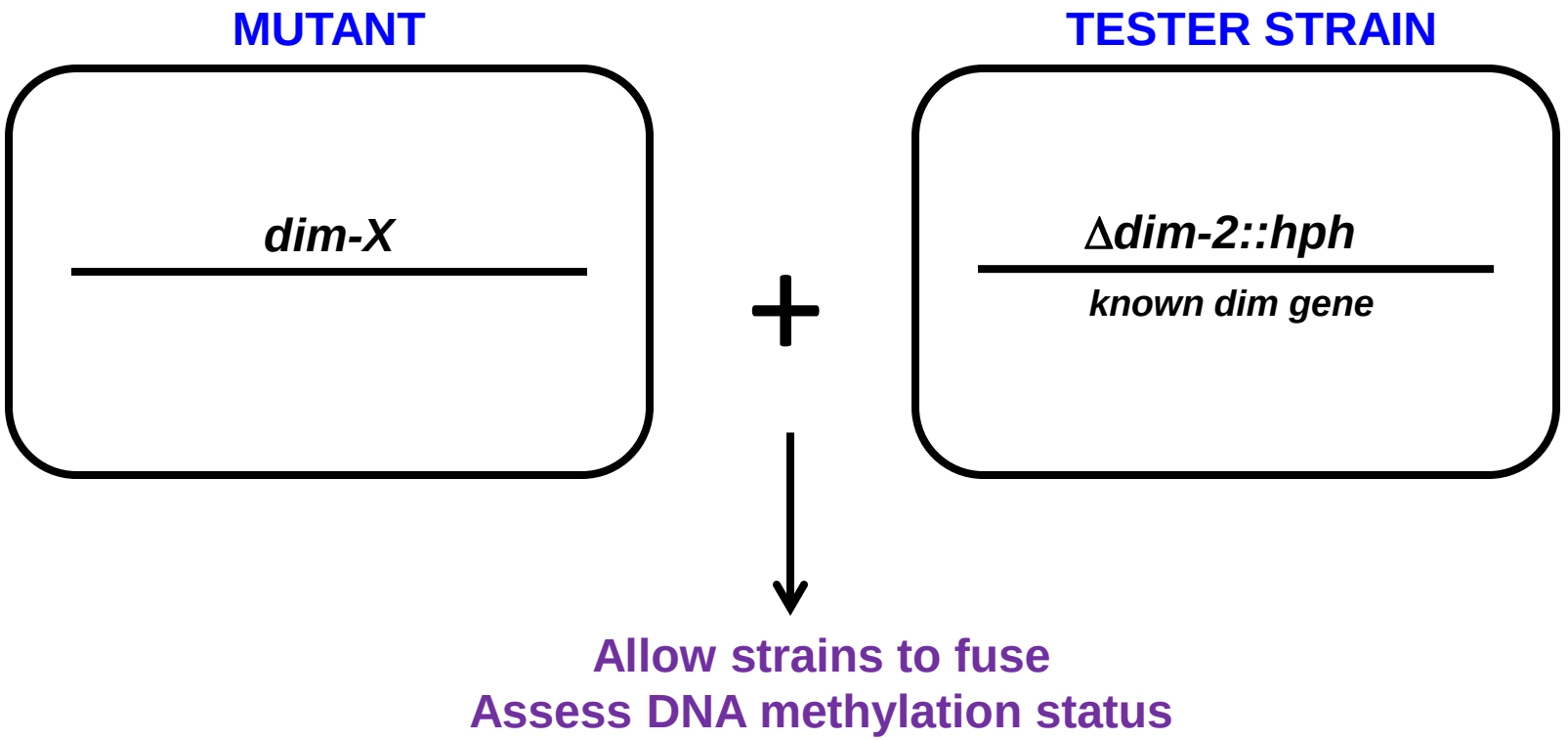
your mutants



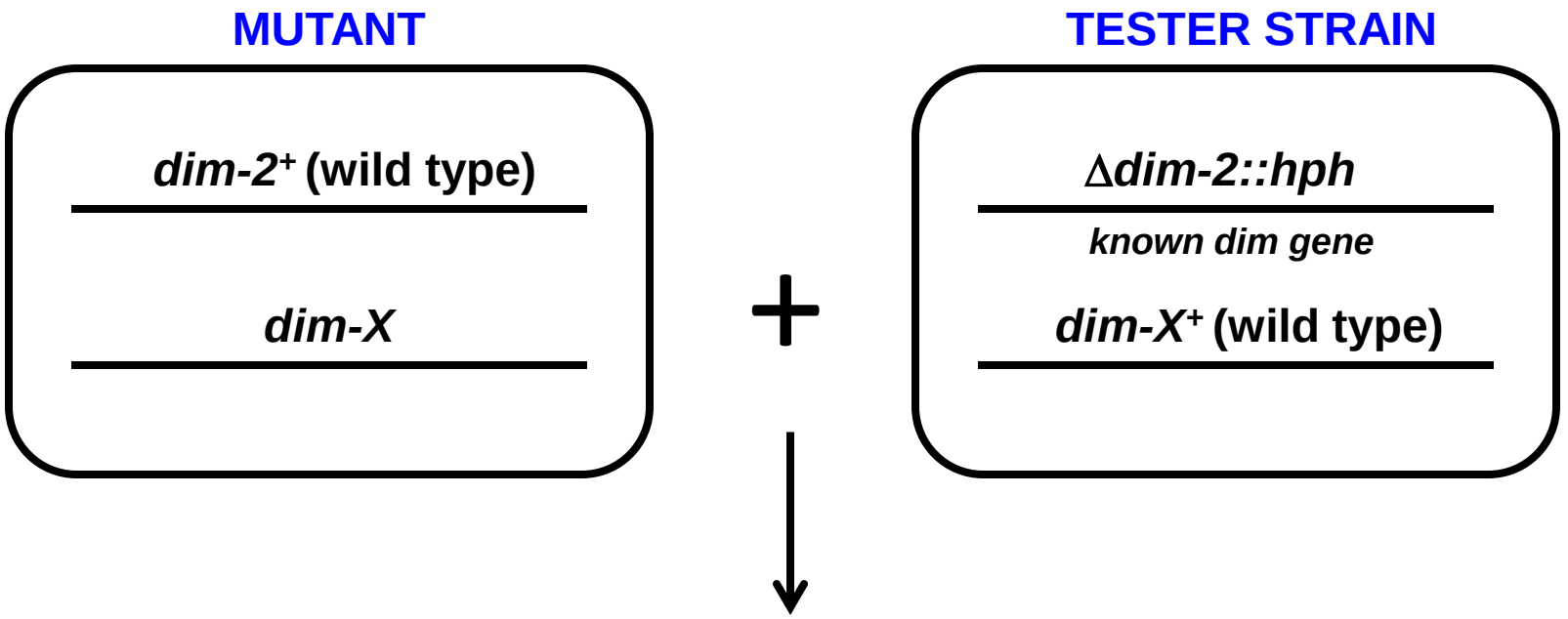
- Mutate genes involved in DNA methylation
- drug resistance cassettes are activated (strains grow with drugs)

- You will test your strains for DNA methylation by Southern blotting
- You will try to determine the gene mutated…
- Is your strain mutated in a KNOWN *dim* gene?

Complementation tests: determining if mutants are novel

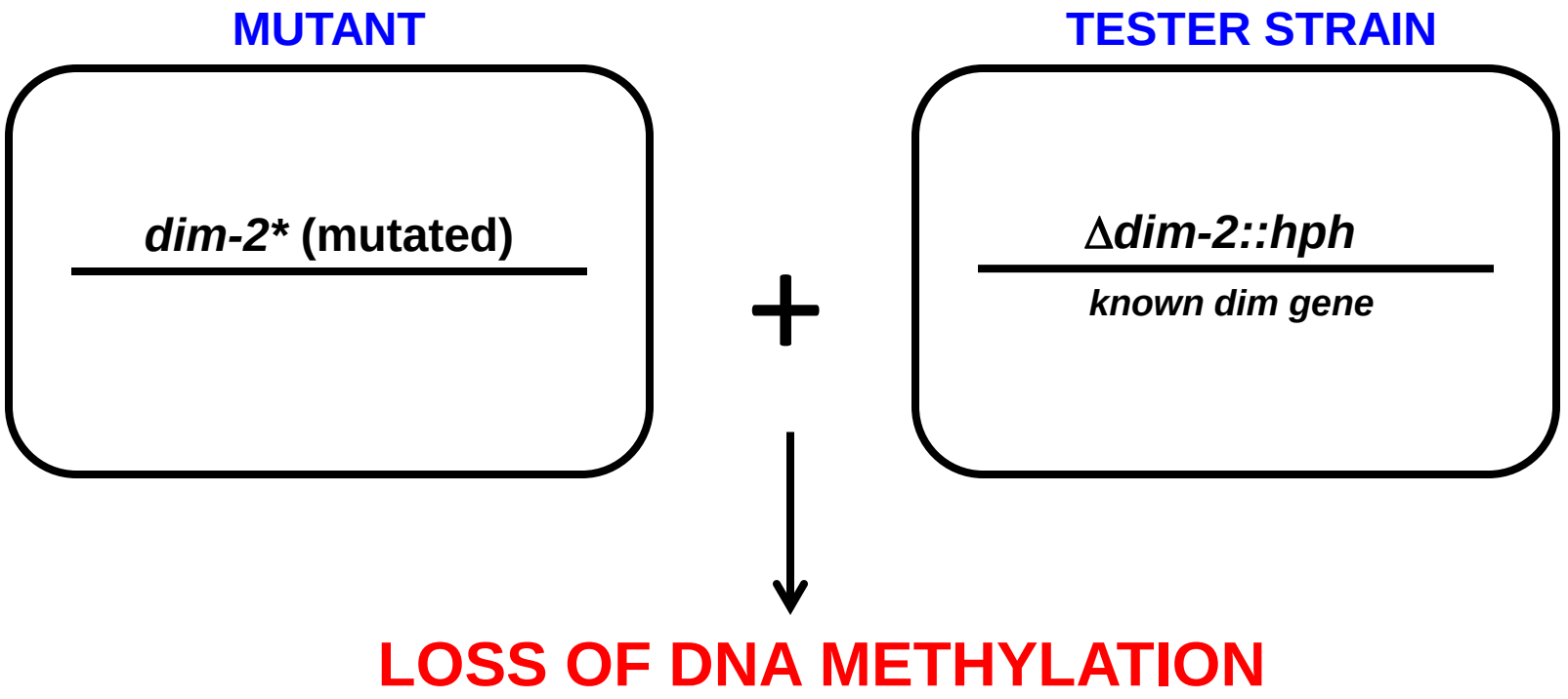


Complementation tests: determining if mutants are novel



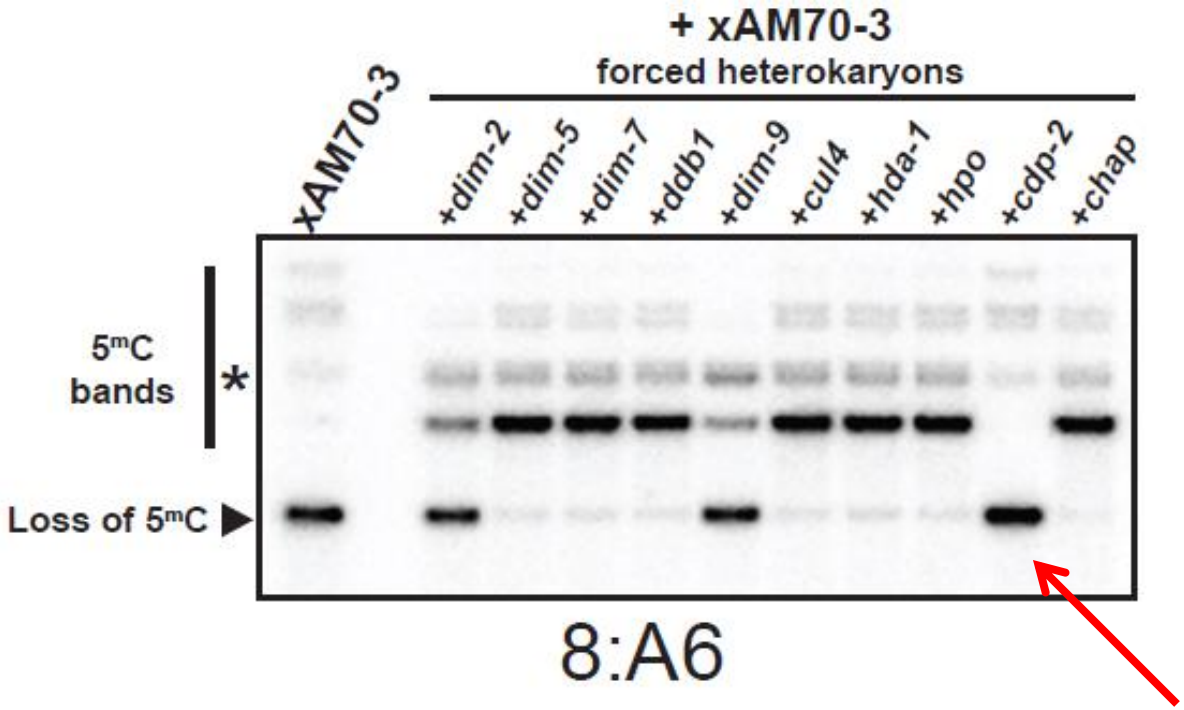
- If DNA methylation is restored, the genes “complement”. Your mutant provides a good *dim-2*, The tester strain provides a good “*dim-X*” gene.
- MUTATION IS NOT IN THIS KNOWN GENE!

Complementation tests: determining if mutants are novel



- If DNA methylation is still lost (because both genes are altered), the genes did not complement.
- MUTANT STRAIN HAS A MUTATION IN THE KNOWN *DIM* GENE.

Complementation tests: determining if mutants are novel



- Forced heterokaryons with known *dim* mutants shows that xAM70-3 has a mutation in *cdp-2*.

- If your mutant is novel (and does not have a mutated known *dim* gene), **what do you want to know next?**

You want to know the identity of the gene that is causing the loss of methylation!

- How do you do that? "Map" the mutant to it's genomic locus
- Many current and past mapping strategies require you to cross your mutant strain to a different strain that contains **SNPs across the genome**
- Crossing your N2977 *dim* mutant strain (Oakridge background) to the wild type N51 Mauriceville strain (has normal DNA methylation) is an example of SNP mapping cross.

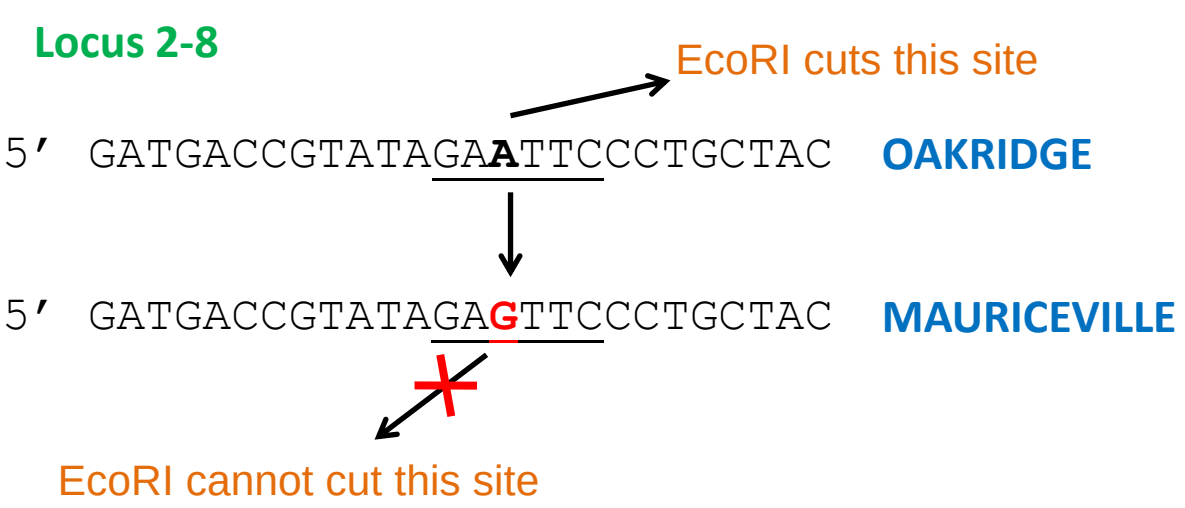
SNP = Single Nucleotide Polymorphism
(essentially a naturally occurring base pair change)

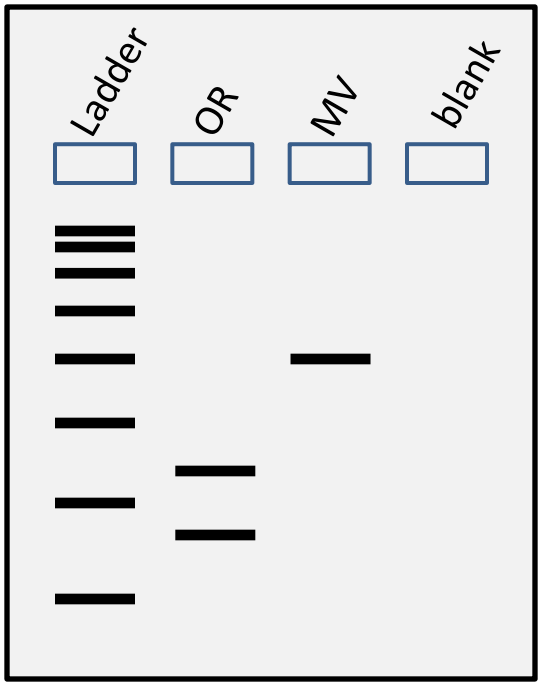
On Linkage Group II (chromosome II), locus: "2-8"

Classical SNP mapping of mutations…

- Classical mapping strategies are highly reliable, yet time consuming. For example: “RFLP mapping”:

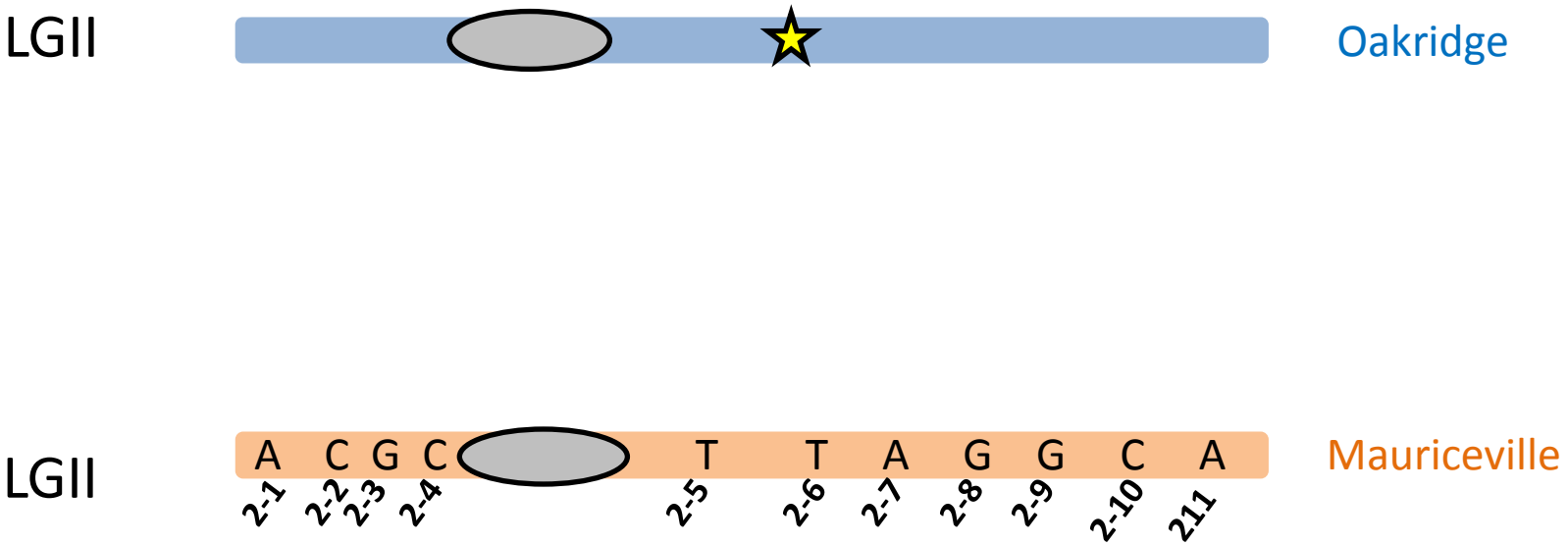
- RFLP = Restriction Fragment Length Polymorphism
  - Basically, the SNP at a particular locus creates or destroys a restriction site
  - So, if you PCR amplify this region and digest the DNA with an enzyme, it will cut or not cut based on whether the originating DNA is Mauriceville or Oakridge





The key to SNP mapping:

- Your mutation is in a known SNP background!
- SNPs are known in each background, and you know your mutated *dim* gene was generated in one of these backgrounds!



Classical RFLP mapping continued

- You can test **individual progeny** from a N2977 (OR) x N51 (MV) cross for "linkage" between your *dim* mutant (as determined by Southerns and individual SNPs)

dim mutation is LINKED to LGII SNPs

Oakridge Mauriceville

See majority of *dim* mutants with Oakridge SNPs

Regardless of crossover (recombination) events

dim mutation is UNLINKED to LGVII

Oakridge Mauriceville

random distribution of SNPs independent of crossovers

Remember, these progeny were classified as dim by a Southern blot!

Classical RFLP mapping continued

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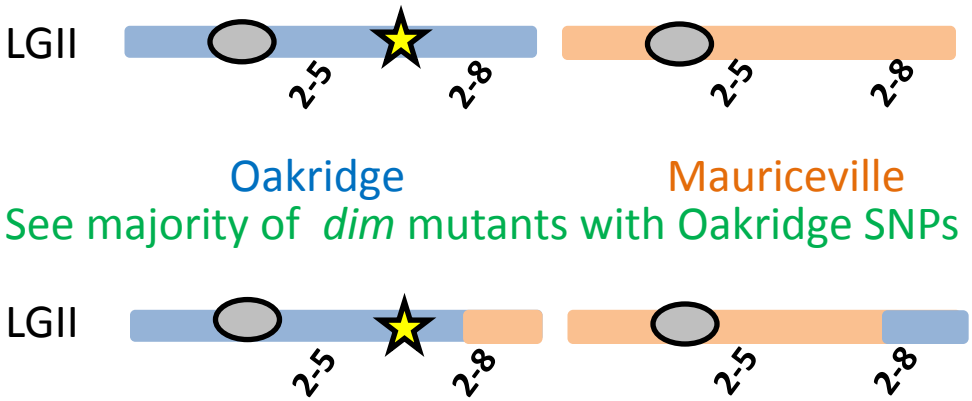

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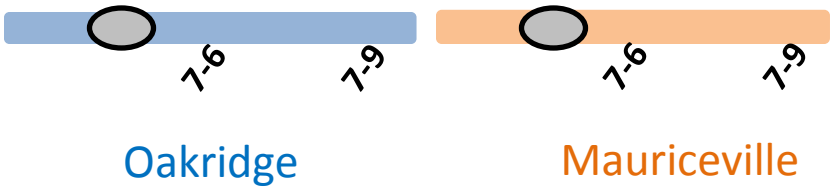
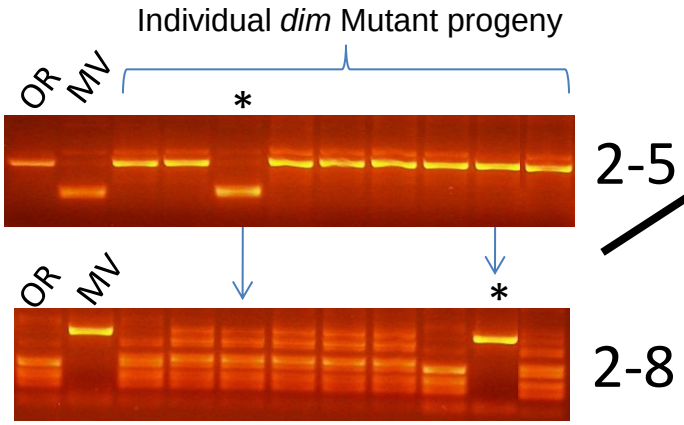


random distribution of SNPs independent of crossovers



<u>Calculating the Linkage distance between your SNPs and your mutation</u>

"CentiMorgans" are defined as the percentage of recombinants between two loci and are a measurement of genomic distance.

Try it!

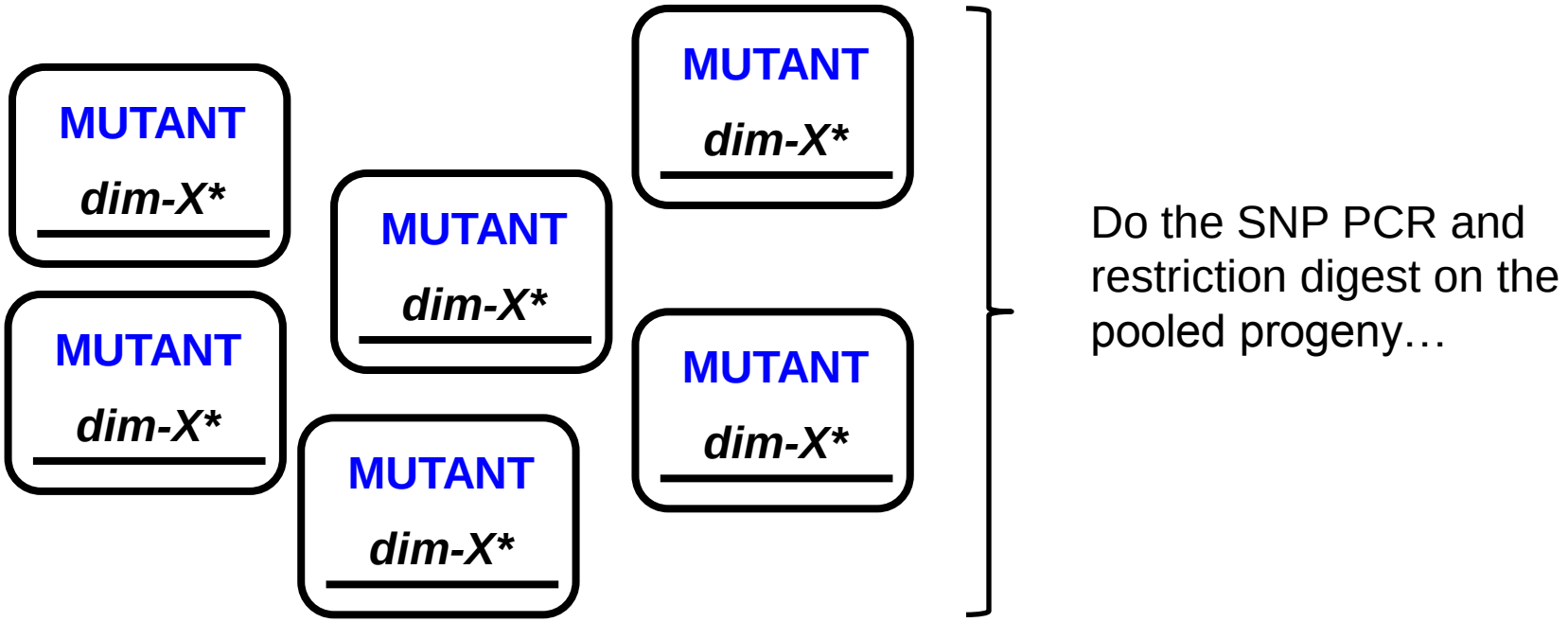
This *dim* mutant is 11cM from each marker

Laurel Payne

Classical RFLP mapping, continued

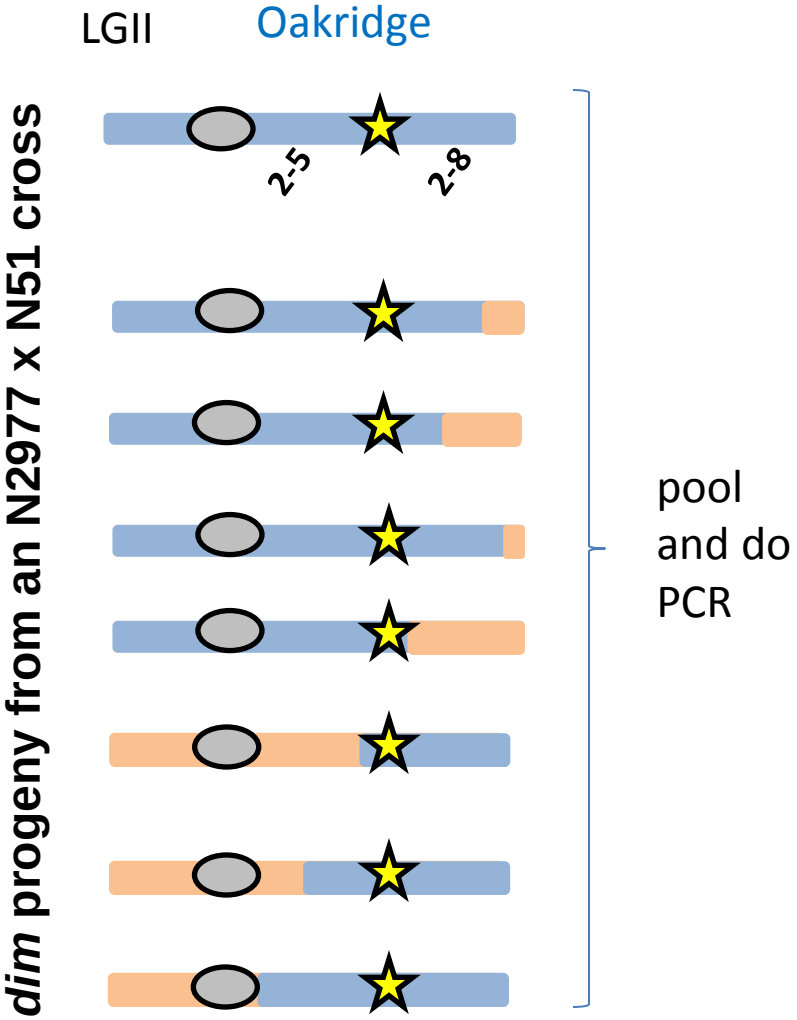
- Test EACH *dim* mutant progeny for the 14 markers across the genome…
  - Each progeny has SEVEN chromosomes, two markers each…
  - test 30 progeny, each with 14 PCR/restriction digests = 420 PCR and digest reactions
  - Quite time consuming

Another strategy: POOL your *dim* mutant progeny (each having SNPs across the genome!)

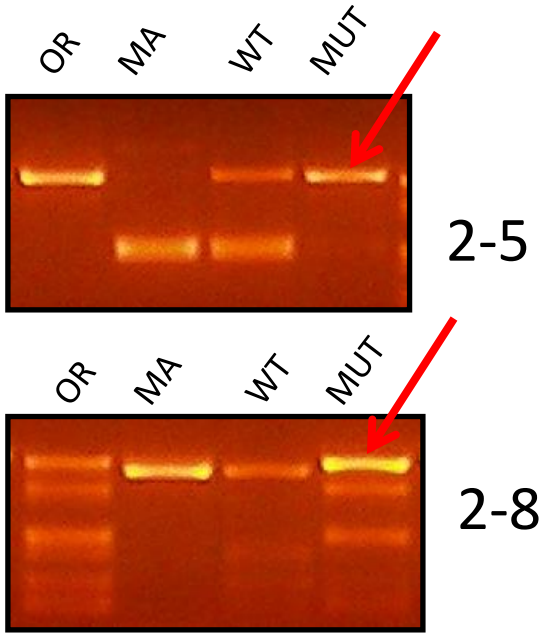


Bulked Segregant classical RFLP mapping

- Mutant progeny from a N2977 (OR) x N51 (MV) cross can be pooled and tested in bulk for “linkage”



Pooled WT and *dim* Mutants



The large majority of the pooled *dim* progeny will look like the Oakridge SNP at the location of the mutation!

Laurel Payne

Classical mapping is the gold standard, but…

- New technologies have been developed which use SNPs and pooled progeny known to be mutant
- The genomes of Oakridge (N2977) and Mauriceville (N51) have been sequenced
  - The location of each SNP between the two is known
- Pool the progeny and sequence ALL the genomes together…

New school mapping technique: Bulk segregant analysis coupled with whole genome sequencing….

locus: 7-1
(unlinked to *dim* mutation)

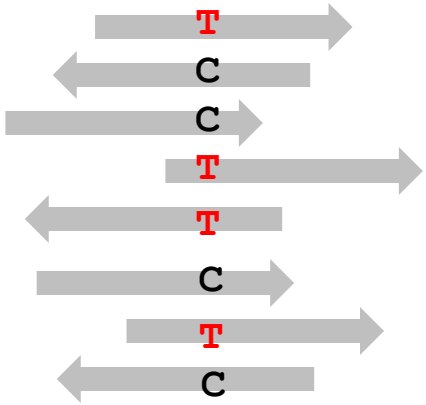
OAKRIDGE 5′ CCGCTAAATTAAGCG**C**AATATGCTCGA

MAURICEVILLE **T**

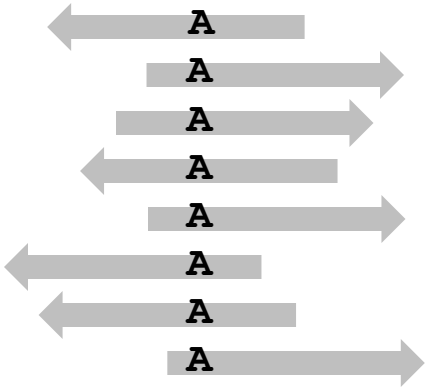
locus: 2-8
(linked to *dim* mutation)

OAKRIDGE 5′ GATGACCGTATAGA**A**TTCCCTGCTAC

MAURICEVILLE **G**

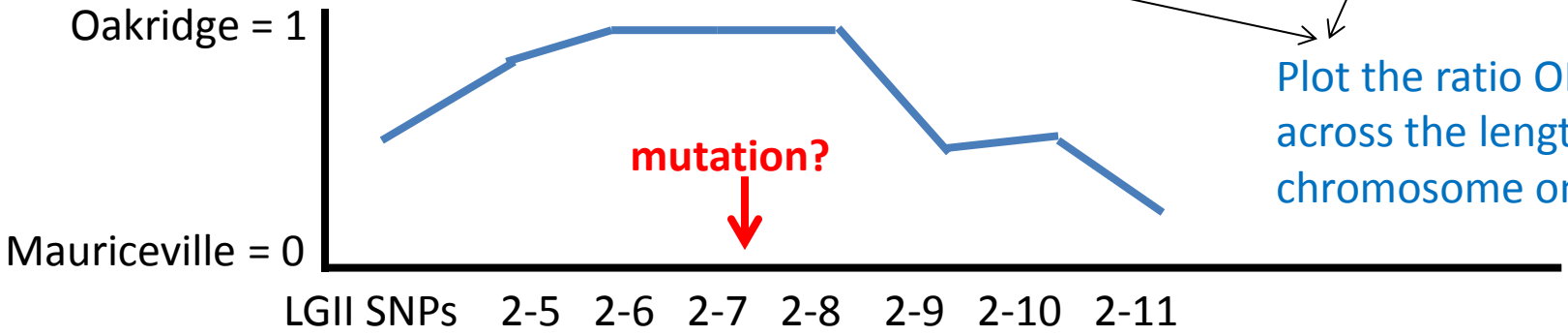


WGS reads (gray)



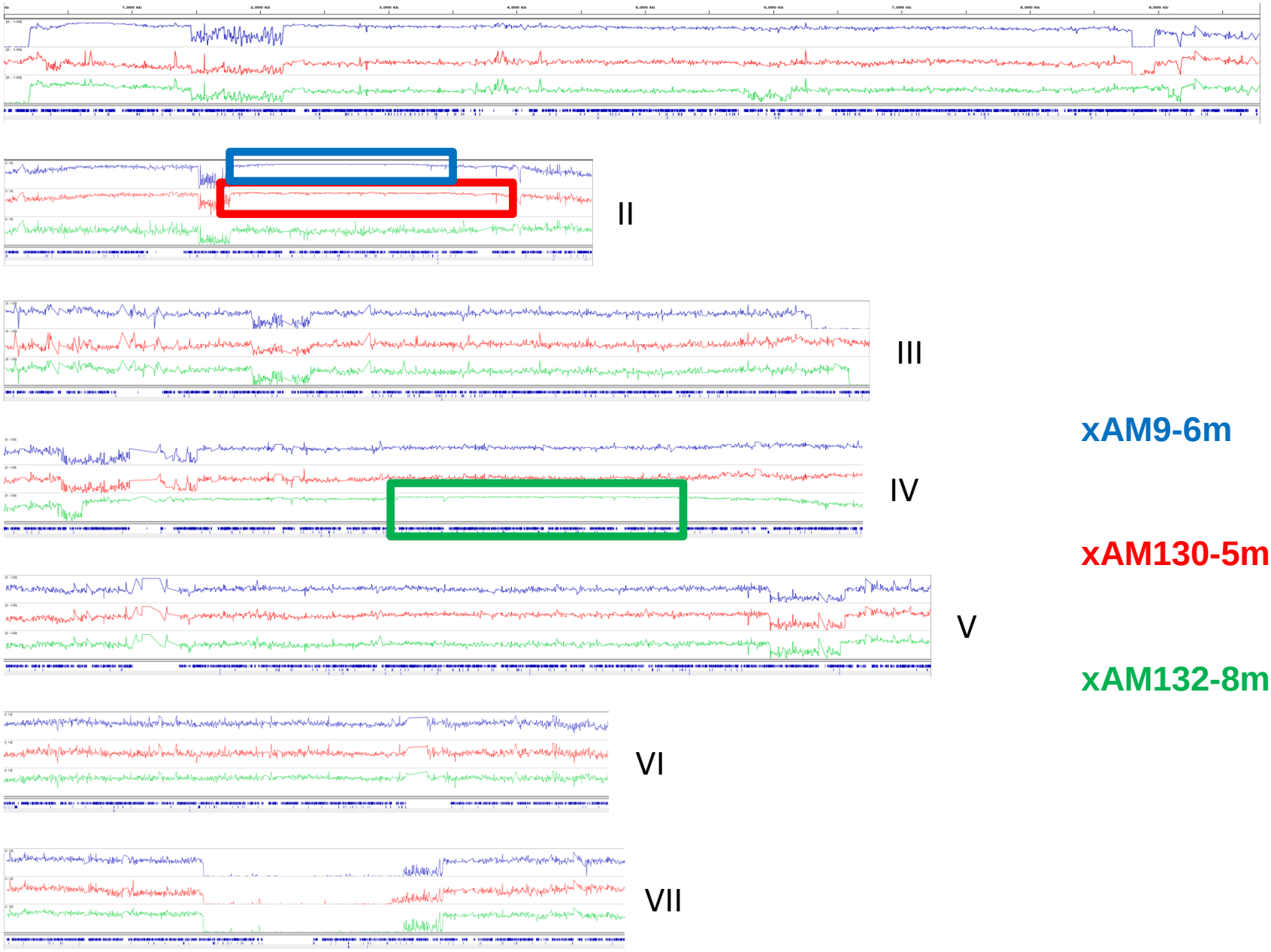
Ratio OR: 0.5

Ratio OR: 1.0



Plot the ratio OR SNP across the length of a chromosome on a graph

Bulk Segregant Analysis with Whole Genome Sequencing of three mutants



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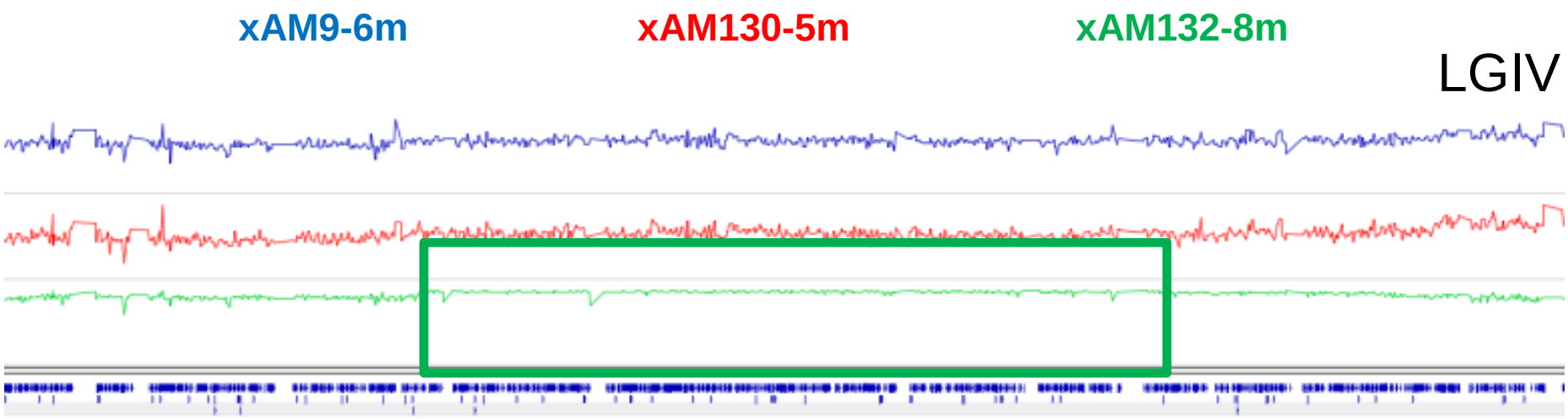
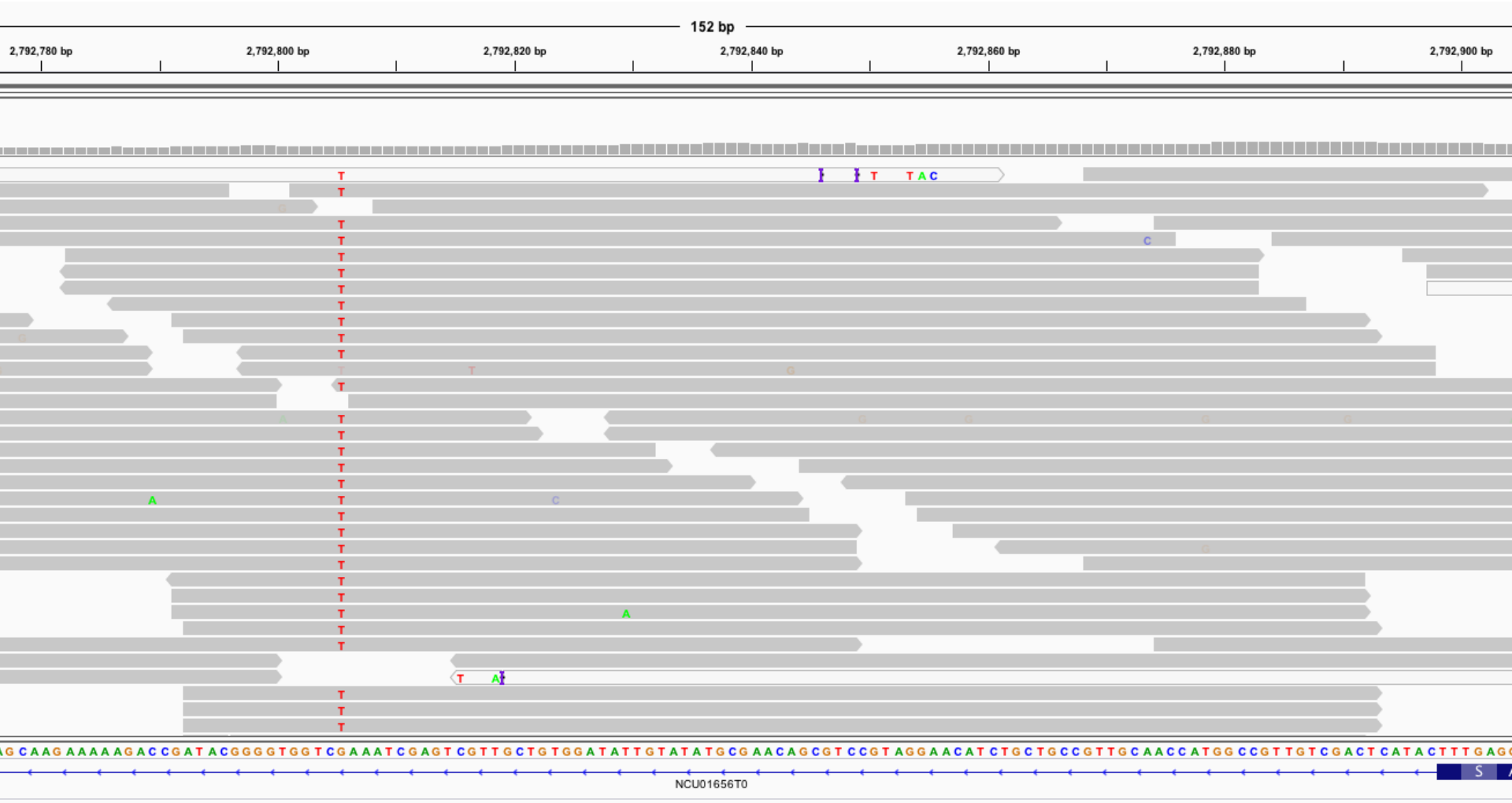


The xAM132-8m mutation seemed to map to a large region of LGIV.

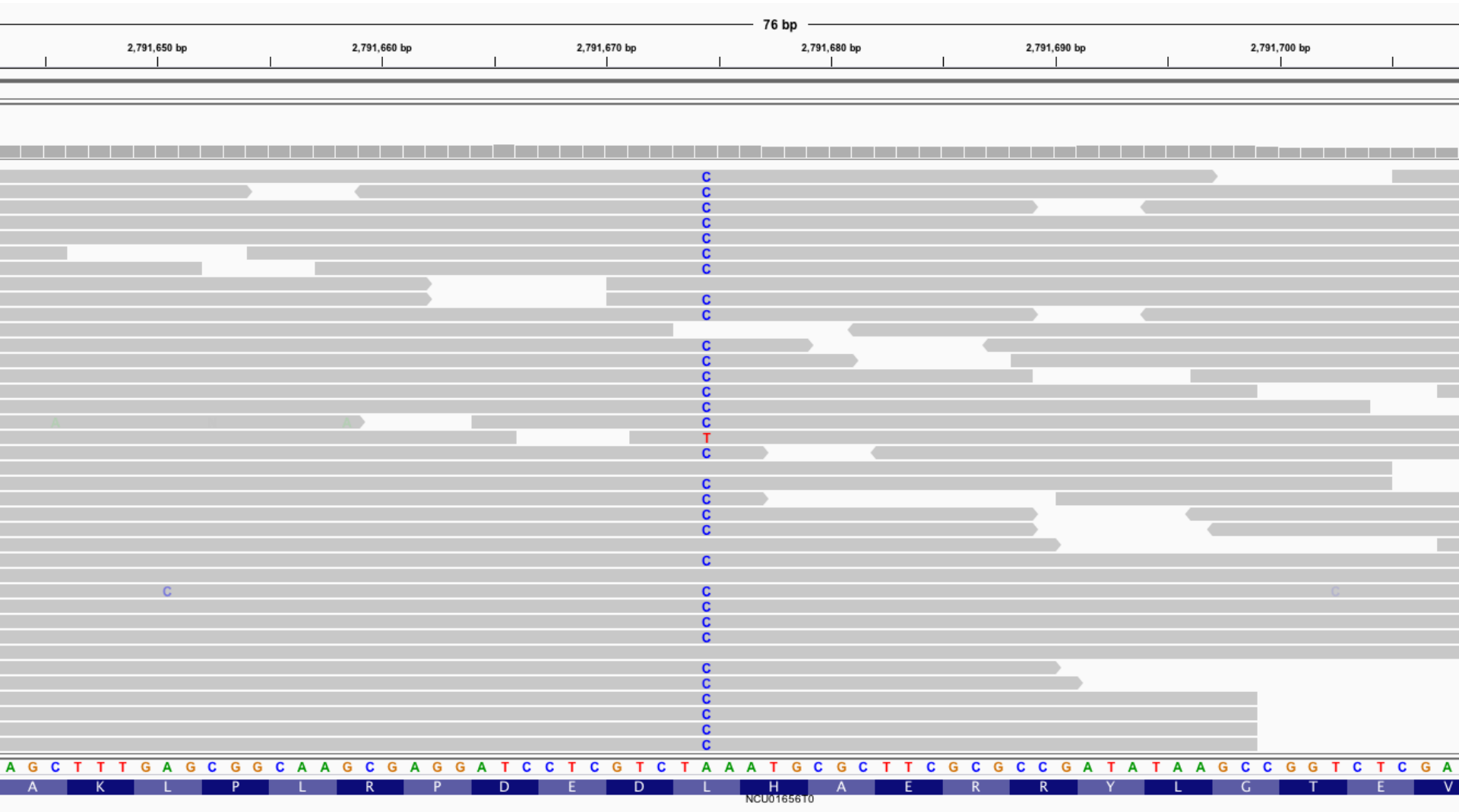
Because we also **get the sequence** back out of this protocol, we can run computer programs to identify the differences between the WT genome and the mutant genome, possibly shedding light upon the causative mutation in your mutant…

It turns out that these genes were not as interesting (KNOWN *dim* genes) (probably due to errors in the *dim-9* complementation tester strain and incomplete complementation)

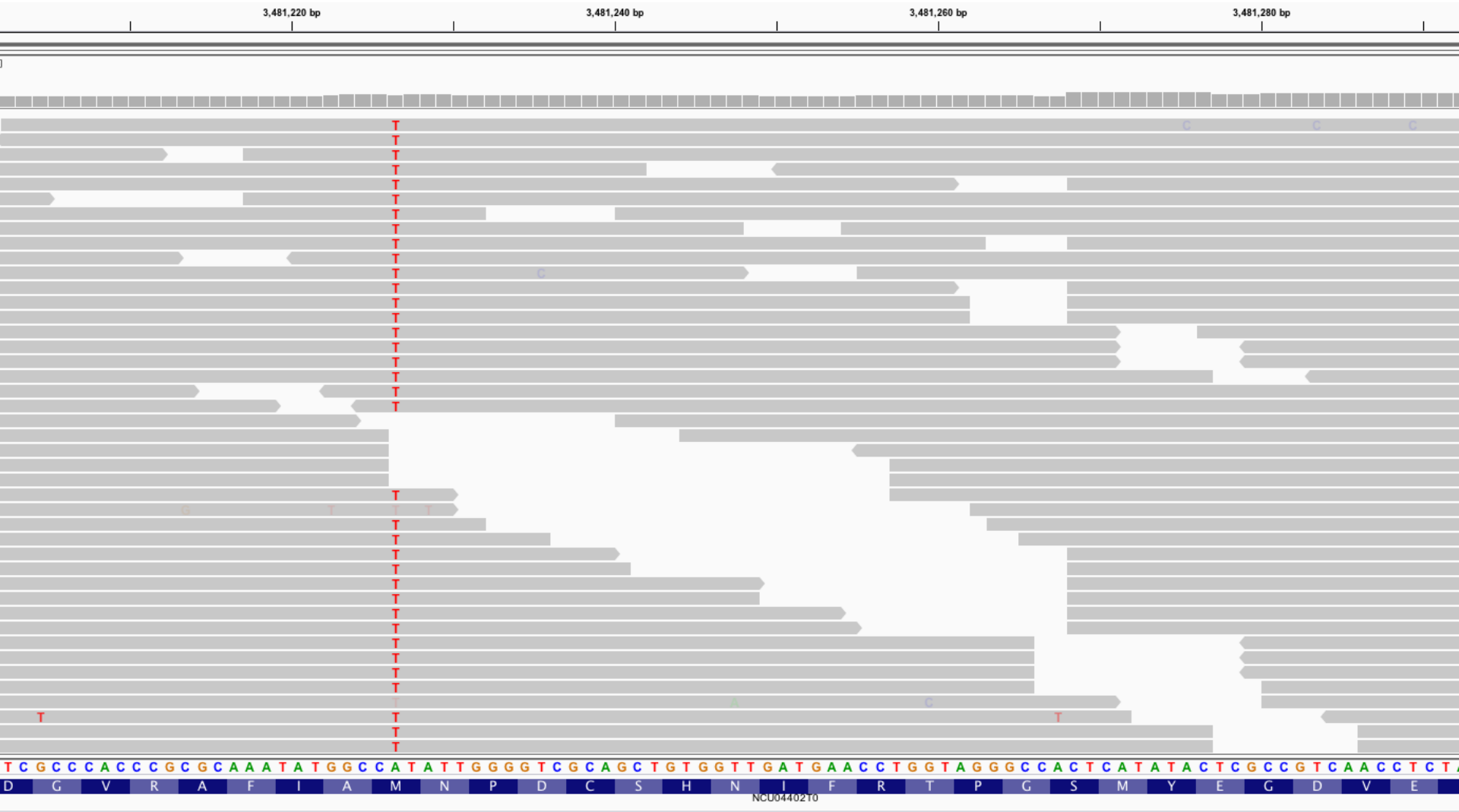
xAM9-6m mutation in NCU01656 (*dim-9*) intron



xAM130-5 mutation in NCU01656 (*dim-9*): L825stop



xAM132-8 mutation in NCU04402 (*dim-5*): M261K



Classical vs. Whole Genome Sequencing Mapping

- Classical mapping is a very well defined and experimentally proven procedure for finding the genomic locus of mutant genes

- Whole genome sequencing should work in principle, but it is still being developed (I am still validating it). This procedure is totally new (yet not a trivial procedure)!