The Maize TILLING Project (http://genome.purdue.edu/maizetilling/) Updates and EcoTILLING Industrial Germplasm

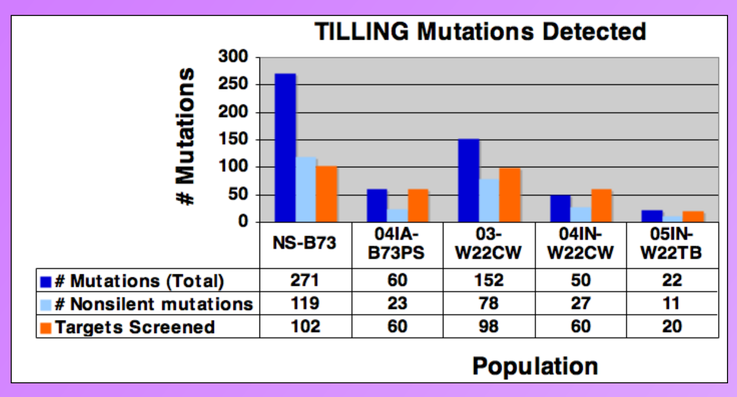
Rita-Ann Monde (1), Dacia Daniel (1), Leonie Leduc (1), Heather Sahm (1), Courtney Chambers (1), Tara Breen (1), Michael Eichstedt (1), Alyssa McCray (1), Ashlee McCray (2) Nirmita Patel (1), Theresa Xavier (1), Elizabeth Greene (2), Jorja Henikoff (2), and Clifford Weil (1).
(1) Purdue University, (2) Fred Hutchinson Cancer Research Center

160 TILLING Requests
34 Orders Completed
105 Targets with mutations
126 Orders in Progress

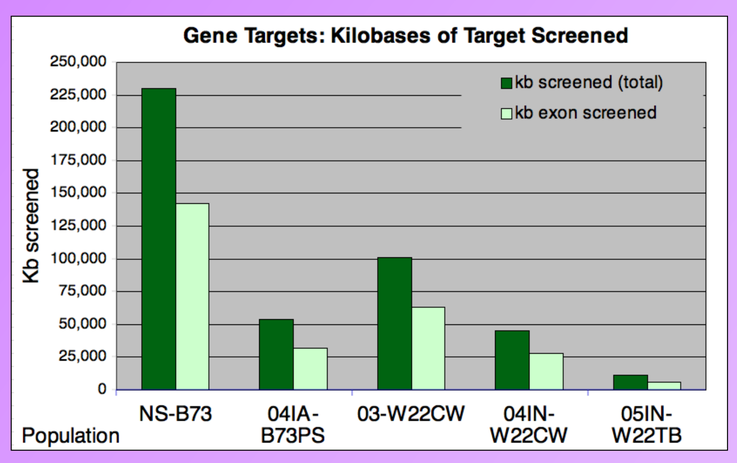
REMEMBER: The "beauty" of a mutation is in the "eyes and hands" of the mutant holder; *you* are the best one to determine whether the base change conditions a phenotype!

Population	# Individuals in current screening population	Potential number of lines to screen*
NS-B73	2304	2304
04IA-B73PS	1152	3400
03-W22CW	1152	1152
04IN-W22CW	1152	4000
05IN-W22TB	1152	1152
08IN-B73CW	Fall 2008	TBD
08IN-W22TB	Fall 2008	TBD
08ES-B73PR	Fall 2008	TBD

*** Lines must have a minimum of 40 M2 seed to be included in the screening population**

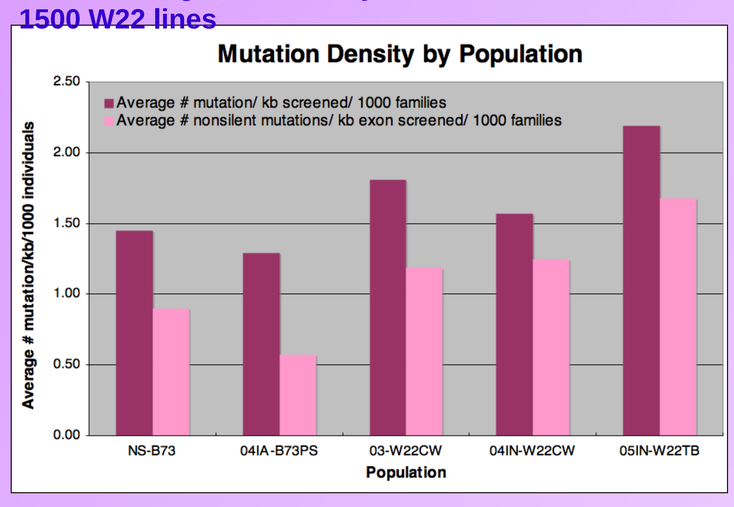


- **14 % of the mutations predicted to be damaging**
- **Average ~ 5.5 mutations/target**



The important (interesting) mutations will be found in the exons. The amount of exon TILLed varies based on the gene target and averages to ~ 60 % exon sequence.

For each target, we initially screen ~ 1500 B73 and 1500 W22 lines



We continue to generate (and accept) additional EMS populations. The 08IN-B73CW population should have a higher mutation density than our current group. The Brutnell Lab is also contributing another W22 population.

TRUMPing

Targeted Resequencing Using Massively Parallel Methods

ABI SOLiD:

Advantages of Sequencing by Oligo Ligation Detection

- **Lower cost! < $1000/target**
- **Sequence entire gene**
- **> 3 GB sequence/run**
- **48-fold pooling of individuals**
- **Flexibility with PCR conditions**
- **2 Base encoding, minimizes errors**
- **Homopolymers not a problem**
- **Higher throughput (50 targets/run)**

Logistical challenges:

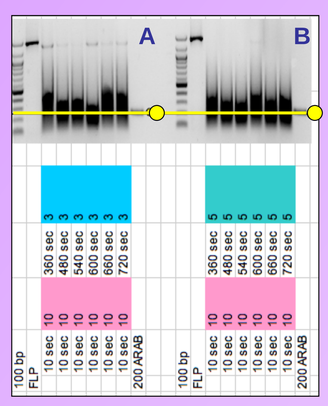
1) Shearing 1-6 kb PCR products to ~ 200 bp fragments
2) Barcoding DNA fragments in each well of a microtiter plate
3) Optimize library creation and amplification for 96 samples
4) Explore ways to multiplex PCR reactions

The Scheme:

1) Build PCR plate: Starting with 36 plates arrayed 8x8 with DNA from indivduals, create a 2D arrayed 96 well plate The 2304 individuals are pooled 48-fold and each is re-presented twice and with a unique set of co-ordinates.

2) Shear DNA and attach barcoded adapters. With DNA in the 96 well plate, use the Covaris E series sonicator to randomly shear PCR products to ~150-200 bp fragments. DNA is in a 96 well plate and the bottom of each well is subjected to a focused acoustic beam. This is a non-invasive way to randomly generate small DNA fragments from gDNA and PCR products.

We have attached a unique 5 base sequence to the 3' end of the ABI SOLiD P1 primers so DNA in a given well will have a specific tag that will be read in the subsequent sequencing reactions.



Sonication optimization using a 1.4 kb PCR product (FLP). Two protocols were tested each with 6 different time points. The first program had a 10 sec burst at intensity setting 10 followed sonication at intensity setting 3 (A) or 5 (B) for varying times ranging from 360-720 seconds. The yellow line and dot indicate the desired 200 bp size.

Variables to be optimized include type of plate (V or round bottom), beam intensity and time.

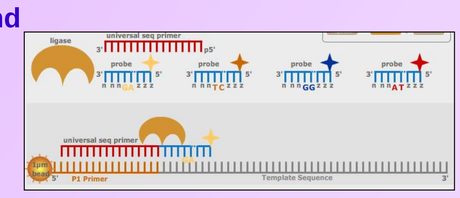
Modified ABI SOLiD adaptors (barcode tag denoted with N):

P1-aBC cca cta cgc ctc cgc ttt cct ctc tat ggg cag tcg gtg at NNNNN

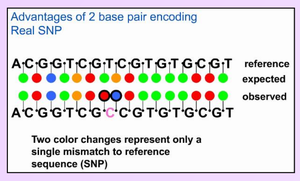
P1-bBC tt ggt gat cgc gag gcg aaa gga gag ata ccc gtc agc cac ta NNNNN

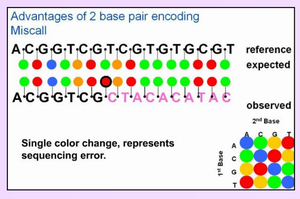
3) Pooling, amplification and emulsion PCR (ePCR). ThePCR products for each target are pooled and enriched for the ~ 150 bp fragments. This library is amplified then is the template for emulsion PCR (ePCR). During ePCR, each DNA molecule is attached to a bead and suspended in a droplet of oil containing all the reagents to further amplify that molecule. After ePCR, beads containing amplifed target are separated from the "empty" beads.

4) Bead attachment to slide and sequencing by ligation. The beads are attached to the slide and a 35 bp sequence is generated by 5 distinct cycles of primer binding and ligation. The primers contain fluoroprobes that specify theidentity of the dinucleotide. This type of sequencing chemistry has several features that are beneficial to the maize EMS populations:



- **Ligation reactions are not affected by homopolymers**
- **Dephosporylation steps eliminate multiple ligations**
- **System is reset after each cycle, reduces background**
- **If a cycle fails, it can be repeated**
- **Each base is read twice and as part of a dinucleotide, thus a true SNP/mutation is represented by two color changes.**
- **Only need ~ 3X depth due to 2D arraying and reduced error rate**



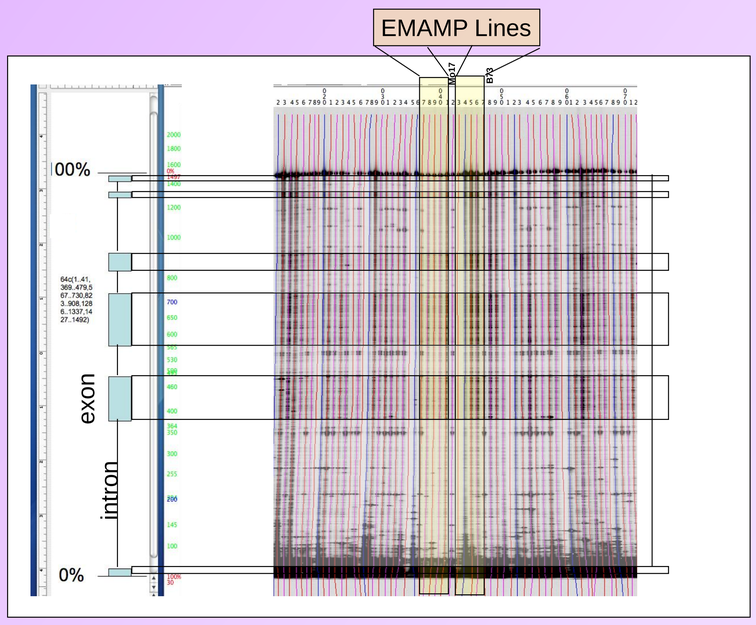


EcoTILLING Industrial Germplasm

HAVE CURRENT BREEDING PROGRAMS REALLY BEGUN TO EXHAUST THEIR GENETIC DIVERSITY?

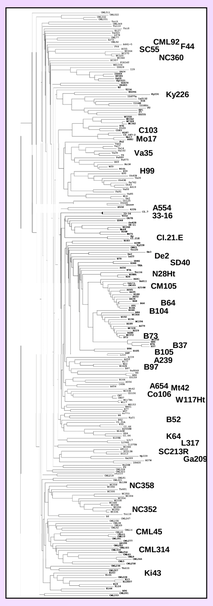
While gains continue to be made, concern is growing about how long those gains can continue. Maize EcoTILLING queries natural variation for specific gene targets in comparison to the B73 reference genome. We also know the intron-exon structure of the target sequence and can compare exon sequence diversity found among older, widely diverse inbreds from the Maize Diversity Lines and that found among ten, recently off-patent, industrial inbreds (the Elite Maize Association Mapping Panel or EMAMP lines).

While diversity is usually assessed using SSR or other markers chosen because they are both polymorphic and occur throughout the genome, the relevant interest is that within coding exons (or regulatory sequences). We compared a broad, representative set of inbreds from the Maize Diversity Lines to the ten EMAMP lines, looking at haplotype variation within exon sequence.



	# EMAMP exon haplotypes	# exon haplotypes in other MDLs
TARGET 1	1	2
TARGET 2	1	1
TARGET 3	2	4
TARGET 4	3	4
TARGET 5	3	4
TARGET 6	3	3
TARGET 7	1	1
TARGET 8	2	4
TARGET 9	2	2
TARGET 10	1	2
TARGET 11	4	8
TARGET 12	3	12
TARGET 13	1	2
TARGET 14	2	2
TARGET 15	2	2
TARGET 16	2	2
TARGET 17	1	5
TARGET 18	1	2
TARGET 19	3	6
TARGET 20	3	4

MDL Lines



EMAMP

PHG39
PHG35
PHZ51
PHJ40
PH207
PHG84
PHG47
LH1
LH123T
LH82

These data suggest that the commercial lines examined retain only slightly less exon diversity than the germplasm overall. The genes tested are not believed to have been overt targets of selection during domestication, though some may have been selected in modern breeding programs. The relatedness of some of the EMAMP lines may also explain the reduction in haplotypes for Targets 11 and 12. Note that, while in several cases all the EMAMP lines have only one haplotype relative to B73, this does not necessarily mean that they are identical to one another.

While the alleles of some genes in commercial germplasm may be fixed, the germplasm remains healthily diverse for many other genes. Genes for which allelic diversity has not been lowered by choice (selection) are prime candidates for directed mutagenesis

Diagrams depicting sequencing by ligation obtained from http://appliedbiosystems.cnpg.com/lsca/webinar/rhodes/chemistry/20070618/ and http://appliedbiosystems.cnpg.com/lsca/webinar/rhodes/2be/20070618/