Theor Appl Genet (2010) 121:697–704
DOI 10.1007/s00122-010-1341-6

ORIGINAL PAPER

Distributed simple sequence repeat markers for efficient mapping from maize public mutagenesis populations

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Received: 22 December 2009 / Accepted: 3 April 2010 / Published online: 18 April 2010

Abstract The genome sequence of the B73 maize inbred enables map-based cloning of genetic variants underlying phenotypes. In parallel to sequencing efforts, multiple public mutagenesis resources are being developed predominantly in the W22 and B73 inbreds. Efficient platforms to map mutants in these genetic backgrounds would aid molecular genetic analysis of the public resources. We screened 505 simple sequence repeat markers for polymorphisms between the B73, Mo17, and W22 inbreds. Using common thermocycling conditions, 47.1% of the markers showed co-dominant polymorphisms in at least one pair of inbreds. Based on these results, we identified 85 distributed markers for mapping in all three inbred pairs. For each inbred pair, the distributed set has 64–71 polymorphic markers with a mean distance of 27–29 cM between markers. The distributed markers give nearly complete coverage of the genetic map for each inbred pair. We demonstrate the utility of the marker set for efficient placement of mutants on the maize genetic map with an example mapping experiment of a seed mutant from the UniformMu mutagenesis resource. We conclude that these distributed molecular markers enable rapid mapping of phenotypic variants from public mutagenesis populations.

Communicated by M. Bohn.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-010-1341-6) contains supplementary material, which is available to authorized users.

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Introduction

Map-based cloning is a reliable method to identify the genetic cause of mutations of interest (Jander et al. 2002). The recently completed B73 genome sequence greatly facilitates map-based cloning (Schnable et al. 2009), making this a practical approach to clone individual genes of interest (Bortiri et al. 2006; Erhard et al. 2009; Gallavotti et al. 2008; Thompson et al. 2009). In parallel to genome sequencing, multiple genomics projects have developed public mutagenesis collections using both transposons and EMS as mutagens (Settles 2009). Two of these populations were generated in mixed genetic backgrounds (Fernandes et al. 2004; May et al. 2003), but the majority of mutagenized resources are in the W22 and B73 inbred backgrounds (Ahern et al. 2009; Cowperthwaite et al. 2002; Kolkman et al. 2005; McCarty et al. 2005; Till et al. 2004).

Mapping and positional cloning of mutants from defined genetic backgrounds can be simplified relative to mapping from undefined or mixed backgrounds (Jander et al. 2002). The first step of positional cloning is identifying a map position to bin level resolution. This is most efficiently completed using a core set of distributed markers (Bortiri et al. 2006). The MaizeGDB database maintains integrated genetic maps with thousands of markers that are anchored relative to the Intermated B73 × Mo17 (IBM) mapping population (Lawrence et al. 2008; Lee et al. 2002). The most current map includes a mix of restriction fragment length polymorphisms (RFLPs), simple sequence repeats (SSRs), insertion/deletions (indels), and single nucleotide polymorphisms (SNPs) that have been mapped in multiple recombinant inbred populations (Burr et al. 1988; Davis et al. 1999; Falque et al. 2005; Fu et al. 2006; Lee et al. 2002; Liu et al. 2009; McMullen et al. 2009; Sharopova et al. 2002). By integrating multiple maps and marker

types, researchers have access to more markers at higher density. However, a subset of these markers do not show polymorphisms between the B73 and Mo17 parents of the IBM population, and most of the markers have not been tested for the W22 inbred.

The two most widely used molecular marker systems in plant breeding and genetics are SNPs and SSRs (Appleby et al. 2009). SNP marker detection systems can provide higher accuracy allele calls and can be completed at higher throughput (Jones et al. 2007). To be economical, SNP genotyping requires access to expensive infrastructure and a high throughput application (Appleby et al. 2009). High throughput SNP detection relies on multiplexing, which both reduces the cost per marker but also decreases the flexibility of marker selection once a SNP assay has been set-up. SNPs contain less allelic diversity than SSRs with most SNPs being biallelic (Hamblin et al. 2007). Consequently, SNP genotyping assays need to employ more markers than SSRs to achieve the same number of polymorphic alleles for comparisons of multiple genetic backgrounds. In maize, 1,016 SNP markers have been identified that can be applied to BSA mapping (Liu et al. 2009). A subset of 124 markers was identified for genome-wide bulk segregant analysis (BSA) in B73/Mo17 F_2 mapping populations. An entry-level SNP genotyping experiment with the 124 markers requires genotyping 45 bulk pools. To apply these markers to other inbred pairs, Liu et al. (2009) recommend genotyping for all 1,016 markers to ensure sufficient coverage with co-dominant markers. In contrast to SNPs, SSRs are more amenable to small scale experiments such as a mapping a single mutant using BSA (Gallavotti et al. 2008; Thompson et al. 2009). Individual SSR markers can be more easily added or removed from a mapping experiment and a smaller number of samples are generally analyzed. The cost of SSR mapping can be further reduced by identifying marker sets that are most useful for the specific mapping experiment.

BSA mapping requires co-dominant markers. The Maize Mapping Project characterized hundreds of SSRs and deposited images of inbred screening gels on the MaizeGDB database (Sharopova et al. 2002). These screening gels included F_1 or mixed DNA samples to test for co-dominant alleles for only 1–2 inbred pairs of the 11 inbreds screened. Similarly, Fu et al. (2006) developed and characterized insertion–deletion polymorphism (IDP) markers within 24 inbreds but did not screen mixed or F_1 DNA samples. In addition, the IDP markers were not screened for the predominant inbred used in public mutagenesis projects, W22. Our anecdotal experience suggested that size differences in SSR alleles were not sufficient to predict co-dominant markers. Here we analyze 505 SSR markers for co-dominant polymorphisms between the B73, W22, and Mo17 maize inbred lines to identify a distributed marker set for efficient mapping of mutants from B73 and W22 public mutagenesis experiments. The Mo17 inbred was chosen as a third reference mapping parent due to the extensive genetic map data for this inbred (Fu et al. 2006; Lee et al. 2002; Liu et al. 2009; Sharopova et al. 2002). In addition, Mo17 is currently being sequenced using a genome shotgun approach, and significant sequence information will be available for this inbred in the near future (http://www.jgi.doe.gov).

Materials and methods

Genomic DNA from the B73, W22, and Mo17 inbreds was extracted from 2 week old seedlings as described (Settles et al. 2004). Purified DNA was diluted to a concentration of ~20 ng/μL just prior to PCR. Each SSR marker was amplified from the three inbreds and from 1:1 mixes of B73/W22, Mo17/W22, and B73/Mo17 DNA. SSR markers were tested from a commercial primer set of 480 primer pairs with 477 non-redundant markers (M8818-1SET, Sigma-Aldrich Co., St. Louis, MO). The remaining 28 primer pairs were selected to fill gaps in the distributed sets from the IBM2 2008 Neighbors Frame 2 genetic map at the MaizeGDB database (http://www.maizegdb.org). The complete list of markers screened is available in Supplemental Table 1. Primer sequences for these markers are available at MaizeGDB. Each marker was tested under common PCR conditions (0.25 μM primers, 150 μM each dNTP, ~40 ng DNA, and GoTaq® PCR mix, Promega Co., Madison, WI). Thermocycling conditions were 94°C for 40 s, 57°C for 45 s, and 72°C for 40 s with 34 cycles. Amplified fragments were visualized by electrophoresis on 14 cm, 4% agarose gels (0.5× TBE) at 90 V for ~2 h and stained in ethidium bromide solution (~0.1 μg/mL). Co-dominant polymorphisms were scored visually from the gel images. If a marker failed to amplify or gave multiple products within an inbred DNA, the PCR was repeated 2–3 times to confirm the amplification pattern.

The screening gel images produced by the Maize Mapping Project (Sharopova et al. 2002) were scored for each marker independently. The gel image was accessed on MaizeGDB, and co-dominant polymorphisms were predicted by estimating the size difference between alleles in the B73, Mo17, and W22 inbreds. When one inbred was missing from the screening gel (178 markers) or when the screening gel appeared to have a technical problem (68 markers), no predictions were made for the specific inbred pairs affected.

The proof-of-concept mapping experiment was completed by crossing a UniformMu *defective kernel* (*dek*) isolate, *dek*-9700*, to the B73 and Mo17 inbreds. UniformMu is a public, transposon mutagenesis population in the W22

inbred background (McCarty et al. 2005). F_2 mapping populations were generated by self-pollinating F_1 plants. Mature mutant and normal seeds were selected visually from segregating ears of the B73/UniformMu and Mo17/UniformMu populations. For DNA extraction, the seeds were imbibed in water overnight, and the pericarp was removed. For normal seeds, the embryo was dissected for the extraction. For the *dek* mutant seeds, the entire endosperm and embryo was used in reduced grain-fill mutants. In the Mo17/UniformMu population, 11/42 *dek* kernels had very severe grain-fill defects, and the pericarp was included in the DNA extraction for these kernels. DNA extraction was completed as described (Settles et al. 2004), except that 1 mL of extraction buffer was used and the homogenized sample was centrifuged prior to phenol:chloroform:isoamyl alcohol extraction. This step sedimented the starch gel formed by the DNA extraction buffer. The supernatant was transferred to a new microcentrifuge tube prior to completing the remaining steps of the extraction. For the BSA PCR, the individual samples were pooled to create normal and mutant bulks. The samples were amplified with the distributed marker set. Individual mutant DNA samples were tested by PCR with the linked SSR markers, and map distances were calculated using Haldane's map function (Haldane 1919).

Results

Each of the 505 SSR markers was tested for co-dominant polymorphisms between B73/Mo17, B73/W22, and Mo17/W22 as shown in Fig. 1a. We defined a co-dominant marker as useful for F_2 mapping when it amplified easily resolved size polymorphisms between at least two inbreds and showed a novel banding pattern in a 1:1 mix of a pair of inbred DNA samples (Fig. 1a, umc1538). Based on these criteria, 238 (47.1%) of the markers amplified co-dominant markers with each pair of inbreds having 154–170 useful markers (Fig. 1b; Table 1). Less than 10% of the 505 markers had three distinct alleles for the inbreds, such as umc1538 (Fig. 1). 31.9% of the markers had two alleles, while 8.5% of the markers showed co-dominance in just one of the three inbred pairs. The remaining 267 markers that were not useful for BSA gave the following amplification patterns: 76 amplified a single allele in all inbreds (Fig. 1a, umc1288); 129 amplified alleles that were difficult to resolve or score (Fig. 1a, umc1590 and phi402893); and 62 failed to amplify (see Supplementary Table 1).

All of the SSRs we tested were screened for size polymorphisms with multiple inbreds by the Maize Mapping Project (Sharopova et al. 2002). These prior screening gels are available at the MaizeGDB database (Lawrence et al. 2008). To compare our results with the Maize Mapping

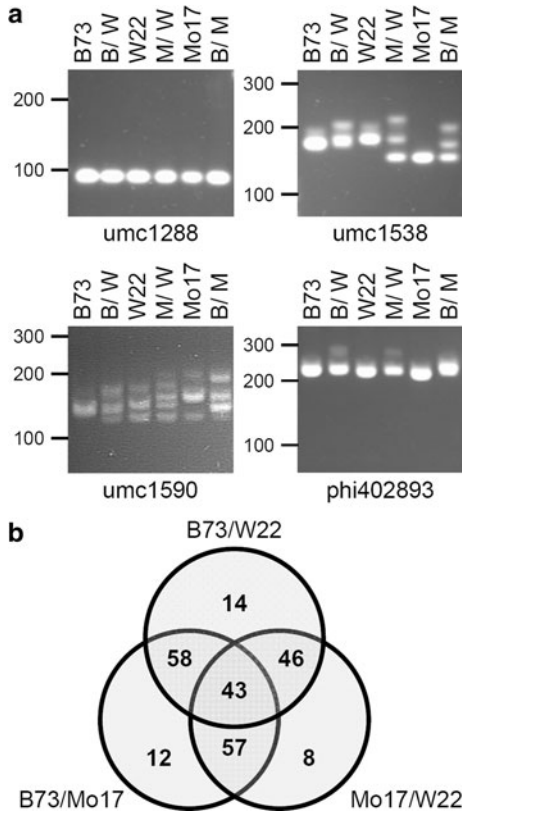

Fig. 1 Screen for polymorphic SSR markers in B73, Mo17, and W22. **a** Examples of PCR scores: umc1288 amplifies a single allele; umc1538 has three co-dominant alleles; umc1590 and phi402893 have polymorphic alleles that are not suitable for BSA. DNA size markers (bp) are indicated for each image. B/W, M/W, and B/M indicate 1:1 mixes of B73:W22, Mo17:W22, and B73:Mo17 inbred DNA. **b** Venn diagram showing the number of co-dominant markers found for each pair of inbreds. Supplementary table 1 gives scores for each of the 505 markers evaluated

Project screening gels, we scored the screening gels for allele size differences to predict markers that were expected to give co-dominant polymorphisms for combinations of B73, Mo17, and W22. With 505 markers, up to 1,515 predictions could be made for combinations of B73/Mo17, B73/W22, and Mo17/W22. W22 was not included in all screening gels, and the MaizeGDB images contained data for 955/1,515 pair-wise predictions. These scores predicted 338 of the 505 markers would produce useful co-dominant markers. However, the allele size differences predicted co-dominance correctly in only 61.4% of 955 comparisons. We found 369 instances where allele sizes were not useful predictors due to multiple factors including: unexpected dominant alleles, alleles that were difficult to resolve, co-dominant alleles that resolved well even though the MaizeGDB image indicated little to no size polymorphism, and markers that did not amplify reliably. Of the 338 markers that were predicted to be co-dominant, 169 (50%) did not produce co-dominant polymorphisms in any of the

Table 1 Number of co-dominant markers identified for each inbred pair from 505 SSR markers

Inbred comparison	Total polymorphic markers				Distributed marker set			
	No. of markers	Average map interval (cM)	SD (cM)	% Map covered	No. of markers	Average map interval (cM)	SD (cM)	% Map covered
B73/Mo17	170	12.0	12.3	91.7	70	26.9	13.2	91.1
B73/W22	161	13.0	11.8	94.6	64	29.1	12.3	90.4
Mo17/W22	154	13.5	11.6	94.5	71	26.6	12.9	92.7
Union of 3 inbred pairs	238	8.9	9.1	95.6	85	22.7	12.0	94.7

three inbred pairs. Without making selections for co-dominant markers, we identified a similar frequency (47.1%) of co-dominant markers ($p = 0.41$; χ^2 test for independence). From these data, we conclude that size polymorphisms between inbreds are not good predictors of co-dominant alleles.

Based on our co-dominance screens, we selected a distributed marker set that could be used for all of the inbred pairs (Supplementary Table 2). For this analysis, we divided the IBM2 2008 Neighbors map coordinates by a factor of 4 to account for the genetic expansion of the IBM population (Lee et al. 2002). This is the average conversion factor for marker coordinates between the Genetic 2008 and IBM2 2008 Neighbors maps at MaizeGDB. Using these predicted genetic distances, the average distance between all polymorphic markers for each inbred pair was <14 cM (Table 1). Most SSR markers amplify both alleles robustly when the bulk segregant map distance is ≥25–35 cM (Carson et al. 2004; Jones et al. 2007). A minimally redundant set of distributed markers would be spaced at 50 cM intervals to ensure that all phenotypic variations can be detected by at least one marker. Based on the size of the IBM2 2008 Neighbors map, 50 markers would be needed for a minimal distributed set. However, we only observed 43 markers that contained three distinct alleles in B73, Mo17, and W22 and these markers are not distributed uniformly. To account for these issues, we selected a distributed set of 85 markers that includes some redundancy (Fig. 2). 64–71 of the distributed markers are polymorphic for each inbred pair, and these polymorphic markers are spaced at a mean genetic interval of 27–29 cM (Table 1).

The distributed markers contain some gaps in which the closest marker is >25 cM (Fig. 2). The majority of the gaps in coverage are at the ends of chromosomes with the largest gap located on the long arm of chromosome 8. This gap is likely to be caused by an error in the IBM2 2008 Neighbors map. The terminal locus of this arm is annotated as *Empty pericarp4* (*Emp4*) and adds approximately 46 cM of genetic distance to the map. *Emp4* has been mapped to chromosome 1 via translocations, molecular markers, and molecular cloning (Gutierrez-Marcos et al. 2007). After excluding the *Emp4* locus from the map, 7.3–9.6% of the genome is predicted to be located >25 cM from a marker depending upon the inbred pair (Table 1).

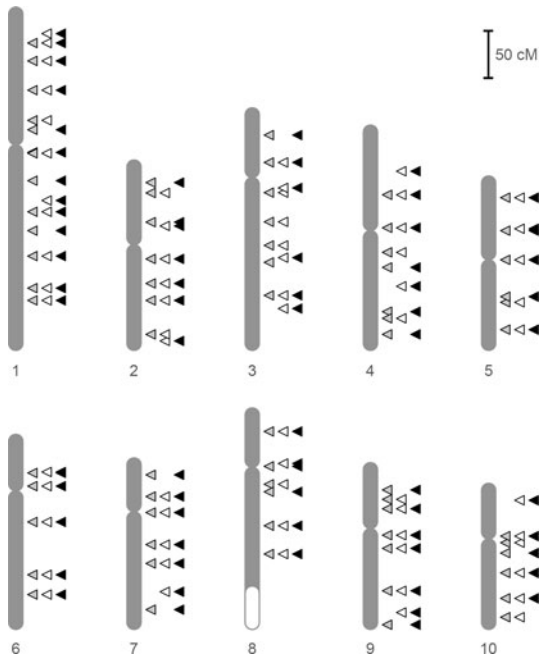

Fig. 2 Genetic map of the distributed marker sets. Markers are positioned based on their genetic coordinates on the IBM2 2008 Neighbors genetic map. *Gray*, *open*, and *black arrowheads* are polymorphic markers for the B73/Mo17, B73/W22, and W22/Mo17 inbred pairs, respectively. The open oval on chromosome 8 indicates the genetic distance added to the map due to misplacement of *Emp4*. Supplementary Table 2 gives map locations and expected co-dominant polymorphisms for the distributed marker set

To test the effectiveness of the distributed marker set, we completed BSA for a *dek* mutant (*dek*-9700*) from the UniformMu mutagenesis population. UniformMu is a transposon-tagging population in the W22 inbred (McCarty et al. 2005). We tested Mo17/UniformMu and B73/UniformMu F_2 mapping populations and found segregation distortion on chromosome 6 in both populations (Fig. 3, Supplementary Figs. 1, 2). Not all markers behaved as expected in both populations. In the B73/UniformMu population, eight markers amplified a single allele when they were expected to be polymorphic based on our screening gels. Likewise, three of the markers in the Mo17/UniformMu population

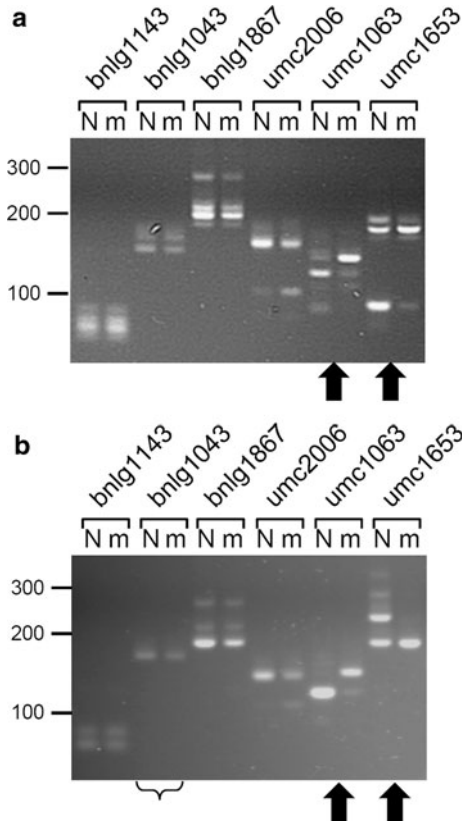

Fig. 3 BSA of F_2 mapping populations for a novel UniformMu *dek* locus. **a** BSA with a Mo17/UniformMu population with pools of 11 normal (N) and 42 *dek/dek* mutant (m) DNA samples. **b** BSA with a B73/UniformMu population with pools of 8 normal (N) and 24 *dek/dek* mutant (m) DNA samples. Markers for chromosome 6 are shown, and the BSA results for all 85 markers are given in supplementary Figs. 1 and 2. *Black arrows* indicate markers that showed the greatest segregation distortion. The *brace* indicates a marker that is not expected to be co-dominant for the mapping population. DNA size markers (bp) are indicated in each image

amplified unexpected non-polymorphic products. PCRs of *dek* mutant individuals from the Mo17/UniformMu population were used to refine the map position (Fig. 4a). Some of the *dek* mutant kernels in this experiment were exceptionally small, and we extracted DNA from whole dried kernels (see "Materials and methods"). Surprisingly, these had relatively little maternal DNA contamination, and recombinants could be scored with reasonable confidence (Fig. 4a, lanes 34–45). These experiments mapped the *dek* mutation to a 10.6 cM interval between umc1063 and umc1653 (Fig. 4b). The predicted genetic distance between these markers is 20.5 cM suggesting that the conversion factor between IBM2 2008 Neighbors map and the Genetic 2008 consensus map coordinates may be an underestimate. We scored the mapping population for bnlg345 to obtain a larger sample of recombinants. When bnlg345 is included, the total interval observed between bnlg345 and umc1653 is similar to the expected distance with 32.3 cM observed versus 39 cM expected. These data suggest the IBM 2008 Neighbors map provides accurate map distance estimates when the W22 inbred is used as a mapping parent.

Discussion

The development of common mutagenesis resources in the B73 and W22 inbreds creates the opportunity for map-based cloning in well-defined genetic backgrounds. By using defined inbreds, a smaller number of markers can be selected to ensure that mutants are mapped in a BSA (Liu et al. 2009). A recent SNP marker system has been developed for BSA mapping of maize mutants. The authors recommend 1,016 markers be used for mapping mutants in uncharacterized inbred combinations such as those involving W22 (Liu et al. 2009). The large number of markers required, high initial set-up costs, and requirement to complete hundreds of BSA mappings before SNP genotyping becomes cost-effective makes this technology less accessible for small research groups that are interested in mapping a few mutants at a time. Consequently, we focused on identifying distributed markers with technology that is more suitable for lower throughput genotyping.

Despite the large number of maize molecular markers that have been developed and characterized, it is not simple to identify distributed, co-dominant markers for pairs of inbreds. Our data suggest approximately 30% of mapped SSRs will be useful for BSA of any given pair of divergent inbreds. This is twice the frequency at which maize SNP markers are expected to produce quantitative co-dominant markers for a pair of divergent inbreds (Liu et al. 2009) and is consistent with the higher information content of SSR loci (Hamblin et al. 2007). It is important to note that we restricted our analysis to 4% TBE agarose gel electrophoresis. An additional 25% of the markers analyzed in this study amplified products that potentially could be scored using higher resolution agarose and capillary electrophoresis techniques.

Similar to the SNPs characterized by Liu et al. (2009), we found polymorphic alleles between two inbreds to produce co-dominant markers suitable for BSA in approximately 50% of cases. Multiple factors are likely to influence whether a SSR amplifies co-dominant polymorphisms useful for BSA. For example, non-specific primer binding can produce complex amplification products such as umc1590 (Fig. 1a). These markers can be mapped in recombinant inbred populations but are not suitable for BSA. Similarly, differential or weak primer binding to the SSR locus could lead to one allele amplifying predominantly when both alleles are present in a DNA sample (e.g., umc1657, Fig. 1a). Based on our survey and Liu et al. (2009), we conclude that co-dominant markers for BSA

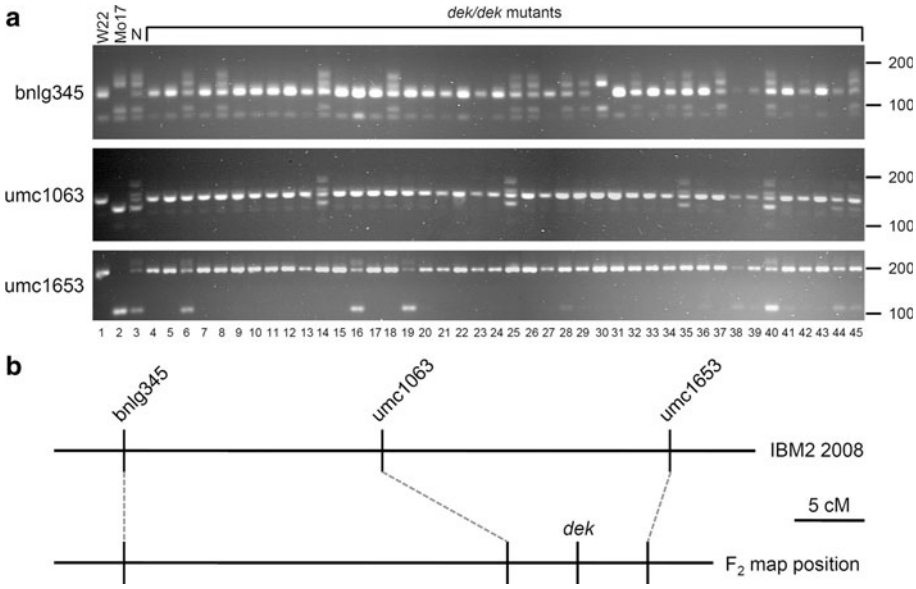

Fig. 4 Fine map of the UniformMu *dek* locus. **a** Recombination frequencies were scored using 42 individual *dek/dek* individuals from the Mo17/UniformMu F_2 mapping population. *Lanes 34–45* show PCR products from severe *dek* seeds in which maternal pericarp tissue was included in the DNA extraction. Weak amplification of the Mo17 allele was attributed to contaminating parental DNA in these samples. DNA size markers (bp) are indicated in each image. **b** Comparison of the predicted genetic distances based on IBM2 2008 Neighbors map coordinates and the observed Haldane genetic distance relative to the *dek* locus

need to be empirically identified irrespective of the marker technology used.

We also found 6.5% of the SSRs failed to amplify in all inbred samples of our survey. This subset of primers may be sensitive to PCR conditions. Alternatively, the failed markers may represent stochastic failure of SSRs to amplify. An earlier study focused on the reliability of maize SSRs observed that 13.8% of markers fail to amplify in any one replicate (Jones et al. 2007). Based on this expected frequency, <2% of markers are predicted to fail in all of the replicates that we completed. Maize molecular mapping studies generally optimize thermocycling conditions for each marker to map as many loci as possible (Fu et al. 2006; Sharopova et al. 2002). Thus, we interpret the failed amplifications as likely to be a result of the single set of thermocycling conditions and PCR reagents. Our goal was to identify markers that could be used under a common PCR regime to enable BSA by amplifying all markers simultaneously. Indeed, the two BSA proof-of-concept experiments contained only 8% missing data suggesting the distributed set of SSRs are more reliable than those assayed by Jones et al. (2007).

The distributed markers we selected provide a simple marker technology to enable mapping of mutants from public mutagenesis resources. By surveying for W22 polymorphisms, mutants from multiple public transposon mutagenesis populations and one EMS mutagenesis population can now be mapped more readily (Ahern et al. 2009; Cowperthwaite et al. 2002; Kolkman et al. 2005; McCarty et al. 2005; Till et al. 2004). We demonstrate the usefulness of the distributed markers by mapping the *dek*-9700* mutant isolate from the UniformMu transposon mutagenesis population to an approximately 10 cM interval in bin 6.07. Eleven of the SSR markers did not show the expected polymorphisms in the two BSA experiments (Supplemental Figs. 1, 2). However, the *dek*-9700* mutant derived from an early generation of the UniformMu introgression and had been backcrossed into W22 for only three generations prior to developing the mapping populations. Thus, the *dek* mutant is expected to retain 12.5% of the genetic variation derived from the *Mutator* parent of the UniformMu pedigree. Each marker assays roughly 1% of the genetic map. If the *Mutator* parent shared common SSR alleles with the B73 and Mo17 parents, 12–13 markers would be expected to be non-polymorphic based on the *dek* mutant pedigree and is consistent with the 11 non-polymorphic markers we observed.

The map position of *dek*-9700* can now guide further genetic experiments to determine whether this is a novel seed mutant locus. Two seed mutants, *su2* and *dek*-1104*, have recombination data that place these loci >20 cM proximal to *dek*-9700* (Scanlon et al. 1994). Nine other seed mutant isolates including two named loci, *dek19* and *emb3*, have been mapped to the long arm of chromosome 6 using B–A translocations (Chang and Neuffer 1994; Heckel et al. 1999; Neuffer and England 1995; Scanlon et al. 1994). Complementation tests with *dek19* and *emb3* will be needed prior to assigning *dek*-9700* a locus name.

It is useful to note that our tissue sampling for the *dek*-9700* mapping experiments was non-conventional (Carson et al. 2004). Instead of extracting DNA from immature developing endosperm tissues, we extracted from mature dried seeds. The maternal pericarp surrounds the mature kernel (Kiesselbach 1949), and there is some potential for maternal DNA contamination. Surprisingly, we found maternal DNA contamination not to be a significant factor even when no special precautions are taken to exclude the pericarp from the DNA extraction. We speculate that DNA

degradation within the pericarp is far more extensive after seed drying than DNA degradation resulting from endosperm programmed cell death (Consonni et al. 2005). In our laboratory, we find maternal DNA contamination to be more common when DNA is extracted from developing endosperm tissues even with careful dissection from maternal tissues (FM and AMS, unpublished). Our tissue sampling approach with dried kernels may be generally applicable for mapping and other molecular genotyping of maize seeds.

Finally, we observed recombination at frequencies close to expected for markers on chromosome arm 6L. These results suggest the predicted genetic distances between the distributed SSR markers are likely to be accurate for B73/W22 and Mo17/W22 mapping populations. When selecting the distributed marker set, we used a conservative bound of <25 cM to account for potential inaccuracies in the IBM genetic map. We estimate that 7.3–9.6% of the genome, depending on the inbred combination, is likely to be outside the range of the distributed marker set to detect linkage in a BSA. Thus, we expect 90–93% of mutants should be able to be mapped with a single F_2 mapping population using the marker set reported here. However, segregation distortion for many SSR markers is readily apparent when one allele constitutes 75% of the DNA sample (Carson et al. 2004; Jones et al. 2007), which corresponds to a Haldane genetic distance of 35 cM. Assuming this upper bound is a realistic expectation for BSA, the coverage of the distributed markers will be greater and the success rate in using this marker system should approach 96–98%. The frequency of success could be increased further by generating two F_2 populations with crosses between all three inbreds.

Acknowledgments We thank Chi-Wah Tseung for technical assistance, Romain Fouquet for critical reading of the manuscript, and the two anonymous reviewers for their helpful comments. This work was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 2007-35304-18439.

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