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High output genetic mapping of polyploids using PCR-generated markers

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Summary. The polymerase chain reaction (PCR) with arbitrarily selected primers has been established as an efficient method to generate fingerprints that are useful in genetic mapping and genomic fingerprinting. To further increase the productivity of mapping and fingerprinting efforts, we have altered existing protocols to include the use of the Stoffel fragment, which is derived from genetically engineered *Taq* polymerase. We also optimized the thermal profile of the reaction to increase the number of useful primers. In mapping of the genome of *Saccharum spontaneum* 'SES 208', a polyploid wild relative of sugarcane, these modifications allowed for an increase of 30% in the number of loci screened per primer, and an 80% increase in the number of polymorphisms per primer. Furthermore, the enzyme cost per reaction was decreased approximately 1.6-fold. Finally, there was an increase from about 70% to about 97% in the number of primers that were useful (i.e., gave a reproducible fingerprint) using our protocol. We have placed some of these markers into linkage groups.

Key words: AP-PCR – RAPD – Sugarcane – *Saccharum* – Stoffel fragment – Genetic mapping – MapMaker – Molecular markers

Introduction

The polymerase chain reaction (Saiki et al. 1985) has become an increasingly important tool in molecular biology (for a recent review see Erlich et al. 1991). The applications of the PCR to problems in molecular biology and genetics have increased dramatically over the last few years. One of these new applications combines the PCR with primers of arbitrary sequence to amplify a 'fingerprint' of different loci from any genome. This method was independently and simultaneously developed by Welsh and McClelland (1990) and Williams et al. (1990) and has been called AP-PCR or RAPDs, respectively. For any primer, the resulting pattern of amplified genomic fragments is highly reproducible and can be used as a 'fingerprint' for: (1) varietal identification and parentage determination (Welsh et al. 1991a, 1992); (2) genetic mapping, because they are inherited in a Mendelian manner (Williams et al. 1990; Welsh et al. 1991b), and (3) for generating phylogenetic trees, especially at the intraspecific level (Welsh et al. 1992).

The AP-PCR technique has allowed the rapid construction of genetic linkage maps. As an example of the high throughput of the method, Sederoff and co-workers recently constructed a 191-marker map in loblolly pine in just 2 months (Neale and Sederoff 1991). Similar work using RFLPs would have taken at least ten-fold longer to complete. In addition, many species of pine trees have very large genomes, thus it is difficult to load enough DNA on the gel to allow for the detection of single, or low-copy, sequences using Southern hybridization. So, the AP-PCR has extended the number of species that are amenable to genetic mapping with molecular markers, in addition to offering increased mapping output in every other species.

We are constructing a genetic map of the genome of a 'wild' form of sugarcane, *Saccharum spontaneum*, using AP-PCR. Because sugarcane is a complex polyploid plant with variable ploidy, the only markers

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that can be mapped are those present in one parent, absent in the other, and that segregate 1:1 in the progeny, such as in single-dose restriction fragments (Wu et al. 1992). Polymorphic fragments that have other segregation ratios cannot be mapped because there is no statistical method for determining their linkages. Therefore, high numbers of polymorphic fragments are required to saturate the map with those that segregate 1:1. Because of high output and the requirement for single-dose markers, AP-PCR is the method of choice for genetic mapping in polyploids such as sugarcane. However, it would be useful to optimize protocols to maximize the number of loci screened in each experiment, with the intent of increasing the number of polymorphisms detected with each primer.

Materials and methods

Plant materials

S. spontaneum 'SES 208' is a 2n = 64 form of this species. Fitch and Moore (1983) derived haploids from SES 208 by tissue culture of anthers. *S. spontaneum* 'ADP 85-0068' is one such a haploid that underwent spontaneous doubling of chromosomes during regeneration (Paul Moore, USDA-ARS Aeia, Hawaii, personal communication). A sexual cross was made between SES 208 and ADP 85-0068, from which over 100 progeny were derived at the Hawaiian Sugarcane Planters' Association (Aeia, Hawaii). This constitutes our mapping population. Total genomic DNAs from *S. spontaneum* SES 208, ADP 85-0068, and the derived progeny were extracted according to Honeycutt et al. (1992). DNA concentrations were determined by averaging three spectrophotometric readings at 260 nm. DNA concentrations were standardized at 50 ng μl^{-1} in H_2O and finally checked on an agarose gel, after which final corrections were made as necessary.

DNA amplification protocols

All amplifications were done in 30-µl reaction volumes in a System 9600 cycler (Perkin-Elmer). Amplifications using *Taq* polymerase contained 1 U *Taq* polymerase (AmpliTaq, Perkin-Elmer), 30 ng template DNA, 0.1 mM of each dNTP (Pharmacia), 2.0 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 8.3. Amplifications using the AmpliTaq Stoffel fragment (Perkin-Elmer) contained 2 U Stoffel fragment, 30 ng template DNA, 0.1 mM of each dNTP, 4.0 mM $MgCl_2$, 10 mM KCl, and 10 mM Tris-HCl, pH 8.3. Amplifications using the *Pfu* polymerase (Stratagene Cloning Systems) contained 1 U polymerase in 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, and 100 µg ml^{-1} bovine serum albumin. Oligonucleotide primers (10-mers, Operon Technologies; 12-mers synthesized by Genosys) were used at 0.22 µM in all reactions.

Unless otherwise noted, the temperature profile to which all reactions were submitted was: 94 °C/3 min, followed by 40 cycles of 94 °C/1 min, 35 °C/1 min, then increase to 72 °C with a ramp of 0.41 °C extension for 7 min. Amplification products were maintained at 12 °C until loaded onto a gel.

When amplification products were labelled for subsequent autoradiography, 1 µCi of α^{32}[P]dCTP (3,000 mCi/mmol – NEN) was included in each reaction. Unless otherwise noted, agarose gels were composed of 1.4% (full-length gels) or 2% (half-length) LE agarose (FMC) dissolved in 0.5x or 1x TBE (Maniatis et al. 1982) and were run in a model HRH gel box (International Biotechnologies Incorporated). Full-length agarose gels were run for 1,300 V × h; half-length gels were run for 550 V × h. Optimal resolution of products was obtained using voltage gradients of 5–8 V cm^{-1}. Denaturing polyacrylamide-gel electrophoresis was done in a sequencing apparatus (Hoefer Scientific Instruments) using gels composed of 4% polyacrylamide, 8 M urea in 1x TBE. Polyacrylamide gels were 0.35 mm thick and were run at a constant power (50 W) for 3.5–4 h, after which autoradiography was done for 12–24 h without the need for gel drying. For agarose gels, 15–20 µl of reaction products were loaded into each well. For polyacrylamide gels, 4 µl of reaction products were added to 14 µl of stop dye containing 50% formamide, the products were denatured at 85 °C for 5 min, immediately put on ice, then 2 µl were loaded into each well.

Automated reaction preparation

Besides manually preparing and loading amplification products, we also used a Tecan RSP 5032 (Tecan/SLT) two-arm robot liquid handling station to prepare and load reactions. In this case, the robot first took 10 µl aliquots of template (30 ng total) from an array and loaded them into the System 9600 array. Then, it took 20 µl from a master reaction mix that was composed of the previously described components at 1.5x concentration. After thermal cycling was complete, the tubes were uncapped and the robot loaded the samples onto agarose gels. Programs were also written to allocate primers into tubes for screening against SES 208 and ADP 85-0068 DNAs. For primer-pair amplifications (Welsh and McClelland 1991), the robot assembled arrays containing equimolar concentrations of two primers, then two reaction mixes containing the appropriate template DNAs were added.

Genetic mapping of AP-PCR single-dose polymorphisms

To determine the linkage relationships of markers that were detected by our approach, we ran MapMaker (Lander et al. 1987) using a minimum L.O.D. score of 4.00 and a maximum θ value of 0.25.

Results and discussion

In a pilot study we compared the output of AP-PCR (Welsh and McClelland, 1990) and RAPD (Williams et al. 1990) protocols, as originally reported by the authors, except that the temperature profile to which the reactions were submitted was altered as described in Material and methods. Using the temperature profile and conditions reported by Williams et al. (1990), nearly one-half of the 10-mers we screened against the mapping parents failed to give products; similar results were observed by Klein-Lankhorst et al. (1991) in genetic mapping of tomato using AP-PCR. This may be caused by differences in the temperature profiles of different cyclers. We fixed primer length at ten bases and tested various modifications to the temperature profile using 20 different primers. Data for optimization of the temperature profile were acquired using recombinant AmpliTaq polymerase. Results using

native *Taq* polymerase were not as reliable, and we therefore discontinued its use. In summary, temperature profile optimization showed that: (1) annealing temperatures of 15 °C, 30 °C, 35 °C, and 40 °C did not cause significant changes in the fingerprint, in agreement with Welsh and McClelland (1990), although some fragments were lost or gained at the temperature extremes; (2) there was a major effect of ramp time from the annealing step to the extension step, in agreement with Klein-Lankhorst et al. (1991), i.e., the longer it takes for the temperature to change from 35 °C to 72 °C, the more primers give reproducible, robust fingerprints; (3) extension times longer than 90 s did not significantly improve resulting fingerprints; (4) 40 cycles were sufficient for good results in 30 µl reactions; and (5) the fingerprints were extremely reliable (we have repeated some primers as many as ten times on two genotypes of sugarcane without observing significant differences).

Primer length and concentration are the most striking differences between the original AP-PCR and RAPD protocols. We fixed primer length at 12 and 20 bases and studied the effect of a 50-fold greater primer concentration in the initial steps of AP-PCR (10 µM) when compared to the RAPD (0.2 µM) protocol. Representative results of a primer titration for 12-mers are shown in Fig. 1. Decreasing the primer concentration caused a decrease in the number of products observed, and the fragments lost as primer concentration decreases were mostly in the lower molecular weight range. This has also been observed with longer primers (John Welsh, CIBR, personal communication; Sobral, unpublished data). In addition, fingerprints produced at lower primer concentrations were more erratic, in that more failed lanes were observed. In general, when using *Taq* or *Pfu* polymerases, primer concentrations of 0.10 µM caused significant failures, and some primers did not work reliably at this concentration.

More amplification fragments might be expected to yield more polymorphic fragments, if the ratio between total number of products and number of polymorphic products is constant for a given pair of DNA templates. Therefore, we might expect that having more fragments would increase the output of polymorphisms in mapping experiments, or the number of informative characters for phylogenetic experiments. Table 1, which summarizes the results of the pilot study, shows that this was not the case. Although more fragments were observed at higher primer concentrations, the number of polymorphisms per primer was approximately the same for both protocols despite the much larger number of fragments observed using higher primer concentrations and autoradiography.

We do not understand why the extra fragments generated using higher primer concentration, as in the original AP-PCR protocol, do not yield higher numbers of polymorphisms per primer. This result suggests that these fragments are products that are not sensitive to sequence variation in the template genome, which is rather bewildering. If these additional fragments were strictly a product of sequence variation of the genome being surveyed, then it would be expected that the number of polymorphisms per primer would be directly related to the total number of fragments amplified. Some of the additional fragments observed in the original AP-PCR setup are a direct consequence

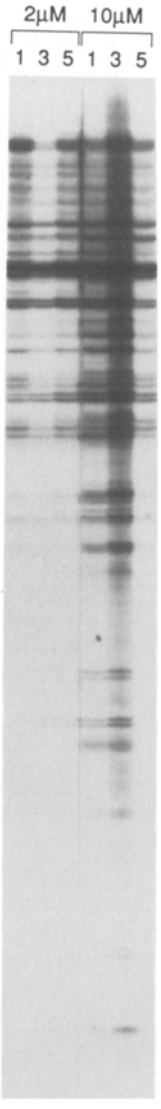

Fig. 1. Effect of primer concentration on the AP-PCR fingerprint. A 12-mer was used at two different concentrations on three different concentrations of template DNA (*S. spontaneum* 'ADP 85-0068'): 1, 3, and 5 ng μl^{-1}. Titration of primer to 0.2 µM caused all but the most intense amplification products to disappear (data not shown). PCR conditions are described in Materials and methods

Table 1. Comparative output of AP-PCR and RAPD protocols

Item	AP-PCR	RAPD
Number of primers tested	18	41
Number of loci screened	482	355
Number of informative primers	12	30
Number of polymorphisms	31	67
Single-dose polymorphisms	16[a]	15[b]
Average polymorphisms per primer	2.58	2.23
Average single-dose polymorphisms per primer	1.33	1.88

[a] The number of single-dose polymorphisms was derived from the 12 informative primers, each tested on 35 progeny from the mapping population

[b] The number of single-dose polymorphisms was derived from the eight most informative primers, each tested on 22 progeny from the mapping population

of the higher resolution and sensitivity of the gel and detection systems, but other fragments are clearly a product of the higher primer concentrations (Fig. 1). It is possible that some of these smaller products may be caused by interactions that do not reflect the level of polymorphism of the genome.

What was desired, then, was a protocol that would increase the total number of loci screened and the number of polymorphisms per primer. To increase the number of polymorphisms per primer, we tested alternate thermostable polymerases using the optimized temperature profile. We also tested pairs of thermostable polymerases in the same reaction. The Stoffel fragment is a 61 kDa modified form of recombinant AmpliTaq polymerase from which the 289 N-terminal amino acids have been deleted such that it lacks 5′–3′ exonuclease activity. The manufacturer claims that the Stoffel fragment is two-fold more thermostable than AmpliTaq and that it displays optimal activity over a broader range of Mg^{++} concentrations. *Pfu* polymerase is a thermostable polymerase from *Pyrococcus furiosis* that has 5′–3′ DNA polymerase activity as well as 3′–5′ exonuclease activity.

Reaction products obtained using the Stoffel fragment ranged from 0.1 to 1.5 kb in size, whereas the AmpliTaq and *Pfu* products generally ranged from 0.5 to 2.5 kb. This reduction in average size of products obtained with the Stoffel fragment might be expected because of the lower processivity of the enzyme (Erlich et al. 1991). In addition, the number of useful primers increased with the Stoffel fragment, in relation to both AmpliTaq and *Pfu*. We screened a total of 40 primers using AmpliTaq, 96 primers using *Pfu*, and 144 primers using the Stoffel fragment. Twelve of forty primers (30%) gave no products with AmpliTaq, 33 of 96 (34%) failed with *Pfu*, and 4 of 144 (2.8%) failed with the Stoffel fragment. DNA fragments amplified by the Stoffel fragment, being smaller, are more suited to being resolved on high-resolution polyacrylamide gels (with visualization using autoradiography or silver staining) than are AmpliTaq or *Pfu* products.

Because the products from Stoffel reactions and AmpliTaq or *Pfu* reactions were of different sizes, we tried using both Stoffel and *Pfu* or AmpliTaq in the same reaction to see whether all or most of the fragments amplified by each enzyme individually could be obtained from one reaction. Typical results for three primers are displayed in Fig. 2. Briefly, the use of Stoffel in conjunction with *Pfu* or AmpliTaq did not allow the amplification of all the products that each enzyme yields when used alone. We tried two different amounts of enzyme per reaction, though we kept the Stoffel: *Pfu* ratio constant at 2:1. The absolute amounts of enzyme influenced the resultant fingerprint; some polymorphic fragments were lost, others were gained, though the overall pattern was similar. The buffer used also affected the results. We used the Stoffel + *Pfu* combination in Stoffel buffer as well as in *Pfu* buffer, and some differences were observed. Given the results using combinations of thermostable polymerases, we do not think that mixing polymerases is a useful strategy.

We chose the ten primers that gave the most polymorphisms with AmpliTaq and the 20 primers that gave most polymorphisms with the Stoffel fragment and used them against the mapping population. With the Stoffel fragment, the average number of loci screened per primer increased by 30% and, even more surprisingly, the average number of polymorphisms per primer increased by 80% (Table 2) relative to AmpliTaq. Note that four of the primers used to compile the data in Table 2 have been tested with both enzymes. Four primers, an average of 11 loci were screened and an average of 2.3 polymorphisms were observed if AmpliTaq was used, while averages of 15.8 loci screened and 3.8 polymorphic fragments were obtained using the Stoffel fragment in place of AmpliTaq. Figure 3 shows a comparison of the finger-

Table 2. Comparative mapping output using AmpliTaq and Stoffel fragment

Item	AmpliTaq	Stoffel
Number of primers tested	7	14
Number of loci screened	80	207
Number of polymorphisms observed[a]	14	57
Average number of loci per primer	11.4	14.8
Average number of polymorphisms per primer	2.0	3.6

[a] Polymorphisms were identified by comparing the fingerprints of SES 208 with those of ADP 85-0068, the other mapping parent

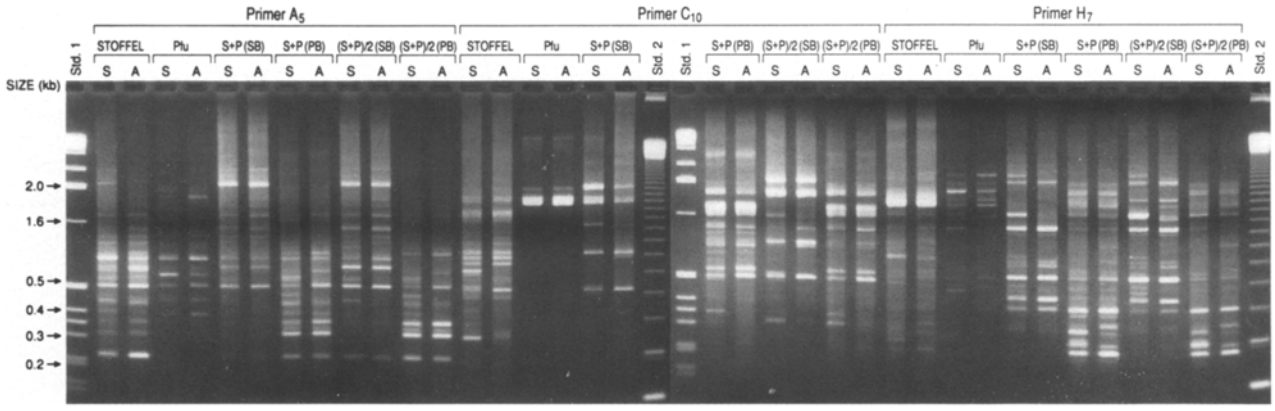

Fig. 2. Effect of different polymerases and buffers on AP-PCR fingerprints. Three 10-mers, A5, C10, and H7, and two template DNAs [mapping parents, SES 208 (*S*) and ADP 85-0068 (*A*)] were used with either the Stoffel fragment alone, *Pfu* alone, the Stoffel fragment plus *Pfu* in Stoffel fragment buffer [$S + P(SB)$], or the Stoffel fragment plus *Pfu* in *Pfu* buffer [$S + P(PB)$]. The Stoffel fragment was used at a concentration of 2 U per reaction and *Pfu* was used at a concentration of 1 U per reaction, except in the lanes $(S + P)/2$, in which 1 U of Stoffel fragment and 0.5 U of *Pfu* per reaction were used. Std. 1 is the 1-kb DNA ladder (BRL) and Std. 2 is the 123-bp ladder (BRL); standard lanes contained 1 μg of DNA. Thermal cycling parameters and other information about the reactions is given in the Materials and methods

prints produced by AmpliTaq, *Pfu*, and the Stoffel fragment using one representative primer. The majority of the products migrated to different positions on the gel, suggesting they represent different loci that are selected for amplification by the different enzymes. This has been a general observation when we have used the same primer with different polymerases. However, one amplified polymorphic DNA fragment that migrated to the same position on the gel when AmpliTaq and the Stoffel fragment were used. These polymorphisms mapped very close to each other (Fig. 4) and, the slight discrepancy in their map positions is most likely due to differences in failed lanes.

To show that the single-dose AP-PCR polymorphisms could be used to construct a genetic linkage map, we selected some of the more informative primers and used them on the entire mapping population. In each PCR run, there were four repetitions each of the two parents, SES 208 and ADP 85-0068, at template concentrations that covered a five-fold range. We only scored the progeny for polymorphisms that were reliably observed in all four repetitions. We discarded the 'sporadic' bands that were occassionally observed in some of the repetitions because these were usually influenced by template concentration. This approach allows the confidence level to be increased, especially because the progeny are only scored once for each polymorphism in mapping situations. The segregation data was used to run MapMaker and the resulting linkage groups are shown in Fig. 4. Approximately 70% of all polymorphisms present in SES 208 yet absent in ADP 85-0068 were single-dose fragments. This number agrees with the ratio obtained using the RFLP approach in this same population (Burnquist 1991). However, RFLP single-dose fragments require 300-fold more DNA per lane, plus a 7–10 day exposure of autoradiograms, in addition to the normally long time spent on gel preparation, blotting, etc. (Burnquist 1991). Linkage was also detected between single-dose RFLPs (Burnquist 1991) and AP-PCR fragments. A high density AP-PCR map of this cross will be published elsewhere.

The reason for increased mapping output using the Stoffel fragment is unknown. However, Erlich et al. (1991) pointed out that genetically engineered variants of *Taq* polymerase might reveal properties that would be valuable to specific applications. It may be that the Stoffel fragment is more sensitive to mismatches between primer and template than are AmpliTaq of *Pfu* polymerases, although it would be expected that fewer fragments would be amplified if this were ture. There are differences in Mg^{++} concentration of Stoffel reactions relative to AmpliTaq reactions, but we have also done Stoffel reactions in AmpliTaq and *Pfu* buffers, with lower Mg^{++} levels, and higher output with Stoffel was still observed.

Not only is the output using the Stoffel fragment and an optimized temperature profile nearly two-fold higher than that of reported protocols (Welsh and McClelland 1990; Williams et al. 1990), but the cost of data acquisition is significantly reduced because 1 U of AmpliTaq currently costs $0.62, whereas 2 U of Stoffel cost $0.39 (Perkin-Elmer catalog). Further savings can be obtained by reducing the volume of the reaction. Successful reactions have been done in 13-μl volumes (Ron Sederoff, North Carolina State, personal communication). Such a reduction in volume

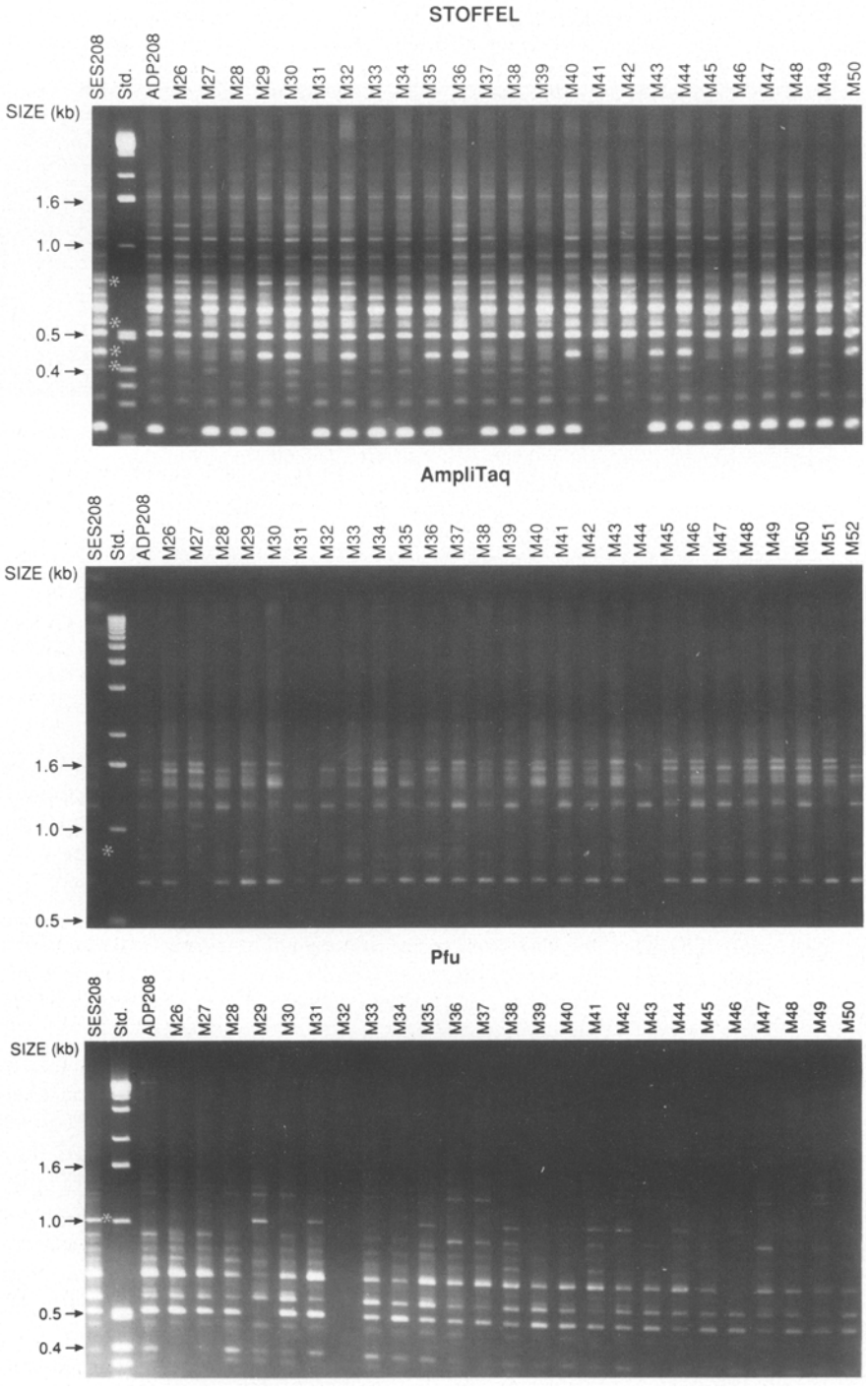

Fig. 3. Comparison of Stoffel, AmpliTaq, and *Pfu* polymerases using one 10-mer. Using the optimized thermal cycling profile and reaction conditions described in the text, primer A5 was used with all three polymerases and the entire mapping progeny; one of the four gel panels is shown here with the polymorphisms indicated by white asterisks to the right of the SES 208 lane. In this case, the Stoffel fragment allowed the scoring of four polymorphisms, and AmpliTaq and *Pfu* each revealed one polymorphism. None of these polymorphisms have the same map location (Al-Janabi et al., submitted)

would lower the polymerase cost per reaction to approximately $0.17. In addition, the next generation of liquid handling robots will be capable of accurately handling even smaller volumes (David Juranas, Tecan/SLT, personal communication).

Although we have increased the output of polymorphisms significantly, the bottleneck is rapidly becoming data acquisition. If data acquisition becomes automated, then using our protocol and a robot it would be possible to make a 200-marker genetic map for a diploid organism that had a similar level of polymorphism with less than 60 useful primers, without even using primers in pairwise combinations. In sugarcane, these 60 primers could be picked from screening

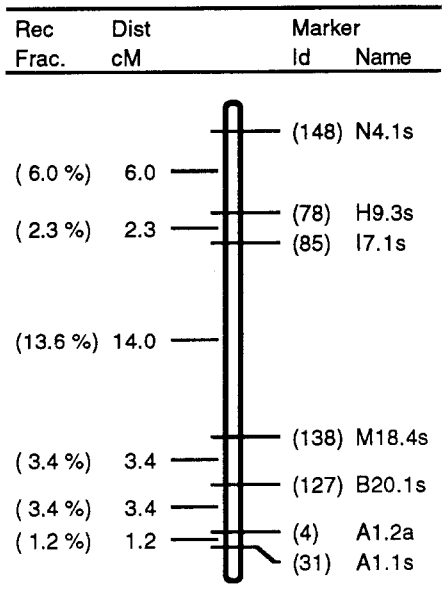

Fig. 4. One linkage group from a map being generated (Al-Janabi et al., in preparation) using AP-PCR and RFLP single-dose polymorphisms. This linkage group is composed exclusively of AP-PCR polymorphisms and includes the two similar sized polymorphisms that were produced by AmpliTaq and Stoffel fragment, *A1.2a and A1.1s*, respectively

approximately 100–180 primers in less than three full runs of the robot (much less than 1 day's work). Note that in our case with sugarcane, we can only map polymorphisms derived from one of the parents (SES 208). Furthermore, if a robot were to work at the rate of five runs/day (less than 5 h to setup reactions and load gels), then the primary data would be ready in 12 days, even with our output. Such potential productivity is especially important to those wishing to apply methodologies that have been developed to study marker-trait associations, such as bulked segregant analysis (Michelmore et al. 1991) and the use of near-isogenic lines (Martin et al. 1991; Yu et al. 1991), in combination with AP-PCR technology. Hundreds of primers, or pairwise combinations of primers, would allow the screening of thousands of loci in a large number of individuals in very few days. Marker-trait associations could be established with data from more individuals than before because of the increased output and lower cost per rection.

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References

Burnquist WL (1991) Development and application of restriction fragment length polymorphism technology in sugarcane (*Saccharum* spp.) breeding. PhD dissertation, Cornell University

Erlich HA, Gelfand D, Sninsky JJ (1991) Recent advances in the polymerase chain reaction. Science 252:1643–1651

Fitch MM, Moore PH (1983) Haploid production from anther culture of *Saccharum spontaneum* L. Z Pflanzenphysiol S:197–206

Honeycutt RJ, Sobral BWS, Keim P, Irvine JE (1992) A rapid DNA extraction method for sugarcane and its relatives. Plant Mol Biol Rep 10:66–72

Klein-Lankhorst RM, Vermunt A, Weide R, Zabel P (1991) Isolation of molecular markers for tomato (*L. esculentum*) using random amplified polymorphic DNA (RAPD). Theor Appl Genet 83:108–114

Lander ES, Green P, Abrahamson, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174–181

Maniatis T, Fritsch E, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Martin GB, Williams JGK, Tanksley SD (191) Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. Proc Natl Acad Sci USA 88:2336–2340

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Neale D, Sederoff R (1991) Genome mapping in pines takes shape. Probe 1:1–3

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 10:1350–1354

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18:7213–7218

Welsh J, McClelland M (1991) Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combinations of primers. Nucleic Acids Res 19:5275–5279

Welsh J, Honeycutt RJ, McClelland M, Sobral BWS (1991a) Parentage determination in maize hybrids using the arbitrarily-primed polymerase chain reaction (AP-PCR). Theor Appl Genet 82:473–476

Welsh J, Petersen C, McClelland M (1991b) Polymorphisms generated by arbitrarily-primed PCR in the mouse: application to strain identification and genetic mapping. Nucleic Acids Res 19:303–306

Welsh J, Pretzman C, Postic D, Saint-Girons T, Baranton G, McClelland M (1992) Genomic fingerprinting by arbitrarily-primed PCR resolves *Borrelia burgdorferi* into three distinct phyletic groups. Int J Syst Bacteriol 42:370–377

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Wu KK, Burnquist W, Sorrells ME, Tew TL, Moore PH, Tanksley SD (1992) The detection and estimation of linkage in polyploids using single-dose restriction fragments. Theor Appl Genet 83:294–300

Yu ZH, Mackill DJ, Bonman JM, Tanksley SD (1991) Tagging genes for blast resistance in rice via linkage to RFLP markers. Theor Appl Genet 81:471–476