Generation of Sequence Signatures from DNA Amplification Fingerprints with Mini-Hairpin and Microsatellite Primers

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ABSTRACT

DNA amplification fingerprinting (DAF) with mini-hairpins harboring arbitrary “core” sequences at their 3’ termini were used to fingerprint a variety of templates, including PCR products and whole genomes, to establish genetic relationships between plant taxa at the interspecific and intraspecific level, and to identify closely related fungal isolates and plant accessions. No correlation was observed between the sequence of the arbitrary core, the stability of the mini-hairpin structure and DAF efficiency. Mini-hairpin primers with short arbitrary cores and primers complementary to simple sequence repeats present in microsatellites were also used to generate arbitrary signatures from amplification profiles (ASAP). The ASAP strategy is a dual-step amplification procedure that uses at least one primer in each fingerprinting stage. ASAP was able to reproducibly amplify DAF products (representing about 10–15 kb of sequence) following careful optimization of amplification parameters such as primer and template concentration. Avoidance of primer sequences partially complementary to DAF product termini was necessary in order to produce distinct fingerprints. This allowed the combinatorial use of oligomers in nucleic acid screening, with numerous ASAP fingerprinting reactions based on a limited number of primer sequences. Mini-hairpin primers and ASAP analysis significantly increased detection of polymorphic DNA, separating closely related bermudagrass cultivars and detecting putatively linked markers in bulked segregant analysis of the soybean (Glycine max) supernodulation (nitrate-tolerant symbiosis) locus.

INTRODUCTION

Genomic scanning strategies use arbitrary oligodeoxynucleotide primers to target discrete sites characteristic of a nucleic acid template and to generate molecular markers for genome mapping and general fingerprinting applications (6,22,23). DNA amplification fingerprinting (DAF), one of such strategies, uses primers as short as 5 nucleotides (nt) in length to produce DNA profiles with high multiplex ratios (6). Extraordinarily stable mini-hairpin primers harboring a “core” arbitrary sequence at the 3’ terminus can also be used to amplify a wide range of templates ranging from plasmid DNA to plant and animal genomes (5). These arbitrary mini-hairpin primers increase detection of polymorphic DNA and direct the controlled amplification of small template molecules, thereby generating “sequence signatures” from plasmids, yeast artificial chromosomes (YAC), cloned DNA and polymerase chain reaction (PCR)-amplified fragments. In this study we explore the use of mini-hairpin primers with short arbitrary cores in fingerprinting, and generate arbitrary signatures from amplification profiles (ASAP) using both mini-hairpin primers or primers complementary to simple sequence repeats (SSR). This ASAP approach produced “fingerprints of fingerprints” that significantly increased the detection of polymorphic DNA, distinguishing closely related bermudagrass cultivars and detecting linked markers in bulked segregant analysis (BSA: 16) of the soybean nitrate-tolerant symbiosis (nts-1) locus.

MATERIALS AND METHODS

Centipedegrass (Eremochloa ophiuroides) cultivars have been described previously (20). Bermudagrass cultivars were Cynodon dactylon (Tifton 10, Texturf 10, Vamont and Common), C. magennisii (Sunturf), two african C. transvaalensis selections (Ctr2570 and Ctr2747) and interspecific C. dactylon x C. transvaalensis crosses (Tifgreen, Tifdwarf, Tiffine, Tifway II and Midiron) (10). Discuta destructiva fungal isolates were GA-1, TN-1, MA-11 and SC-101 (18). Glycine soja PI468397 was crossed with G. max nts382, a supernodulating ethylmethane sulfonate (EMS)-induced mutant of soybean cv. Bragg, to produce F₂ and F₃ segregating populations. DNA was extracted using established protocols (cf. Reference 5), and the concentrations were measured using a TKO100 fluorometer by fluorescence enhancement of dye H33258 (Hoefer Scientific, San Francisco, CA, USA).

DAF reactions were in a total volume of 10–25 µL (usually 20 µL) (4) and contained 3 µM primer, 0.3 units/µL AmpliTaq® Stoffel Fragment DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), 200 µM of each deoxynucleoside triphosphate (United States Biochemical, Cleveland, OH, USA), 0.1 to 5 ng/µL of template DNA, 1.5 mM MgCl₂ and Stoffel buffer (10 mM KCl, 10 mM Tris-HCl; pH 8.3) when using unstructured primers, or 4 mM MgSO₄ and TTNK10 buffer (10 mM KCl, 4 mM (NH₄)₂SO₄, 0.1% Triton® X-100, 20 mM Tris-HCl; pH 8.3) (9) when using mini-hairpin primers. The mixture was amplified in a recirculating hot-air thermal cycler (Bios Laboratories, New Haven, CT, USA) for 35 cycles of 30 s at 96°C, 30 s at 30°C and 30 s at 72°C. ASAP reactions contained the same components as DAF reactions, but primer concentrations were usually kept at 9 µM and template concentrations at about 0.1 ng/µL (usually corresponding to a final 1:1000 dilution of a standard DAF reaction). ASAP
reactions with SSR primers were amplified for 35 cycles of 30 s at 96°C, 60 s at 55°C and 30 s at 72°C and using 3 µM primer concentration.

Amplification products were separated in polyester-backed 5% polyacrylamide–7 M urea slab mini-gels (4). Wells were generally loaded with 3 µL of a 1:5 dilution of each amplification mixed with 3 µL of loading buffer (10 M urea, 0.08% xylene cyanol FF), and electrophoresis was run at 120 V for approximately 75 min. ASAP reactions using SSR primers were diluted 1:50 prior to electrophoresis. DNA was detected by silver staining (2) according to a slightly modified protocol (1), and backed gels were preserved by drying at room temperature. Amplification products were isolated directly from the silver-stained polyacrylamide gel (19). Denaturing 20% polyacrylamide–7 M urea gels and silver staining were used to determine the electrophoretic mobilities of oligomers (14). Data obtained from segregating populations were analyzed using Mapmaker Version 1.0 for Macintosh® (E.I. DuPont de Nemours, Wilmington, DE, USA). Genetic relationships between cultivars were examined by phylogenetic analysis using parsimony (PAUP, Version 3.1; D.L. Swofford, Illinois Natural History Survey, Champaign, IL, USA). DAF bands (50–700 bp in length) in gels loaded with diluted amplification reactions were scored as present [1] or absent [0], entered as unordered, nondirected and unweighted characters (Wagner mode for binary characters), and analyzed with an exhaustive search for minimal trees. Distance matrices showing absolute and relative number of nonshared bands, and homoplasy values were calculated.

RESULTS

DAF with Mini-Hairpin Primers with Short Arbitrary Cores

Closed sets of primers harboring a constant mini-hairpin at their 5’ termini and an arbitrary core of only 3 nt were synthesized and used to direct amplification of a wide variety of templates, ranging from amplification products isolated directly from polyacrylamide gels to whole genomes of fungal and plant origin. DNA profiles obtained from several DAF fragments isolated from fingerprints of dogwood

![Figure 1. Genetic relationships between five centipedegrass cultivars. Each phylogram represents a single minimal tree generated using parsimony analysis (PAUP) from amplification bands (in the 50–700-bp range) produced with 15 unstructured octamers or 9 mini-hairpin primers. Cultivars examined were Tennessee Hardy (TH), Tennessee Tuff (TT), Oklawn (Ok), Centennial (Ct) and Tifton common (Tc). The descriptors of branch length are proportional to the number of inferred changes averaged across all possible character reconstructions.](image-url)
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*(Cornus florida)* cvs. “Cherokee Princess” and “Santamour” (ranging from 100 to 250 bp), fungal isolates of the dogwood-anthracnose pathogen *D. destructiva*, flowering dogwood, several centipedegrass and bermudagrass cultivars, and soybean cv. Bragg were relatively complex, containing about 15–80 amplification products in the 50–700-bp range. The large differences in template complexity (from a few hundred to billions of base pairs) were not followed by corresponding changes in the number of amplification products. This unexpected behavior can only be explained by selective amplification of only a few of the possible targeted genomic sites, rather than by mismatch priming (3), as mismatching is reduced to a minimum with the use of short arbitrary core sequences (5).

The existence of hairpin-turn structures (12) formed on the 5′ terminus of the primers causes them to have abnormally rapid electrophoretic mobilities (5,13,14). A detailed study of the primer series HP7-NNN [HP7 corresponds to the highly stable *(Tm) = 76.5* mini-hairpin GCGAAGC (12,13); N = A, G, C or T] showed the absence of correlation between primer electrophoretic mobility and efficiency of amplification in DAF analysis of soybean cv. Bragg and bermudagrass cv. Tifway (data not shown). Mini-hairpin decamers migrated as hexamer or heptamer oligonucleotides (with apparent lengths ranging 5.7–7.8 nt). Mobilities varied with the sequence of the arbitrary core, and increased in the order A>C>>T=G, A=C>T=G and G>>A>T=C for the first, second and third nucleotide from the 3′ terminus, respectively. Despite mobility fluctuations that paralleled melting temperatures, which were indicative of differ-

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**Table 1. Analysis of Plant DNA with Unstructured Octamers and Mini-Hairpin Decamer Primers**

<table>
<thead>
<tr>
<th>Primers (Number Tested)</th>
<th>DAFb Loci</th>
<th>Loci/Primer</th>
<th>Polymorphic Loci Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soybean Bulked DNAb</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstructured octamers (103)c</td>
<td>2424</td>
<td>24.2 ± 5.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mini-hairpin decamers (64)d</td>
<td>1808</td>
<td>30.6 ± 6.6</td>
<td>2</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Centipedegrass Cultivarsc</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstructured octamers (15)</td>
<td>332</td>
<td>22.1 ± 7.6</td>
<td>45</td>
<td>13.6</td>
</tr>
<tr>
<td>Mini-hairpin decamers (9)</td>
<td>287</td>
<td>31.9 ± 5.2</td>
<td>65</td>
<td>22.6</td>
</tr>
</tbody>
</table>

aAmplification products scored were in the 50–700-bp range.
bComparison of wild-type and supernodulating plants from a soybean F2 population.
cOctamers include the series GTCCANNN. Only 3 primers tested failed amplification.
dPrimers correspond to the series GCGAAGCNNN. Only 5 primers failed amplification.
eData obtained from the analysis of 5 cultivars in the experiment described in Figure 1.

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**Figure 2. Effect of primer concentration on ASAP analysis.** Panel A: Replicate DAF amplifications of soybean cv. Bragg DNA with primer GTCCATTT (lanes 1) and GTCCAAATT (lanes 2). The replicated amplifications were from DNA obtained in separate extractions, and were further used in Panels B and C. Panel B: Effect of primer concentration during reamplification of DAF profiles generated with primer GTCCATTT (Panel A), with primers GTAACCCC or the mini-hairpin decamer HP7-TCC (HP7=GCGAAGC). Note inconsistent minor amplification products at low octamer primer concentrations. Panel C: Effect of primer concentration during reamplification of DAF profiles, generated with primer GTCCAAATT (panel A), with mini-hairpin primer HP7-GTC. Molecular weight standards (M) are given in kb.
ences in the stability of terminal mini-
hairpins (14), the majority of primers
produced complex fingerprints with a
balanced number of amplification
products of high and low intensity
(ranging 15–43 in the 50–700-bp inter-
val). Only 5 out of the 64 primers test-
ed failed to amplify soybean or
bermudagrass DNA or did so ineffi-
ciently.

Mini-hairpin primers distinguished
plant taxa at the interspecific and in-
traspecific levels. For example, a con-
siderable number of polymorphisms
were observed between and within C.
dactylon, C. transvaalensis and C. ma-
gennissii cultivars, as well as C. dacty-
lon x C. transvaalensis hybrids (data
not shown).

Mini-Hairpin Primers Enhance
Detection of Polymorphic DNA

On average, the number of amplifi-
cation products in the 50–700-bp inter-
val, obtained with mini-hairpin primers
and separated on polyacrylamide mini-
gels, was 20–60% higher than the
number produced with unstructured
primers. For example, mini-hairpin
primers generated 26% more products
in soybean than standard DAF primers
(Table 1). Mini-hairpin primers also de-
tected polymorphic DNA with high ef-
iciency (Table 1). Higher (twofold to
fourfold) levels of polymorphic DNA
were found in a previous study of cen-
tipedegrass (5) and in the identification
of closely related bermudagrass culti-
vars (10). Similarly, several fungal iso-
lates of D. destructiva (GA-1, TN-1,
MA-11 and SC-101) were distin-
guished using a few mini-hairpin
primers (not shown). These isolates
were very difficult to separate, even af-
ter the use of a considerable number of
standard arbitrary primers (18). The
usefulness of mini-hairpin primers was
particularly evident in BSA. A screen
of soybean BSA phenotypic pools of
the supernodulating nts-1 locus with
103 octamer primers (Table 1) failed to
produce new polymorphic markers. In
contrast, the HP-7NNN series (64
primers) revealed two clear polymor-
phisms.

Fingerprinting analysis of five cen-
tipedegrass cultivars with 15 unstruc-
tured octamers and 9 mini-hairpin
decamers showed again that mini-hair-
pin primers can detect polymorphic

![Figure 3. ASAP with mini-hairpin decamers with all possible single-base substitutions in the arbi-
trary core. DAF profiles originally generated from soybean cv. Bragg DNA with the mini-hairpin
primer HP-7-AGA (HP-7=GCGAAGC) were reamplified with mini-hairpin decamers with all possible single-
base substitutions (underlined) in the arbitrary core AGA sequence. Mismatching in amplicon termini
appears favored over scanning of internal sequences. Molecular weights standards (M) are given in kb.](image)
DNA with high efficiency, distinguishing plant material at the intraspecific level (Table 1). Parsimony analyses of amplification loci generated with these primers were used to delineate genetic relationships between these established cultivars. Comparison of resulting phylograms showed that the inferred genetic relationships between the cultivars, obtained using either unstructured or mini-hairpin primers, were identical (Figure 1). Centipedegrass phylograms were also comparable to those obtained in a previous study with unstructured primers (20). Results suggest that mini-hairpin decamers are unbiased in their targeting of amplicons and behave similarly to standard DAF primers.

**Fingerprints of Fingerprints: Sequence Signatures Amplified from DAF Profiles**

Specific signatures (ASAP) were obtained from amplification products by reamplification of DAF profiles with mini-hairpin or standard arbitrary primers. ASAP from DAF profiles generated with mini-hairpin decamers or standard octamers were obtained reliably both from a same or replicated DAF amplification or from different DNA extractions (Figure 2). However, primer concentration appeared crucial for ASAP reproducibility. Standard octamers required at least 9 µM concentrations. Mini-hairpin decamers required 6–9 µM primer concentrations (see Figure 2C), unless primers had core sequences partially complementary to DAF product termini. In such cases a 3 µM primer concentration suffices. Higher primer levels appeared to increase fingerprint complexity (Figure 2B), and sometimes better separation procedures were needed to produce resolvable fingerprints. Slight changes in fingerprint patterns were sometimes observed at high primer levels. ASAP was quite tolerant of template concentration within the range 0.001 to 1 ng/µL. Tolerance was also observed in ASAP produced at low primer concentration from amplifications with mini-hairpin primers with core sequences partially complementary to product termini (not shown). In contrast, ASAP produced with more distinct core sequences required higher primer concentrations in order to avoid restriction of the range of optimal template concentrations.

Similar profiles were obtained when mini-hairpin decamers with all possible single-base pair substitutions in the arbitrary core were used to amplify DAF patterns generated from soybean (Figure 3) or bermudagrass DNA (not shown) with the mini-hairpin primers HP$_7$-CTG or HP$_7$-AGA. This suggests that mismatching in amplicon termini is favored over scanning of internal sequences. Therefore, the use of ASAP primers with sequences partially complementary to DAF product termini should be avoided in fingerprinting applications.

**ASAP Enhances Detection of Polymorphic DNA**

The usefulness of ASAP was again demonstrated in BSA of the nts-1 locus. The nts-1 region appears highly conserved, as demonstrated by the scarcity of markers obtained from the coupling of BSA and DAF (Table 1). Despite this fact, ASAP, with just a few mini-hairpin primers, identified several polymorphisms between the bulks (Figure 4, A and B). The use of a limited number of primer sequences provided numerous fingerprinting reactions. About 8 putative markers were detected with 10 ASAP, produced with 7 octamers and mini-hairpin decamers, from DAF profiles generated with 2 octamer primers; three of them were tested in a small segregating population of ten individuals and behaved as Mendelian markers. The different combinations tested (10 out of 81 possible ASAP)
Figure 5. Effect of length of mini-hairpin primer core on the amplification of products with defined termini. DAF products obtained from soybean cv. Bragg DNA using an octamer and mini-hairpin primers with derived arbitrary cores (lanes 1) [their sequences are indicated above bars and over lanes], were amplified in ASAP reactions with primers CCGAGCTG (lanes 2), HP_{7}-CCGAGCTG (lanes 3) and HP_{7}-CTG (lanes 4) and other related primers (data not shown). Primers HP_{7}-TG, HP_{7}-G and HP_{7} were incapable of amplifying any of the engineered templates. A 1:100 dilution of amplification products was used as template in all cases. Amplification products were subjected to a 1:10 dilution prior to electrophoresis, because of the high reamplification efficiencies. Profiles did not result from the amplification of the low levels of original soybean DNA template present in the reaction, since such low concentrations were unable to produce fingerprints per se. Similarly, failing to add primer during initial amplification did not allow reamplification. Profiles do not result either from contaminant DNA present in enzyme preparations, because no product was generated when template was omitted in the reaction. Molecular weights standards (M) are given in kb.
always rendered distinct fingerprints.

ASAP were also generated by reamplification of DAF fingerprints with degenerate 5′-anchored SSR primers, such as NN(AG)$_6$, NN(CT)$_6$ and NN(TG)$_6$. The primers produced informative profiles, but amplification of arbitrary sequences unrelated to the SSR motif (cf. Reference 25) was considerably decreased when only a few abundant amplification products were obtained (in about 50% primer combinations tested). Figure 4B shows a 185-bp polymorphism between BSA pools that was produced using the SSR primer NN(AG)$_6$, segregated as a Mendelian marker, and mapped 40 cM from marker pa-381, away from the nts-1 locus. ASAP with SSR primers also differentiated closely related bermudagrass cultivars (Figure 4C). In a previous study, cultivars Tifway II (a γ-irradiation-induced mutant of Tifway) and Tifdwart (a natural somatic mutant from Tifgreen) proved very difficult to distinguish from their close parents (10). An extended screen of 81 octamer primers showed only 4 primers capable of separating these lines. ASAP analysis with only 3 primers identified several polymorphisms, some co-dominant (Figure 4C) and useful for the identification of a group of 13 established bermudagrass cultivars (data not shown).

### Importance of the Terminal Symmetry of Amplification Products Used as Templates in ASAP Reactions

ASAP was finally used to investigate the role of the mini-hairpin and arbitrary core sequence during amplification. Mini-hairpin primers with cores of varying length were used to produce DAF products engineered to have terminal symmetries of between 7 and 15 nt. DNA amplifications were then used as templates in ASAP reactions using the original primer sets (Figure 5). Primer GCCGACTG successfully reamplified fragments with terminal symmetries homologous to, and thus originally generated by, this octamer (Figure 5, top panel). Primer HP$_7$-CCGAGCTG also produced the same DNA pattern, but with products expressing the 14-nt mobility shift towards high molecular weight expected from the 5′-terminal mini-hairpin. In contrast, HP$_7$-CTG reamplified inefficiently only a few of the products generated with the octamer primer, despite having perfect complementarity to the last three 3′-terminal nucleotides. These results highlight the important role of an 8-nt core sequence (7) in amplicon selection.

All primers were able to reamplify DNA originated with HP$_7$-CCGAGCTG, HP$_7$-GCTG and HP$_7$-CTG (Figure 5, top and middle panels). However, the original amplification pattern was only reproduced when the primer and the product terminal sequence were complementary. The absence of the hairpin or a shorter or longer core sequence resulted in variant fingerprints. Results emphasize the importance of both hairpin and arbitrary core sequence in primer-template interaction.

While HP$_7$-TG, HP$_7$-G and HP$_7$ oligonucleotides were incapable of producing DAF or ASAP profiles from either original or engineered templates, the failed amplifications were successfully used as templates by primers with longer core sequences (Figure 5). The appearance of new amplification products appears to result from the successful amplification of first-round products that were originally poorly amplified. A similar explanation was found for the observation that amplification of engineered templates with 5-base terminal symmetry using sequence-related octamers generated many more products than expected (7).

### DISCUSSION

Mini-hairpin primers can be used to fingerprint a wide variety of templates, ranging from plasmids and PCR products to complex plant and animal genomes. The reliable amplification of small template molecules was tested using plasmid DNA through proper assignment of amplification products to predicted amplicons (5). Since then, we have used these “structured” primers in the fingerprinting of several plant genomes and in genetic mapping applications, where DNA polymorphisms generally segregated as dominant Mendelian markers. We have shown that both arbitrary core and mini-hairpin sequences were determinants of amplification (Figure 5). As previously suggested (5), our results confirm that the mini-hairpin is somehow involved in selecting annealing sites during the primer-template screening phase of the amplification reaction. Genetic relationships established with the use of mini-hairpin primers compared well with those found using standard arbitrary primers (Figure 1), providing functional proof that both primer types are comparable in their ability to screen genomes. Our results also validate the use of amplification products obtained with mini-hairpin primers as estimators of genetic diversity or similarity, provided they depict actual nucleotide divergence by following established criteria (11). Finally, we have confirmed that mini-hairpin primers detect polymorphic DNA with high efficiency (Table 1). The enhanced resolving power of mini-hairpin primers may result from an increase in the size of the genome being probed, as the annealing of these primers appears to be influenced by secondary structure of DNA and interactions between amplicon termini (5).

The present study introduces ASAP analysis as a novel “fingerprint-tailoring” strategy that increases detection of polymorphic DNA. The technique is based on the ability of mini-hairpin primers harboring short arbitrary cores to fingerprint genomes indirectly through the generation of reliable “sequence signatures” from DAF profiles. These ASAP constitute “fingerprints of fingerprints” that provide additional scanning of primary sequences. The ASAP approach produced reproducible fingerprints that were, however, dependent on appropriate optimization of amplification reaction components, mainly primer and template concentration, and markers that were predominantly dominant.

ASAP analysis was used here to generate markers for the positional cloning of the supernodulation nts-1 locus in soybean, a trait that segregates as a single recessive Mendelian locus and controls root nodule number and mass by a shoot-mediated systemic mechanism. Markers associated with a trait or genomic region can be directly...
identified by BSA with pooled DNA samples from individuals in a segregating population that express or fail to express a particular phenotype. We have coupled BSA and DAF-derived genome-scanning techniques to find \textit{nts-1}-associated amplification markers with which to screen soybean YAC and bacterial artificial chromosome (BAC) libraries (under construction) and anchor initial contigs. A screen of phenotypic pools of the nitrate-tolerant symbiosis (\textit{nts-1}) locus (containing segregants homozygous for \textit{nts-1}-flanking markers pA-381 and pA-36, and tightly linked marker pUTG-132a) with 181 arbitrary 8-mer to 30-mer primers (representing about 4000 loci) (unpublished) produced two polymorphic markers that were converted into sequence-characterized amplified regions (SCARs) and screened on a large F\textsubscript{2} segregating population. Only one of them was scorable and linked (24 cM distant from \textit{nts-1} and 7.6 cM from marker pA-36). The screening of an additional 103 octamers failed to produce additional linked markers (Table 1). Despite the extremely conserved nature of the \textit{nts-1} region, ASAP analysis with only a few primers detected several markers putatively linked to \textit{nts-1}. Bonafide and tightly linked markers are now being converted into SCARs.

The arbitrary selection of primers can be biased to include recognition of particular sequence motifs within the genome. Primers can be derived from known sequences representing dispersed DNA (such as mammalian \textit{Alu} elements or bacterial REP and ERIC motifs) or structural chromosomal domains (such as telomeric sequences). Primers can also derive from sequence arrays in repetitive DNA, such as the simple sequence repeats (SSR) present in microsatellites. The advantage of targeting these sequence motifs is that they usually represent highly polymorphic regions. Furthermore, generated markers are usually co-dominant and express many allelic variants. Several strategies based on the direct but arbitrary amplification of microsatellites are now available. Initially, primers complementary to SSR loci were used directly for genomic fingerprinting (15,17). However, amplification with these microsatellite primers resembled closely that with true arbitrary primers (21). Anchoring of primers was then proposed as a vehicle to diminish mismatch priming to putative primer annealing sites other than true SSRs. For example, random amplified microsatellite polymorphism (RAMP) analysis used primers that consist of a 5′ arbitrary anchor and a 3′ repeat sequence complementary to an SSR motif, usually in combination with arbitrary decamers (24). Similar in concept, inter-SSR PCR was used to amplify regions that span two closely spaced SSRs with one primer with 5′ or 3′ arbitrary anchors (25). However, in all these alternatives the amplification of the SSR motif coexists with the amplification of unrelated arbitrary sequences, and the true nature of microsatellite markers requires confirmation by hybridization to an SSR oligonucleotide. Here we show yet another use for microsatellite primers, the generation of relatively simple sequence signatures by reamplification of DNA fingerprints with 5′-anchored SSR primers. These primers were anchored at their 5′ termini with ambiguous (degenerate) bases, because the primers had to target about 10–15 kb of DAF-amplified sequence that contained only a few SSR annealing sites. Moreover, the amplification reaction had to occur under stringent
conditions to avoid mismatch priming. These ASAP were reproducible, heritable and able to distinguish pooled DNA in BSA and closely related bermudagrass cultivars (Figure 4).

ASAP is a dual-step amplification procedure that requires the use of at least one primer in each experimental stage. Provided the sequence of the primers differs substantially from each other, distinct fingerprints can be generated in each combination. Therefore, it is possible to apply the principle of combinatorial use of oligomers to ASAP fingerprinting. For example, a set of 10 oligonucleotides can be used in 100 different pairwise combinations, while if amplification is performed using two arbitrary primers (6) in the first step, up to 10,000 different fingerprinting reactions are possible. This feature will allow extensive screening of nucleic acids with only a limited number of primer sequences. It should be noted that an ASAP is designed to fingerprint a collection of amplification products produced during the first step of the screening reaction. Therefore, priming during the second step will only occur within the subselected fragments, and the number of targeted regions in a genome will only depend on the number of primers or combination of primers used in the first step of the ASAP reaction. Together with tec-MAAP (8) and other tailoring strategies, ASAP reaction. Together with tec-primers used in the first step of the screening reaction. Therefore, priming produced during the first step of the reaction is possible. This feature enables in 100 different pairwise combinations, while if amplification is performed using two arbitrary primers (6) in the first step, up to 10,000 different fingerprinting reactions are possible. This feature will allow extensive screening of nucleic acids with only a limited number of primer sequences. It should be noted that an ASAP is designed to fingerprint a collection of amplification products produced during the first step of the screening reaction. Therefore, priming during the second step will only occur within the subselected fragments, and the number of targeted regions in a genome will only depend on the number of primers or combination of primers used in the first step of the ASAP reaction. Together with tec-MAAP (8) and other tailoring strategies, ASAP analysis adds yet another versatile tool for those trying to dissect closely related organisms or DNA templates.

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