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Hybridization of microsatellites to RAPD: a new source of polymorphic markers

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Genetic analyses of plant and animal populations and species for taxonomic, evolutionary and ecological studies tremendously benefited from the development of various molecular marker techniques which reveal polymorphisms at the DNA level. In general, DNA-based markers have important advantages such as selective neutrality, abundance, high variability and independence of environmental effects. Among the most widely applied techniques in plants are genomic RFLP analysis (1), multilocus DNA fingerprinting with mini- or microsatellite complementary probes (2–3), PCR amplification of individual microsatellite loci (4) and PCR with arbitrary or semispecific primers that amplify anonymous regions of the genome (5–7). RFLPs and PCR-amplified microsatellites are locus-specific, codominant markers which proved highly useful for population genetic studies and the establishment of high density linkage maps. However, such locus-specific approaches require molecular cloning procedures whereas multilocus fingerprinting and anonymous PCR strategies do not. Therefore, the latter techniques are preferred tools to study species where little or no DNA sequence information is available.

Here we report a new method which combines arbitrarily or semispecifically primed PCR with microsatellite hybridization to produce several independent and polymorphic genetic fingerprints per electrophoretic gel. No prior sequence information is needed. This novel method which we call RAMPO (random amplified microsatellite polymorphisms) is generally applicable for plants, and most probably for animals and humans as well. Genomic DNA is first amplified with a single arbitrary or microsatellite-complementary PCR primer. After electrophoretic separation and staining of the PCR products, the gel is either dried or blotted onto a nylon membrane, and subsequently hybridized to a [^{32}P]-labelled, microsatellite-complementary oligonucleotide probe (e.g. $[CA]_8$). Autoradiography detects reproducible and polymorphic fingerprint profiles which do *not* correspond to the staining patterns, and which are completely different for each probe.

The presence of dinucleotide repeats in anonymous genomic PCR products was detected incidentally during the use of microsatellite-complementary oligonucleotides as PCR primers (MP–PCR) for genomic fingerprinting. We found that hybridization of Southern-blotted MP–PCR or RAPD products of tomato and kiwi fruit template DNA to ^{32}P-labelled dinucleotide repeat probes $[GT]_8$ or $[GA]_8$ resulted in strong and distinct signals after autoradiography (6). We suspected that such banding patterns might represent an additional source for polymorphic markers in eukaryotes. In the present study, we tested this prediction with a variety of species and cultivars of the genus *Dioscorea* (yams), as well as some other plant and one fungal species. PCR products generated by different microsatellite or arbitrary primers were electrophoresed, blotted and successively hybridized to $[CA]_8$ and $[GA]_8$. Since PCR with arbitrary primers is known to be sensitive to variations in experimental conditions we performed four replicate experiments for each template/primer combination to ensure reproducibility. Ethidium bromide staining of electrophoresed PCR products revealed distinct banding patterns both with arbitrary and microsatellite primers. Hybridizing the RAPD and MP–PCR products with $[CA]_8$ and $[GA]_8$ yielded novel and highly reproducible fingerprinting profiles (data submitted but not shown). These profiles were completely different from the staining patterns, and strongly depended upon the specific primer/probe combination. Distinctly different fingerprints were produced when individual genotypes were probed with the same oligonucleotide following amplification with different primers. Thus, hybridization signals resulted from PCR products and not from unamplified genomic DNA which might be present in the gel. We interpret the occurrence of these bands as follows: any RAPD or MP–PCR reaction probably creates many thousand different products of various abundance. However, staining only visualizes the most abundant amplicons, whereas the majority of minor fragments will remain below the detection level or form a background smear. The ubiquitous presence of dinucleotide repeats in eukaryotic genomes provides a means of visualizing a subset of such minor amplification products by hybridization. The signal intensity of fragments harboring a certain microsatellite motif will depend both on the length of this motif and the abundance of the fragment.

To test whether the banding patterns revealed by this novel type of DNA fingerprinting were also variable between closely related genotypes, we hybridized a set of RAPD gels comprising 21 accessions of *D.bulbifera* to $[CA]_8$ and $[GA]_8$. Results obtained for the primer OPG-15 are shown in Figure 1. Several controls were included to test the specificity of the reaction. Neither RAPD products nor hybridization signals occurred in lanes x–z (no primer and/or no template). As expected, *E.coli* template DNA (lane w) resulted in RAPD products which did *not* hybridize to $[CA]_8$ or $[GA]_8$, consistent with the view that microsatellites are generally absent from prokaryotic genomes. $[CA]_8$- and $[GA]_8$-generated hybridization signals provided new polymorphisms in addition to those seen after ethidium bromide staining. A total of three complementary DNA profiles were therefore detectable after one single round of PCR and gel electrophoresis. We do not know yet whether the polymorphisms revealed after hybridization are derived from sequence variation in the primer binding sites, or from insertion/deletion of microsatellite motifs

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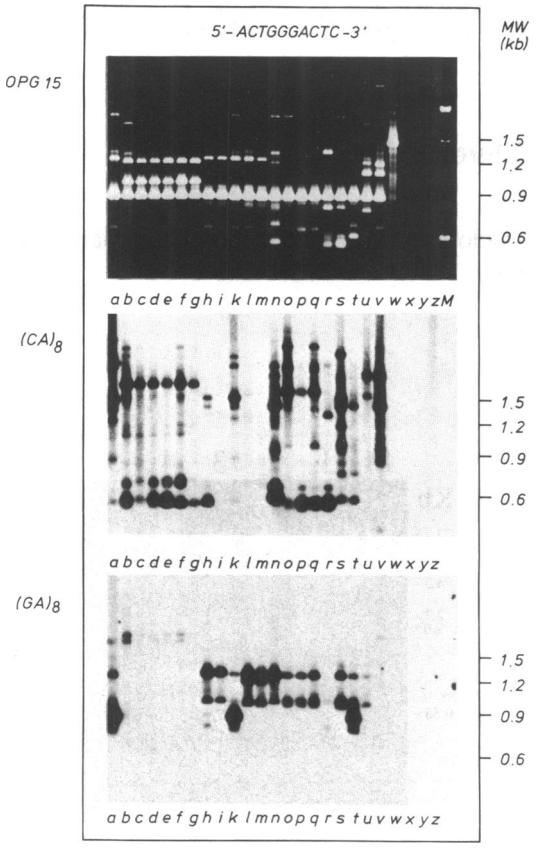

Figure 1. RAMPO analysis of 21 accessions of *Dioscorea bulbifera* (lanes a–v) obtained by RAPD primer OPG-15 (5'-ACTGGGACTC-3') in combination with a $[CA]_8$ or a $[GA]_8$ probe. The ethidium bromide staining pattern of the RAPD profile is shown in the upper panel. Control experiments: *E.coli* DNA as template (lane w); no template (lane x); neither primer nor template (lane y); no primer (lane z). Positions of molecular weight markers (lane M) are indicated in kilobases.

and/or their neighbouring sequences. Since the variability is detected in random genomic target sequences and visualized by microsatellite hybridization, we tentatively call this new technique RAMPO (random amplified microsatellite polymorphisms). However, we would like to stress that the approach may also work with other kinds of ubiquitous probes.

The RAMPO approach is by no means restricted to kiwi fruit, tomato and yams, but worked with all tested plant species so far. For example, $[GT]_8$ hybridized to fragments produced by the arbitrary primer UBC 352, generating distinct fingerprints for each of five plant genera tested (data submitted but not shown). However, we detected no signal by hybridizing $[GT]_8$, $[CT]_8$ or $[CAC]_5$ with the products of RAPD amplifications (UBC 429, UBC 352) from a fungus (*Ophiostoma novo-ulmi*). This may reflect the lower complexity and lower microsatellite content of fungal genomes relative to most plants and animals (3). We have not yet tested the full range of useful probes. So far, no signals were obtained with tetranucleotide repeats, probably because they are relatively rare. $[AT]_n$ and $[GC]_n$ probes are problematic because of self-complementarity. $[CAC]_5$ as the only trinucleotide tested up till now produced a few weak bands with some *Dioscorea* species, indicating that trinucleotide repeats may well represent an additional reservoir of useful RAMPO probes.

Taken together, the RAMPO technique combines several advantages of oligonucleotide fingerprinting (2,3), RAPD–PCR (5) and MP–PCR (6,7), i.e. the speed of the assay, the high sensitivity, high level of variability detected and no requirement for *a priori* DNA sequence information. By replacing enzyme digestion of genomic DNA with RAPD– or MP–PCR to generate the pool of DNA fragments subjected to microsatellite probing, the RAMPO procedure will be applicable for studies where only trace amounts of DNA are obtainable. It may prove especially useful in species where little or no intraspecific variation is detected by RAPDs alone, such as red pine (8) and peanut (9).

DNA was extracted from *Dioscorea*, *Pinus*, *Podocarpus*, *Agathis*, *Vitex* and *Nestegis* species using a modified CTAB protocol (3), from *Ophiostoma* by the procedure of Raeder and Broda (10). RAPD analysis was performed in 25 µl reaction volumes containing 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.4 µM primer (= 10 pmol/reaction), 0.8–1.8 U *Taq* DNA polymerase (Gibco-BRL) and 15–40 ng template DNA. After initial denaturation (94°C, 3 min), PCR was run for 39 cycles each consisting of a 95°C denaturing step (1 min), a 37°C annealing step (1 min), and a 72°C elongation step (90 s) in a Hybaid Omnigene or a Perkin Elmer TC 9600 thermocycler.

A modified touch-down protocol (11) was used for MP–PCR. Samples were separated on 1.5% agarose gels in TBE buffer, stained with ethidium bromide and photographed.

Gels were either dried or blotted onto a nylon membrane, and hybridized to ^{32}P-labelled, microsatellite-complementary oligonucleotide probes as previously described (2,3). T_m values were calculated according to Miyada and Wallace (12), and hybridization and stringent washes carried out at T_m –5°C. Hybridization signals were visualized by autoradiography using intensifying screens.

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