Theor Appl Genet (1992) 83:871–877

THEORETICAL AND APPLIED GENETICS

Genetic fingerprinting of *Theobroma* clones using randomly amplified polymorphic DNA markers

J. Wilde, R. Waugh, and W. Powell*

Cell and Molecular Genetics Department, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland

Received April 5, 1991; Accepted July 9, 1991
Communicated by J.W. Snape

Summary. Randomly amplified polymorphic DNA (RAPD) markers have been used to characterise cocoa clones representing the three main cultivated subpopulations: Criollo, Forastero and Trinitario. The use of single primers of arbitrary nucleotide sequence resulted in the selective amplification of DNA fragments which were unique to the individual cocoa clones studied. The use of a single primer allowed each of the clones evaluated to be unequivocally characterised. The application of RAPD markers for the evaluation of germplasm and cocoa improvement programmes are discussed.

Key words: Cocoa – RAPDs – DNA – Polymorphism – Genetic diversity

Introduction

Cocoa (*Theobroma cacao*) is an important tropical tree crop, whose centre of diversity is considered to be the equatorial slopes of the Andes in the Colombian, Peruvian and Ecuadorian border area (Lass and Wood 1985). Of the cocoa species used for cultivation, three major types can be distinguished: the Criollos, Forasteros and Trinitarios. The Trinitarios are considered to be either relatively recent hybrids between Criollos and Forasteros or intermediate types. This classification is, however, based almost exclusively on morphological characters which are known to be influenced by a range of environmental factors.

Although considerable genetic diversity exists in cocoa, the current cultivated crop tends to be based on a small portion of the available gene pool (Cope 1976). This factor, together with the threat to wild cocoa posed by wide-spread deforestation in South and Central America, has resulted in the designation of cocoa as a priority crop for conservation (Anon. 1987). In an effort to broaden the genetic base and preserve wild cocoa species, the International Board for Plant Genetic Resources (IBPGR) has identified two international "base collections" sited in Trinidad and Costa Rica. Collection and evaluation is an important feature of the history of the cocoa crop (Allen and Lass 1983) and many of the accessions collected are maintained by the Cocoa Research Unit, University of West Indies (Kennedy 1984). The maintenance, characterisation and evaluation of cocoa germ plasm is an important but costly and time-consuming process.

Traditionally, cocoa genetic resources have been characterised using a combination of morphological and agronomic traits. The effectiveness of using these to estimate genetic diversity has been questioned by several workers (Gottlieb 1977; Brown 1979). The long generation time of most perennial crops also indicates that many of the morphological descriptors can only be assessed at maturity. Molecular and biochemical markers that are not subject to environmental influences provide an opportunity to examine more precisely the genetic relationships between accessions (Bernatzky and Tanksley 1989). Isozymes have been used to characterise cocoa germ plasm (Lanaud and Berthaud 1984; Atkinson et al. 1986; Yidana et al. 1987), but the level of polymorphism detected is a limiting factor for cultivar discrimination.

Restriction fragment length polymorphism (RFLP) has the potential to produce a large number of genetic markers, since any cloned DNA sequence can be used as a probe (Tanksley et al. 1989). Nuclear RFLPs (for a review see Helentjaris and Burr 1989) have been used extensively to fingerprint a range of crop plants, but their

* To whom correspondence should be addressed

exploitation in characterising perennial crops has been limited. Mini-satellite DNA sequences identified by hybridisation to defined segments of bacteriophage M13 have been used to distinguish between *Malus*, *Prunus* and *Rubus* genotypes (Nybom et al. 1990) and in paternity analyses in apple (Nybom and Schaal 1990). Waugh et al. (1990) have used ribosomal DNA (r-DNA) and chloroplast-specific probes to investigate intra- and interspecific diversity in *Rubus*. However, RFLP technology is labour intensive and its successful exploitation, particularly in developing countries, may be hampered by the availability of the radionucleotides required for the labelling of probes. Although non-radioactive detection methods have been developed (Ishii et al. 1990) their sensitivity, especially for low copy sequences, can be problematic. Recently, procedures for the identification of DNA polymorphisms based on the polymerase chain reaction (PCR) which are not dependent on hybridisation analysis with isotopically labelled probes have been described (Williams et al. 1990; Welsh and McClelland 1990). In this manuscript we report the use of the random amplified polymorphic DNA or 'RAPD' marker system (Williams et al. 1990), for the characterisation and fingerprinting of *Theobroma* genotypes.

Materials and methods

Plant material

Pods from the cocoa species listed in Table 1 were obtained from the germplasm collection maintained at the Cocoa Research Unit, University of West Indies, Trinidad. Seeds were germinated and established as plants at SCRI. Leaf material was harvested after 8 weeks from plants originating from single seeds and genomic DNA was extracted according to the procedure described by Dellaporta et al. (1983).

Polymerase chain reaction (PCR)

PCR reactions (100 µl final volume) contained 150 ng genomic DNA, dATP, dCTP, dGTP and dTTP, each at 100 µ*M* final concentration, 200 n*M* primer, 1 × *Taq* polymerase buffer and 3 units of *Taq* DNA polymerase (NBL). Each reaction mix was overlaid with 100 µl of mineral oil to prevent evaporation. The random sequence decamer oligonucleotide primers used in this study were synthesised on an Applied Bio-Systems 391 PCR-mate oligonucleotide synthesiser and are listed in Table 2. For enzymic amplification, samples were subject to 45 repeats of the following thermal cycle: 1 min at 92 °C followed by 1 min at 35 °C, then 2 min at 72 °C. After the final cycle, samples were incubated for a further 3 min at 72 °C, then held at 16–20 °C prior to analysis. Fragments generated by amplification were separated according to size on 5% polyacrylamide gels run in 1 × TBE [89 m*M* TRIS HCl (pH 8.3), 89 m*M* boric acid, 5 m*M* EDTA], stained with ethidium bromide and visualised by illumination with UV light (312 nm).

Hybridisation analysis

Amplified DNA fragments separated according to their length by polyacrylamide gel electrophoresis were transferred electrophoretically to Biodyne Nylon Membrane (PALL) in a Biorad electroblotting apparatus in 0.4 × TAE at 1.5 A constant current for 1 h. Fragments to be used as probes were excised and the DNA was isolated using a unidirectional electroelution apparatus (IBI). Isolated fragments were labelled by random priming (Feinberg and Vogelstein 1984) with ^{32}P dCTP (3,000 Ci/mmol, ICN Biomedicals), and used to probe the prepared blots by standard procedures (Maniatis et al. 1982). After hybridisation, blots were washed in several changes of 0.1 × SSC, 0.1% SDS at 65 °C, and hybridising fragments were revealed by autoradiography.

Table 1. *Theobroma* genotypes studied together with their country of origin

Genotype	Geographical origin	Population type
Accession Trinidad (ACT 1-7)	Trinidad	Trinitario
Amazon (AMAZ 6-3)	Ecuador	Forastero
Catonga	Brazil	Comum
Cacao Centre (CCII)	Costa Rica	Hybrid
Cocoa Research Unit (CRU 8-9)	Unknown	Forastero
Estacion Experimental Tropical (EET 399)	Ecuador	Hybrid
Iquitos mixed cabacillo (IMC 55)	Peru	Forastero
Matina	Costa Rica	Forastero
Parinari (PA 107)	Peru	Forastero
Taujica (TJ 1)	Honduras	Criollo
Theobroma microcarpum L.		
Herrania camargoana L.		
Herrania sp. L		

Results

To determine the suitability of the RAPD technique for 'fingerprinting' cocoa, 13 different primers of arbitrary nucleotide sequence (Table 2) were used to amplify specific sequences from the cocoa clone PA 107. Figure 1 shows that amplification products of different sizes, which are easily resolved by electrophoresis and visualised by staining with ethidium bromide, are generated for each primer. The amplification products are up to 3 kb in length. The distribution of product sizes after amplification with nine of the primers is shown in Fig. 2. PCR-generated RAPD markers have been shown to be as effective in identifying useful polymorphisms in repetitive DNA as in low-copy DNA sequences (Williams et al. 1990). The type of cocoa DNA (low, middle or high copy) amplified by the 14 primers was examined by probing a Southern blot of the amplified sequences with total ^{32}P-labelled cocoa DNA. Total genomic DNA did not

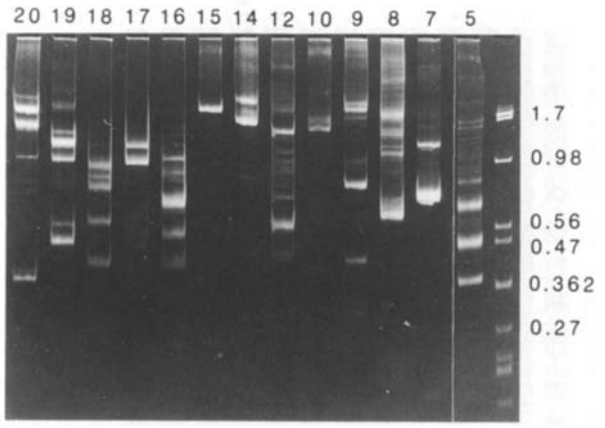

Fig. 1. Amplification of genomic DNA from the Forastero clone, PA 107, with the 13 different primers of arbitrary sequence listed in Table 2. Fragment were fractionated on 5% polyacrylamide gels alongside Lambda DV1 *Hae*III markers

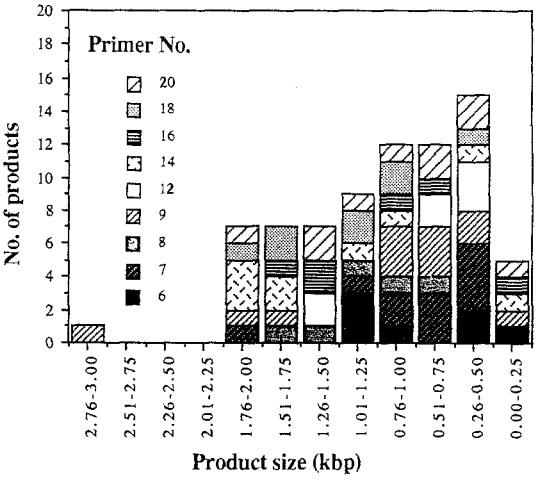

Fig. 2. Frequency distribution for the size of genomic amplification products generated from 13 cocoa genotypes with a total of eight primers (primers 6, 7, 8, 9, 12, 14, 16, 18 and 20). The nucleotide sequence information for each primer is given in Table 2

Table 2. Base sequence of primers and number of phenotypes identified with each

Primer No.	Sequence	No. of phenotypes identified
5	TCGGAGTGGC	ND
6	ACTCAGGAGC	ND
7	CCACCGCCAG	12
8	AGAGATGCCC	4
9	CAGTTCTGGC	10
10	CGTGCTAGCA	ND
12	GTTTCCGGTG	7
14	TCCCGACCTC	6
15	GCTCGTCAAC	ND
16	CCTGGCGAGC	8
17	GTTAGCGGCG	ND
18	GCCCTACGCG	13
19	CGTCCGTCAG	ND
20	ACTCGTAGCC	10

ND – not determined for all 13 genotypes

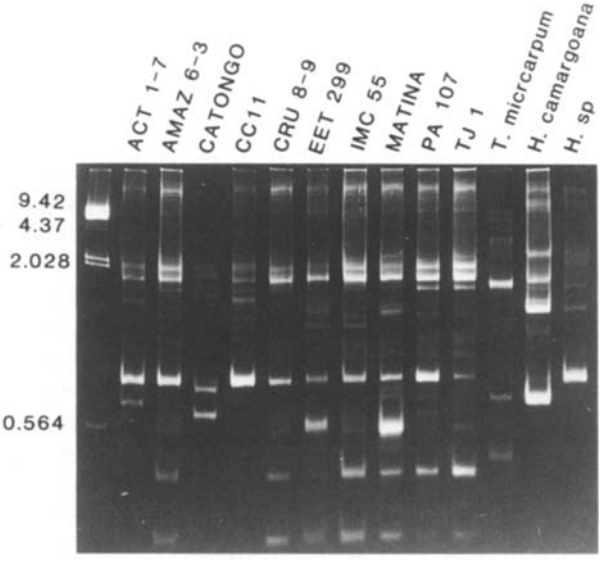

Fig. 3. Amplification of genomic DNA from 13 cocoa genotypes with primer 9. *Hin*dIII was used as size markers

hybridise strongly to any of the cocoa amplification products, which would suggest that low-copy sequences are being amplified (results not shown). Reassociation kinetic studies have indicated that cocoa has a small genome with little repetitive DNA (P. Fritz, personal communication). The amplification of low-copy sequences is therefore not unexpected.

Of the 13 primers tested, 4 produced complex patterns that proved difficult to interpret. Nine were therefore used to analyse the extent and nature of the polymorphism in cocoa. The range of different phenotypes identified with primer 9 is illustrated in Fig. 3. To establish whether the different sized amplification products were length variants containing a similar core DNA sequence or whether they were distinct sequences, amplified products were excised from gels, labelled with ^{32}P dCTP and used to probe Southern blots of the amplified products. Figure 4a shows a gel of the amplified products obtained using primer 7. Figure 4b shows clearly that the labelled fragment from AMAZ 6-3 specifically hybridises to a subset of the products which are similar in length to the labelled fragment. Outside the primer binding sites, the other amplified products bear no sequence homology to the radiolabelled band. Thus, the size variant amplified bands must be derived from distinct regions of the genome.

The number of unique phenotypes identified with each of the ten primers is given in Table 2. Primers differ

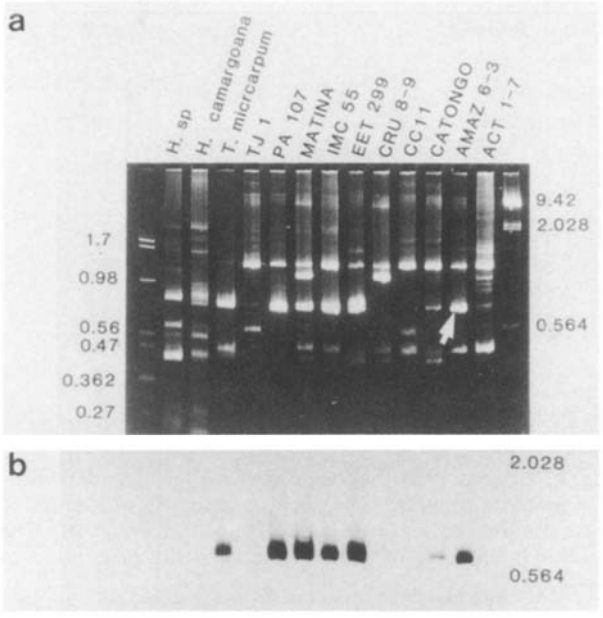

Fig. 4. **a** Amplification products obtained with primer 7 alongside λ *Hin*dIII markers. The *arrow* indicates the amplification product used as a probe for the Southern hybridisation analysis shown in Fig. 4b. **b** Southern blot of the same gel using the indicated AMAZ 6-3 products as probe

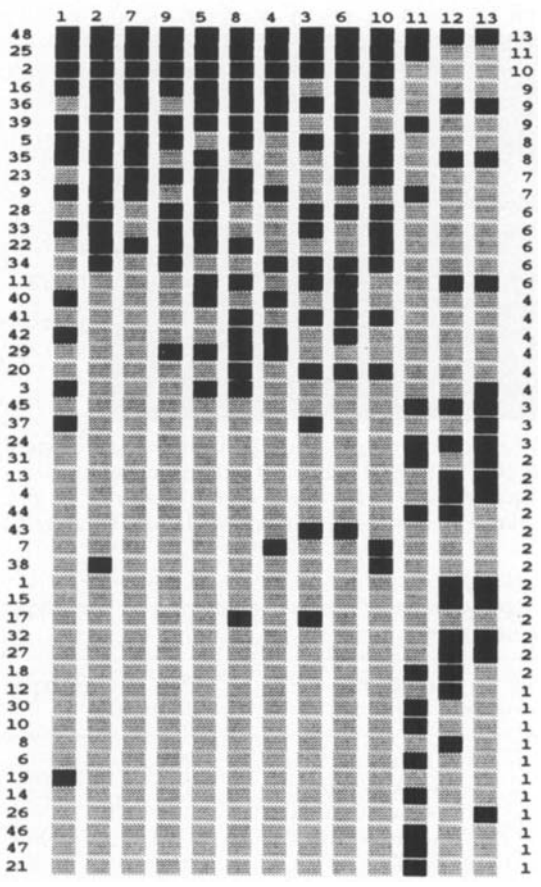

Fig. 5. 'Bandmap' of shared and polymorphic amplification products. A total of 48 amplification products were generated and these are indicated individually in the *left margin*. ■ represents the presence of an amplification product. Primer 7 amplified bands 1–11, primer 9 bands 12–23, primer bands 24–30, primer 18 bands 31–38 and primer 20 bands 39–48. Numbers at the *top* of each column represent the individual cocoa genotypes studied as listed in Table 1. Numbers in the *right margin* indicate the number of genotypes sharing a given amplification product

significantly ($X^2_{(7)} = 22.34***$) in their capacity to detect polymorphism, and primer 18 is capable of uniquely identifying each of the 13 genotypes studied. In order to quantify the level of polymorphism detected, Nei's estimate of similarity, based on the probability that an amplified fragment from one plant will also be found in another ($D_{AB} = 2 \times$ number of shared fragments/number of fragments$_A$ + number of fragments$_B$) (Nei and Li 1979), was used to generate a similarity matrix (Table 3). The matrix highlights the distinction between the wild cocoa species and the cocoa cultivars. Within the cocoa cultivars the proportion of shared fragments range from 0.51 to 0.86. Overall the lowest degree of similarity between cultivars tends to be associated with Catonga, which is a Forastero type originating from the Belem region of Brazil.

An alternative graphical method of recording the RAPD data, which allows the differences and similarities between genotypes to be simultaneously presented, is shown in Fig. 5. The 'bandmap' retains the original RAPD information, highlights similarities between genotypes by placing those which share the same amplification products adjacent to each other, draws attention to the most and least commonly occurring product and immediately makes clear whether or not two similar genotypes are in fact identical with respect to their amplification products (Powell et al. 1991). It also has the advantage of presenting graphically individual data scores for each cocoa clone. One can therefore easily reference the more informative amplified fragments which can distinguish the cocoa cultivars examined. This figure amply illustrates the extensive DNA polymorphism detectable in cocoa using a PCR approach. Furthermore, genotypes 2, 7 and 9, which are all Forastero types, tend to be grouped together and are adjacent to genotype 8 (Matina), which is also classified as a Forastero type but possesses an Amelanado pod shape.

Discussion and conclusions

This paper is the first report of the use of a DNA-based polymorphism assay to assess the level of variability within *Theobroma*. A relatively small subset of cocoa genotypes was examined which included the Forastero, Trinitario and Criollo groups. The polymerase chain re-

Table 3. Similarity matrix based on Nei's estimate of similarity

	ACT 1-7	AMAZ 6-3	Catonga	CCII	CRU 8-9	EET	IMC 55	Matina	PA 107	TJI	T. micro	H. cam	H.
ACT 1-7	1.00												
AMAZ 6-3	0.73	1.00											
Catonga	0.51	0.62	1.00										
CCII	0.65	0.68	0.65	1.00									
CRU 8-9	0.76	0.86	0.54	0.64	1.00								
EET	0.63	0.73	0.75	0.73	0.73	1.00							
IMC 55	0.68	0.82	0.51	0.62	0.75	0.60	1.00						
Matina	0.62	0.71	0.65	0.64	0.71	0.69	0.75	1.00					
PA 107	0.72	0.89	0.64	0.71	0.81	0.75	0.73	0.70	1.00				
TJI	0.63	0.86	0.67	0.62	0.72	0.74	0.72	0.69	0.86	1.00			
T. micro	0.34	0.37	0.24	0.33	0.27	0.30	0.33	0.33	0.35	0.29	1.00		
H. cam	0.26	0.29	0.20	0.20	0.29	0.22	0.28	0.22	0.23	0.25	0.34	1.00	
H. sp	0.36	0.35	0.34	0.35	0.35	0.36	0.31	0.28	0.29	0.34	0.29	0.64	1.0

Mean = 0.59
1 SD = 0.24

action has been used previously to amplify wheat genomic DNA sequences (D'Ovidio et al. 1990) using primers designed from the published sequence of a wheat storage protein gene. The appraoch described by Williams et al. (1990) and used in this manuscript to fingerprint cocoa clones utilises short oligonucleotide primers of arbitrary sequence. This approach allowed reliable discrimination both between and within *Theobroma* groups. Importantly, the methodology used does not depend on the use of radioisotope and is therefore more readily transferable to developing countries. Conventional RFLP analysis requires relatively large quantities of uncontaminated DNA. Extraction of high quality, uncontaminated DNA from cocoa and other tropical tree crops has been shown to be difficult (Couch and Fritz 1990) and often requires expensive caesium chloride centrifugation. PCR based assays are technically simple, easily automated and require small quantities of DNA which can be extracted using mini-prep procedures. Indeed, extraction of DNA from apices within dormant buds is also possible in *Theobroma* (J. Wilde, unpublished results), and such an approach would be particularly suitable for genetic fingerprinting of cocoa genotypes based on RAPDs.

Bailey (1983) has identified three basic criteria for cultivar identification: distinguishable intervarietal variation, minimal intravarietal variation, environmental stability and experimental reproducibility. Considering the first criterion, the level of variation detected in the *Theobroma* samples studied with RAPDs is amongst the highest revealed with any known marker system. Although we have not examined the level of intraclonal variability in detail, the experiments reported are highly reproducible and therefore fulfil the third criterion. Present methods of cocoa classification, which are mainly based on morphological characters, are time consuming. Furthermore, young plants cannot be fully classified until their reproductive structures have fully developed, which can take several years. RAPD marker analysis may be performed on DNA extracted from juvenile tissue and but apices (Kreike 1990).

Atkinson et al. (1986) have suggested that the slow incorporation of cocoa germplasm maintained in gene banks into ongoing cocoa improvement programmes may reflect the poor descriptive data available for cocoa. Williams et al. (1990) have stressed that each RAPD marker is the equivalent of a sequence tagged site and a set of well-defined primers could represent a series of descriptors that would facilitate the characterisation and evaluation of cocoa genetic resources in developing countries. The exploitation of RAPDs would assist in the elimination of duplicates, allow rationalisation of existing gene banks and allow future cocoa collecting expeditions to be targeted towards geographical areas possessing maximum levels of genetic diversity.

Further genetical analyses, involving the use of well-defined crosses, are required to determine the number of loci associated with a particular primer in *Theobroma*. However, specific amplified DNA fragments are transmitted to progeny seedlings (J. Wilde, unpublished results). Such an observation has direct relevance for studies of gene flow. Outcrossing in *Theobroma* is promoted by a pollen incompatibility system (Knight and Rogers 1955), and the unequivocal identification of the parentage of seedlings would be an immediate application of the technique used in this paper. Heterosis has been observed in crosses between unrelated cocoa clones, and the phenomenon of interpopulation heterosis provides the basis for almost all modern cocoa breeding programmes (Toxopeus 1969; Kennedy et al. 1987). A specific objective is therefore to identify cocoa clones with good combining ability. The field evaluation of F_1 hybrids has been the main method used to identify and predict heterotic combinations. The relationship between diversity at the DNA level, measured by RFLP analysis and heterosis,

has been examined in maize (Lee et al. 1989; Godshalk et al. 1990). Such studies have indicated that RFLP analysis is of value in allocating maize genotypes to heterotic groups, and similar studies (based on RAPDs) with cocoa could be initiated.

The use of RAPDs to measure the extent and nature of variability in *Theobroma* does not depend upon the availability of genetic linkage maps. Similarly, RAPDs can be used to measure gene flow, identify heterotic parental combinations or monitor somaclones, all without prior knowledge of the detailed chromosomal genetics of RAPDs. Although RAPD markers may be used for genetic mapping studies, they tend to be inherited as dominant markers (Williams et al. 1990). This mode of inheritance does not allow heterozygous and homozygous dominant individuals to be identified without subsequent crosses. Such problems can be circumvented by the use of doubled haploids (Powell 1990), and doubled haploid populations of *Theobroma cacao* have been generated (Lanaud 1987a, b). Doubled haploids, representing fixed homozygous material together with RAPDs, may therefore represent an attractive strategy for both linkage map creation and exploitation in *Theobroma*.

In conclusion, we have demonstrated that RAPD markers may be used to genetically fingerprint cocoa clones. It is anticipated that the methods described in this paper will have a major impact on the management of cocoa genetic resources and will act as a catalyst for an expansion in the utilisation of genetic marker-based technology with tropical perennial crops.

Acknowledgements. The authors gratefully acknowledge financial support from the Biscuit, Cake, Chocolate and Confectionery Alliance, UK. The research presented is part of a PhD programme being pursued by J. Wilde in the Department of Agricultural Botany, the University of Reading. We are grateful to Prof. J. Spence, Cocoa Research Unit, University of West Indies, for the supply of germ plasm and to Dr. B. Reavy for synthesis of the oligonucleotide primers.

References

Anon. (1987) Genetic resources of cocoa. In: Proc 1st Meeting Cacao Working Group, IBPGR, Rome. AGP/IBPGR/80/56

Allen J, Lass RA (1983) London Cocoa Trade Amazon Project, Final Report, Phase 1. Cocoa Growers Bulletin, p 34

Atkinson MD, Withers LA, Simpson MJA (1986) Characterisation of cacao germplasm using isoenzyme markers. Euphytica 35:741–750

Bailey DC (1983) Isozymic variation and plant breeders' rights. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, Part A. Elsevier, Amsterdam, pp 425–441

Bernatzky R, Tanksley SD (1989) Restriction fragments as molecular markers for germplasm analysis and utilisation. In: Brown ADH, Marshall DR, Franket OH, Williams JT (eds) The use of plant genetic resources. Cambridge University Press, Cambridge, pp 353–362

Brown AHD (1979) Enzyme polymorphism in plant populations. Theor Popul Biol 15:1–42

Cope FW (1976) Cacao, *Theobroma cacao* (Sterculiaceae). In: Simmonds NW (ed) Evolution of crop plant. Longman, London New York, pp 285–289

Couch JA, Fritz PJ (1990) Isolation of DNA from plants high in polyphenolics. Plant Mol Biol Rep 8:8–12

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: Version II. Plant Mol Biol Rep 1:19–21

D'Ovidio R, Tanzarella OA, Porceddu E (1990) Rapid and efficient detection of genetic polymorphism in wheat through amplification by polymerase chain reaction. Plant Mol Biol 15:169–171

Feinberg AP, Vogelstein B (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 137:266–267

Godshalk LB, Lee M, Lamkey KR (1990) Analysis of the relatedness of restriction fragment length polymorphisms to maize single-cross hybrid performance. Theor Appl Genet 80:273–280

Gottlieb LD (1977) Electrophoretic evidence and plant systematics. Ann Mo Bot Gard 64:161–180

Helentjaris T, Burr B (eds) (1989) Development and application of molecular markers to problems in plant genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor/NY

Ishii T, Panaud O, Brar DS, Khush GS (1990) Use of non-radioactive digoxigenin-labelled DNA probes for RFLP analysis in rice. Plant Mol Biol Rep 8(3):167–171

Kennedy AJ (1984) The international cocoa genebank, Trinidad: its role in future cocoa research and development. In: Proc 9th Int Cocoa Res Conf, Lorne, Togo, pp 63–69

Kennedy AJ, Lockwood G, Mossu WG, Simmonds NW, Tan GY (1987) Cocoa breeding: past, present and future. Cocoa Growers Bull 38:5–22

Knight R, Rogers HH (1955) Incompatibility in *Theobroma cacao*. Heredity 9:69–77

Kreike J (1990) Genetic analysis of forest tree populations isolation of DNA from spruce and fir apices. Plant Mol Biol 14:877–879

Lanaud C (1987a) Doubled haploids of cocoa (*Theobroma cacao* L.). 1. Observations of fertility. Plant Breed 99:187–195

Lanaud C (1987b) Doubled haploids of cocoa (*Theobroma cacao* L.). 2. Observations of monogenic and polygenic characters. Plant Breed 99:196–202

Lanaud C, Berthaud J (1984) Mise en evidence de nouveaux marqueurs genetiques chez *Theobroma cacao* L. Par les techniques d'electrophorese. In: Proc 9th Int Cocoa Res Conf, Togo, pp 249–253

Lass RA, Wood GAR (1985) Cocoa production, present constraints and priorities for research. World Bank Techn Paper No. 39

Lee M, Godshalk EB, Lamkey KR, Woodman WW (1989) Association of restriction fragment length polymorphisms among maize inbreds with agronomic performance of their crosses. Crop Sci 29:1067–1071

Maniatis T, Fritsh EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor/NY

Nei M, Li WH (1979) Mathematical model for study genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 74:5267–5273

Nybom H, Schaal BA (1990) DNA "fingerprints" applied to paternity analysis on apples (*Malus* × *Domestica*). Theor Appl Genet 79:763–768

Nybom H, Rogstad SH, Schaal BA (1990) Genetic variation detected by the use of the M13 "DNA fingerprint" probe in

Malus, *Prunus* and *Rubus* (Rosaceae). Theor Appl Genet 79:153–156

Powell W (1990) Environmental and genetical aspects of pollen embryogenesis. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry 12. Haploids in crop improvement 1. Springer, Berlin Heidelberg New York, pp 45–65

Powell W, Phillips MS, McNicol JW, Waugh R (1991) The use of DNA markers to estimate the extent and nature of genetic variability in *Solanum tuberosum* cultivars. Ann Appl Biol 118:423–432

Tanksley SD, Young ND, Patterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for an old science. Biotechnology 7:257–264

Toxopeus H (1969) Cocoa *Theobroma cacao* L. In: Ferwerda FP, Wit F (eds) Outlines of perennial crop breeding in the tropics. Jeeman, Wageningen, pp 79–109

Waugh R, Ven WTG van de, Phillips MS, Powell W (1990) Chloroplast DNA diversity in the genus *Rubus* (Rosaceae) revealed by Southern hybridisation. Plant Syst Evol 172:65–74

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18, 24:7213–7218

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, 22:6531–6535

Yidana JA, Kennedy AJ, Withers LA (1987) Variation in peroxidase isozymes of cocoa (*Theobroma cacao* L.). In: Proc 10th Int Cocoa Res Conf, Dominican Rep., pp 719–723