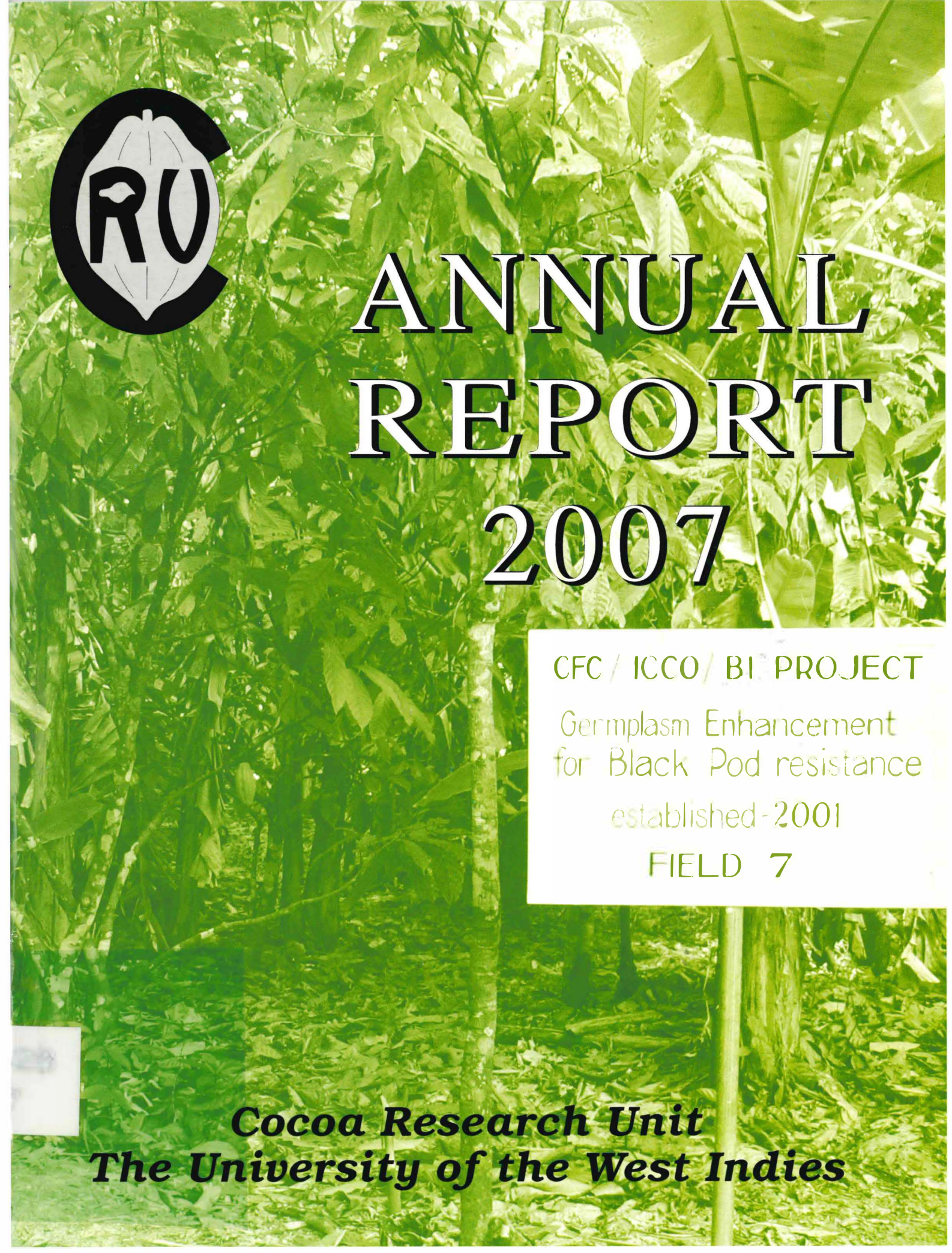

ANNUAL REPORT 2007

CFC / ICCO / BI PROJECT

Germplasm Enhancement for Black Pod resistance

established - 2001

FIELD 7

Cocoa Research Unit
The University of the West Indies

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Cover photograph. Field trial planted in 2001 with enhanced germplasm for Black Pod disease resistance

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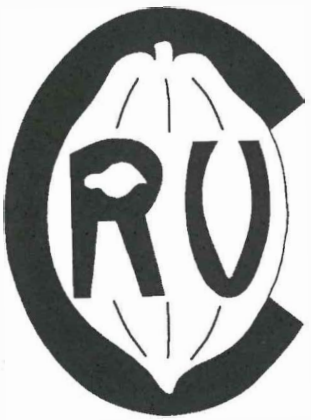

Cocoa Research Unit
The University of the West Indies
St. Augustine, Trinidad and Tobago
2008

Fingerprinting cacao trees in the International Cocoa Genebank, Trinidad with microsatellites

L.A. Motilal, P. Umaharan, D. Zhang and M. Boccara

Introduction

Verification of tree identities within the ICG,T began in the late 1980s under the aegis of V. Mooleedhar, F. Bekele, F. Hosein, E. Johnson, Y. Christopher, and O. Sounigo. A methodology combining morphological description, isoenzyme characterisation and DNA amplification was used as a tool to match sample trees to a reference tree. Initially, DNA profiles were produced with randomly amplified polymorphic DNA (RAPD) products from several primers. However in 2001, RAPDs were phased out in favour of SSRs which give more informative and reliable amplification of small duplicated segments of DNA. Specific primers for these microsatellite loci were designed (Lanaud *et al.*, 1999) and many other primers are now available. More recently, the agarose system for fingerprinting was outmoded when the medium-throughput capillary sequencer system at the USDA, in Beltsville, Maryland became available.

Identity resolutions in cacao genebanks by multilocus microsatellite fingerprinting have commonly used 15 loci (Saunders *et al.*, 2004; Zhang *et al.*, 2006). Suggestions to increase the rate of output have been forwarded (Motilal *et al.*, 2007). The present contribution builds on earlier work and provides an early look into tree fingerprinting within plots in UCRS.

Materials and methods

DNA extraction, quantification, amplification and sizing of amplified products were carried out as described in Motilal *et al.* (2007).

Primer assessment

Table 1. Cacao accessions used for determination of best microsatellites for verification studies in field germplasm collections.

Accession	Location (fingerprinting code, fp)	Group	Country of Origin (Status)
AC 2 [BLZ]	Greenhouse, T1 (fp1026)	Criollo	Belize (wild)
AC 20 [BLZ]	Greenhouse, T1 (fp1032)	Criollo	Belize (wild)
B 9/10-25 [POU]	Marper Farm, C1078	Refractario	Ecuador (cultivated)
BC 3 [BLZ]	Greenhouse, T1 (fp1019)	Criollo	Belize (wild)
COCA 3348/44 [CHA]	UCRS, Field 6B, E374 T2 (fp1047)	Forastero	Ecuador (wild)
CRIOLLO 22 [CRI]	UCRS, Field 4A C276 T3	Criollo	Costa Rica (cultivated)
EET 400 [ECU]	UCRS, Field 6B, F455 T1	Forastero	Ecuador (cultivated)
ELP 1	Greenhouse, T6 (fp950)	Forastero	French Guiana (wild)
GU 241/P	UWI Campus Field 1A, x2y33 (fp500)	Forastero	French Guiana (wild)
H 1	Not available	Forastero	Peru (cultivated)
HF 8 [BLZ]	Greenhouse, T1 (fp987)	Criollo	Belize (wild)
IB 2 [BLZ]	Greenhouse, T1 (fp1020)	Criollo	Belize (wild)

IB 9 [BLZ]	Greenhouse, T1 (fp996)	Criollo	Belize (wild)
ICS 75	San Juan Estate Block 2	Trinitario	Trinidad (cultivated)
ICS 97	San Juan Estate Block 1	Trinitario	Trinidad (cultivated)
ICS 100	San Juan Estate Block 2	Trinitario	Trinidad (cultivated)
IMC 3	UWI Campus Field 3 x1y3	Forastero	Peru (wild)
IMC 12	Marper Farm, C1056	Forastero	Peru (wild)
IMC 16	Marper Farm, D603	Forastero	Peru (wild)
IMC 67	La Reunion Estate	Forastero	Peru (wild)
JA 5/4 [POU]	Marper Farm, C526 (fp2307)	Refractario	Ecuador (cultivated)
JA 5/5 [POU]	Marper Farm, C324 (fp1351)	Refractario	Ecuador (cultivated)
LCT EEN 31	UCRS, Field 6A, A6 T3 (fp450)	Forastero	Ecuador (wild)
LCT EEN 162 S1010	UCRS, Field 5B, C216 T2 (fp2945)	Forastero	Ecuador
MO 9	Marper Farm, D835 (fp253)	Forastero	Peru (wild)
MO 20	Marper Farm, D809 (fp254)	Forastero	Peru (wild)
MOQ 6/95	Marper Farm, C1 (fp582)	Refractario	Ecuador (cultivated)
MXC 67	UWI, Campus Field 12, x3y6	Criollo	Mexico (cultivated)
NA 184	UCRS, Field 5B, G612 T1	Forastero	Peru (wild)
NA 241	UCRS, Field 4A, D383 T4 (fp2716)	Forastero	Peru (wild)
NA 244	UCRS, Field 5B, E400 T3 (fp16)	Forastero	Peru (wild)
NA 266	UCRS, Field 5B, G634 T3 (fp25)	Forastero	Peru (wild)
NA 331	Marper Farm, D477 (fp383)	Forastero	Peru (wild)
NA 406	UCRS, Field 5B, F447 T1 (fp23)	Forastero	Peru (wild)
NA 432	Marper Farm, D717 (fp271)	Forastero	Peru (wild)
NA 435	Marper Farm, D760 (fp260)	Forastero	Peru (wild)
NA 504	Marper Farm, D465 (fp167)	Forastero	Peru (wild)
NA 528	Marper Farm, D774 (fp112)	Forastero	Peru (wild)
NA 680	UCRS, Field 5A, D337 T3 (fp649)	Forastero	Peru (wild)
NA 702	Marper Farm, D104 (fp819)	Forastero	Peru (wild)
NA 705	Marper Farm, C102 (fp1280)	Forastero	Peru (wild)
NA 733	Marper Farm, D721 (fp274)	Forastero	Peru (wild)
NA 734	Marper Farm, D546 (fp377)	Forastero	Peru (wild)
NA 771	UCRS, Field 5B, F478 T4 (fp27)	Forastero	Peru (wild)
NA 773	UCRS, Field 5B, F547 T3 (fp1266)	Forastero	Peru (wild)
NA 831	Marper Farm, D741 (fp267)	Forastero	Peru (wild)
NA 833	Marper Farm, D640 (fp297)	Forastero	Peru (wild)
NAPO 2 [CHA]	UWI, Campus Field 7, x8y9 (fp1922)	Forastero	Ecuador (wild)
PA 279 [PER]	Marper Farm, D59 (fp426)	Forastero	Peru (wild)
PA 299 [PER]	Marper Farm, C936 (fp571)	Forastero	Peru (wild)
POR 1 [TTO]	UWI, Campus Field 2, x2y12 (fp1897)	Criollo	Venezuela
POUND 7/B [POU]	UCRS, Field 6B, F407 T3 (fp521)	Forastero	Peru (wild)
SCA 12	Marper Farm, D205	Forastero	Peru (wild)
SCA 24	Marper Farm, D569	Forastero	Peru (wild)
SPA 5 [COL]	UWI, Campus Field 2, x1y15 (fp1817)	Forastero	Colombia or Peru
U 1	Not Available	Forastero	Peru (cultivated)
UF 613	UCRS, Field 4A, A93 T2 (fp1237)	Trinitario	Costa Rica (cultivated)
YAL 6	Not Available	Forastero	French Guiana

The work reported here builds on that of Motilal *et al.* (2007) by including NA accessions that were not resolved by the fifteen recommended loci (Saunders *et al.*, 2004). The full set of 60 accessions is provided in Table 1. Summary statistics including the polymorphism information

content (PIC; Botstein *et al.*, 1980) were obtained with PowerMarker v3.25 (Liu and Muse, 2005). The probability of identity among full siblings (PID_{sib}; Waits *et al.*, 2001) from each SSR

Table 2. Information about microsatellite loci from sixty cacao accessions.

[1]Locus	Rank	[2]Sepn	[3]N_a	Allele range	[4]PID_{sib}	[5]PIC
CIR1	31	8 (13.3)	7	127-151	0.51	0.52
CIR3	1	21 (35.0)	15	211-279	0.33	0.85
CIR6	23	14 (23.3)	8	229-251	0.43	0.66
CIR7	29	11 (18.3)	6	148-162	0.50	0.55
CIR8	25	15 (25.0)	7	289-307	0.46	0.63
CIR9	12	15 (25.0)	9	258-296	0.39	0.73
CIR10	17	12 (20.0)	6	206-216	0.41	0.70
CIR11	13	20 (33.3)	13	282-320	0.39	0.73
CIR12	11	18 (30.0)	14	164-216	0.38	0.75
CIR15	4	27 (45.0)	14	232-260	0.35	0.80
CIR17	35	7 (11.7)	5	271-289	0.63	0.39
CIR18	14	17 (28.3)	9	331-355	0.39	0.73
CIR22	28	12 (20.0)	8	273-291	0.50	0.57
CIR24	33	11 (18.3)	7	186-204	0.55	0.49
CIR26	15	12 (20.0)	8	272-308	0.40	0.71
CIR29	21	15 (25.0)	9	159-187	0.42	0.68
CIR30	18	10 (16.7)	5	172-186	0.41	0.69
CIR33	3	25 (41.7)	15	273-347	0.35	0.81
CIR37	6	25 (41.7)	14	134-178	0.36	0.78
CIR40	16	21 (35.0)	12	258-296	0.41	0.71
CIR42	5	20 (33.3)	11	202-238	0.35	0.80
CIR43	8	17 (28.3)	8	202-216	0.38	0.75
CIR45	36	8 (13.3)	4	288-294	0.64	0.37
CIR55	34	5 (8.3)	3	240-252	0.60	0.40
CIR56	22	14 (23.3)	10	314-364	0.43	0.67
CIR57	24	10 (16.7)	5	247-257	0.46	0.62
CIR58	7	22 (36.7)	15	208-324	0.38	0.76
CIR60	10	18 (30.0)	10	189-215	0.38	0.75
CIR184	20	16 (26.7)	8	117-147	0.42	0.68
CIR210	26	10 (16.7)	7	138-152	0.47	0.60
CIR229	27	16 (25.0)	8	309-325	0.47	0.60
CIR243	9	16 (26.7)	7	125-141	0.38	0.75
CIR244	2	21 (35.0)	13	240-270	0.34	0.82
CIR274	19	21 (33.3)	11	186-224	0.42	0.70
CIR278	37	5 (8.3)	4	98-118	0.65	0.34
S012	32	9 (15.0)	6	264-285	0.53	0.52
S016	30	8 (13.3)	5	201-221	0.51	0.52
Average ± s.e.m.			8.8 ±0.6		0.44 ± 0.01	0.65 ± 0.02

[1]Microsatellite code; [2]Separation ability; [3]Number of alleles;
[4]Probability of identity of siblings (Waits *et al.*, 2001)
[5]Polymorphism information content (Botstein *et al.*, 1980);
N_a, range and PIC obtained from PowerMarker v3.25 (Liu and Muse, 2005).
PID_{sib} and separation ability obtained from GIMLET v1.3.3 (Valière, 2002).

was obtained with GIMLET v.1.3.3 (Valière, 2002).

Varying combinations of primers (244 sets) were prepared and the corresponding allelic datasets were analysed with GIMLET v.1.3.3 (Valière, 2002). The separation success, of each of the 244 primer sets, as a function of the separation ability of the full complement of the 37 loci was calculated. The 244 datasets were examined for the minimal combination of loci that would give resolution identical to the full complement of 37 loci.

Plot homogeneity assessment in UCRS

Fifty-two plots (51 accessions) containing at least two trees were assessed with six loci (mTcCIR1, mTcCIR6, mTcCIR7, mTcCIR8, mTcCIR33 and mTcCIR60). Trees with missing data were excluded from subsequent analysis. Genotype data were analysed with GIMLET v.1.3.3 (Valière, 2002) for individual plot homogeneity using the regroup option.

Results

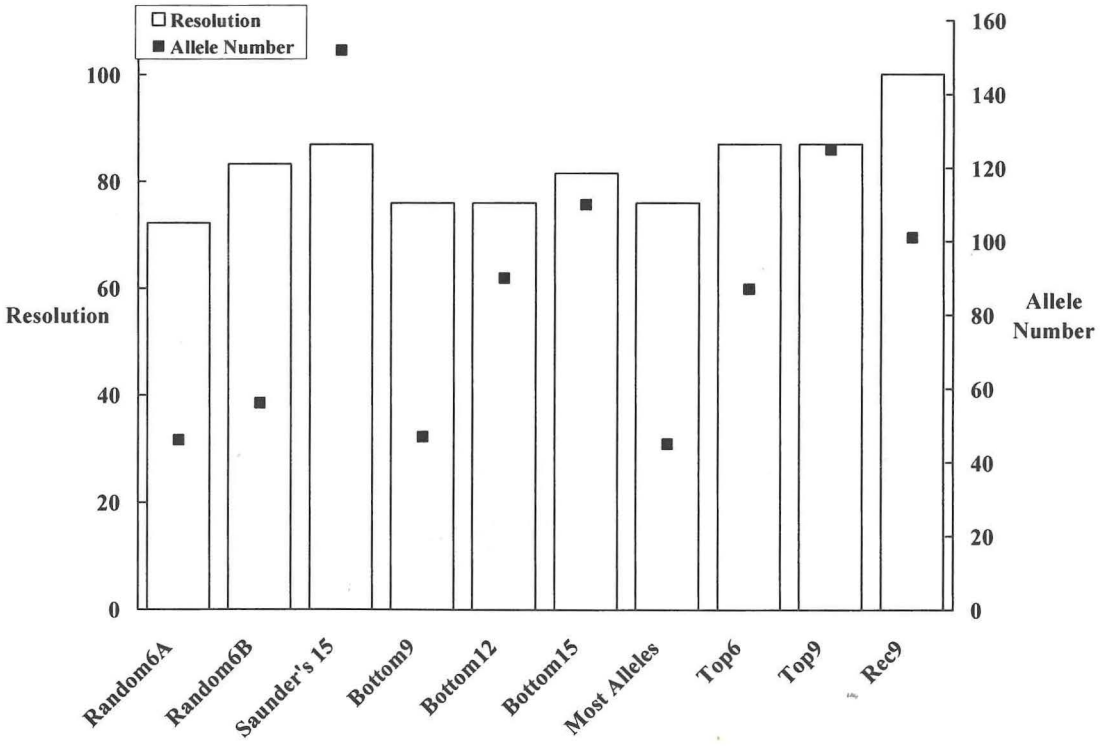

Figure 1. Comparison of resolution ability with allele number in ten primer combinations. Resolution ability was relative to that obtained with all (37) loci on sixty *Theobroma cacao* L. accessions. Top and bottom loci are as ranked with GIMLET v.1.3.3 (Valière, 2002). Saunder's 15 is the set recommended by Saunders *et al.* (2004). Most Alleles are three loci with 15 alleles each. Rec9 is the recommended set of nine primers from this study.

Primer assessment

Characteristics of the individual SSR loci based on the sixty accessions utilised in this study are provided in Table 2. A total of 326 alleles were obtained from 37 loci which resolved the 60 cacao accessions into 54 (90%) groups. Six pairs of accessions were unresolved: AC 20 [BLZ] vs. IB 9 [BLZ], BC 3 [BLZ] vs. HF 8 [BLZ], CRIOLLO 22 vs. IB 2 [BLZ], NA 184 vs. NA 331, NA 432 vs. NA 860 and NA 831 vs. NA 833. The set of primers currently in use for cacao fingerprinting (Saunders *et al.*, 2004) separated the 60 accessions into 47 (78.3%) groups. The accessions that were unresolved with the latter primer set were only NA accessions which are known to be comprised of several sib families: one additional pair was added (NA 406 vs. NA 528) and six other NA accessions (NA 266, NA 435, NA 504, NA 734, NA 773 and NA 860) were lumped into the same group as NA 184 and NA 331.

The separation ability of a primer set was influenced by its composition. Primer combinations comprising the most informative loci as ranked by GIMLET v.1.3.3 (Valière, 2002) performed as well as the set recommended by Saunders *et al.* (2004) even though the numbers of loci and alleles were fewer (Figure 1). An equivalent separation of the sixty accessions with nine loci as compared to that with 37 loci was achieved. These loci were: (a)

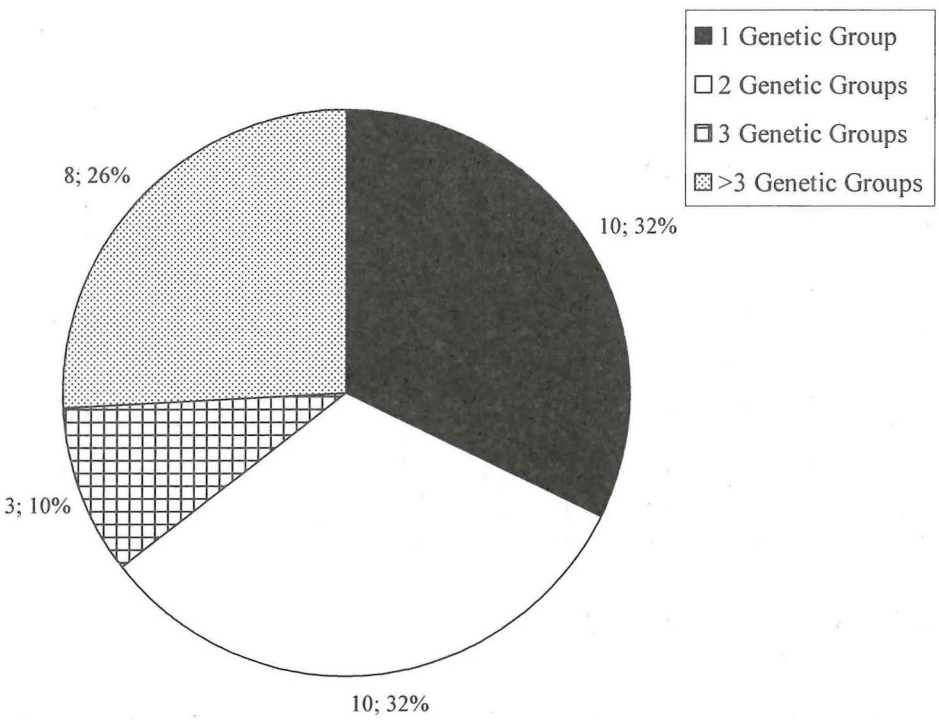

Figure 2. Plot homogeneity assessment in the University Cocoa Research Station of the International Cocoa Genebank, Trinidad. Thirty-one plots with genotype data from six microsatellite loci on at least four trees were evaluated with GIMLET v.1.3.3 (Valière, 2002).

Group 1 – mTcCIR15, mTcCIR26, mTcCIR37; (b) Group 2 – mTcCIR33, mTcCIR57, mTcCIR42 and (c) Group 3 – mTcCIR12, mTcCIR243, mTcCIR244. Each group represents a trio suitable for post-PCR[1] multiplexing based on allele ranges obtained in this study. This set of nine loci had a total of 101 alleles, a combined PID of 8.886×10^{-12} and a combined PID_{sib} of 1.437×10^{-4}; the latter being a hundred-fold increase to that obtained (2.233×10^{-6}) from the set of 15 recommended by Saunders *et al.* (2004).

UCRS plot homogeneity

The 52 plots examined contained 22 homogenous samples (42.3%) and 30 (57.7%) mixed plots with sixteen plots (30.8%) having two genetic groups (Table 3). Analysed plots which contained at least four trees had a mixed composition of genetic identities in 67.7% (21 of 31 plots) of the plots (Figure 2).

Table 3. Plot homogeneity of accessions in the International Cocoa Genebank, Trinidad.

Accession	Field, plot in UCRS	# Trees in plot	Trees studied	# Genetic groups
AM 1/19	5B, I771	8	8 (T1-8)	1
AM 1/28	6A, A1	8	7 (T2,3,4,7,9,12,14)	1
AM 1/53	6A, A2	7	5 (T1,2,13,15,16)	1
AM 1/54	5B, I811	11	4 (T1,2,6,7)	1
AM 1/60	5A, A26	3	3 (T1,3,6)	1
AM 1/70	4A, F549	2	2 (T2,3)	1
AM 1/85	4A, F538	3	2 (T1,2)	1
AM 2/12	5B, B95	4	4 (T1,4,5,8)	1
AM 2/18	5B, H679	2	2 (T2,15)	1
AM 2/61	5B, H716	3	2 (T7,9)	1
AM 2/62	5B, B105	13	5 (T2,4,6,10,15)	2 (T2,4,6,10); (T15)
AM 2/65	5B, I810	8	5 (T5,7,8,11,14)	2 (T14); (T5,7,8,11)
AM 2/82	5B, I806	4	3 (T1,3,4)	1
AM 2/83	5B, B108	15	9 (T1,2,3,4,6,8,9,11,12)	1
AM 2/96	5B, I819	8	3 (T3,5,7)	2 (T3,T5); (T7)
B 12/1	6B, F461	9	9 (T1,2,6,10-15)	4 (T2,13); (T12); (T1,10); (T6, 11,14,15)
B 13/7	5B, I728	12	9 (T3,4,5,6,7,8,11,12)	2 (T3,4,5,6,712,14); (T8,11)
B 17/17	5B, I784	10	7 (T2,3,5,6,7,9,10)	2 (T3); (T2,5,6,7,9,10)
B 18/4	6B, F457	14	10 (T1,2,5,6,8,12-16)	3 (T6); (T1,5,15); (T2,8,12,13,14,16)
B 4/8	6B, F439	5	3 (T1,3,7)	3 (T1); (T3); (T7)
B 7/21	6B. F438	9	8 (T2,3,5,6,7,12,13,14)	7 (T2); (T5); (T6); (T7); (T13); (T14); (T3,12)
CL 10/5	5B, A4	4	4 (T3,4,5,6)	2 (T3,4); (T5,6)
CL 10/14	5A, A1	11	7 (T2,4,5,6,7,13,14)	4 (T7); (T13); (T5,6); (T2,4,14)
CL 13/27	5B, A24	9	6 (T2,10,11,12,13,14)	2 (T12); (T2,10,11,13,14)
CL 27/50	5B, I743	12	9 (T2,3,4,6,9,11,12,13,14)	1
CL 91/5	5B, A64	2	2 (T4,7)	2(T4); (T7)
CL 9/17	5B, A24	12	12 (T1-10,12,16)	4 (T6); (T9,12); (T2,3,4,8);(T1,5,7,10,16)

[1] Polymerase chain reaction

CRUZ 7/8	6B, B83	6	3 (T1,9,10)	2 (T1,9)*; (T10)*
DOM 27	4A, B203	2	2 (T1,2)	2 (T1); (T2)
ICA 70	4A, C290	3	3 (T1,2,3)	1
JA 1/9	6A, A51	3	3 (T3,6,12)	1
JA 5/27	5B, F483	6	5 (T1,4,6,8,9)	5 (T1); (T4); (T6); (T8); (T9)
JA 5/39	5B, D234	14	11 (T1-8,10,12,15)	2 (T6,8); (T1,2,3,4,5,7,10,12,15)
JA 10/16	5B, E411	2	2 (T1,2)	1
LP 1/21	5B, I746	4	4 (T4,6,8,13)	2 (T4); (T6,8,13)
LP 1/21	5B, I779	5	4 (T3,4,5,8)	3 (T4); (T8); (T3,5)
LP 3/4	5B, A33	16	14 (T1-4, T6-9, T11-16)	4 (T12); (T13); (T1,2,14); (T3,4,6,7,8,9,11,15,16)
LP 4/12	5B, I803	10	9 (T1-7,9,10)	1
LP 4/48	5B, B140	10	9 (T1-3, T5-10)	8 (T2); (T3); (T5); (T6); (T7); (T8); (T10); (T1,9)
LP 5/19	6A, B95	3	3 (T2,8,9)	1
LX 38	5B, C206	8	7 (T2,3,4,5,6,8,9)	4 (T4); (T5); (T9); (T2,3,6,8)
LX 43	5B, C201	16	12 (T1, T3-9, T11, T14-16)	2 (T9); (T1, T3-8, T11,T14-16)
MOQ 6/95	5B, C221	5	3 (T4,6,8)	3 (T4); (T6); (T8)
NA 176	4A. D389	3	3 (T1,2,4)	1
NA 669	4A, D418	4	3 (T1,2,4)	3 (T1); (T2); (T4)
PA 169	6B, C180	11	6 (T1,4,7,10,12,15)	3 (T7)*; (T12)*; (T1,4,10,15)
PA 293	4A, F516	4	3 (T1,2,4)	2 (T4); (T1,2)
SLA 16	5B, D242	8	5 (T1,3,7,8,14)	1
SLC 4	5B, A39	6	4 (T1,2,5,6)	1
SLC 18	5B, A13	9	5 (T5,6,7,8,9)	2 (T5,7,8,9); (T6)
TRD 15	4A, A43	2	2 (T1,3)	2 (T1); (T3)
TRD 111	4A, A87	3	3	1

*May be one group as one difference of 2 base pairs is responsible for the separation

Discussion

Fingerprinting a germplasm collection with the aim of detecting mislabelling errors relies on the use of loci that can differentiate among present holdings and future acquisitions. These loci should be able to maximise differences amongst accessions. The present study demonstrated that the composition of the set of loci used for fingerprinting will affect the resolution efficiency. Furthermore, a set of nine loci (mTcCIR12, 15, 26, 33, 37, 42, 57, 243 and 244) was identified that was superior to that of Saunders *et al.* (2004) and supersedes those recommended in an earlier report (Motilal *et al.*, 2007).

This study found a high level (58%) of plots in UCRS that putatively contained replicated clonal material but instead contained more than one genetic group. This is nearly twice the percentage of off-types reported previously for cacao germplasm collections (Figueira, 1998; Risterucci *et al.*, 2001; Motilal and Butler, 2003) including the ICG,T (Sounigo *et al.*, 2001). However, this is still a relatively small sample from the 2,300 accession in the ICG,T, so it can only serve as an approximate estimate of the error rate in the whole collection. Nevertheless, the importance of recording the tree number when samples are taken and maintaining up-to-date records for information on individual trees cannot be over-emphasised.

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