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Variability of butterfat content in cacao (*Theobroma cacao* L.): combination and correlation with other seed-derived traits at the International Cocoa Genebank, Trinidad

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Abstract

Cocoa butterfat and cocoa powder are key economic products from the seeds of the cacao tree (*Theobroma cacao* L.). In this study, 323 accessions (comprised mainly of Upper Amazon Forasteros and Refractarios) from the International Cocoa Genebank, Trinidad were characterized for one biochemical and five morphological seed-derived traits. The data were analysed using non-parametric statistics including correlation analysis to identify promising parental candidates for future cacao breeding programmes. The Upper Amazon Forastero group had the greatest proportion of accessions with high butterfat content in cotyledons, whereas Refractario and Trinitario groups tended to contain more accessions with high butterfat content per fruit. The correlation of butterfat content of cotyledons with the dry mass of cotyledons was inconsistent in significance and direction. However, consistent significant positive correlations between butterfat content per fruit, cotyledon size and dry mass of cotyledons were found. The results suggested that butterfat content is a likely trait for independent selection but that selection for increased cotyledon size could lead to the selection of genotypes for high butterfat yield. Several promising accessions exhibited favourable levels of multiple traits and MATINA 1/7, CRU 51, AM 2/91 [POU], CRU 133, EET 58 [ECU] and POUND 18/A [POU] could be recommended as good choices for parental stock in breeding programmes for improving cacao butterfat content.

Keywords: breeding; butterfat; cacao; correlation; seed traits

Introduction

The seeds of the cacao plant (*Theobroma cacao* L.) are processed to obtain cocoa liquor, cocoa powder and cocoa butterfat for use in the pharmaceutical and food industries. World production of cocoa beans in 2005/2006 was 3.5 million tonnes (www.icco.org/statisitcs) and is expected to reach 3.7 million tonnes in 2010 (FAO, 2003). Chocolate confectionery retailed at £2.53 billion in 2003 in the UK (http://www.bccca.org.uk/) and USD 27.9 billion in 2005 in USA (http://www.ecandy.com/ecandyfiles/2005_Confectionery_Review.ppt#6).

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The yield of edible material, particularly bean 'weight' (mass) and cocoa butterfat content are two industry quality parameters used in price determination (Biscuit, Cake, Chocolate and Confectionery Alliance (BCCCA), 1996). Plants whose fruits contain many large heavy seeds with high butterfat content are therefore desirable as farmers would obtain greater returns whilst satisfying industry standards. High butterfat content would be beneficial to the industry as fewer nibs would need to be ground to obtain the same amount of butterfat. The primary focus of breeding programmes in the past, however, has been to improve seed yield and disease resistance (Kennedy *et al.*, 1987; Lockwood and Yin, 1993), with limited emphasis placed on improving product quality. Screening for butterfat content conducted in Malaysia [51.5–60.3% in 126 accessions; End *et al.*, 1991 (cited in Pires *et al.*, 1998)], Trinidad and Tobago (46.1–59.8% in 247 accessions; Khan, 1998) and Brazil (45.4–60.3% in 490 accessions; Pires *et al.*, 1998) has identified sources of high butterfat content. However, these are yet to be exploited in breeding for increased seed yield and butterfat content.

A potentially diverse collection of primary and secondary germplasm from the South American Amazon (see Kennedy and Mooleedhar, 1993) housed in the International Cocoa Genebank, Trinidad (ICG,T) is yet to be fully exploited through breeding. Recent studies have shown considerable variation for disease resistance (Iwaro *et al.*, 2003; Surujdeo-Maharaj *et al.*, 2004; Thevénin *et al.*, 2005), morphology (Bekele *et al.*, 2006) and butterfat content (Khan, 1998) within this genebank, which can be exploited in pre-breeding programmes to accumulate genes for desirable traits. The identification of superior accessions useful for breeding programmes was initiated in the Common Fund for Commodities/International Cocoa Organization/International Plant Genetic Resources Institute project (CFC/ICCO/IPGRI project; Sounigo *et al.*, 2006) and has paved the way for broadening the genetic base of farmed cacao. The present study was therefore undertaken to complement earlier studies at the ICG,T by investigating the interrelationships between butterfat content and other key seed traits and to identify superior accessions with good combinations, which would be beneficial for future breeding programmes focussed on improving seed yield and butterfat content.

Materials and methods

Data collection

Ripe, well-developed, disease-free, open-pollinated fruits were collected from accessions in the ICG,T. Pod index (the number of fruits required to produce 1 kg of dried cocoa), seed number and lengths and widths of dried unfermented seeds without testae (i.e. embryo enclosed in cotyledons; hereafter referred to as cotyledons) were recorded over the period 1992–2005 in the Morphological Characterization Project at the Cocoa Research Unit of the University of the West Indies (CRU/UWI) [see Bekele *et al.* (2006) for methodology].

Additionally, dry mass (g) and butterfat content (%) data from dried cotyledons were recorded over the period December 1996 to June 1998 in the Butterfat Project at CRU/UWI (Khan, 1998). To determine butterfat content, two samples (20 dried, paired cotyledons) were taken from each accession and ground separately using a coffee mill (Procter Silex) via two 30-s pulses. The ground samples were then placed in foil boats and dried for 16 h at 110°C in a natural convection oven (Lab-Line Instruments Inc., Melrose Park, IL), cooled and stored in a desiccator at 25°C until analysis. A 2 g subsample from each of the dried ground samples was boiled for 30 min in 100 mL of 4.0 N HCl. After filtration, the residue was washed with distilled water until it was clear of the pigment and then placed in an extraction thimble and dried for 16 h at 60°C in a natural convection oven (Lab-Line Instruments Inc.). Butterfat was then extracted using the Soxtec Soxhlet System HT6 (Tecator, Sweden). Each dried subsample was extracted under reflux for 1 h using petroleum ether as the solvent (40–60 fraction), which was later removed by rotary evaporation. The butterfat extract was then dried in a natural convection oven for 30 min at 100°C, cooled in a desiccator and the percentage butterfat in the cotyledons determined on a dry matter basis.

Cotyledon size (cm^2) was estimated as the product of length and width of the dried cotyledons. Cotyledon mass per fruit (g) was estimated as the product of mean dry mass of cotyledons and seed number; butterfat content per fruit (%) was estimated as the product of cotyledon mass per fruit and mean butterfat content of a seed. A common subset of accessions from these two datasets were identified and retained for subsequent analysis. Accessions arising from ICG,T field plots with known misidentifications were discarded.

Accession stratification

In cacao, clones are usually named according to their origin resulting in various accession groups with broad similarities of origin and history. These accession groups can be conveniently classed into Criollo, Forastero, Refractario and Trinitario categories. The Forastero category has been traditionally subdivided into Lower Amazon Forastero and Upper Amazon Forastero.

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The accessions GU 241/P and GU 305/P would form part of the GU accession group and could be included in the Lower Amazon Forastero category. However, the fruits of the GU material are quite different from other Forastero material and the distinctiveness of the GU accession group has been described (Lachenaud *et al*., 2001 and references therein). These GU accessions were therefore grouped with the unknown material into a Various category to obtain a more homogenous Forastero category. The details of each accession group can be obtained from ICGD (2007).

The final dataset consisted of 323 accessions and comprised 147 Forasteros (1 Lower Amazon Forastero and 146 Upper Amazon Forasteros), 115 Refractarios, 15 Trinitarios and 46 Various accessions of differing origins (Table 1).

Table 1. Documentation of germplasm accessions used in this study

Category[a]	Accession group	Number of accessions
LAF	CRUZ	1
REF	AM	12
	B	15
	CL	13
	CLM	2
	JA	37
	LP	15
	LV	1
	LX	4
	LZ	2
	MOQ	6
	SJ	4
	SLA	2
	SLC	2
TRI	DOM	1
	GS	1
	ICS	6
	TRD	3
	UF	4
UAF	AMAZ	3
	COCA	1
	IMC	17
	LCT EEN	5
	MO	3
	NA	75
	PA	34
	POUND	5
	SCA	3
VAR	CRU	23
	DOPOL	3
	E	1
	EET	5
	EQX	1
	GU	4
	M	1
	MATINA	1
	SP	1
	SPA	2
	SPEC	4

[a] LAF, Lower Amazon Forastero; REF, Refractario; TRI, Trinitario; UAF, Upper Amazon Forastero; VAR, Various.

Statistical analyses

Descriptive statistics

Descriptive statistics for butterfat content of cotyledons, butterfat content per fruit, cotyledon size, dry mass of cotyledons, cotyledon mass per fruit, seed number and pod index were generated using the Instat Plus freeware program (Statistical Services Centre, 2005) for the entire dataset, for the groups Upper Amazon Forastero, Refractario and Trinitario and for accession groups containing at least five accessions. The top 25 ranked accessions for each trait were identified.

Comparison testing

Trait data over the 323 accessions were tested for normality with the Shapiro–Wilk test using the freeware program PAST (Hammer *et al*., 2001). With the exception of seed number, significant ($P < 0.01$) deviations were observed. Arcsine transformation of butterfat content of cotyledons did not normalize the data. The traits were therefore tested using non-parametric statistics. Lower and upper quartiles from the entire dataset were used to group Refractario and Upper Amazon Forasteros for butterfat content of cotyledons, butterfat content per fruit, cotyledon mass per fruit and cotyledon size into low (≤lower quartile), medium (lower quartile to upper quartile) and high (>upper quartile) categories. The distribution of these classes was tested with Chi-square using Instat Plus (Statistical Services Centre, 2005).

Pairwise comparison between the Refractario, Trinitario and Upper Amazon Forastero categories for butterfat content of cotyledons, butterfat content per fruit, cotyledon mass per fruit and cotyledon size were tested using the Kruskal–Wallis test, which generated Mann–Whitney U statistics in PAST (Hammer *et al*., 2001). Accession groups containing more than seven accessions (AM, B, CL, JA, LP, CRU, IMC, NA) were similarly analysed for these traits.

Correlation analysis and trait combination

The level of correlation among the traits: butterfat content of cotyledons, butterfat content per fruit, cotyledon size, dry mass of cotyledons, cotyledon mass per fruit, seed number and pod index was assessed with Spearman's r_s in PAST (Hammer *et al*., 2001) for the Refractario and Upper Amazon Forastero categories. The description of correlation effect magnitude followed Hopkins (2002).

Exclusion limits of less than 55% for butterfat content of cotyledons, 24% for butterfat content per fruit, 2.8 cm^2 for cotyledon size, 1.1 g for dry mass of cotyledons, 45 g for cotyledon mass per fruit, 45 for seed number and more than 23 for pod index were applied to obtain the top-ranked accessions.

Results

Trait combination in accessions

Butterfat content of cotyledons averaged 53.51 ± 0.12% over the 323 accessions (Table 2) and ranged from 44.4% (CL 27/50) to 59.8% (CRUZ 7/8), while butterfat content per fruit was 21.0 ± 0.3% and ranged from 9.0% (NA 92) to 37.7% (MATINA 1/7).

The top 25 ranked accessions per trait are presented in Table 3 and selected trait combinations in Table 4. A high dry mass of cotyledons was observed in 124 accessions, whereas 84 accessions had a large cotyledon size. Twenty-five (30%) of the latter accessions were not included in the high dry mass of cotyledons class. Generally, top-ranked accessions for butterfat content of cotyledons were not included in the top 12 ranked list for other traits (Table 3) and only 3 accessions (CRU 133, CRU 127 and POUND 18/A [POU]) were selected for other traits when the top 25 accessions were considered. A high butterfat content of cotyledons and per fruit was observed in 68 and 78 accessions, respectively; 17 accessions (25 and 22%, respectively) possessed a combination of these two traits. Two accessions (CRU 127 and POUND 18/A [POU]) were present in the top 25 list for both butterfat content of cotyledons (56.5%) and butterfat content per fruit (30 and 34%, respectively). Similarly, only four varieties with high (56–57%) butterfat content in cotyledons (CRU 133, JA 3/5 [POU], PA 157 [PER] and POUND 18/A [POU]) were considered as good candidates for pod index with pod indices of 19, 24, 23 and 21, respectively.

Table 2. Trait descriptive statistics of *Theobroma cacao* L. germplasm

Trait[a]	Group[b]	Mean	s.e.m.[c]	Range
BFC	REF	52.46	0.19	44.4–57.0
	TRI	52.50	0.49	47.7–54.6
	UAF	54.46	0.12	50.9–58.0
	Global	53.51	0.12	44.4–59.8
BFF	REF	21.88	0.44	9.6–32.4
	TRI	23.35	1.79	11.7–34.0
	UAF	19.79	0.35	9.0–34.4
	Global	20.98	0.27	9.0–37.7
CDM	REF	1.08	0.02	0.5–1.4
	TRI	1.15	0.08	0.7–1.6
	UAF	0.91	0.01	0.5–1.4
	Global	1.00	0.01	0.5–1.9
CMF	REF	41.65	0.80	19.0–60.9
	TRI	44.22	3.17	23.7–62.8
	UAF	36.31	0.63	17.5–62.2
	Global	39.23	0.50	17.5–68.7
CS	REF	2.66	0.04	1.7–3.7
	TRI	2.83	0.12	2.1–3.7
	UAF	2.32	0.03	1.0–3.5
	Global	2.52	0.03	1.0–3.9
SNO	REF	38.36	0.48	25.6–51.1
	TRI	38.91	1.42	26.9–49.5
	UAF	39.86	0.56	21.2–57.8
	Global	39.35	0.34	35.3–43.5
PI	REF	26.84	0.61	15.5–51.8
	TRI	27.10	1.69	16.0–39.1
	UAF	30.17	0.53	17.0–56.8
	Global	28.33	0.37	15.5–56.8

[a] BFC, butterfat content in cotyledons (%); BFF, butterfat content per fruit (%); CDM, dry mass of cotyledons (g); CMF, cotyledon mass per fruit (g); CS, cotyledon size as cotyledon length × width from seed without testa (cm^2); SNO, seed number; PI, pod index.
[b] Accession groupings were REF (Refractario, $n = 115$), TRI (Trinitario, $n = 15$), UAF (Upper Amazon Forastero, $n = 146$), Global ($n = 323$).
[c] Standard error of the mean.

CRUZ 7/8 with the highest butterfat content in cotyledons (59.8%) had a low cotyledon size (1.89), low dry mass of cotyledons (0.657 g) and high pod index (32.38) and was at the lower end of the medium butterfat content per fruit category. Within the dataset of 323 accessions there were no accessions that had a combination of high butterfat content of cotyledons, high seed number, large cotyledon size and high dry mass of cotyledons. Four accessions (CRU 133, EET 58 [ECU], JA 10/16 [POU] and LP 4/12 [POU]) combined high butterfat content of cotyledons, large cotyledon size, high dry mass of cotyledons and low pod index. CRU 133 was in the top 25 accessions for butterfat content of cotyledons and pod index; EET 58 [ECU] for cotyledon size, dry mass of cotyledons and pod index and JA 10/16 [POU] for pod index. LP 4/12 [POU] was excluded from the top 25 ranked accessions for all traits. MATINA 1/7 with 54.9% butterfat content in cotyledons was just excluded from the top 25 ranked accessions for this trait although it was the best performer for butterfat content per fruit, dry mass of cotyledons, cotyledon size and cotyledon mass per fruit and was also included among the top-rated individuals for low pod index.

Based on inclusion in the top 25 accessions, AM 2/91 [POU], CRU 51, ICS 68, IMC 10, IMC 65, JA 1/10 [POU], JA 1/17 [POU], JA 5/24 [POU], JA 5/31 [POU], JA 5/36 [POU], JA 10/16 [POU] and MATINA 1/7 were good parental candidates for low pod index and high butterfat content per fruit (Table 4). Superior individuals for parental candidates for improvement of butterfat content (parental mean value of butterfat content in cotyledons ≥54%; parental mean value of butterfat content per

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Table 3. Top 25 ranked accessions for the traits examined

BFC[a] > 55%	BFF[b] > 24%	CS[c] > 2.8 cm^2	CDM[d] > 1.1 g	CMF[e] > 45 g	SNO[f] > 45	PI[g] < 23
CRUZ 7/8 (59.8 ± 0.4)	MATINA 1/7 (37.7)	MATINA 1/7 (3.90)	MATINA 1/7 (1.93 ± 0.06)	MATINA 1/7 (68.7)	IMC 10 (57.8 ± 6.1)	JA 5/36 (15.5)
SPEC 194/75 (58.8 ± 0.4)	CRU 51 (35.9)	EET 58 (3.84)	ICS 39 (1.60 ± 0.03)	CRU 51 (65.5)	POUND 18/A (53.4 ± 7.3)	ICS 68 (16.0)
PA 132 (58.0 ± 0.7)	IMC 10 (34.4)	CRU 51 (3.82)	UF 168 (1.57 ± 0.02)	UF 168 (62.8)	NA 137 (53.3 ± 3.4)	CRU 51 (16.0)
PA 293 (57.3 ± 0.6)	POUND 18/A (34.0)	CRU 105 (3.80)	UF 677 (1.57 ± 0.07)	ICS 39 (62.6)	IMC 6 (52.9 ± 5.8)	IMC 10 (17.0)
NA 702 (57.2 ± 0.4)	ICS 39 (34.0)	CRU 89 (3.79)	CRU 51 (1.48 ± 0.07)	IMC 10 (62.2)	CRU 59 (52.9 ± 8.3)	JA 5/35 (17.0)
NA 13 (57.2 ± 0.1)	UF 168 (33.7)	UF 668 (3.71)	UF 668 (1.44 ± 0.02)	EET 95 (61.7)	IMC 53 (52.0 ± 13.1)	CRU 105 (17.2)
NA 312 (57.2 ± 0.1)	JA 5/34 (32.4)	JA 5/35 (3.69)	NA 21 (1.44 ± 0.09)	UF 677 (61.6)	POUND 25/A (52.0 ± 4.4)	JA 5/31 (17.4)
NA 176 (57.1 ± 0.0)	UF 677 (32.1)	JA 5/41 (3.54)	LP 3/40 (1.45 ± 0.08)	JA 1/17 (60.9)	IMC 55 (51.6 ± 5.2)	AM 2/91 (17.5)
JA 3/5 (57.0 ± 0.2)	JA 1/17 (32.2)	AMAZ 6 (3.51)	JA 5/18 (1.42 ± 0.02)	POUND 18/A (60.2)	IMC 85 (51.5 ± 7.1)	LP 3/40 (17.9)
CRU 133 (56.9 ± 0.0)	EET 95 (31.8)	AM 2/91 (3.48)	EET 19 (1.40 ± 0.12)	JA 5/34 (59.8)	B 17/10 (51.1 ± 14.5)	EET 58 (18.1)
NA 739 (56.8 ± 0.3)	IMC 65 (31.3)	TRD 86 (3.45)	AM 2/91 (1.39 ± 0.06)	IMC 65 (58.1)	IMC 94 (50.8 ± 3.3)	JA 1/17 (18.3)
PA 279 (56.8 ± 0.1)	ICS 68 (31.2)	LP 4/48 (3.44)	JA 1/17 (1.38 ± 0.08)	ICS 68 (57.7)	IMC 68 (50.7 ± 10.3)	JA 5/24 (18.4)
CRU 127 (56.5 ± 0.0)	DOPOL 6/1510 (30.5)	JA 10/4 (3.34)	ICS 66 (1.36 ± 0.01)	DOPOL 6/1510 (57.5)	IMC 59 (50.3 ± 9.8)	CL 27/50 (18.7)
PA 179 (56.5 ± 0.5)	AM 2/91 (30.0)	JA 5/31 (3.33)	JA 5/34 (1.35 ± 0.03)	JA 5/31 (56.5)	IMC 65 (50.2 ± 9.4)	B 6/8 (18.7)
POUND 18/A (56.5 ± 0.4)	JA 1/10 (30.0)	CRU 136 (3.25)	MOQ 6/102 (1.34 ± 0.09)	JA 5/36 (55.9)	NA 62 (50.1 ± 4.6)	JA 5/41 (18.8)
MOQ 6/95 (56.5 ± 0.2)	CRU 127 (30.0)	SJ 1/19 (3.25)	EET 95 (1.34 ± 0.01)	JA 1/10 (55.1)	CRU 95 (50 ± 10.2)	IMC 65 (18.8)
PA 157 (56.4 ± 0.3)	JA 5/36 (29.7)	UF 168 (3.24)	GU 241/P (1.34 ± 0.04)	AM 2/91 (55.1)	IMC 38 (49.9 ± 4.4)	CRU 133 (18.9)
SCA 19 (56.4 ± 1.0)	IMC 6 (29.4)	JA 10/41 (3.23)	EET 58 (1.32 ± 0.10)	JA 5/24 (54.5)	IMC 60 (49.6 ± 8.9)	CL 10/5 (19.1)
PA 173 (56.3 ± 0.2)	JA 5/31 (29.3)	SP 1 (3.21)	JA 1/9 (1.30 ± 0.04)	JA 5/27 (54.3)	JA 5/23 (49.5 ± 10.4)	JA 1/10 (19.2)
NA 387 (56.3 ± 0.2)	B 17/10 (29.1)	LP 3/4 (3.20)	LZ 13 (1.30 ± 0.05)	B 17/10 (53.6)	ICS 68 (49.5 ± 4.4)	LP 4/48 (19.2)
NA 475 (56.3 ± 0.4)	EET 19 (29.0)	SPEC 138/15 (3.19)	JA 1/10 (1.29 ± 0.03)	LP 3/40 (53.6)	NA 833 (49.4 ± 3.3)	MATINA 1/7 (19.2)
CRU 80 (56.3 ± 0.3)	IMC 55 (28.6)	CRU 137 (3.19)	JA 5/27 (1.29 ± 0.03)	EET 19 (53.6)	POUND 16/B (49.2 ± 5.8)	CRU 57 (19.2)
IMC 11 (56.2 ± 0.6)	JA 5/24 (28.4)	EET 195 (3.18)	DOPOL 6/1510 (1.29 ± 0.05)	IMC 6 (53.6)	NA 739 (49 ± 4.7)	UF 29 (19.3)
NA 528 (56.2 ± 0.3)	JA 10/16 (28.1)	ICS 39 (3.17)	LCT EEN 73/A (1.28 ± 0.06)	JA 1/9 (53.2)	NA 831 (49 ± 7.5)	SJ 2/22 (19.3)
NA 46 (56.1 ± 0.2)	JA 5/27 (27.8)	LP 5/19 (3.16)	CL 9/17 (1.28 ± 0.05)	CRU 127 (53.1)	IMC 51 (48.6 ± 5.7)	JA 10/16 (19.4)

Suffix identifiers of [ECU] for EET; [PER] for PA; [POU] for AM, AMAZ, B, JA, LP, POUND and SJ; and [VEN] for SP accession groups are present.
[a] Butterfat content in cotyledons (%).
[b] Butterfat content per fruit (%).
[c] Cotyledon size as cotyledon length × width (cm^2).
[d] Cotyledon dry mass (g).
[e] Cotyledon mass per fruit (g).
[f] Seed number.
[g] Pod index.

Table 4. Cacao accessions with selected combinations of seed-derived traits

Trait combination[a]	Number of accessions	Selected accessions[b]	Traits in top 25 accessions
CDM & PI & SNO & BFC	3	POUND 18/A [POU]	BFF, BFC, CMF, SNO
		IMC 10	BFF, CMF, PI, SNO
CDM & PI & BFC	5	EET 58 [ECU]	CDM, CS, PI
		JA 10/16 [POU]	BFF, PI
BFF & PI & CMF & CS	31	AM 2/91 [POU]	BFF, CDM, CS, PI
		CRU 51	BFF, CDM, CS, PI
		MATINA 1/7	BFF, CDM, CMF, CS, PI
		ICS 39	BFF, CDM, CMF, CS
		UF 168	BFF, CDM, CMF, CS
		ICS 68	BFF, CMF, PI, SNO
		JA 1/10 [POU]	BFF, CDM, CMF, PI
		JA 1/17 [POU]	BFF, CDM, CMF, PI
		JA 5/24 [POU]	BFF, CMF, PI
		JA 5/31 [POU]	BFF, CMF, CS, PI
BFF & CMF & PI	25	B 17/10 [POU]	BFF, PI, SNO
		IMC 65	BFF, CMF, PI, SNO
		JA 5/36 [POU]	BFF, CMF, PI

[a] BFC, butterfat content in cotyledons (%); BFF, butterfat content per fruit (%); CDM, cotyledon dry mass (g); CMF, cotyledon mass per fruit (g); CS, cotyledon size as cotyledon length × width (cm^2); PI, pod index; SNO, seed number.
[b] Two or more entries in ranked traits of top 25 accessions.

fruit ≥30%) along with other seed-derived traits were: AM 2/91 [POU], CRU 51, CRU 133, ICS 39, ICS 68, IMC 10, IMC 65, JA 1/10 [POU], MATINA 1/7 and POUND 18/A [POU] with AM 2/91 [POU], CRU 51, CRU 133, ICS 39, IMC 10, MATINA 1/7 and POUND 18/A [POU] as the best parental candidates having at least 55% mean butterfat content.

Accession groups

Descriptive statistics for the traits examined for 14 accession groups are given in Table 5. The accession group CL had the lowest butterfat content in cotyledons and second lowest butterfat content per fruit whilst the POUND group had the highest butterfat content in

Table 5. Means of cotyledon size, butterfat content in cotyledons, butterfat content per fruit and cotyledon mass per fruit in selected accession groups

Accession group[a]	Number of accessions	CS[b]	BFC[c]	BFF[d]	CMF[e]
AM*	12	2.70 ± 0.11	52.03 ± 0.53	19.76 ± 1.53	37.79 ± 2.69
B*	15	2.51 ± 0.50	52.14 ± 0.52	20.81 ± 1.04	39.93 ± 1.93
CL*	13	2.63 ± 0.12	51.29 ± 0.74	18.86 ± 1.42	36.67 ± 2.65
CRU†	23	2.70 ± 0.12	53.38 ± 0.49	22.60 ± 0.95	42.41 ± 1.77
EET†	5	3.21 ± 0.17	53.52 ± 0.65	25.69 ± 2.75	48.17 ± 5.50
ICS‡	6	2.70 ± 0.13	53.37 ± 0.54	26.20 ± 2.16	48.98 ± 3.73
IMC#	17	2.55 ± 0.07	54.73 ± 0.19	24.71 ± 1.07	45.15 ± 1.95
JA*	37	2.75 ± 0.06	53.23 ± 0.25	24.81 ± 0.58	46.65 ± 1.11
LCT EEN#	5	2.22 ± 0.21	53.40 ± 0.28	19.47 ± 2.55	36.49 ± 4.81
LP*	15	2.71 ± 0.13	52.13 ± 0.56	20.66 ± 1.16	39.70 ± 2.26
MOQ*	6	2.69 ± 0.14	53.39 ± 0.71	22.40 ± 2.10	42.11 ± 4.17
NA#	75	2.30 ± 0.04	54.32 ± 0.18	18.59 ± 0.42	34.25 ± 0.78
PA#	34	2.24 ± 0.06	54.92 ± 0.26	20.16 ± 0.42	36.68 ± 0.72
POUND#	5	2.37 ± 0.14	55.06 ± 0.44	22.47 ± 3.70	40.63 ± 6.41

[a] Accession group (*Refractario, †Various, ‡Trinitario, #Upper Amazon Forastero).
[b] Cotyledon size (cm^2) as cotyledon length × width.
[c] Butterfat content in cotyledons (%).
[d] Butterfat content per fruit (%).
[e] Cotyledon mass per fruit (g).

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cotyledons but medium butterfat content per fruit. A reversal of top performers in butterfat content of cotyledons to low butterfat content per fruit also occurred with NA and PA accession groups. Low performers in butterfat content of cotyledons could also become high butterfat content per fruit performers and included the accession groups B, LP, MOQ, but was particularly apparent in CRU, EET, ICS and JA accession groups (Table 5).

Significant pairwise comparisons for nine selected accession groups are provided in Table 6. Twenty accession group pairs had a significant difference in butterfat content of cotyledons but only ten of these accession pairs (AM/IMC, B/IMC, B/NA, CL/IMC, CL/JA, CRU/PA, JA/NA, JA/PA, LP/IMC and NA/PA) returned significant butterfat content per fruit effects. Conversely, seven accession group pairs (AM/JA, B/JA, CRU/JA, LP/JA, IMC/NA, CRU/NA and IMC/PA) revealed significant differences for butterfat content per fruit although butterfat content of cotyledons was not significantly different in these pairs.

Accession categories

Descriptive statistics for the traits studied in Refractario Trinitario and Upper Amazon Forastero categories are presented in Table 2. *H* statistics corrected for ties (*Hc*) statistics from the Kruskal–Wallis test for the traits' butterfat content in cotyledons and per fruit, cotyledon mass per fruit and cotyledon size were all highly significant ($P < 0.001$) when Refractario, Trinitario and Upper Amazon Forastero categories were compared. Significant pairwise differences between individual accession categories are presented in Table 7. Cotyledon size, cotyledon mass per fruit, butterfat content of cotyledons and butterfat content per fruit distributions had similar medians in Refractario and Trinitario categories. However, the latter two categories were significantly different from the Upper Amazon Forastero category.

The proportion of genotypes in low, medium and high classes was significantly different ($P < 0.001$) between Refractario and Upper Amazon Forastero categories for cotyledon size, cotyledon mass per fruit, butterfat content of cotyledons and butterfat content per fruit (Table 8). The Refractario category contained a higher percentage of accessions with large cotyledon size and cotyledon mass per fruit than the Upper Amazon Forastero category. Furthermore, the Upper Amazon Forastero category contained more accessions with high butterfat content in cotyledons than the Refractario category but the latter had a greater proportion of accessions with high butterfat content per fruit than the Upper Amazon Forastero category.

Correlations

Correlation coefficients and their significance are provided in Table 9. As expected, cotyledon size was significantly ($P < 0.01$) correlated with dry mass of cotyledons, cotyledon mass per fruit, butterfat content per fruit and pod index in Refractarios, Upper Amazon Forasteros and the entire dataset. Significant ($P < 0.01$) correlations (moderate–large; positive) irrespective of grouping were also obtained for cotyledon size and butter fat content per fruit.

Significant correlations obtained over the entire dataset for size and butterfat content of cotyledons, dry mass and butterfat content of cotyledons and dry mass of cotyledons with seed number became non-significant within the Refractario and Upper Amazon Forastero categories except for butterfat content and dry mass of cotyledons in Upper Amazon Forasteros.

Discussion

The present study evaluated the interrelationship of butterfat content and other seed-derived traits of 323 accessions from the ICG,T to determine which traits could be co-selected and to identify promising candidate parental genotypes for future breeding programmes. The main trait of interest, cotyledon butterfat content from dried unfermented seeds, varied from 44 to 60% across all 323 accessions. Butterfat content in cotyledons of 14 accession groups was below the required range of 55–58% of commercial fermented seed (BCCCA, 1996). However, Pires *et al.* (1998) suggested that this would be due in part to losses in seed mass that might occur during fermentation. Increased progeny fat content is known to occur when parental accessions have high fat content (Alvarado and Bullard, 1961; Pires *et al.*, 1998). Thus, the results of the present study have added valuable information on the variability of an important industry trait within the ICG,T collection. We have also identified superior individuals (Tables 8 and 9) for use in breeding programmes. In this regard, the accessions CRUZ 7/8 (Lower Amazon Forastero), SPEC 194/75 (Various) and PA 132 [PER] (Upper Amazon Forastero) are promising parental candidates for butterfat content of cotyledons. Likewise, the accessions MATINA 1/7 (Various), CRU 51 (Various) and IMC 10 (Upper Amazon Forastero) are promising parental candidates for butterfat yield as measured as butterfat content per fruit.

The variability in trait data enabled distinction between cacao categories, especially between the Refractario and the Upper Amazon Forastero categories (Tables 4 and 5). This is in agreement with previous work (Iwaro *et al.*, 2003; Bekele *et al.*, 2006). The Refractario category

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Table 6. Significant pairwise comparisons of four seed traits between nine accession groups

	AM[a]	B[a]	CL[a]	JA[a]	LP[a]	CRU[b]	IMC[c]	NA[c]	PA[c]
AM	–								
B		–							
CL			–						
JA	b**, d**	b**, d**	a*, b***, d**	–					
LP				b**, d*	–				
CRU			a*	b*, d*		–			
IMC	a***, b*, d*	a***, b*	a***, b**, d*	a***	a***, b*	a*	–		
NA	a***, c**	a***, b*, c*, d**	a***, c**	a***, b***, c***, d***	a***, c**, d*	b***, c**, d***	b***, c**, d***	–	
PA	a***, c**	a***, c*	a***, c**	a***, b***, c***, d***	a***, c**	a*, b*, c**, d**	b***, c**, d***	a*, b*, d*	–

Seed traits were (a) butterfat content in cotyledons, %; (b) butterfat content per fruit, %; (c) cotyledon size as cotyledon length × width, cm^2 and (d) cotyledon mass per fruit, g. Significance levels are: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Blank cells below the diagonal have no significant pairwise differences.
[a] Refractario, [b] Various, [c] Upper Amazon Forastero.

contained fewer accessions than the Upper Amazon Forastero category when high butterfat content of cotyledons was considered. However, when high butterfat content per fruit was considered the reverse was found. A similar effect was noticed particularly within the CRU, EET, ICS and JA accession groups where accessions with low butterfat content in cotyledons became high butterfat content per fruit performers. Pires *et al.* (1998) also report a similar effect with seed fat content and total fat yield per plant. This suggests that a consideration of the average butterfat content of the cotyledons of the cacao seed may not truly reflect the butterfat yield of the cacao plant. It also indicates that cacao accessions may have a maximum limit to the resources allocated to butterfat accumulation.

The butterfat content from the 323 accessions in this study was similar from the set of 490 accessions of Pires *et al.* (1998) who employed a similar extraction method. Nine accession groups were common to both studies and six of these (EET, ICS, IMC, NA, PA and POUND) could be compared. Generally, higher butterfat content was obtained in the present study but the number of accessions per group was either low or dissimilar in both the studies. The PA accessions with reasonably similar numbers (25 vs. 34 in this study) returned the same value of 54.9% for butterfat content in cotyledons. The top-ranked candidate for butterfat content in cotyledons, CRUZ 7/8, originated as the progeny of open pollinations of C SUL 7 and C SUL 8 (ICGD, 2007) and the accession group C SUL was the top ranked of the 28 groups assessed by Pires *et al.* (1988).

The search for linked traits by correlation analysis revealed differing correlations among cacao categories. Refractario, Upper Amazon Forastero and Global datasets were similar in having no correlation between dry mass of cotyledons (estimate of economic seed mass) and seed number. Iwaro *et al.* (2003) reported a weak correlation between 'bean number' and 'bean weight' ($r = -0.19$). This suggests that dry mass of cotyledons and seed number are traits for independent selection.

There was also an agreement among categories for significant ($P < 0.001$) positive correlation of cotyledon size with dry mass of cotyledons ($r \sim 0.3$, moderate in Refractario and Upper Amazon Forastero) and for significant ($P < 0.001$) negative correlation between cotyledon size and pod index ($r \sim -0.6$). However, large cotyledon size was no guarantee for inclusion with high mass. This suggests that care should be taken when using the term large for cacao seeds, which traditionally has been taken to mean mass (BCCCA, 1996). Pod index is a highly heritable trait (Ramírez and Enríquez, 1988) as is dry seed mass (Lockwood and Pang, 1995; Fallo and Cilas, 1998). Clement *et al.* (2004) reported a major

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Table 7. Pairwise comparison (Mann–Whitney test) of three cacao categories for four seed traits

Trait	Differences for contrasted pairs
Cotyledon size (cm^2)	Refractario vs. Trinitario (n.s.); Refractario vs. Upper Amazon Forastero***; Trinitario vs. Upper Amazon Forastero***
Cotyledon mass per fruit (g)	Refractario vs. Trinitario (n.s.); Refractario vs. Upper Amazon Forastero***; Trinitario vs. Upper Amazon Forastero**
Butterfat content in cotyledons (%)	Refractario vs. Trinitario (n.s.); Refractario vs. Upper Amazon Forastero***; Trinitario vs. Upper Amazon Forastero***
Butterfat content per fruit (%)	Refractario vs. Trinitario (n.s.); Refractario vs. Upper Amazon Forastero***; Trinitario vs. Upper Amazon Forastero*

n.s., not significant; *, ** and *** significant at 5, 1 and 0.1% levels, respectively.

quantitative trait locus (QTL) co-localizing bean size (probably as bean length) with bean 'weight' on chromosome four. Clement *et al.* (2003) reported three mapping populations having major QTLs for bean length on chromosomes 2 and 4 and for bean mass on chromosome 4 with two mapping populations exhibiting QTL for bean mass also on chromosomes 6 and 9. These results and the present study suggest that selection for larger bean size may be a useful trait for inclusion in breeding programmes that aim to achieve greater seed yield.

CRUZ 7/8 was the top candidate for butterfat content in cotyledons but generally performed poorly when the other traits were considered. A correlation analysis for butterfat content in cotyledons with cotyledon size, cotyledon mass per fruit, pod index and seed number revealed non-significant interactions in the Refractario and Upper Amazon Forastero categories. A similar effect was observed in the Global dataset except for cotyledon size, which was weak but significant. These traits may not be amenable for co-selection with butterfat content of cotyledons. A significant ($P < 0.01$) correlation between butterfat content in cotyledons and dry mass of cotyledons was obtained in the Upper Amazon Forastero category (positive, small; $r_s = 0.2$) but not in the Refractario category. A small, negative but significant ($P < 0.01$) correlation was found in the Global dataset. Williams and Olaniran (1981) found a general trend of increase in butterfat content with 'bean weight' and further stated that their finding was in agreement with that of Egbe and Owolabi (1972). Wood and Lass (1985) reported a positive correlation between bean weight (mass) and fat content but did not provide any values. Pires *et al.* (1998) found a significant but low negative association ($r = -0.149$) in 490 accessions, whereas Beek *et al.* (1977) found a significant positive association in only one cross but not between crosses for butterfat content and dry seed mass. These conflicting results may be due to the differences in size and accession composition of the various correlation studies. Lanaud *et al.* (2003) in one mapping population found a QTL for seed butterfat content on chromosome nine, which also carried a major QTL for bean weight. Taken together, the correlation results suggest that butterfat in cotyledons and dry mass of cotyledons may be traits for independent selection although Pires *et al.* (1998) suggested that it would be possible to select both traits

Table 8. Percentage of genotypes in three classes for cotyledon size, cotyledon mass per fruit, butterfat content in cotyledons and butterfat content per fruit in Refractario and Upper Amazon Forastero cacao

Trait	Class (rating)	Refractario ($n = 115$)	Upper Amazon Forastero ($n = 146$)	Chi-square value
Cotyledon size	High (> 2.81 cm^2)	38.3	6.2	34.7***
	Medium (2.25–2.81 cm^2)	47.0	54.8	
	Low (≤ 2.24 cm^2)	14.8	39.0	
Cotyledon mass per fruit	High (≥ 45 g)	36.5	8.2	26.4***
	Medium (32.9–45 g)	48.7	57.5	
	Low (≤ 32.8 g)	14.8	34.3	
Butterfat content in cotyledons	High (> 55%)	5.2	25.3	42.4***
	Medium (52.3–55%)	48.7	66.4	
	Low (≤ 52.2%)	46.1	8.2	
Butterfat content per fruit	High (> 24%)	32.2	11.6	13.9***
	Medium (17.5–24%)	49.6	56.2	
	Low (≤ 17.4%)	18.3	32.2	

***Significant at the 0.1% level.

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Table 9. Spearman's r_s correlation coefficients and their significance among selected traits

Traits[a]	Refractario ($n = 115$)	Upper Amazon Forastero ($n = 146$)	Global accessions ($n = 323$)
BFC & CS	−0.03 (n.s.)	0.15 (n.s.)	−0.18***
BFC & BFF	0.29**	0.20*	0.08 (n.s.)
BFC & CDM	0.14 (n.s.)	0.25**	−0.15**
BFC & CMF	0.14 (n.s.)	0.12 (n.s.)	−0.08 n.s
BFC & PI	−0.06 (n.s.)	−0.01 (n.s.)	0.10 (n.s.)
BFC & SNO	0.08 (n.s.)	−0.06 (n.s.)	0.08 (n.s.)
BFF & CDM	0.74***	0.56***	0.70***
BFF & CS	0.28**	0.46***	0.40***
CS & CDM	0.32***	0.34***	0.49***
CS & CMF	0.30***	0.42***	0.43***
CS & PI	−0.65***	−0.69***	−0.69***
CS & SNO	0.06 (n.s.)	0.20*	0.00 (n.s.)
CDM & SNO	0.08 (n.s.)	−0.15 (n.s.)	−0.14*

Significance levels: n.s., not significant; *, ** and *** significant at 5, 1 and 0.1% levels, respectively.
[a] BFC, butterfat content in cotyledons (%); BFF, butterfat content per fruit (%); CDM, cotyledon dry mass (g); CMF, cotyledon mass per fruit (g); CS, cotyledon size as cotyledon length × width (cm^2); PI, pod index; SNO, seed number.

based on the low negative correlation these authors observed. Furthermore, the selection of genotypes for high butterfat production was recommended to be based exclusively on selection for increased dry seed yield/plant.

Refractario and Upper Amazon Forastero categories had small and positive but significant correlations of butterfat content in cotyledons with butterfat content per fruit, whereas the correlation was not supported in the pooled Global dataset. This suggests that butterfat content per fruit may be dependent on other factors. Pires *et al.* (1998) found that seed fat content was not significantly correlated with fat yield per plant, which may in part be due to the use of a heterogeneous dataset as was the Global dataset of this study. Butterfat content of cotyledons may then be utilized as a selection criterion for butterfat yield when breeding within a genetic group but would not be supported for use if crosses are made between different groups. In this regard, a trait with more consistent correlation across groupings should be utilized for selection of genotypes with increased seed butterfat content.

Butterfat content per fruit was significantly and positively correlated with cotyledon size in the Refractario (small; $r_s = 0.28$), the Upper Amazon Forastero (moderate; $r_s = 0.46$) and the Global datasets (moderate; $r_s = 0.40$). When dry mass of cotyledons was utilized, the correlations with butterfat content per fruit were much stronger. Correlations within groupings were: Refractario, very large, $r_s = 0.74$; Upper Amazon Forastero, large, $r_s = 0.56$ and Global, very large, $r_s = 0.70$. This suggests that selection for either increased cotyledon size or cotyledon dry matter may be utilized for the selection of genotypes with high butterfat yield. Either of these traits would be economic alternatives to measuring butterfat content. Furthermore, the moderate and positive significant correlation observed between cotyledon size and dry mass of cotyledons suggests that seed size may be utilized as an indicator of dry mass. Furthermore, our findings indicate that selection for larger cotyledon size would lead to a favourable selection of genotypes with low pod index. In addition, since cacao farmers bulk seeds for fermentation from numerous fruits of different trees and bulked samples are subsequently used in the industry for butterfat extraction, it would seem more promising to select for high butterfat content per fruit than for high butterfat content in cotyledons.

Nevertheless, within the accessions studied, there were several candidates that favourably combined multiple seed-derived traits and which suggested that improvement to butterfat content together with other seed-derived traits is possible. Six accessions (AM 2/91 [POU], CRU 51, CRU 133, ICS 39, IMC 10, MATINA 1/7 and POUND 18/A [POU]) were identified as excellent parental candidates for butterfat content in combination with high cotyledon mass and low pod index. MATINA 1/7 was also the only accession from these candidates that was included in the 100 clones of the CFC/ICCO/IPGRI project collection (Sounigo *et al.*, 2006). The disease resistance of the suggested six parental candidate accessions should be assessed before embarking on any breeding programme with these accessions.

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