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RESEARCH ARTICLE

Assessment of polyclonal derivatives for morphological traits and hybridity analysis using SSR markers in cocoa (*Theobroma cacao* L.)

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Abstract

Cocoa (*Theobroma cacao* L.) is increasingly cultivated as an intercrop in South India, necessitating the development of high-yielding, region-specific varieties. As a self-incompatible crop, establishing polyclonal orchards with cross-compatible varieties and producing full-sib hybrids through controlled pollination is vital for genetic improvement. Evaluation of half-sib and full-sib progenies was undertaken in polyclonal cocoa gardens situated at Coconut Research Station, Aliyar Nagar and Horticulture Research Station, Thadiyankudisai, under the auspices of Tamil Nadu Agricultural University. Pods developed from the natural cross and cross-made using caging techniques were evaluated and seeds were sown to assess the performance of the hybrids. The findings illustrated the highest germination percentage in the half-sib cross CCRP 5 × X (89.66 %), followed by CCRP 4 × X (85.57 %). The highest value for plant morphological characters like plant height (2.11 m), stem girth (15.7 cm) and jorquette height (1.58 m) was recorded in CCRP 5 × X. Among the full-sib progenies, the highest germination percentage (86.1) was recorded by FS 17 (CCRP 3 × CCRP 5), followed by FS 18 (CCRP 5 × CCRP 3) (81.8). The hybridity of the seedlings was tested using SSR markers, which confirmed that out of 26 full-sib crosses, 16 exhibited a heterozygous nature. These findings demonstrate the potential of selected progenies to serve as valuable genetic resources for breeding programs aimed at enhancing cocoa productivity and sustainability in South India.

Keywords

cocoa; full-sib; half-sib; hybridity test; morphological traits

Introduction

One of the most coveted commodity crops is cocoa (*Theobroma cacao* L.), a member of the Malvaceae family cultivated in the world's humid tropical regions. Cocoa's origination centre is the equatorial Americas tropical rain forest (1). Cocoa is cultivated in tropical areas between 10 to 20 degrees latitude north and south of the equator (2). Cocoa is primarily grown for chocolate production and contributes to various byproducts used in skincare products, sweets, fragrances and medications. Cocoa, introduced to India in 1798, is predominantly cultivated in Kerala, Andhra Pradesh, Tamil Nadu and Karnataka (3). Cocoa is categorized into three primary groups: Criollo, Forastero and Trinitario (4, 5). Despite recognizing

these three varietal types, only Forastero types have shown optimal performance under Indian conditions. Since 1979, breeding initiatives at Kerala Agricultural University have developed and released seven enhanced Forastero clones. The improved Forastero clones released are CCRP 1, 2, 3, 4, 5, 6 and CCRP 7, along with three hybrids: CCRP 8, 9 and 10 (6).

Cocoa is a self-incompatible crop and incompatibly is primarily gametophytic. In this type of incompatibility, pollen tubes usually grow, but the male gamete fails to fuse with the female gamete. This widespread incompatibility poses a significant obstacle to utilizing many genotypes in cocoa breeding programs (7). Self-incompatible genotypes are being used for the production of hybrids. Establishing seed gardens with multiple cross-compatible clones are called polyclonal orchards and seedlings developed from pods formed by natural hybridization in the polyclonal orchard are called half-sib progeny (8). The conventional method of manual pollination in cocoa is known to be time-consuming and inefficient. Similar challenges were addressed in mango cultivation following the discovery of self-incompatibility in varieties such as Dashehari, Langra, Chausa and Bombay Green. This led to the development of the "caging technique" at IARI (Indian Agricultural Research Institute, Delhi, India). In this technique, grafted plants of self-incompatible varieties are planted alongside those of male parents within an insect-proof cage. Pollination is facilitated by freshly reared house flies, eliminating the need for tedious hand pollination (9). The same principle was tried in cocoa as it is self-incompatible. Thus, seven cocoa clones with cross-compatible clones were caged inside an insect-proof net and the resultant pods were harvested to raise the full-sib progeny. Research indicates that single pollen plant donors are commonly involved in cocoa pollination (10). This approach produces a substantial percentage of full-sib progenies (24-70 %) in natural crosses.

Molecular markers, particularly Simple Sequence Repeats (SSRs), are extensively utilized in agricultural science and plant breeding to assess hybridity (11). SSRs are particularly popular in breeding programs for speciality crops due to their high polymorphism and PCR-based assay capabilities. These markers comprise tandem nucleotide repeats of varying lengths, which are examined through electrophoresis, facilitating high-throughput analysis (12). SSRs have been utilized in cocoa to study off-type purity resulting from hybridization. Research indicates that eleven highly polymorphic SSR markers: mTcCir6, mTcCir9, mTcCir12, mTcCir15, mTcCir17, mTcCir18, mTcCir21, mTcCir24, mTcCir25, mTcCir26 and SHRSTc23 (13). These markers helped to assess the genetic composition and purity of cocoa plants derived from hybrid crosses. The study entitled Evaluation of Polyclonal Derivatives for Morphological Traits and Hybridity Assessment Using SSR Markers aimed to develop half-sib and full-sib progenies of cocoa (*Theobroma cacao* L.) through controlled pollination. Morphological traits were analyzed to evaluate their agronomic performance and SSR markers were employed to confirm hybridity and ensure genetic integrity. This research enhances cocoa breeding programs by integrating phenotypic evaluation with molecular tools.

Materials and Methods

Evaluation of hybrid seeds (half-sibs) developed from the natural cross in polyclonal garden

Materials

Polyclonal garden maintained with CCRP 1 to CCRP7 at Coconut Farm, Coconut Research Station, Tamil Nadu Agricultural University, Aliyar Nagar and Horticultural Research Station, Thadiyankudisai (2023) was used for the experiment. The pods developed due to natural crossing in the polyclonal garden were collected, sown and evaluated for morphological traits.

The possible parentages are:

1. CCRP1 × X
2. CCRP2 × X
3. CCRP3 × X
4. CCRP4 × X
5. CCRP5 × X
6. CCRP6 × X
7. CCRP7 × X

In each half-sib, 45 plants were planted and evaluated for various traits.

Matured Forastero type (green- immature, yellow- ripe) pods were collected from a polyclonal garden at CRS, Aliyar Nagar. The seeds from each cross were collected and sown in black polythene bags measuring 6" × 9" with a thickness of 250 gauge. Each bag was fitted with four drainage holes and filled with a potting mix in a 2:1:1 ratio of soil, sand and farmyard manure (FYM). This arrangement ensures an ideal environment for seed germination and early growth.

Experimental design

The experiment was laid out in Completely Randomized Block Design with 7 treatments along with three replicas.

Observations at the nursery-stage

Germination (up to 15 days) and seedling characteristics, including height, girth and number of leaves, were recorded every two weeks from the 60th to the 120th day after germination.

Germination percentage

The number of seeds germinated was noted on the 15th day under each half-sib. The seed viability and germination success rate under controlled conditions were measured using this parameter and the formula in Equation 1.

Germination (%) = (No. of seeds germinated/ Total no. of seeds sown) ×100 (Eqn. 1)

Seedling height

Seedling height was quantitatively assessed by measuring from the ground level to the tip of the main stem, recorded in centimetres. The seedling girth was measured 5 cm above the ground level, as an indication of stem diameter was measured using a scale and recorded in cm. Leaf area was measured in 5 plants per replication, with the fifth leaf from the top used as standard. The leaf area was estimated using the method previously described and recorded in cm^2, as given in Equation 2 (14).

Leaf Area = L × B × 0.666 + 0.73 (Eqn. 2.)

Where, L - Length of the leaf (cm) B - Breadth of the leaf (cm)

Observation at the field level

The plant height was measured as a comprehensive assessment of vertical growth from the ground. The height of the first jorquette was determined by measuring the vertical distance from ground level to the first branching point, which was recorded in meters (m). The number of fan branches from the first jorquette was counted and documented.

To develop F1 hybrids between available clones at Aliyar Nagar using cagging technique

In the polyclonal garden (CCRP 1 to CCRP7), crosses (Table 1) were made using the selected male and female parents using insect-proof pollination cages at Coconut Research Station, Aliyar Nagar, Coimbatore, Tamil Nadu. The experiment was laid out using a completely randomized block design. The experiment consisted of 7 varieties with 26 crosses and three replications. As mentioned, seedling parameters and plant characters at the field level were observed.

Table 1. Crosses made in polyclonal gardens

CCRP 1 × CCRP 2	CCRP2 × CCRP 1
CCRP1 × CCRP3	CCRP3 × CCRP 1
CCRP1 × CCRP6	CCRP6 × CCRP 1
CCRP1 × CCRP7	CCRP7 × CCRP1
CCRP2 × CCRP3	CCRP3 × CCRP 2
CCRP2 × CCRP4	CCRP4 × CCRP2
CCRP4 × CCRP6	CCRP6 × CCRP4
CCRP3 × CCRP4	CCRP4 × CCRP3
CCRP3 × CCRP5	CCRP5 × CCRP 3
CCRP4 × CCRP5	CCRP5 × CCRP 4
CCRP 5 × CCRP 6	CCRP 6 × CCRP 5
CCRP 5 × CCRP 7	CCRP 7 × CCRP 5
CCRP 6 × CCRP 7	CCRP 7 × CCRP 6

Molecular characterization of hybrids using SSR markers

Plant material and DNA extraction

Young leaves were harvested from full-sib hybrid cocoa seedlings produced in the study. Genomic DNA was isolated from these leaves using the Qiagen DNeasy Plant Mini Kit, per the manufacturer's instructions. The extracted DNA was quantified using 0.8 % agarose gel electrophoresis and dilutions were prepared in TE buffer (pH 8.0).

PCR amplification of SSR markers

SSR primers specific to cocoa obtained from Xcelris Labs Ltd., Ahmedabad, were used for PCR amplification. PCR reactions were prepared in 200 µL microcentrifuge tubes with a total volume of 15 µL per reaction (15). Each reaction included 5 ng of template DNA, 0.2 µM of each forward and reverse primer, 0.25 mM of each dNTP, 0.3 mM $MgCl_2$, 0.75 µL of Taq polymerase (Bangalore Genei Pvt. Ltd., Bangalore) and 1x reaction buffer. PCR amplification was conducted using a Mycycler thermal cycler (Bio-Rad Laboratories, California) with the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min. A final extension step was performed at 72 °C for 7 min.

Gel electrophoresis and visualization

PCR products were mixed with loading buffer and resolved on 3 % agarose gels prepared in 1x TBE buffer at 90V for appropriate separation. DNA bands were visualized by staining with ethidium bromide and photographed under UV transillumination using the Alpha Imager™ 1200 Documentation and Analysis System (Alpha Innotech Corporation, USA).

Data analysis

Fragment lengths and SSR marker patterns were analyzed to confirm hybridity and assess genetic diversity among parental trees and full-sib hybrid cocoa seedlings. The experiment results were statically analyzed using the previously described procedure (16).

Results

In the present study, all the selected clones were subjected to self-pollination. All the pollinated flowers were shed on the fourth day, confirming their self-incompatibility.

Evaluation of half-sibs at nursery stage

The mean value of the number of pods collected among the 7 half-sib crosses of cocoa showed marked differences. It ranged from 9 to 34 numbers with a CV of 35.0 %. A maximum number of pods collected was observed in CCRP 1 × X (34 numbers) and lowest in CCRP 7 × X (9 numbers). The mean number of pods harvested observed was 23.3 (Table 2). The number of beans per pod ranged from 32.8 to 43.1, with a mean value of 38.6. The coefficient of variation value of 9.60 % was observed among the half-sib crosses of cocoa. Gametophytic self-incompatibility in cocoa results from the failure of gamete fusion (17). Self-incompatible clones with known performance and parentage assembled in bi-clonal or polyclonal orchards will result in F1 hybrids (18). When both parents are self-incompatible, all pods resulting from cross-pollination are suitable for seed production. Seed gardens should be isolated from other cocoa gardens to minimize unintended cross-pollination (19). In the cross-pollinated species, random natural pollination with known maternal clones receiving pollen from unknown sources results in half-sib progenies (20).

Table 2. Number of pods collected from the natural cross in the polyclonal garden

Parentage	No. of pods harvested	No. of beans per pod
CCRP1 × X	34	38.2
CCRP2 × X	29	41.0
CCRP3 × X	17	43.1
CCRP4 × X	25	37.5
CCRP5 × X	24	42.0
CCRP6 × X	25	35.6
CCRP7 × X	9	32.8
Mean	23.3	38.6
Maximum	34	43.1
Minimum	9	32.8
SE(d)	8.1	3.70
CV	35.0	9.60

The germination percentage of different half-sibs seedlings exhibited significant differences 15 days after sowing. Among the different half-sibs seedlings, CCRP 5 × X registered the highest percentage of germination (89.66 %), followed by CCRP 4 × X (85.57 %) and CCRP 2 × X (83.36 %). The lowest germination percentage was observed in CCRP 7 × X (74.03 %) half-sibs seedlings (Table 3). Assessment of seedling vigour can be a pre-selection method in cocoa to choose the hybrids with high yield and abiotic stress resistance. Significant differences in seedling height were observed at monthly intervals from 60 days to 120 days after sowing among the various half-sib seedlings (Table 4). At 60, 90 and 120 days after sowing, the half

Table 3. Germination percentage of half-sibs at 15 days after sowing (DAS)

Parentage	Germination percentage (15 DAS)
CCRP1 × X	75.11
CCRP2 × X	83.36
CCRP3 × X	74.18
CCRP4 × X	85.57
CCRP5 × X	89.66
CCRP6 × X	73.52
CCRP7 × X	74.03
Mean	79.4
SE(d)	1.53
CD (0.05)	3.29**

Table 4. Seedling height of half-sibs at nursery stage

Parentage	Seedling height (cm)		
	60 DAS	90 DAS	120 DAS
CCRP1 × X	22.2	26.2	33.5
CCRP2 × X	22.7	25.7	37.9
CCRP3 × X	21.9	24.9	34.5
CCRP4 × X	22.7	26.7	32.7
CCRP5 × X	23.0	28.0	39.8
CCRP6 × X	22.9	25.9	35.1
CCRP7 × X	21.0	24.0	34.7
Mean	22.4	25.9	35.5
SE(d)	0.34	0.51	0.76
CD (0.05)	0.72**	1.09**	1.64**

-sibs, CCRP 5 × X, exhibited significantly the highest seedling height (23.0 cm, 28.0 cm and 39.8 cm, respectively). The lowest seedling height was observed in the cross CCRP 7 × X (21.0 cm, 25.9 cm and 32.7 cm, respectively). The seedling girth (Table 5) recorded at 60, 90 and 120 days after sowing exhibited significant differences, with the highest seedling girth in CCRP 5 × X (2.07 cm, 2.39 cm and 2.61 cm, respectively) and lowest in CCRP 7 × X (1.71 cm, 1.92 cm and 2.18 cm) half-sibs.

Table 5. Seedling girth of half-sibs at the nursery stage

Parentage	Seedling girth (cm)		
	60 DAS	90 DAS	120 DAS
CCRP1 × X	1.88	2.10	2.30
CCRP2 × X	1.93	2.12	2.41
CCRP3 × X	1.75	1.96	2.22
CCRP4 × X	1.90	2.17	2.56
CCRP5 × X	2.07	2.39	2.61
CCRP6 × X	1.84	2.12	2.23
CCRP7 × X	1.71	1.92	2.18
Mean	1.87	2.11	2.36
SE(d)	0.03	0.05	0.04
CD (0.05)	0.07**	0.12**	0.08**

The leaf area recorded in the different half-sib seedlings of cocoa showed significant variations among them at various stages of observation after sowing (Table 6). At 60 days after sowing, the half-sibs, CCRP 5 × X, recorded significantly the highest leaf area (45.42 cm^2), followed by CCRP 2 × X, which registered 43.89 cm^2. The leaf area was lowest in half-sibs of CCRP 7 × X (39.69 cm^2). At 90 and 120 days after sowing, half-sib CCRP 5 × X registered the highest leaf area (61.15 cm^2 and 74.80 cm^2), which was followed by CCRP 3 × X 55.87 $cm^2$and 64.09 cm^2) half-sibs. The lowest leaf area was observed in CCRP 7 × X (41.25 cm^2 and 47.47 cm^2) half-sibs. Results show that half-sib CCRP 5 × X performed well for all the morphological characters at the nursery stage. This can be attributed to heterosis when interclonal hybrids are developed (21). In cocoa, a positive correlation between vegetative characteristics and yield has been observed (22).

Table 6. Leaf area of half-sibs at nursery stage

Parentage	Leaf area (cm^2)		
	60 DAS	90 DAS	120 DAS
CCRP1 × X	42.38	53.12	61.87
CCRP2 × X	43.89	51.81	61.67
CCRP3 × X	43.09	55.87	64.09
CCRP4 × X	40.21	49.96	59.87
CCRP5 × X	45.42	61.15	74.80
CCRP6 × X	40.81	50.17	55.87
CCRP7 × X	39.69	41.25	47.47
Mean	42.21	51.91	60.80
SE(d)	1.17	1.01	1.34
CD (0.05)	2.51**	2.17**	2.87**

Evaluation of half-sibs at the field

The seedlings were planted in the field to assess their growth performance. In seasons I and II, half-sib CCRP 5 × X registered the maximum plant height (1.97 m and 2.26 m), followed by CCRP 3 × X (1.94 m and 2.21 m) half-sibs. The minimum value was registered in CCRP 7 × X (1.70 m and 1.93 m) half sibs (Table 7). Half sib CCRP 5 × X recorded the lowest time taken for the formation of jorquette (215.0 days), which was followed by CCRP 3 × X (220.0 days) half sibs whereas the maximum days were recorded in CCRP 6 × X and CCRP 7 × X (240.0 days) half-sibs. Half -sib CCRP 5 × X registered the maximum forgetting height (1.58 m), followed by CCRP 2 × X (1.51 m) half-sibs. The minimum value was recorded in CCRP 7 × X (1.20 m) half sibs (Table 8)

Table 7. Plant height of half-sibs in the field

Parentage	Plant height (m)		
	Season I	Season II	Mean
CCRP1 × X	1.77	2.02	1.89
CCRP2 × X	1.81	2.12	1.96
CCRP3 × X	1.94	2.21	2.07
CCRP4 × X	1.91	2.15	2.03
CCRP5 × X	1.97	2.26	2.11
CCRP6 × X	1.89	2.15	2.02
CCRP7x X	1.70	1.93	1.82
Mean	1.86	2.12	1.99
SE(d)	0.03	0.04	0.03
CD (0.05)	0.06**	0.09**	0.07**

Table 8. Time taken for Jorquette formation and Jorquette height of half-sibs in the field

Parentage	Time taken for first jor-quette formation (days)	Jorquette height (m)
CCRP1 × X	230.0	1.46
CCRP2 × X	225.0	1.51
CCRP3 × X	220.0	1.50
CCRP4 × X	230.0	1.35
CCRP5 × X	215.0	1.58
CCRP6 × X	240.0	1.40
CCRP7× X	240.0	1.20
Mean	228.6	1.43
SE(d)	5.58	0.03
CD (0.05)	12.2**	0.06**

Evaluation of F1 hybrids developed through caging technique

Parents selected for seed gardens are chosen based on progeny trials and these selected individuals are propagated through vegetative methods. They are planted so that crosses can be made using caging techniques. The caging technique was first used in self-incompatible mango varieties at IARI, New Delhi (9). Research indicates that in cocoa, pollinations typically involve a single pollen donor plant, leading to a substantial portion of full-sibling families in natural crosses (24 -70 %) (10). Approximately 500 full-sib progenies, comprising around 30,000 trees, were established between 1993 and 2010 as foundational populations for CEPEC's breeding program

(23).

The mean value of the number of pods collected among the twenty-six crosses of cocoa showed marked differences. It ranged from 2 to 5 numbers with a CV of 25.0 % (Table 9). Maximum of pods collected was noted in FS 1(CCRP1 × CCRP2), FS 4 (CCRP3 × CCRP 1) and FS 5 (CCRP1 × CCRP6) (5 numbers). The germination percentage of different full-sib seedlings exhibited significant differences 15 days after sowing (Table 10). Among the different full-sib seedlings, FS 17 (CCRP3 × CCRP 5) registered the highest percentage of germination (86.1 %), which was followed by FS 18 (CCRP 5 × CCRP 3) (81.8 %) and FS 23 (CCRP5 × CCRP7) (81.1%). The lowest germination percentage was registered in FS 8 (CCRP7 × CCRP 1) (68.4 %). Seedling height recorded 30 days from the 60th day of sowing presented significant differences among the full sibs. The cross FS 18 (CCRP5 × CCRP3) recorded maximum seedling height at 60, 90 and 120 days after sowing (Table 11). Leaf area varied significantly among the full-sib seedlings and the crosses FS 17 (CCRP3 × CCRP 5) recorded the maximum leaf area (62.96 cm^2 at 60 days after sowing, 88.48 cm^2 at 90 days

Table 9. Number of pods collected from caging technique

Parentage	No. of pods harvested
FS 1(1 × 2)	5
FS 2 (2 × 1)	4
FS 3 (1 × 3)	4
FS 4 (3 × 1)	5
FS 5 (1 × 6)	5
FS 6 (6 × 1)	4
FS 7 (1 × 7)	3
FS 8 (7 × 1)	3
FS 9 (2 × 3)	4
FS 10 (3 × 2)	4
FS 11 (2 × 4)	3
FS 12 (4 × 2)	3
FS 13 (4 × 6)	3
FS 14 (6 × 4)	4
FS 15 (3 × 4)	2
FS 16 (4 × 3)	3
FS 17 (3 × 5)	3
FS 18 (5 × 3)	3
FS 19 (4 × 5)	4
FS 20 (5 × 4)	4
FS 21(5 × 6)	2
FS 22 (6 × 5)	2
FS 23 (5 × 7)	3
FS 24 (7 × 5)	3
FS 25 (6 × 7)	3
FS 26 (7 × 6)	3
Mean	3.4
Maximum	5
Minimum	2
SE(d)	0.86
CV	25.0

Table 10. Germination percentage (%) of full sibs at nursery stage

Parentage	Germination percentage at 15 DAS
FS 1(1 × 2)	80.0
FS 2 (2 × 1)	78.3
FS 3 (1 × 3)	76.3
FS 4 (3 × 1)	78.0
FS 5 (1 × 6)	72.7
FS 6 (6 × 1)	70.0
FS 7 (1 × 7)	70.6
FS 8 (7 × 1)	68.4
FS 9 (2 × 3)	73.6
FS 10 (3 × 2)	74.2
FS 11 (2 × 4)	72.8
FS 12 (4 × 2)	71.3
FS 13 (4 × 6)	72.0
FS 14 (6 × 4)	71.8
FS 15 (3 × 4)	77.1
FS 16 (4 × 3)	76.9
FS 17 (3 × 5)	86.1
FS 18 (5 × 3)	81.8
FS 19 (4 × 5)	75.3
FS 20 (5 × 4)	71.1
FS 21(5 × 6)	75.3
FS 22 (6 × 5)	72.3
FS 23 (5 × 7)	81.1
FS 24 (7 × 5)	77.1
FS 25 (6 × 7)	72.0
FS 26 (7 × 6)	70.4
Mean	74.9
SE(d)	1.35
CD (0.05)	2.70**

Table 11. Seedling height of full sibs at nursery stage

Parentage	Seedling height (cm)		
	60 DAS	90 DAS	120 DAS
FS 1(1 × 2)	22.8	28.2	37.0
FS 2 (2 × 1)	23.4	27.5	36.1
FS 3 (1 × 3)	22.0	25.8	34.5
FS 4 (3 × 1)	21.5	26.0	35.7
FS 5 (1 × 6)	21.0	24.8	34.0
FS 6 (6 × 1)	20.6	25.1	31.2
FS 7 (1 × 7)	19.7	24.0	31.0
FS 8 (7 × 1)	20.3	23.8	29.5
FS 9 (2 × 3)	21.4	24.9	32.8
FS 10 (3 × 2)	22.1	26.0	30.3
FS 11 (2 × 4)	20.8	23.8	29.8
FS 12 (4 × 2)	19.2	23.0	28.1
FS 13 (4 × 6)	18.7	22.5	26.5
FS 14 (6 × 4)	20.1	23.0	26.3
FS 15 (3 × 4)	21.0	24.1	28.6
FS 16 (4 × 3)	19.5	22.8	25.4
FS 17 (3 × 5)	23.8	27.8	37.5
FS 18 (5 × 3)	24.3	28.5	38.2
FS 19 (4 × 5)	20.7	22.1	30.2
FS 20 (5 × 4)	20.1	23.5	32.8
FS 21(5 × 6)	21.3	24.1	33.0
FS 22 (6 × 5)	20.5	23.7	32.1
FS 23 (5 × 7)	23.0	28.0	36.8
FS 24 (7 × 5)	22.4	27.1	35.5
FS 25 (6 × 7)	21.5	25.1	33.1
FS 26 (7 × 6)	21.0	25.7	34.6
Mean	21.3	25.0	32.3
SE(d)	0.36	0.56	0.70
CD (0.05)	0.72**	1.13**	1.40**

Table 10. Germination percentage (%) of full sibs at nursery stage

Parentage	Leaf area (cm^2)		
	60 DAS	90 DAS	120 DAS
FS 1(1 × 2)	53.54	70.25	83.85
FS 2 (2 × 1)	51.51	68.33	80.95
FS 3 (1 × 3)	42.85	54.84	62.31
FS 4 (3 × 1)	35.97	44.69	52.88
FS 5 (1 × 6)	36.21	51.03	57.37
FS 6 (6 × 1)	38.83	52.68	58.54
FS 7 (1 × 7)	43.17	53.48	61.34
FS 8 (7 × 1)	36.69	49.02	54.68
FS 9 (2 × 3)	36.53	48.35	55.58
FS 10 (3 × 2)	40.02	54.68	63.50
FS 11 (2 × 4)	41.02	51.52	57.87
FS 12 (4 × 2)	32.20	45.49	52.88
FS 13 (4 × 6)	35.70	44.39	52.20
FS 14 (6 × 4)	36.69	46.76	52.68
FS 15 (3 × 4)	42.12	53.54	60.40
FS 16 (4 × 3)	37.36	46.52	57.04
FS 17 (3 × 5)	62.96	88.48	98.54
FS 18 (5 × 3)	59.01	81.65	95.83
FS 19 (4 × 5)	42.03	53.18	60.97
FS 20 (5 × 4)	40.81	53.08	61.57
FS 21(5 × 6)	46.28	55.58	64.80
FS 22 (6 × 5)	44.69	57.01	64.13
FS 23 (5 × 7)	43.99	60.90	70.34
FS 24 (7 × 5)	41.32	55.58	66.53
FS 25 (6 × 7)	37.36	52.68	62.83
FS 26 (7 × 6)	39.79	50.17	56.67
Mean	42.25	55.54	63.95
SE(d)	0.86	1.24	1.46
CD (0.05)	1.73**	2.49**	2.94**

after sowing and 98.54 cm^2 at 120 days after sowing). The cross FS 13 (CCRP4 × CCRP6) recorded the lowest leaf area (Table 12)

Genetic purity test by SSR markers

This study assessed the genetic purity of full-sib cocoa hybrids developed using a caging technique using microsatellite (SSR) markers (Table 13 and Fig 1). A survey of parental polymorphism among seven parents (CCRP 1 to CCRP 7) involved in the cross revealed that out of 16 SSR markers tested, only six markers (mTcCIR6, mTcCIR7, mTcCIR11, mTcCIR12, mTcCIR15 and mTcCIR19) showed polymorphism. These six polymorphic markers were then utilized to verify the hybridity of 26 full-sib hybrids from the specific crosses based on the heterozygous nature of F1 hybrids. The surveys of F1 hybrids using markers mTcCIR6, mTcCIR7, mTcCIR11, mTcCIR12 and mTcCIR15

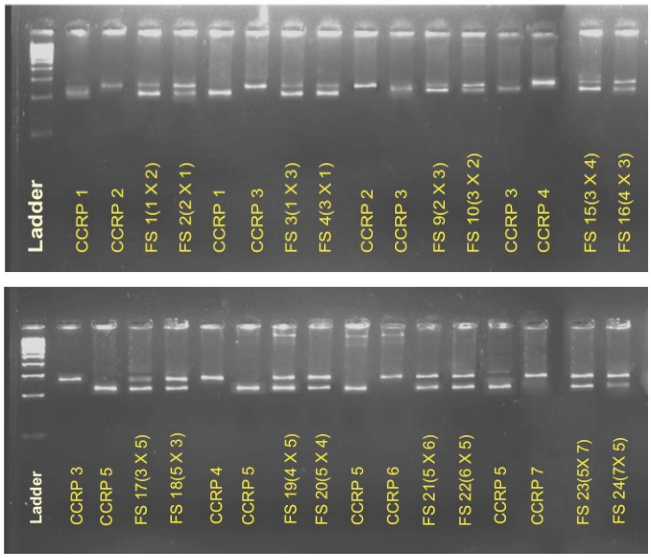

Fig. 1. SSR marker profile of cocoa A-B. Full sib hybrids generated by the primer mTcCIR12.

Table 13. List of primers used for SSR analysis

S.No.	Primer		Sequence	Annealing temperature (°C)
1	mTcCIR 1	F	GCAGGGCAGGCTCAGTGA	51
		R	TGGGCAACCAGAAAACGA T	
2	mTcCIR 3	F	CATCCCAGTATCTCATCCATTCA	46
		R	CTGCTCATTTCTTTCATATCA	
3	mTcCIR 6	F	TTCCCTCTAAACTACCCTAAAT	46
		R	TAAAGCAAAGCAATCTAACATA	
4	mTcCIR 7	F	ATGCGAATGACAACTGGT	51
		R	GCTTTCAGTCCTTTGCTT	
5	mTcCIR 8	F	CTACTTTCCCATTTACCA	46
		R	TCCTCAGCATTTTCTTTC	
6	mTcCIR 9	F	ACCATGCTTCCTCCTTCA	51
		R	ACATTTATACCCCAACCA	
7	mTcCIR11	F	TTTGGTGATTATTAGCAG	46
		R	GATTCGATTTGATGTGAG	
8	mTcCIR12	F	TCTGACCCCAAACCTGTA	46
		R	ATTCCAGTTAAAGCACAT	
9	mTcCIR15	F	CAGCCGCCTCTTGTTAG	46
		R	TATTTGGGATTCTTGATG	
10	mTcCIR 17	F	AAGGATGAAGGATGTAAGAGAG	51
		R	CCCATACGAGCTGTGAGT	
11	mTcCIR18	F	GATAGCTAAGGGGATTGAGGA	51
		R	GGTAATTCAATCATTTGAGGATA	
12	mTcCIR 19	F	CACAACCCGTGCTGATTA	46
		R	GTTGTTGAGGTTGTTAGGAG	
13	mTcCIR 21	F	GTCGTTGTTGATGTCGGT	46
		R	GGTGAGTGTGTGTGTTTGTCT	
14	mTcCIR 22	F	ATTCTCGCAAAAACTTAG	46
		R	GATGGAAGGAGTGTAAATAG	
15	mTcCIR 24	F	TTTGGGGTGATTTCTTCTGA	46
		R	TCTGTCTCGTCTTTTGGTGA	
16	mTcCIR 25	F	CTTCGTAGTGAATGTAGGAG	46

confirmed heterozygosity in 16 cross combinations. SSR markers have effectively identified cultivars and hybrids in various plant species (24, 25).

Conclusion

The study focused on developing superior cocoa hybrids with enhanced agronomic traits to support future breeding initiatives to improve yield, quality and stress tolerance in cocoa (*Theobroma cacao* L.). Cocoa breeding programs often prioritize the identification of progenies with desirable characteristics, including vigorous growth, disease resistance and high productivity, through careful selection and evaluation. Thus, among the seven half-sib crosses, the cross CCRP 5 × X performed well in the nursery stage and for morphological traits in field conditions. Among full-sib progenies, FS 17 (CCRP 3 × CCRP 5) and FS 18 (CCRP 5 × CCRP 3) showed maximum vigour at the seedling stage. Further, the hybridity of 16 crosses was confirmed using mTcCIR6, mTcCIR7, mTcCIR11, mTcCIR12 and mTcCIR15- SSR markers.

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Authors' contributions

JV wrote the original draft, including methodology, data analysis, review and editing. VKR provided instrumentation facilities, resources and project administration. PK did the review & editing, visualization, formal analysis and software. SJ did the conceptualization, investigation, funding acquisition, review & editing, supervision and resources and validation.

Compliance with ethical standards

Conflicts of Interest: The authors declare no conflict of interest.

Ethical approval: No data falsification, deformation, or modification was used to present the results and the article was not submitted anywhere else. There is no risk to national security or public health from research.

Declaration of generative AI and AI-assisted technologies in the writing process

While preparing this work, the author(s) used Grammarly to reduce grammatical errors. After using this tool/service, the author(s) reviewed and edited the content as needed and take (s) full responsibility for the publication's content.

References

1. Bailey BA, Strem MD, Bae H, De Mayolo GA, Guiltinan MJ. Gene expression in leaves of *Theobroma cacao* in response to mechanical wounding, ethylene and/or methyl jasmonate. Plant Sci. 2005;168 (5):1247-58. https://doi.org/10.1016/j.plantsci.2005.01.002
2. Motamayor JC, Risterucci A-M, Lopez PA, Ortiz CF, Moreno A, Lanaud C. Cacao domestication I: the origin of the cacao cultivated by the Mayas. Heredity. 2002;89(5):380-86. https://doi.org/10.1038/sj.hdy.6800156
3. Ratnam R. Introduction of Criollo cacao into Madras state. S Ind Hortic. 1961;9(4):24-29.
4. Cheesman E. Notes on the nomenclature, classification and possible relationships of cacao populations. Trop Agri. 1944;21:144-59.
5. Simmons J. Cocoa production, economic and botanical perspectives. New York: Praeger Publishers; 1976.
6. Prasannakumari Amma S, Nair R, Lalithabai E, Malika V, Minimol J. Koshy Abraham. Cocoa in India. Kerala: Kerala Agricultural University; 2009.
7. Bartley B, Cope F. Practical aspects of self-incompatibility in *Theobroma cacao* L. Agric Genet Sel Top R Moav; 1973.
8. Wood G, Lass R. Cocoa tropical agriculture. London: Longman; 1985.
9. Sharma D, Singh R. Self-incompatibility in mango (*Mangifera indica* L.). Hortic Res. 1970; 10:108-18.
10. Yamada MM, Guries RP. Mating system analysis in cacao (*Theobroma cacao* L.). Agrotrópica. 1988;10(3):165-76.
11. Walters T, Posluszny U, Kevan P. Isozyme analysis of the grape (*Vitis*). I. A practical solution. Can J Bot. 1989;67(10):2894-99. https://doi.org/10.1139/b89-371
12. Kuhn DN, Motamayor JC, Meerow AW, Borrone JW, Schnell RJ. SSCP markers provide a useful alternative to microsatellites in genotyping and estimating genetic diversity in populations and germplasm collections of plant-speciality crops. Electrophoresis. 2008;29(19):4096-108. https://doi.org/10.1002/elps.200700937
13. Livingstone DS, Motamayor JC, Schnell RJ, Cariaga K, Freeman B, Meerow AW, et al. Development of single nucleotide polymorphism markers in Theobroma cacao and comparison to simple sequence repeat markers for genotyping of Cameroon clones. Mol Breed. 2011;27:93-106. https://doi.org/10.1007/s11032-010-9416-2
14. Mensah-Brako B. Effect of watering regime and cocoa pod husk on soil fertility and growth of hybrid cocoa seedlings in the semi-deciduous forest zone of Ghana. MSc [Thesis]. Kumasi: Kwame Nkrumah University of Science and Technology; 2011. Available from: https://ir.knust.edu.gh/handle/123456789/3987
15. Saunders JA, Mischke S, Leamy EA, Hemeida AA. Selection of international molecular standards for DNA fingerprinting of *Theobroma cacao*. Theo App Gene. 2004;110:41-47. https://doi.org/10.1007/s00122-004-1762-1
16. Panse VG, Sukhatme PV. Statistical methods for agricultural workers. New Delhi: India. Indian Council of Agricultural Research; 1954.
17. Cope F. Incompatibility in *Theobroma cacao*. Nature. 1958;181 (4604):279. https://doi.org/10.1038/181279a0
18. Apshara ES. Performance of selected cocoa (*Theobroma cacao* L.) clones under arecanut and coconut. J Plant Crops. 2013;41 (2):24246.
19. Takrama J, Cervantes-Martinez C, Phillips-Mora W, Brown J, Motamayor J, Schnell R. Determination of off-types in a cocoa breeding programme using microsatellites. Ingenic Newslett. 2005;10:2-8.
20. Asolkar T, Desai AR, Singh NP. Molecular analysis of cashew genotypes and their half-sib progeny using RAPD marker. Biotechnol Bioinf Bioeng. 2011;1:495-504.
21. Montserin B, Verteuil Ld. Hybridization work of the Department of Agriculture (Trinidad and Tobago) for the control of witches' broom disease of cacao. Trinidad and Tobago: Extension Training and Information Services Division Ministry of Food Production;1956.
22. Paulin D, Mossu G, Lachenaud P, Cilas C. Cocoa breeding in Côte-d'Ivoire. Performance analysis of sixty-two hybrids in four localities. Café, Cacao, Thé. 1993;37(1):3-20.

23. Pires J, Monteiro W, Pinto L, Figueira A, Yamada M, Ahnert D, editors. A proposal for cocoa breeding. In: 12th International Cocoa Research Conference, Lagos, Nigéria, Cocoa Producers' Alliance; 1996, p. 287-92.

24. Becher S, Steinmetz K, Weising K, Boury S, Peltier D, Renou JP, et al. Microsatellites for cultivar identification in Pelargonium. Theoret App Gene. 2000;101:643-51. https://doi.org/10.1007/s001220051526

25. Esselink G, Smulders M, Vosman B. Identification of cut rose (*Rosa hybrida*) and rootstock varieties using robust sequence tagged microsatellite site markers. Theoret App Gene. 2003;106:277-86. https://doi.org/10.1007/s00122-002-1122-y