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Confirmation of top cross hybrids in guava using morpho-molecular markers

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Abstract: The study was conducted to confirm the genetic diversity and hybridity of seventeen guava progenies developed from top-crossing between genetically distinct green and purple guava varieties. Morphological, biochemical, and molecular markers effectively identified hybrids exhibiting phenotypes from both parents. Moreover, remarkable genetic diversity was revealed among these segregants. Biplot analysis demonstrated a strong positive relationship between: (1) chlorophyll and anthocyanin content, (2) leaf length-to-width ratio, (3) leaf area, and (4) petiole length, identifying G15 and G16 genotypes as superior top-cross hybrids. A set of 10 simple sequence repeat (SSR) markers identified 36 alleles with a mean of 3.6 alleles per primer. The polymorphism percentage was 80.83 %, with pairwise dissimilarity ranging from 0.071 to 0.357. Four SSR primers (mPgCIR03, mPgCIR08, mPgCIR11, and mPgCIR19) specifically confirmed the top-cross hybrid status of G6, G8, G9, G10, G15, and G16 genotypes. These diverse genetic resources will be maintained for homozygous plant production through selfing and subsequent guava improvement programs.

Key Words: genetic diversity, top cross, polymorphism, dissimilarity index, molecular marker, segregates

Določitev najboljših križancev gvajave z morfološkimi in molekularnimi markerji

Izvleček: Namen raziskave je bil potrditi genetsko raznolikost in hibridnost sedemnajstih potomcev gvajave pridobljenih s križanjem genetsko različnih zelenih in škrlatnih sort. Morfološki, biokemični in molekularni markerji so potrdili, da izražajo križanci fenotipe obeh staršev, pri čemer je bila med njimi ugotovljena opazna genetska raznolikost. Biplotna analiza je pokazala močne pozitivne povezave med lastnostmi kot so: (1) vsebnost klorofila in antocianov, (2) razmerje med dolžino in širino listov, (3) v listni površini in (4) dolžini listnih pecljev. Pri tem sta bila genotipa G15 in G16 prepoznana kot najboljša križanca. Z naborom 10 markerjev enostavnih ponavljajočih se zaporedij (SSR) je bilo določenih 36 alelov, s poprečjem 3,6 alela na marker. Odstotek polimorfizma je bil 80,83 %, parna različnost je bila med 0,071 in 0,357. Štirje SSR primerji (mPgCIR03, mPgCIR08, mPgCIR11 in mPgCIR19) so še posebej potrdili najboljše križance med genotipi kot so G6, G8, G9, G10, G15 in G16. Ta raznolik genetskih vir bo vzdrževan za vzgojo homozigotnih rastlin preko samooprševanja v bodočih programih žlatnenja gvave.

Ključne besede: genetska raznolikost, vrhunski križanci, polimorfizem, indeks različnosti, molekularni marker, segregacija

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1 INTRODUCTION

Guava (*Psidium guajava* L., 2n = 22), a member of the Myrtaceae family, is widely cultivated in tropical and subtropical regions worldwide for its fleshy fruits (Grattapaglia et al., 2012; Morton, 1987). The genus *Psidium* comprises approximately 150 species, of which only 20 produce edible fruits (Jitendra et al., 2017). Due to its wide adaptability, nutritional value, and medicinal importance, guava has gained global popularity as a profitable crop (Medina & Herrero, 2016).

Although guava is well grown in almost all parts of Bangladesh, little attention has been paid to varietal improvement. Only four released varieties are available, while different obsolete varieties like Swarupkathi, Kanchannagar, Mukundapuri, Alahabad, Strawberry guava are still under cultivation at the farmers' level. Therefore, we assumed that a new guava variety could be developed with the introgression among the widespread varieties with desirable traits. Because of its vegetative propagation means facilitates the genetic purity of the successive generations. Guava is an allogamous fruit crop and self-pollination has been recorded to the extent of about 80 %. Singh (2007) reported that self-pollination in guava varies between 35–60 %, depending on the variety. For instance, 'Allahabad Safeda' shows a 50–60 % fruit set through self-pollination, while other varieties like 'Red Flesh' can achieve higher success rates (up to 70–80 %) under optimal conditions (Pommer & Murakami, 2009). However, 35 % natural cross-pollination occurs that creates the opportunity to develop a heterozygous population with an adequate genetic variation for selecting desirable commercial improved variety (Purseglove, 1968).

The determination of genetic diversity and hybridity among breeding materials using morphological markers represents a traditional approach that has been successfully employed for decades. However, this method presents significant constraints for breeding strategies, particularly in perennial crops (Chandra et al., 2005). Consequently, molecular markers have emerged as a superior alternative for varietal improvement programs, offering applications at multiple stages: (1) germplasm evaluation at variety or species level (Valdés-Infante et al., 2003; Rodríguez et al., 2004), (2) hybridity estimation (Barbour et al., 2010), (3) trait-specific association mapping (Feria-Romero et al., 2009), and (4) linkage mapping, quantitative trait locus (QTL) identification, and marker-assisted selection (Ritter, 2012).

Among various PCR-based techniques used in horticultural crop improvement, simple sequence repeats (SSRs) have proven particularly reliable for hybrid assessment. Compared to morphological markers, SSRs enable accurate hybrid identification at the seedling stage with greater efficiency, requiring smaller population sizes and shorter evaluation periods to select promising genotypes (Risterucci et al., 2010). In the present study, we employed two phenotypically distinct guava genotypes as parents for top-cross hybridization: a superior white-pulp cultivar (IPSA guava) and a local pink-pulp variety. Subsequent evaluation of phenotypic and genetic variability incorporated both traditional and advanced breeding techniques to identify effective molecular markers for precise genetic diversity assessment and top-cross hybrid confirmation.

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS

The experiment was conducted using two cultivated varieties of guava and fifteen offspring developed from the top-crossing of these cultivated varieties. The study was carried out in the nursery and experimental field of the Department of Horticulture at Bangabandhu Sheikh Mujibur Rahman Agricultural University between 2019 and 2022. The experimental materials consisted of a top-cross population, which inherited traits from the parent plants and exhibited significant morphological variation. The parental lines included: G1: A purple guava (open-pollinated female) and G2: IPSA guava (purebred male). Additionally, 15 segregants (G3 to G17) were derived from hybridization. The plant materials are described in detail in Table 1.

2.2 EXPERIMENT DESIGN

The experiment was laid out in the Randomized Complete Block Design (RCBD) with three replications where 17 genotypes were allocated randomly in each experimental unit as the independent variables. For morphological parameters determination, nine leaves from each genotype were used in one replication and repeated three times. Different morphological markers were used following the guidelines of the International Union for the Protection of New Varieties of Plants (UPOV, 1987) and Alam et al. (2019).

2.3 QUALITATIVE CHARACTERS

Fully developed leaves of the fifth and sixth position from the apex of a shoot were selected for the evalua-

Table 1: Characteristics feature with the accession number of the parents and the derived segregates exploited in the study

Sl. No.	Genotypes	Salient feature of the genotypes	Variety /Accession
1	G1 (Female)	Purple colored plant having purple colored fruit (both skin and flesh), fruit medium in size with hard seeds and astringency taste	Purple peyara (inferior)
2	G2 (Male)	Green colored plant having less seeded, sweet and medium size fruit	IPSA peyara (superior)
3	G3	Purple plant	F1 Segregates of the crossing between G1 and G2
4	G4	Green plant	
5	G5	Purple plant	
6	G6	Green plant	
7	G7	Green plant	
8	G8	Purple plant	
9	G9	Green plant	
10	G10	Purple plant	
11	G11	Green plant	
12	G12	Green plant	
13	G13	Green plant	
14	G14	Green plant	
15	G15	Purple plant	
16	G16	Purple plant	
17	G17	Green plant	

tion of qualitative phenotypic characters based on the leaf base and apex shape (Alam et al. 2019; UPOV 1987), the color of the leaf, twigs and vein (IBPGR 1993), leaf surface nature (curvature or twisting type) (Methela et al., 2019; Nagar et al., 2018a) and plant habitus (erect or spreading type) features (Patel 2006; Sharma et al. 2010; Nagar et al., 2018b). All the characters were observed critically in the eye estimation and expressed in descriptive traits.

2.4 QUANTITATIVE CHARACTERS

The quantitative data of each plant of the parents and F1 generations were recorded based on the leaf length, leaf width and petiole length with the help of digital slide calipers and expressed as centimeters (Shiva et al., 2017). The leaf area (cm^2) was measured in leaf length and width, and average data was used to compare the studied accessions. The leaf blade length to width was calculated by the average length of the leaf blade divided by the average width of the respective leaf blade for randomly selected four leaves of each genotype.

2.5 BIOCHEMICAL ASSESSMENT

2.5.1 Total chlorophyll

Chlorophyll content was estimated by the SPAD chlorophyll fluorescence and acetone extraction procedure. A portable chlorophyll meter (SPAD-502 Plus, Minolta Corporation, Ltd., Osaka, Japan) was used to measure the leaf chlorophyll concentration as a rational unit. Measurements were made at a central point on the leaflet between the midrib and the leaf margin of 5^{th} and 6^{th} leaf from the top (Colla et al. 2013). Six random measurements per plant were taken and averaged to a single SPAD value for each treatment. Chlorophyll was extracted from 200 mg of leaf samples in 10 ml of acetone (80 % acetone), and the supernatant was made up to the final volume of 25 ml and preserved in dark condition for 24 hours. The absorbance was recorded at 663 and 645 nm using a UV visible spectrophotometer. Total chlorophyll was estimated using the following formula and expressed as mg/g FW (Khan et al. 2017).

$$Chl\ a = [12.7\ (A_{663}) - 2.69\ (A_{645})]\ [V/(1000 \times M]$$
$$Chl\ b = [22.9\ (A_{645}) - 4.68\ (A_{663})]\ [V/(1000 \times M]$$

TChl (mg/g FM) = Chla + Chlb

Where, Chl= Chlorophyll, V= Volume, M= Mass and TChl= Total chlorophyll

2.5.2 Anthocyanin

Fresh leaf (100 mg) was used for anthocyanin extraction following Chu et al. (2013) procedure with some modifications. The leaf sample was homogenized in 3 ml of acidic ethanol (1 % HCl w/v) on an ice base and the extracted sample was incubated at 4 °C for 1 hour on the shaker with moderate shaking mode. The suspension was centrifuged with 14,000 rpm at 4 °C for 5 minutes for clarified suspension and this suspension was used for further absorbance analysis. The absorption was measured with a UV- visible spectrophotometer at 530 nm and 657 nm wavelength.

Quantification of anthocyanin was performed using the following equation:

$$Q_{Anthocyanin} = (A_{530} - 0.25 \times A_{657}) \times M^{-1}$$

Here, $Q_{Anthocyanin}$ indicated the amount of anthocyanin,

A_{530} and A_{657} were the absorptions at the indicated wavelengths and M was the mass of the plant material used for extraction (g).

2.6 MOLECULAR CHARACTERIZATION

2.6.1 Materials for molecular characterization

For molecular characterization, we analyzed 12 distinct guava genotypes selected from an initial pool of 17, excluding five F1 progenies that exhibited close morphological resemblance to their parental lines. The genetic diversity assessment employed ten carefully selected SSR markers that demonstrated precise amplification across 10 F1 progenies and two parent plants. These markers were chosen based on their proven reproducibility, clear scorable banding patterns (150-320 bp), and prior validation in guava (*Psidium guajava*) as reported by Rodríguez et al. (2007) and Kareem et al. (2018). The selected primers generated distinct polymorphic profiles suitable for genetic differentiation, showed optimal amplification efficiency, and specifically targeted known guava loci.

2.6.2 Isolation of genomic DNA

The genomic DNA from the actively growing young, fresh and healthy leaves of the selected 12 genotypes was extracted following the modified CTAB (Cetyl Trimethylammonium Bromide) method (Chakrabarti et al. 2006). 150 mg of leaf materials were cut into small pieces and kept inside the mortar. Then some sand and 700 µl of DNA extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2 % CTAB solution (w/v), 0.2 % (v/v) 2-Mercaptoethanol) was added and crushed with pestle. Crushed materials were transferred to a 1.5 ml microcentrifuge tube followed by adding 700 µl of DNA extraction buffer. This sample was incubated at 65 ℃ for one hour in a shaker with gentle shaking after thoroughly mixing by vortex mixture for 30 sec afterward, centrifuged for 15 minutes at 15000 rpm. The supernatant of 700 µl was transferred to the microcentrifuge tube, and then added 4 µl of RNase and kept 45 minutes at 37 °C. Then 700 µl of Chloroform: Isoamyl alcohol (24: 1, v/v) was added to the sample and mixed thoroughly in gentle mode. Spinning this sample at 12000 rpm for 10 minutes with the addition of and a 2/3rd volume isopropanol for spinning down the DNA pellet and supernatant was discarded carefully. The pellets were washed with 70 % ethanol and again spinned out at 10000 rpm for 10 minutes. After drying the pellets were dissolved in 100 µl of sterile water and stored at -20℃ until the PCR analysis.

2.6.3 PCR analysis for genotypes selection

Ten SSR primers were used to amplify the DNA sample of 12 genotypes. PCR was conducted in 25 of reaction volume for each reaction and total 12 reactions were done for each 10 SSR primers. The PCR reaction mixture contained template DNA (20 ng), 1 µM forward and 1 µM reverse primers, 200 µM of dNTPs, and 10X PCR buffer, 0.1 U Taq DNA polymerase and $MgCl_2$ solution (1.5 mM). The optimization of conditions was made separately for each marker. PCR was carried out in the thermal cycler with an initial denaturation at 94 °C for 5 min; denaturation at 94 °C for 30 sec; primer annealing at 55 °C for 45 sec; extension at 72 °C for 2 min and final extension at 72 °C for 4 min.

All amplifications were confirmed after running PCR product (10 µl) on agarose gels (1.5%). An 8 µl ladder (100 bp) with 2 µl loading dye was used for comparison. After staining with ethidium bromide (EtBr) gel was visualized with the gel documentation system.

2.6.4 Data analysis

Principal component analysis (PCA) was done using the R-statistical program to distinguish F_1 segregates with respective parents according to their morphological features. Marker (SSR) based data were analyzed, and Roger's genetic distance matrices were calculated be-

tween each pair of lines using DARwin software 6.0 (Perrier and Jacquemoud-Collet 2016).

3 RESULTS

3.1 MORPHOLOGICAL CHARACTERIZATION

3.1.1 Qualitative characters

Morphological qualitative traits exhibited distinct visual differences between parents and their F1 segregants (Table 2, Figure 1). The female parent (G1) displayed oblanceolate leaf shapes, while the male parent (G2) showed elliptical leaves. Among the 15 segregants, we observed various leaf shapes including oblong, elliptical, lanceolate, oblong-to-elliptical, and ovate forms. While both parents shared an obtuse leaf base shape, their segregants exhibited deviations including cordate and rounded bases. Similar variation occurred in leaf apex shapes, with F1 progenies showing apiculate, rounded, and acute forms compared to the parents' obtuse apices. The male parent G2 exhibited leaf twisting and midrib curvature - traits absent in female parent G1 - with intermediate variations appearing among their segregants. Leaf surface texture varied from smooth to rough (Table 2). Ventral surface analysis (Figure 1a) revealed three texture types: smooth, moderately smooth, and rough. Dorsal surfaces (Figure 1b) showed moderately smooth textures only in G2 and G11, with rough textures in all other genotypes. Branching pattern attitudes varied between parents and segregating progenies (Table 2). Spreading growth habits characterized genotypes G1, G4-G5, G7-G8, and G11-G14, while the remaining genotypes exhibited erect growth forms.

Though the female (G1) had greyed dark purple and male (G2) had light green color fully matured leaves, their pogenies showed different leaf colors viz. yellowish green, light green, green, maroon dark purple and greenish-purple. Similarly, G1 had dark red and G2 had light green twigs color while dark red, brownish red, yellowish-green, light green, light green with red streak, green with red streaks and reddish-green were observed among the segregates. Considerable variation for leaf vein color viz. red, dark red, reddish green and green was also noticed among the segregates, whereas G1 had red color leaf vein and G2 had green color leaf vein. Stem color variation viz. reddish brown, greenish brown, brown was found among the segregating progenies through their two parents such as G1 had dark reddish brown and G2 had greenish brown stem.

3.1.2 Quantitative characters

Morphological data on five quantitative traits were showed significant variation among two parent guava lines and their 15 segregates (Table 3). Though, both the parents viz. G1 (9.98 cm) and G2 (9.8 cm) had almost similar fully developed leaf lengths, the segregates showed a slight variation. Among the progenies, the highest leaf length was found in G14 (11.80 cm) and the lowest in G8 (7.85 cm). The highest leaf width was found in G14 (6.70 cm) and the lowest was in G2 (3.93 cm). Although the parents G1 and G2 had identical petiole length (0.75 cm) but remarkable variations were observed among the progenies where G14 (0.88 cm) had the highest and G8 (0.38 cm) had the lowest petiole length. Similarly, the maximum leaf area was observed in G14 (79.57 cm^2) and the minimum in G8 (35.27 cm^2). The highest leaf length width ratio was identified in G2 (4.37) but the lowest was in G6 (1.63)

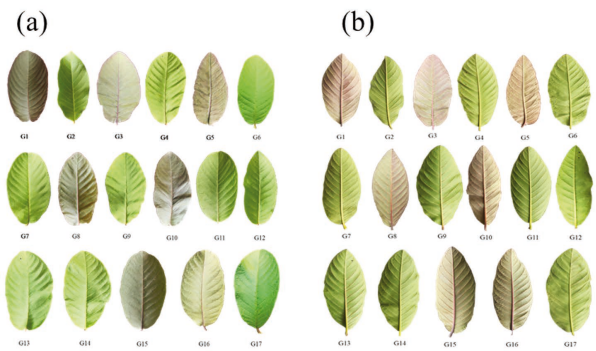

Figure 1: Shape and color of fully developed leaf (a) ventral surface (b) dorsal surface in different guava genotypes.

3.2 BIOCHEMICAL ANALYSIS

3.2.1 Chlorophyll content

The total chlorophyll content estimated by the SPAD meter was statistically identical in both the parents and their progenies (Table 4). However, the highest chlorophyll content (%) was measured in G10 (45.20) and the lowest in G11 (35.45). On the other hand, Chla is almost similar in two parents, viz. G1

Table 2: Morphological characteristics of guava

Genotype	Fully developed leaf shape	Leaf base shape	Leaf apex shape	Fully developed leaf twisting	Curvature in midrib	Leaf surface nature		Tree habit	Fully developed leaf color	Twig color	Leaf vein color	Stem color
						Ventral surface	Dorsal surface					
G1	Oblanceolate	Obtuse	Obtuse	Absent	Absent	Smooth	Rough	Spreading	Greyed dark purple	Dark red	Red	Dark reddish brown
G2	Elliptical	Obtuse	Obtuse	Present	Present	Smooth	Moderately smooth	Erect	Light green	Light green	Green	Greenish brown
G3	Ovate	Cordate	Obtuse	Absent	Absent	Smooth	Rough	Erect	Maroon purple	Dark red	Dark red	Reddish brown
G4	Elliptical	Rounded	Apiculate	Absent	Present	Moderately smooth	Rough	Spreading	Yellowish green	Green with red streaks	Reddish green	Greenish brown
G5	Lanceolate	Cordate	Acute	Present	Present	Moderately smooth	Rough	Spreading	Maroon purple	Dark red	Dark red	Reddish brown
G6	Oblong	Cordate	Obtuse	Absent	Absent	Smooth	Rough	Erect	Yellowish green	Light green with red streak	Green	Greenish brown
G7	Oblong	Rounded	Rounded	Present	Present	Smooth	Rough	Spreading	Light green	Light green	Green	Reddish brown
G8	Oblong	Cordate	Acute	Present	Absent	Smooth	Rough	Spreading	Greenish purple	Brownish red	Red	Reddish brown
G9	Elliptical	Obtuse	Rounded	Present	Absent	Smooth	Rough	Erect	Yellowish green	Light green with red streak	Reddish green	brown
G10	Oblong	Cordate	Obtuse	Present	Absent	Rough	Rough	Erect	Greenish purple	Brownish red	Dark red	Reddish brown
G11	Ovate	Rounded	Apiculate	Absent	Absent	Smooth	Moderately smooth	Spreading	Green	Light green with red streak	Green	Brown
G12	Lanceolate	Cordate	Acute	Present	Absent	Smooth	Rough	Spreading	Green	Light green with red streak	Green	Greenish brown
G13	Elliptical	Obtuse	Rounded	Absent	Present	Smooth	Rough	Spreading	Light green	Light green with red streak	Reddish Green	Greenish brown
G14	Oblong to elliptical	Obtuse	Obtuse	Present	Absent	Moderately smooth	Rough	Spreading	Yellowish green	Light green with red streak	Green	Greenish brown
G15	Oblong	Rounded	Acute	Present	Absent	Smooth	Rough	Erect	Maroon purple	Reddish green	Dark red	Reddish brown
G16	Oblong	Rounded	Acute	Present	Absent	Smooth	Rough	Erect	Dark purple	Dark red	Red	Reddish brown
G17	Ovate	Rounded	Obtuse	Present	Present	Smooth	Rough	Spreading	Light green	Yellowish green	Green	Greenish brown

(1.10 mg g^{-1} FM) and G2 (1.14 mg g^{-1} FM) but remarkable variations were found among the segregates (Table 4). The highest Chla was determined in G16 (1.46 mg g^{-1} FM); which was at par with G10 and G15and the lowest in G13 (0.86 mg g^{-1} FM). Meanwhile, both the parent showed differences in Chlb content denoted as G1 (0.56 mg g^{-1} FM) and G2 (0.45 mg g^{-1} FM). Consequently, wide variation was observed among the segregates Depicted as G16 (0.74 mg g^{-1} FM) had maximum and G13 (0.37 mg g^{-1}FM) had minimum Chlb content. Similar trends of result in the TChl content were observed in the parents G1 (1.65 mg g^{-1} FM) and G2 (1.59 mg g^{-1} FM) and the progenies of G16 (1.65 mg g^{-1} FM) had maximum and G13 (1.24 mg g^{-1} FM) had minimum TChl content.

3.2.2 Anthocyanin content

The results depict that anthocyanin content was varied significantly between the parents where purple parent G1 and the green parent G2 were showed about 14.03 mg g^{-1} FM and 1.86 mg g^{-1} FM, respectively (Figure 2a). So a large variation was found among the segregates where G3 (17.89 mg g^{-1} FM) had maximum anthocyanin, which was at par with G16, G1, G15, G8, G5, and all these genotypes had a different shade of purple leaves. On the contrary, G6 (0.54 mg g^{-1} FM) had minimum anthocyanin, which was statistically similar with the genotypes G4, G11, G14, G17, G13, G2, G9, G7, G12, and all of those had a different shade of green leaves (Figure 2b).

Morpho-biochemical characteristics variation visualize in Biplot of PCA analysis - The biplot (Figure 3) displayed 68.4 % of the total variation observed (PC1 in Dim1 = 46.5 % and PC2 in Dim2 = 21.9 %) in the standardized data of the 17 genotypes for the studied eight morpho-biochemical traits. This biplot was visualized from two perspectives (Yan and Reid, 2008), showing a strong positive correlation among Chla, TChl, Chlb, ChlSPAD and Anth traits due to having an acute angle and covered 46.5% of the variation (PC1). On the other hand, Anth and LWR; LA and PL also had a strong positive correlation that covered 21.9 % of the variation (PC2).

In addition, biplot analysis showed the genotypes' trait profiles, especially those positioned far away from the origin and correlation among the traits. Therefore, the scatter plot helped select genotypes for the yield contributing traits or traits that helped in better qualitative performance. In the present biplot visualization after loading variations by PC1 and PC2, it was evident that genotypes G10 and G12 had better performance for a higher percentage of SPAD value;

Table 3: Variation in leaf length, leaf width, petiole length, leaf area and leaf length width ratio among the guava genotypes

Genotype	Leaf length (cm)	Leaf width (cm)	Petiole length (cm)	Leaf area (cm2)	Leaf length width ratio
G1	9.98 ± 0.40ab	5.18 ± 0.29abcd	0.75 ± 0.10ab	51.71 ± 4.88ab	1.93 ± 0.04a
G2	9.8 ± 0.71ab	3.93 ± 2.02d	0.75 ± 0.06ab	37.83 ± 19.38b	4.37 ± 4.86a
G3	8.72 ± 2.46ab	4.53 ± 1.65abcd	0.53 ± 0.10def	42.52 ± 25.50ab	1.98 ± 0.18a
G4	9.33 ± 1.03ab	4.90 ± 0.35abcd	0.65 ± 0.06bcd	45.91 ± 7.65ab	1.90 ± 0.13a
G5	8.80 ± 2.11ab	4.48 ± 1.01bcd	0.40 ± 0.00ef	40.93 ± 17.93b	1.96 ± 0.11a
G6	10.73 ± 1.21ab	6.60 ± 0.81ab	0.70 ± 0.08bc	71.49 ± 16.50ab	1.63 ± 0.06a
G7	10.60 ± 0.84ab	5.68 ± 0.64abcd	0.50 ± 0.00def	60.41 ± 10.86ab	1.88 ± 0.17a
G8	7.85 ± 2.84b	4.30 ± 0.81cd	0.38 ± 0.05f	35.27 ± 18.05b	1.79 ± 0.44a
G9	10.80 ± 0.84ab	5.92 ± 0.25abcd	0.55 ± 0.10cde	64.12 ± 7.18ab	1.82 ± 0.10a
G10	9.98 ± 1.97ab	4.95 ± 1.01abcd	0.53 ± 0.05def	50.74 ± 20.31ab	2.02 ± 0.17a
G11	9.98 ± 1.97ab	4.95 ± 1.01abcd	0.53 ± 0.05def	50.74 ± 20.31ab	2.02 ± 0.17a
G12	8.90 ± 0.28ab	4.88 ± 0.68abcd	0.40 ± 0.00ef	43.48 ± 7.10ab	1.85 ± 0.24a
G13	10.73 ± 1.74ab	5.68 ± 0.88abcd	0.63 ± 0.05bcd	61.96 ± 18.57ab	1.89 ± 0.10a
G14	11.80 ± 1.06 a	6.70 ± 0.65a	0.88 ± 0.05a	79.57 ± 14.67a	1.76 ± 0.03a
G15	9.23 ± 0.40ab	5.65 ± 0.48abcd	0.55 ± 0.06cde	52.19 ± 5.99ab	1.64 ± 0.13a
G16	10.85 ± 2.71ab	5.45 ± 1.86abcd	0.53 ± 0.05def	62.86 ± 34.62ab	2.04 ± 0.22a
G17	10.70 ± 2.08ab	6.30 ± 0.76abc	0.75 ± 0.06ab	68.57 ± 21.15ab	1.69 ± 0.14a
LSD0.05	3.70	2.19	0.16	37.93	3.08

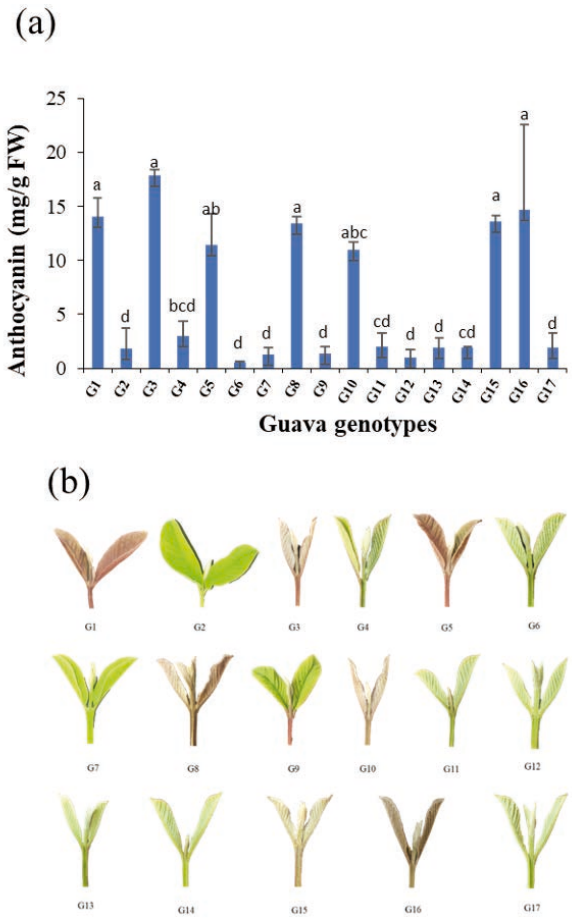

Figure 2: Anthocyanin pigment content (a) and variation in twig color due to having different level of anthocyanin (b) among 17 guava genotypes.

G16 could be a better choice for the maximum TChl; G16, G15, G10 were superior for a higher amount of Chla and Chlb content; G3 could be selected for the highest Anth content.

3.2.3 Analysis of correlation matrix

The correlation matrix analysis among the different morphological traits (Figure 4a and 4b) revealed that a strong positive correlation was observed between Tchl and Chla (0.99) followed by Tchl and Chlb (0.97). Meanwhile, Chla has a significant correlation with Chlb (0.94). Almost similar correlation matrix was noticed between ChlSPAD and Chla (0.65); ChlSPAD and TChl (0.64) whereas it was 0.59 between ChlSPAD and Chlb (0.59). Furthermore, PL was found correlated with LA (0.58) and Chlb with anthocyanin (0.53).

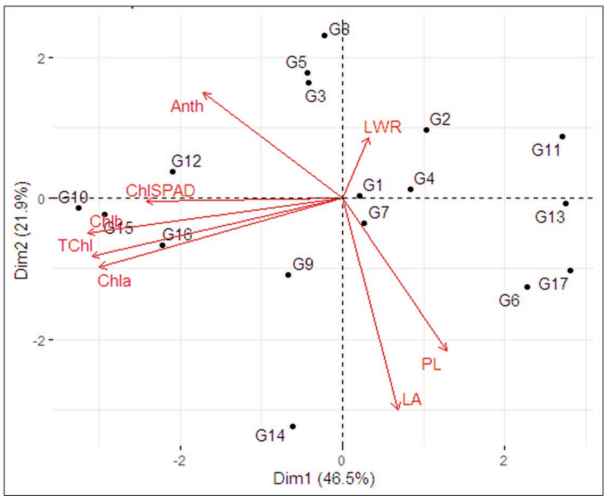

Figure 3: Biplot analysis of guava genotypes for morphological and biochemical character association. (LA = Leaf area (cm^2); PL = Petiole length (cm); LWR = Leaf length width ratio; Chl a = Chlorophyll a (mg g^{-1} FM); Chl b = chlorophyll b (mg g^{-1} FM); TChl = Total chlorophyll (mg g^{-1} FM); ChlSPAD = SPAD value of chlorophyll (%); Anth =Anthocyanin (mg g^{-1} FM).

3.3 MOLECULAR CHARACTERIZATION

3.3.1 Level of polymorphism

Different polymorphism levels were observed among all 12 studied guava genotypes using a set of ten SSR primers (Table 5). A total of 36 alleles were generated by SSR markers, with a mean of 3.6 alleles per primer. Among the ten primers, six (mPgCIR02, mPgCIR05, mPgCIR15, mPgCIR17, mPgCIR21, and mPgCIR25) produced both polymorphic and monomorphic bands, while the remaining four primers exhibited exclusively polymorphic banding patterns. All primers were selected from previously characterized guava loci (Rodríguez et al., 2007; Kareem et al., 2018).

In this study, mPgCIR02, mPgCIR03, mPgCIR08, mPgCIR11, and mPgCIR19 showed clear polymorphism, while the remaining primers displayed lower polymorphism levels. Among the SSR markers, mPgCIR03, mPgCIR08, and mPgCIR11 each produced 4 polymorphic bands, and mPgCIR19 yielded 3 polymorphic bands, with no monomorphic alleles detected. These four markers demonstrated 100 % polymorphism, making them strong candidates for varietal improvement programs.

The highest number of alleles was observed with mPgCIR02, which showed 83.33% polymorphism, while the lowest polymorphism (50%) was recorded for mPgCIR17.

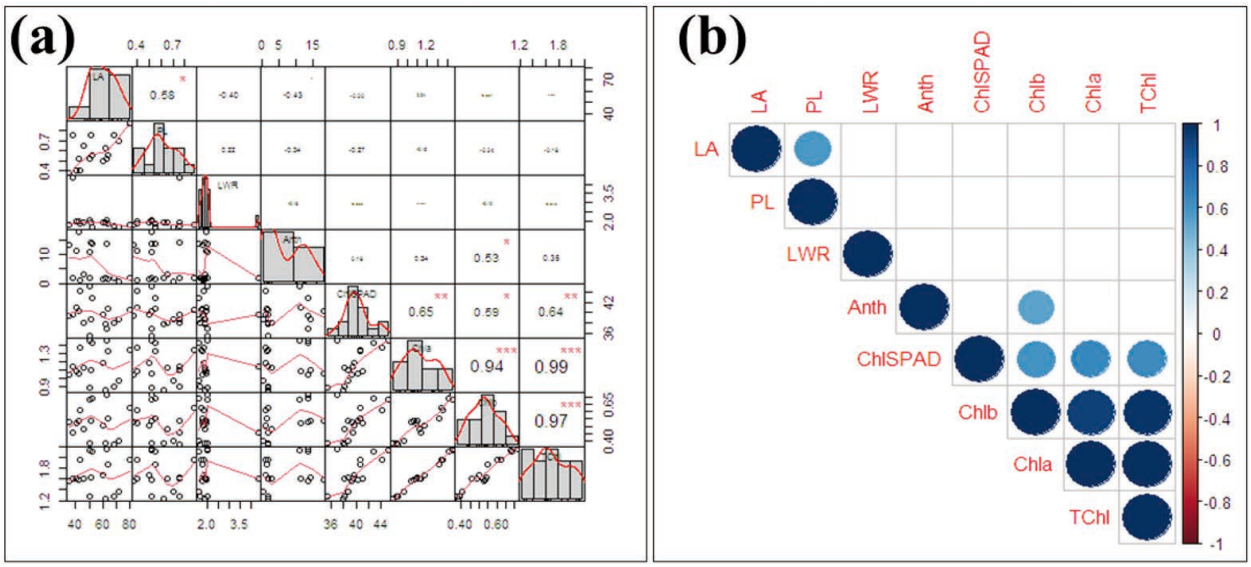

Figure 4: Correlation and visualization of guava genotypes. Correlation matrix with significant value among the different variables of 17 guava genotypes (a). Visualization of correlation matrix among the different variables of guava genotypes. (Blank space indicates insignificant correlation. Cyan to blue and cyan to red colors show significant (p<0.05) positive and negative correlation between traits respectively) (b). (LA = Leaf area (cm^2); PL = Petiole length (cm); LWR = Leaf length width ratio; Chla = Chlorophyll a (mg g^{-1} FM); Chlb = chlorophyll b (mg g^{-1} FM); TChl = Total chlorophyll (mg g^{-1} FM); ChlSPAD = SPAD value of chlorophyll (%); Anth =Anthocyanin (mg g^{-1} FM).

Table 4: Variation in Chlorophyll content (SPAD units), Chla, Chlb and TChl in different guava genotypes

Genotype	Chlorophyll (SPAD units)	Chla (mg g^{-1} FM)	Chlb (mg g^{-1} FM)	TChl (mg g^{-1} FM)
G1	38.75 ± 1.06	1.10 ± 0.004de	0.56 ± 0.02bcde	1.65 ± 0.02de
G2	39.60 ± 2.12	1.14 ± 0.002cd	0.45 ± 0.01ef	1.59 ± 0.02de
G3	40.30 ± 1.56	1.06 ± 0.004e	0.53 ± 0.01cde	1.58 ± 0.02de
G4	38.10 ± 0.57	1.15 ± 0.028cd	0.47 ± 0.01def	1.62 ± 0.01de
G5	41.05 ± 1.34	1.08 ± 0.001de	0.55 ± 0.04bcde	1.63 ± 0.04de
G6	38.75 ± 0.35	0.95 ± 0.021f	0.42 ± 0.02f	1.36 ± 0.00f
G7	39.70 ± 2.83	1.17 ± 0.001c	0.42 ± 0.02cde	1.71 ± 0.04d
G8	39.30 ± 1.56	1.04 ± 0.002e	0.54 ± 0.01cde	1.57 ± 0.01e
G9	40.70 ± 1.41	1.33 ± 0.021b	0.56 ± 0.02bcd	1.90 ± 0.04c
G10	45.20 ± 4.67	1.45 ± 0.014a	0.65 ± 0.04ab	2.10 ± 0.05ab
G11	35.45 ± 4.17	0.88 ± 0.007g	0.37 ± 0.02f	1.25 ± 0.03fg
G12	44.25 ± 4.88	1.37 ± 0.014b	0.62 ± 0.02bc	1.99 ± 0.04bc
G13	37.90 ± 1.84	0.86 ± 0.021g	0.37 ± 0.01f	1.24 ± 0.01g
G14	40.65 ± 1.91	1.35 ± 0.014b	0.65 ± 0.04ab	1.99 ± 0.05bc
G15	42.95 ± 5.16	1.44 ± 0.028a	0.65 ± 0.04ab	2.09 ± 0.02ab
G16	36.95 ± 10.54	1.46 ± 0.021a	0.74 ± 0.03a	2.20 ± 0.05a
G17	38.05 ± 0.64	0.87 ± 0.028g	0.39 ± 0.01f	1.26 ± 0.01fg
LSD0.05	14.79	0.07	0.11	0.13

3.3.2 Polymorphic information

Polymorphic information observed by SSR primers revealed that thirty alleles showed 83.33 % poly-

morphism among the total alleles and six alleles showed monomorphism of 16.67 %. The overall percentage of polymorphic alleles was 80.83 %. All primers produced specific, effective, and measurable alleles. The amplified alleles ranged from 220-1250 bp (Figure 5; Table 5). A representative image of mPgCIR08 primer showed the allelic difference between the parents and segregates (Figure 5). The average polymorphic information content (PIC) was found 0.576 among the genotypes. The primer mPgCIR03 showed highest (0.693) polymorphic information followed by mPgCIR08 and mPgCIR11. Thus, the primer mPgCIR03, mPgCIR11, and mPgCIR19 were shown effective for the selection of top cross hybrids and genetic diversity study.

Determination of genetic relatedness with dissimilarity matrix - A dissimilarity matrix using ten SSR markers was used to estimate the genetic relatedness of analyzed accessions of guava species. The dissimilarity matrix (Figure 6, Table 5) represented the pair-wise dissimilarity value ranged from 0.071 to 0.357. The lowest value was observed between the G8 and G14 (0.071); thus, these are the closest genotypes. Similarly, a lower value (0.097) was found between the genotypes G5 and G16; 0.103 was found between G6 and G8; 0.111 was found for three pairs of G2 and G9; G4 and G9; G6 and G14. So, it can be said that G5 and G16; G6 and G8; G2 and G9; G4 and G9; G6 and G14 were closer genotype pairs. Contrary, the highest dissimilarity matrix value (0.357) was found among G1 and G15; G6 and G15 genotype pairs indicated these genotypes were not closely

Table 5: Polymorphic information of ten SSR markers with their sequences

Sl No.	Name of primer	Sequences (5'-3')	Observed size (bp)	NA	NPA	PIC	QMA	%PA
	mPgCIR02	F: AGTGAACGACTGAAGACC	220-1250	6	5	0.569	1	83.33
1		R: ATTACACATTCAGCCACTT						
2	mPgCIR03	F: TTGTGGCTTGATTTCC	220-800	4	4	0.693	0	100
		R: TCGTTTAGAGGACATTTCT						
3	mPgCIR05	F: GCCTTTGAACCACATC	220-800	3	2	0.567	1	66.67
		R: TCAATACGAGAGGCAATA						
4	mPgCIR08	F: ACTTTCGGTCTCAACAAG	220-800	4	4	0.676	0	100
		R: AGGCTTCCTACAAAAGTG						
5	mPgCIR11	F: TGAAAGACAACAAACGAG	220-800	4	4	0.650	0	100
		R: TTACACCCACCTAAATAAGA						
6	mPgCIR15	F: TCTAATCCCCTGAGTTTC	240-780	3	2	0.576	1	66.67
		R: CCGATCATCTCTTTCTTT						
7	mPgCIR17	F: CCTTTCGTCATATTCACTT	300-700	2	1	0.393	1	50
		R: CATTGGATGGTTGACAT						
8	mPgCIR19	F: AAAATCCTGAAGACGAAC	220-800	3	3	0.671	0	100
		R: TATCAGAGGCTTGCATTA						
9	mPgCIR21	F: TGCCCTTCTAAGTATAACAG	300-1250	4	3	0.476	1	75
		R: AGCTACAAACCTTCCTAAA						
10	mPgCIR25	F: GACAATCCAATCTCACTTT	200-780	3	2	0.546	1	66.67
		R: TGTGTCAAGCATACCTTC						
Total		------	-----	36	30		06	808.34
Percentage/Average*		3.6*	83.3	0.575	16.67	80.83*		

NA number of alleles, NPA number of polymorphic alleles, PIC polymorphism information content, QMA quantity of monomorphic alleles, PPA percentage of polymorphic alleles, SSR simple sequence repeat and *indicates the average values of QAA and PPA produced by each primer

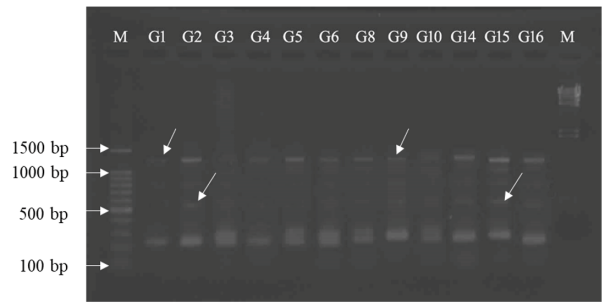

Figure 5: Polymorphic profile of primer mPgCIR08 for 12 guava genotypes. Hybridity testing of guava hybrid using the mPgCIR08 SSR marker. M= 100 bp ladder, Lane 2 and 3 indicated the two parent genotypes and lane 4-13 indicated their offspring genotypes viz. lane 3= G3, Lane 4= G4, lane 5= G5, lane 6= G6, lane 7= G8, lane 8= G9, lane 9= G10, lane 10= G14, lane 11= G14, lane 12= G15 and lane 13= G16. Lane 6, 8 and 12 (arrow) represents top cross hybrids (G6, G9 and G15) guava.

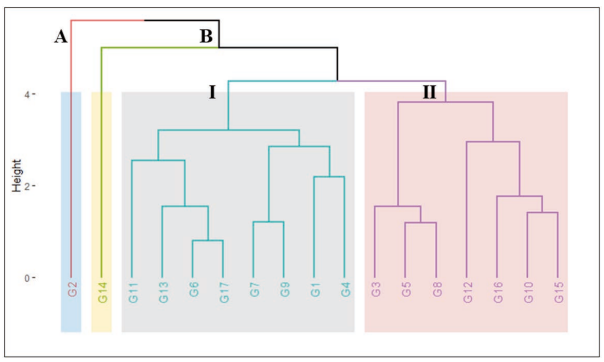

Figure 6: Dendrogram showing genetic relationship among 10 segregating guava progenies and their two parent genotypes based on SSR marker analysis.

related. Furthermore, a higher level of dissimilarity was also observed in several genotypes such as G15 and G14 (0.333); G2 and G15 (0.313); G16 and G15 (0.310).

4 DISCUSSIONS

The effectiveness of SSR markers for early-stage selection and screening of plants has been well established for assessing genetic diversity and identifying pure hybrids (Maravilla et al., 2017; Dawson et al., 2013; Tuler et al., 2015). In this study, we evaluated twelve guava genotypes using ten SSR primer pairs, among which four primers (mPgCIR03, mPgCIR08, mPgCIR11, and mPgCIR19) demonstrated 100 % polymorphism. These results align with previous findings by Ma et al. (2019), Dinesh et al. (2017), Campos-Rivero et al. (2017), and Urquía et al. (2019), who reported 90-97 % polymorphism using SSR markers for genetic diversity analysis and hybrid confirmation. Notably, Kanupriya et al. (2011) identified 23 microsatellite markers that successfully discriminated among nine guava varieties.

Besides molecular markers, morpho-biochemical markers are helpful for variety identification and are reliable in establishing the genetic relationships across more extensive and diverged accessions of guava (Padilla-Ramirez and Gonzalez-Gaona 2008). In this study morphological traits viz. fully developed leaf shape, leaf base and apex shape, leaf twisting, midrib curvature, leaf surface nature, tree habit, the color of fully developed leaf, twig, leaf vein and stem showed remarkable variations. The variations of leaf characteristics in guava were also reported in some recent studies (Alam et al. 2019; Methela et al. 2019; Nagar et al. 2018a; Nagar et al. 2018b). In an experiment, Dubey et al. (2016) found leaf length ranged from 10.75 cm to 13.95 cm, leaf width from 4.36 cm to 7.08 cm, and leaf area from 65.1 cm^2 to 95.71 cm^2. The observed leaf width value of this study was well supported by the findings of El-Sisy (2013) who found that leaf width was varied from 4.0 cm to 6.9 cm. El-Sharkawy and Othman (2009) stated that the leaf petiole length of five guava genotypes ranged from 0.84 cm to 0.55 cm. El-Sisy (2013) also reported that leaf area ranged from 30.67 to 88.33 cm^2 which were similar with the findings of this study.

Chlorophyll and anthocyanin are the two most essential pigments in leaves (Croft and Chen 2017). Chlorophyll, commonly responsible for green color, is an essential pigment for converting light into chemical energy and the increased synthesis of anthocyanins is the main reason leading to purple coloration (Croft et al. 2017). In this study chlorophyll SPAD value among the parent and their segregates were well supported by the previous study done by Afifi et al. (2019), who found about 35.47 % to 47.47 % chlorophyll content variation among the guava genotypes. In all the case, the Chla content in leaf was found higher than the Chlb. The possible reason could be that Chla is the primary pigment while others, including Chlb are accessory pigments (Srichaikul et al. 2011). Anthocyanin is responsible for the colors (red, purple, and blue) of leaves, stems, roots, flowers and fruits (Khoo et al. 2017) that reflect the color variation among the segregates. It might happen because the parents used in the hybridization process possess different colors with the significant anthocyanin variation that strongly influenced the pigmentation variation of their segregates. Again, biplot analysis of morpho-biochemical traits is considered an efficient way of suitable genotype selection and magnitude of the relationship among the agronomic traits (Farshadfar et al. 2013). Sau et al. (2017)

conducted biplot analysis to identify the principal yield attributes and considerable variations were observed in yield and yield contributing characters. From the biplot and correlation matrix analysis, a strong positive correlation was observed between Tchl and Chla (0.99) followed by Tchl and Chlb (0.97) in the present study that is supported by the findings of Santos et al. (2017).

5 CONCLUSIONS

Genetic diversity assessment and top-cross hybrid selection were conducted using morphological, physio-chemical, and molecular markers. Among the SSR primers tested, mPgCIR03, mPgCIR08, mPgCIR11, and mPgCIR19 effectively identified top-cross hybrids derived from the G1 × G2 hybridization scheme. Results revealed that progenies G5, G6, G8, G9, G10, G15, and G16 showed the highest segregation, exhibiting morphological characteristics from both parents. The study demonstrated that morphological variation and anthocyanin pigmentation serve as valuable selection criteria when combined with molecular markers for identifying superior hybrid progenies. These findings provide significant insights for hybridization programs and progeny selection in tropical guava, particularly when based on phenotypic characterization. Furthermore, the developed segregating progenies represent valuable genetic resources that can serve as foundation material for future guava improvement programs targeting desirable traits.

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7 CONFLICT OF INTEREST

The authors declare no conflict of interest.

8 AUTHOR CONTRIBUTIONS

The funding for the work was received through Md. Sanaullah Biswas. Md. Sanaullah Biswas, Mohammad Sharif Raihan and M. Mizanur Rahman conceptualized the initial work and the planned activities of this work. Masuma Zahan Akhi and Md. Sanaullah Biswas carried out the field experiment and performed the laboratory experiments. Masuma Zahan Akhi and Md. Sanaullah Biswas collection of the data. Masuma Zahan Akhi and Jahidul Hassan organized, analyzed and interpreted the data. Masuma Zahan Akhi and Md. Sanaullah Biswas drafted the initial manuscript. Jahidul Hassan and Mohammad Sharif Raihan carried out the review of the manuscript. Md. Sanaullah Biswas finally reviewed and shaped the manuscript. All the authors read and approved the final draft of the manuscript.

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