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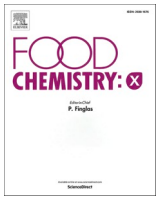

Characterization of fine-flavor cocoa in parent-hybrid combinations using metabolomics approach

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ABSTRACT

Fine-flavored cocoa is generally characterized by fresh bean color and sensory characteristics. However, these methods cannot be applied to progenies/hybrids because their colors may vary depending on their parents. Additionally, sensory evaluation lacks universal quality standards, necessitating robust complementary characterization methods. This study aimed to characterize the fine-flavor cacao in parent-hybrid combinations using widely targeted Gas Chromatography-Mass Spectrometry (GC–MS) and bean phenotype analysis. Fine-flavored cacao exhibits white-bean characteristics and a lighter color than forastero. Conversely, the hybrids displayed varying percentages of fresh bean color. Caffeine and organic acids (malic acid, fumaric acid, citric acid, lactic acid, and tartaric acid) were found to correspond to the characteristics of fine-flavored cacao. Each parent-hybrid combination demonstrated distinct flavor characteristics, with the ICCRI03-hybrid emerging as a promising clone, exhibiting flavor characteristics similar to those of its female parent (fine-flavor cacao). This information on flavor characteristics will be beneficial for further fine-flavored cacao selection.

1. Introduction

Theobroma cacao has evolved into one of the most important agricultural commodities (Castro-Alayo et al., 2019). It has a significant economic value (Bagnulo et al., 2023) because its beans serve as a raw material for the chocolate industry (Moreno-Rojas et al., 2023). From a commercial and industrial perspective, cocoa is classified into two product types: bulk or standard quality, known for its basic flavor, and fine-flavor cocoa, distinguished by outstanding aromatic notes (Rottiers et al., 2019). Fine-flavor cocoa offers superior quality, commanding a higher price in the market compared to bulk cocoa (Escobar et al., 2021). The ICCO (ICCO, 2017) estimates that fine-flavor cocoa typically costs between $5000 and $10,000 per metric ton, while bulk cocoa ranges from $3000 to $3500. Consequently, improving cacao beans as raw materials for fine-flavored cocoa is necessary (Tscharntke et al., 2023).

Fine-flavored cocoa is usually produced by *T.cacao* trees originating from Criollo and Trinitario (Dillon et al., 2023). The Criollo variety stands out for its high flavor quality, but is also accompanied by limitations such as low vigor, low productivity, and susceptibility to pests and diseases (Kongor et al., 2016) (Nguyen et al., 2021). Consequently, Criollo is classified as a rare variety nearing extinction (Lachenaud & Motamayor, 2017), especially in Indonesia, where the original Criollo variety no longer exists. Trinitario, a hybrid resulting from the crossbreeding of the Criollo and Forastero varieties, has high vigor. However, flavor quality and resistance to pests and diseases vary (Colonges et al., 2022) (Kongor et al., 2016). These challenges can be addressed through crossbreeding and selection in plant breeding programs to improve the flavor quality and resistance (Bekele & Phillips-Mora, 2019) (DuVal et al., 2017).

The Indonesian Coffee and Cocoa Research Institute (ICCRI) conducted various crossing combinations to enhance resistance against pests and diseases, flavor quality, and yield. This breeding project resulted in several hybrids, which were subsequently evaluated for their resistance levels and yield. However, the flavor quality of these parent-hybrid combinations has yet to be comprehensively assessed or

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characterized.

The characterization of cocoa breeding materials is essential to provide a reference for the further utilization of these parent-hybrid combinations as genetic resources (Pereira et al., 2017). Additionally, it plays a crucial role in making decisions for further selection or research on the plant breeding of fine-flavored cocoa (Santander Muñoz et al., 2020). Consequently, characterization contributes to the acceleration of the cacao breeding process for fine-flavored cocoa (Dorice et al., 2020).

Currently, fine-flavored cocoa can be distinguished based on its bean appearance or fresh bean color (Kongor et al., 2016). (Oliva-Cruz et al. (2021) emphasized that the fresh color of cacao beans is a crucial characteristic for discriminating between genotypes. Specifically, cacao varieties from the Criollo and Trinitario groups, characterized by up to 80 % white beans, were categorized as fine-flavored cocoa. In contrast, the Forastero and Trinitario varieties with up to 80 % purple beans are classified as bulk cacao (Devy et al., 2019). However, the fresh bean color of hybrids can vary depending on the parentage, because fresh bean color is inherited through additive or incomplete dominance (Lachenaud & Motamayor, 2017).

Another method for distinguishing between fine flavors and bulk cocoa is sensory evaluation (Escobar et al., 2021). According to this approach, fine-flavored cocoa is characterized by outstanding and highly complex notes (Santander Muñoz et al., 2021). In contrast, bulk cocoa is characterized by a basic or simple flavor without aromatic notes (Herrera-Rocha et al., 2024). However, this sensory analysis has limitations, as there is no universal standard of quality owing to the complexity of the attributes and the lack of expert or certified panelists (Krähmer et al., 2015) (Smulders et al., n.d.). This method is impractical when dealing with a large number of samples because there is a limit to the number of samples that can be evaluated by panelists (Fang et al., 2014). Therefore, it is necessary to employ a robust method to complement the existing approach for characterizing fine-flavored cocoa.

Metabolomics is a powerful and robust method for characterizing plants (Grissa et al., 2016), revealing both volatile and non-volatile compounds that determine the flavor quality of cocoa (Herrera-Rocha et al., 2021). The analytical methods employed in metabolomics, such as gas chromatography–mass spectrometry (GC–MS), have led to a wide range of compound detection in cocoa beans and are appropriate for examining metabolite profiles in cacao (Michel et al., 2021). This method can profile different metabolite levels between hybrids and their parents (Le et al., 2023) and provide a high level of precision in identifying and characterizing specific traits associated with the complexity of plant phenotypic diversity, such as cocoa flavor quality (Colantonio et al., 2022).

Previous studies have the metabolomic approaches of Criollo and Forastero. Some of these studies have focused on dynamic postharvest processes (Castro-Alayo et al., 2019) (Moreno-Rojas et al., 2023), while others have characterized unfermented beans (Qin et al., 2017). Most of these studies have revealed the presence of volatile components. However, the outstanding taste of fine-flavored cacao is correlated with non-volatile compounds (Velásquez-Reyes et al., 2023). Additionally, both volatile and non-volatile organic compounds influence the distinct aromas and flavors of cocoa (Velásquez-Reyes et al., 2023) (Castro-Alayo et al., 2019). Only a few studies have specifically highlighted the use of nonvolatile compounds to characterize fine-flavored cocoa. Hence, the present study is the first to report a comprehensive characterization of fine-flavored cocoa resulting from several cross combinations in a plant breeding program, focusing on non-volatile components. The primary objective of this study was to characterize fine-flavor cocoa in parent-hybrid combinations using a metabolomic approach and bean appearance and to correlate the important metabolites responsible for the fine flavor of cocoa with their bean appearance. This information is necessary to determine which of the best crossing combinations could produce hybrids with high flavor quality. The selection of appropriate parent-hybrid combinations for cacao breeding relies on the presence of characterization and evaluation data (Bekele & Phillips-Mora, 2019). Therefore, this characterization information will be valuable for further improvements and plant breeding research on fine-flavored cocoa.

2. Material and methods

2.1. Plant material

Twelve clones of *Theobroma cacao* obtained from the Indonesian Coffee and Cocoa Research Institute (ICCRI) were used. The cacao clones used in this study were planted in Jember, East Java, Indonesia, at an altitude of 45 m above sea level, with an average rainfall of 224 mm, an average temperature of 32 °C, and under the same cultivation management. All cacao trees used in this study were mature (4 years old) and of similar height (2–3 m). The genetic backgrounds of these samples included the Trinitario variety, specifically DR2, TSH 858, SULAWESI 01, SCA 06 (parents), and PNT 12 (non-parent, selected as the representative for white bean color). The Forastero variety comprised KEE 02 (Parent), KW 516 (Non-parent chosen as the representative for dark purple bean color), and hybrids resulting from crosses between Trinitario, namely ICCRI 03 (DR 2 × SCA 06), ICCRI 09 (TSH 858 × SULAWESI 01), KW 733 (TSH 858 × SULAWESI 01), KW 746 (TSH 858 × SULAWESI 01), and Trinitario crossed with Forastero, KW 742 (SULAWESI 01 × KEE 02). The descriptions of the samples are presented in Table 1. The sensory status of this sample was used as a benchmark to characterize the flavor quality by metabolomic analysis. These sensory data were obtained in 2022 using different harvesting batches from this study. Details of the sensory results are shown in Fig. S1.

2.2. Sample collection

Cacao pods were manually pollinated to expedite fruit development and maintain fruit uniformity. In October 2023, approximately six months after pollination, healthy and ripe cacao pods were harvested manually. The collected cacao pods were opened, and the pulp and bean exocarp were removed to observe the bean color. The remaining samples were collected and pooled for fermentation.

2.3. Bean color measurement

Three healthy ripe pods per clone were collected from the different cacao trees and used as replicates. Subsequently, three beans per pod were selected to measure the fresh bean color. The samples were opened, and the pulp was manually removed. After removing the pulp and bean exocarp, the fresh bean color of each clone was measured using the L*a*b* coordinate system, where L signifies lightness, a represents

Table 1
Sample information.

Clones	Genetic background	Status based on sensory analysis
DR 2	Trinitario	Fine flavor cacao
PNT 16	Trinitario	
SULAWESI 01	Trinitario	Bulk cacao
TSH 858	Trinitario	Fine favor cacao
SCA 06	Trinitario	
KEE 02	Forastero	Bulk cacao
KW 516	Forastero	Bulk cacao
ICCRI 03	Hybrid (DR 2 x SCA 06)	
ICCRI 09	Hybrid (TSH 858 x SULAWESI 01)	
KW 733	Hybrid (TSH 858 x SULAWESI 01)	
KW 746	Hybrid (TSH 858 x SULAWESI 01)	
KW 742	Hybrid (SULAWESI 01 X KEE 02)	

the green-to-red spectrum, and b indicates the blue-to-yellow spectrum. This measurement was performed using a calibrated colorimeter (CM-2500d, Minolta Co., Ltd., Osaka, Japan) and a Munsell color chart. Measurements were conducted using the dominant bean color per pod for each clone.

2.4. Fermentation and drying process

Each cacao sample clone was opened and combined into a 5 kg bamboo wood basket. Small-scale fermentation was carried out at an environmental temperature of 27 °C and humidity of 80 % for 98 h. Following the ICCRI fermentation standard, the beans were manually turned off after 48 h to ensure homogeneous fermentation. The endpoint of the fermentation was determined using a cut test. After fermentation, the beans of each clone were spread on a round bamboo wood tray and naturally dried under sunlight for three days. Each day, the beans were manually mixed to achieve a moisture content of 7–8 %. After drying, all beans were cut and only fully fermented beans were selected for metabolomic analysis.

2.5. Sample extraction and derivatization procedures

Seven grams of beans cut from each clone were placed in a polycarbonate tube containing a stainless-steel ball. All tubes were cooled with liquid nitrogen and ground into a powder using a Multi Breads Shocker device from Yasui Kikai, Co. Approximately 5 mg of cacao powder was used for analysis. Sample extractions were conducted by adding 5 mg of cacao powder to 1000 μL of a mixed solvent (composed of methanol, water, and chloroform with a ratio of 5:2:2 *v*/v/v) containing 100 μg/mL of the internal standard (Ribitol) (Hanifah et al., 2022). Blank samples containing only the mixed solvent were also prepared in 2 mL tubes. All the samples and blank were then vortexed and incubated in a shaker at 1200 rpm, 37 °C for 30 min. Centrifugation was performed at 4 °C, 10,000 rpm for 3 min to separate the supernatant. 600 μL of the supernatants were placed into a 1.5 mL microtube, added with 300 μL ultrapure water, and vortexed to homogenize the liquid. 200 μL of supernatant was transferred into a new 1.5 mL microtube, and 200 μL of supernatant in each sample was pooled for Quality Control (QC). All samples, including the QC and blank, were sealed with hole caps and then centrifuged under vacuum conditions using a centrifugal concentrator (TAITEC, Saitama, Japan) for 2 h at room temperature to remove water. The samples were then kept at −30 °C before derivatization. Oximization and silylation were performed as derivatization procedures in this analysis based on a previous study (Hanifah et al., 2022).

2.6. Gas chromatography-mass spectrometry (GC–MS) analysis

A GC–MS-QP2010 Ultra system equipped with an InertCap of 5MS/NP (35 m × 0.18 mm, I.D. 0.18 μm (GL Science, Co., Ltd.) was employed for metabolomic analysis. Before analysis, the mass spectrometer underwent tuning and calibration checks. 1 μL of a derivatized sample was injected in a split mode at a ratio of 25:1 *v*/v, with an injection temperature of 280 °C in a randomized sequence. Hydrogen was used as the carrier gas in this analysis, with a linear velocity of 39.0 cm/s and a flow rate of 1.2 mL/min. The column temperature initiated at 80 °C for 4 min, then increased to 330 °C at a rate of 15 °C/min, and was kept steady for approximately 8 min. The interface and ion source temperatures were consistently maintained at 310 °C and 280 °C, respectively. Electron ionization (EI) was generated at 1.00 kV. The spectra were recorded within the mass range of *m/z* 85–500 were recorded. Prior to the first sample injection, a standard mixture of alkanes (C8–C40) was injected to determine the retention time (instrumental peak identification).

2.7. Data processing

The raw data obtained from the GC–MS analysis were converted to the Andi (AIA) file format using the GC/MS solution software (Shimadzu). The Andi file format was then transformed into an Analysis Base File (ABF) format using the ABF Converter program, which is available for download from the website. Subsequent processes, including peak alignment, peak filtering, and tentative annotation, were performed using MS-DIAL version 4.38 (developed by Riken, Kanagawa, Japan), based on the GL-Science DB spectral database (InertCap 5MS-NP, predicted Fiehn RI). This database was downloaded from the official MS-DIAL website. The annotation was verified by cross-referencing all peaks with data available in the NIST (National Institute of Standards and Technology) library (NIST/EPA/NIH EI-MS Library) within the GC/MS solution software packages. A metabolite similarity of >80 % was used in this analysis. Ribitol was used as an internal standard to normalize the relative intensities of annotated metabolites. Metabolites with a high RSD (Relative Standard Deviation) value above 30 % within the QC samples were excluded from the analysis.

2.8. Statistical analysis

Multivariate data analysis was employed to visualize the data through principal component analysis (PCA) with auto-scaling and no transformation. Variations in the dataset were analyzed using analysis of variance (ANOVA), followed by a post-hoc Tukey's honest significant difference (HSD) test and a volcano plot. Significant differences were determined at *P value* of ≤0.05. OPLS-R analysis (Orthogonal Projection to Latent Structure Regression) was also constructed to identify the correlation between metabolites corresponding to fine-flavor cocoa and fresh bean color. In the PCA and OPLS-R analysis, metabolites served as explanatory variables, while fresh bean color values (L*, a*, b*) were used as response variables for OPLS-R data analysis. From the OPLS-R analysis, VIP score (variable importance in projection) and coefficient values were obtained. A VIP value greater than one was considered to have an important contribution to the model. Model performance was evaluated by R^2 and Q^2 values, indicating the proportion of variance in the data elucidated and predicted by the model (Kim et al., 2023). This OPLS-R model was further validated using CV-ANOVA at *P value* of ≤0.05. Multivariate data analysis and OPLS-R were analyzed using SIMCA-P software ver. 13 developed by Umetrics, Umea, Sweden, while ANOVA was conducted using R software (ver. 2023.12.0 + 369).

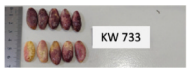
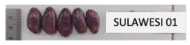
3. Result and discussion

3.1. Bean appearance/fresh bean color of cacao

Fresh bean color is one of the phenotypic criteria used to distinguish between Criollo and Forastero varieties. Criollo, a fine-flavored cocoa, is characterized by white bean cotyledons, whereas Forastero is a bulk cocoa with purple beans (Lachenaud & Motamayor, 2017). Previous studies have primarily described the fresh bean and fruit colors of the Criollo and Forastero varieties (Oliva-Cruz et al., 2021) (Lachenaud & Motamayor, 2017). This report is the first to highlight fresh bean color in parent-hybrid combinations of cacao clones. To provide a more comprehensive description of fine-flavored cocoa in both parents and hybrids, this study assessed fresh bean color based on L*a*b* values and quantified the percentage of fresh bean color, as presented in Table 2 and Fig. S2.

Trinitario varieties, namely DR2 as a fine-flavored cocoa based on sensory analysis (Fig. S1) and PNT 16, demonstrated a dominance of white beans per pod, exceeding 88 % (Fig. S2). These clones also exhibited a more intense lighter color than the other clones. In contrast, beans from the Forastero varieties KEE 02 and KW 516 (Bulk cocoa) were dark-purple beans, greater than 80 % per pod. A previous study noted that white bean color is typically attributed to the rare Criollo

Table 2
Quantitative data and visual appearance of fresh bean color.

No.	Clones	L*	a*	b*	Color	Color code**	Visual appearance
1	DR 2	73.28 a	5.13 de	25.75 a	White	75Y8/2	
2	PNT 16	79.29 a	3.05 e	22.33 a	White	75Y8/2	
3	KW 733 (TSH 858 x SUL 01)	51.80 b	17.05 a	2.95 b	Light purple	5RP66	
4	KW 746 (TSH858 x SUL 01)	40.31 bc	9.69 bcd	4.87 b	Light purple	5RP66	
5	TSH 858	43.80 bc	13.26 ab	2.91 b	Purple	5RP44	
6	ICCRI 03 (DR 2 x SCA 06)	36.04 c	10.48 bcd	3.35 b	Purple	5RP44	
7	KW 742 (SUL 01 x KEE 02)	35.18 c	11.80 abc	2.63 b	Dark purple	5RP3/2	
8	SCA 6	32.26 c	8.76 bcde	1.10 b	Dark purple	5RP3/2	
9	Sulawesi 1	29.89 c	7.01 cde	0.88 b	Dark purple	5RP3/2	
10	ICCRI 09 (TSH 858 x SUL 01)	29.92 c	5.46 de	1.32 b	Dark purple	5RP3/2	
11	KEE 02	31.48 c	7.45 bcde	1.07 b	Dark purple	5RP3/2	
12	KW 516	33.48 c	8.33 bcde	2.26 b	Dark purple	5RP3/2	

Remarks: Different letters in the same column indicate statistically significant differences (Tukey's HSD, p-value <0.05). L*: Lightness; a*: Green to red; b*: Blue to yellow. **) Codes are based on Munsell color chart.

variety and some Trinitario varieties, which are characterized by aromatic notes such as a mild nutty flavor. In contrast, Forastero variety has a purple color, resulting in a stronger and more astringent flavor (Lachenaud & Motamayor, 2017) (Collin et al., 2023). Furthermore, the Djati Roenggo (DR2) clone is widely regarded as having a Criollo trait, as noted by Susilo et al. (2011). This clone has white beans due to the absence of a gene responsible for the synthesis of anthocyanins. Prior studies have qualitatively described the color characteristics of Criollo and Forastero varieties (Lachenaud & Motamayor, 2017) (Collin et al., 2023) (Kongor et al., 2016). However, this study is the first to quantitatively analyze the color of Fine flavor cacao (Trinitario), bulk cacao (Forastero), and their hybrid combinations.

SULAWESI 01, SCA 06, and TSH 858 clones from the Trinitario variety exhibited significantly lower lightness. SULAWESI 01 and SCA 06 had dark purple beans per pod, with percentages of 100 % and 79.89 %, respectively, whereas TSH 858 exhibited a purple bean color exceeding 79 % (Fig. S2). These results indicate that these three clones resembled the Forastero type based on their fresh bean color. However, considering the sensory results (Fig. S1), TSH 858 was noted to have more aromatic attributes, whereas SULAWESI 01 exhibited a more astringent flavor. This suggests that fresh bean color alone may not be the main criterion for characterizing fine-flavored cocoa (Lachenaud & Motamayor, 2017).

This study revealed that the progenies/hybrids exhibited varying percentages of fresh bean color per pod (Fig. S2) and had a significantly lower light color than DR 2 and PNT 16 (Table 2). ICCRI 03, a hybrid derived from a cross between DR 2 (Trinitario white bean) and SCA 06 (Trinitario dark purple), exhibited dark purple (16.67 %), purple (75 %), and light purple (8.33 %) beans per pod. A previous study reported that the inheritance of fresh bean color in cacao follows additive or incomplete dominance (Lachenaud & Motamayor, 2017). Traditional methods of reproduction, such as crossbreeding, introduce considerable variability by combining genes from both parents (Oliva-Cruz et al., 2021). Similar results were observed for the hybrids KW 733, KW 746, and ICCRI 09, resulting from crosses between TSH 858 (Trinitario light purple bean) and SULAWESI 01 (Trinitario dark purple bean). Furthermore, the cross between SULAWESI 01 (Trinitario, dark purple bean) and KEE 02 (Forastero, dark purple bean) produced the hybrid KW 742, the fresh bean color of this hybrid was dominated by dark purple bean. *Theobroma cacao* is a perennial crop with high heterozygosity owing to its cross-pollinated crops and diploidy (2n = 20) (Bekele & Phillips-Mora, 2019). Consequently, crosses between Trinitario plants resulted in hybrids with high heterozygosity. The crossing of two heterozygous plants produces a genetically diverse and non-uniform population (Mixão et al., 2023). According to these results, categorizing these hybrids as fine-flavored cacao based on their fresh bean color would be inadequate. Therefore, this study also characterized all the cacao clones based on metabolomic data.

3.2. Metabolite profiles of all samples

The results of the metabolome profiling of all cacao clones are presented in Fig. 1, with the aim of identifying the overall characteristics of all cacao samples, including parent-hybrid combinations and clones, as representative samples: PNT 16 (Trinitario, white bean) and KW 516 (Forastero, dark purple bean). The PCA score plot of all the cacao samples shown in Fig. 1A strongly indicates that the cacao clones were categorized according to their variety and bean appearance. Trinitario varieties with dark purple beans, namely SULAWESI 01 and SCA 06, were distinctly separated from Trinitario white beans (DR 2 and PNT 16) along PC1, which explained 22.4 % variance. Forastero varieties (KEE 02 and KW 516) were clustered on the negative side of PC1. Interestingly, the Trinitario white beans were clustered in the center between the Forastero and Trinitario dark purple beans. Another notable observation in the PCA score plot is that the hybrids could not be classified according to their bean appearance.

This study performed separate Principal Component Analysis (PCA) for the DR 2 (Trinitario white beans) and Forastero varieties (KEE 02 and KW 516), as illustrated in Fig. 1C and D. This analysis aimed to distinguish the characteristics of fine flavor and bulk cocoa, which were used as a basis for characterizing parent-hybrid combinations. Based on the sensory results obtained in 2022, shown in Fig. S1, clone DR 2 was classified as a fine-flavor cocoa owing to its higher aromatic attributes, including floral, nutty, and fruity notes. In contrast, the Forastero varieties (KEE 02 and KW 516) were categorized as bulk cocoa, exhibiting pronounced bitterness and an astringent flavor. The PCA score plot in Fig. 1C distinctly reveals the separation between fine-flavored cocoa (Trinitario white beans) and bulk cocoa (Forastero dark purple beans) along PC1, explaining 41 % of the variance. This metabolomic result aligns with the sensory analysis, providing a clear distinction between fine-flavor and bulk cocoa.

In the PCA loading plot presented in Fig. 1D and Table S2, the positive side of PC1 contained higher concentrations of caffeine and organic acids, including malic, fumaric, citric, lactic, and tartaric acids. This finding suggests that these compounds are more abundant in fine-flavored cocoa. This result was also supported by volcano plot analysis (Fig. 1E), which showed that 26 metabolites had notable differences, as determined by a *t-test* with significant *p-values* below 0.05 (Fig. 1E). Among these significant metabolites, five compounds, including caffeine and organic acids such as malic acid, fumaric acid, citric acid, and tartaric acid, were found to be twice as high in fine-flavored cocoa. This finding aligns with prior research reporting that Criollo, the finest cacao types are characterized by lower theobromine and higher caffeine contents (Álvarez et al., 2012). Caffeine plays a crucial role in the taste and aroma enhancement of cocoa beans (Luna et al., 2002) (Davrieux et al., n.d.). Caffeine is a member of the alkaloid group responsible for its characteristic bitter flavor. The amount of caffeine varies depending on the cocoa variety (Toker et al., 2020). The characteristic flavor of cocoa is determined by both volatile and non-volatile organic compounds (Castro-Alayo et al., 2019). The reaction of organic acids with ethanol contributes to the development of a fruity aroma (Qin et al., 2017). Organic acids are essential for balancing cocoa's flavor (Holm & Aston, 1993). Similar results have been reported in previous studies on Criollo types, which exhibited higher levels of malic, citric, lactic, and tartaric acids (Velásquez-Reyes et al., 2023). These acids are significantly associated with sour, fruity, and floral flavors in fruits (Kim et al., 2023). Lactic acid, which contributes to the rancid odor of cocoa, is also linked to browned fruit aroma and can be reduced through proper roasting processes (Sari et al., 2023) (Toker et al., 2020). Citric acid, which is known to have a lesser impact on sourness perception than tartaric and malic acids (Kim et al., 2023), has been associated with lower acidity levels (Sari et al., 2023).

In contrast, theobromine, quinic acid, and dopamine levels were found to be higher in the Forastero variety. This result aligns with those of previous studies, which reported that the Forastero variety tends to have higher theobromine and lower caffeine content (Luna et al., 2002) (Davrieux et al., n.d.) (Velásquez-Reyes et al., 2023). Theobromine contributes to the bitter taste of cocoa (Guzmán Penella et al., 2023) (Vázquez-Ovando, Chacón-Martínez, Betancur-Ancona, Escalona-Buendía and Salvador-Figueroa, 2015) Quinic acid, which is known to correlate with astringent flavor, was higher in the Forastero variety. This result is consistent with a previous sensory analysis indicating that the Forastero group and SULAWESI (bulk cocoa) were dominated by an astringent flavor. Additionally, this study identified higher levels of dopamine in Forastero and some Trinitario dark purple beans. Dopamine is a metabolite commonly found in cacao beans and its levels have been observed to increase during cocoa fermentation. It has been reported that dopamine is produced from complex polyphenol degradation and is associated with lower acidity levels (Herrera-Rocha et al., 2021).

3.2.1. Metabolite profiles of Parent-hybrid combinations

To gain a more comprehensive understanding of the characteristics of parent-hybrid combinations, this study conducted separate principal component analyses (PCA) for each crossing combination, as illustrated in Fig. 2. In the PCA score plot (Fig. 2A), three distinct clusters were found from a cross between DR 2 (Trinitario, fine flavor cocoa) as the female and SCA 06 (Trinitario) as the male, resulting in the hybrid ICCRI 03. Female and male parents were clearly distinguished based on PC1, which accounted for 50.7 % of the variance. A notable result from the PCA showed that the hybrid clustered together with its female parent along the negative side of PC1, suggesting that the metabolite profile of this hybrid was close to that of its female parent.

The PCA loading plot (Fig. 2B, Table S3), caffeine, a crucial metabolite contributing to the separation of fine flavors from bulk cocoa, was clustered on the negative side of PC1. This finding suggests that caffeine accumulated in both the female parent and the hybrid. Referring to the bar graph in Fig. 3, the female parent and hybrid exhibited significantly higher levels of caffeine than the male parent and Forastero varieties (KEE 02 and KW 516). Caffeine, a characteristic of the Criollo genotype, plays a crucial role in the enhancement of the taste and aroma of cocoa. Caffeine concentration can be influenced by the genetic origin of cacao (Velásquez-Reyes et al., 2023) (Davrieux et al., n.d.). Additionally, the relative intensities of malic, fumaric, and tartaric acids were significantly higher in the hybrid and female parents than in the Forastero group. A previous study has reported that organic acids are linked to the development of acidic, fruity, and flowery notes (Luna et al., 2002). This result suggests that this type of parent-hybrid combination has the potential to produce hybrids with higher metabolites responsible for fine-flavored cocoa. Furthermore, this finding highlights the fact that the flavor quality of cocoa can be influenced by its female parents. This result is similar with the previous study which reported that the flavor characteristics of cocoa beans are primarily influenced by the genetics of the female parent. The female parent plays a crucial role in determining flavor attributes, such as cocoa, acidity, floral notes, nuttiness, and other flavor elements (Sukha et al., 2017). The likelihood that the pollen donor influences the flavor quality of cacao beans arises from the fact that both male and female gametes play vital roles in determining the endosperm of the hybrid seed. Although the latest study focused on the effect of pollen donors through sensory analysis (Sukha et al., 2017), the present study is the first to characterize parent-hybrid combinations resulting from a plant breeding program using metabolomic analysis.

The other hybrids, ICCRI 09, KW 746, and KW 733, were a cross between TSH 858 (Trinitario light purple beans) as the female parent and SULAWESI 01 (Trinitario dark purple beans) as the male parent. Unfortunately, the beans of KW 746 were not sufficient for the fermentation process; thus, this hybrid was excluded from metabolomic analysis. In the PCA score plot of this crossing, depicted in Fig. 2C, four distinct clusters were observed. PC1, explaining 44.7 % of the variance, highlighted a clear separation between female and male parents. Interestingly, the ICCRI 09 hybrid grouped with its female parent on the

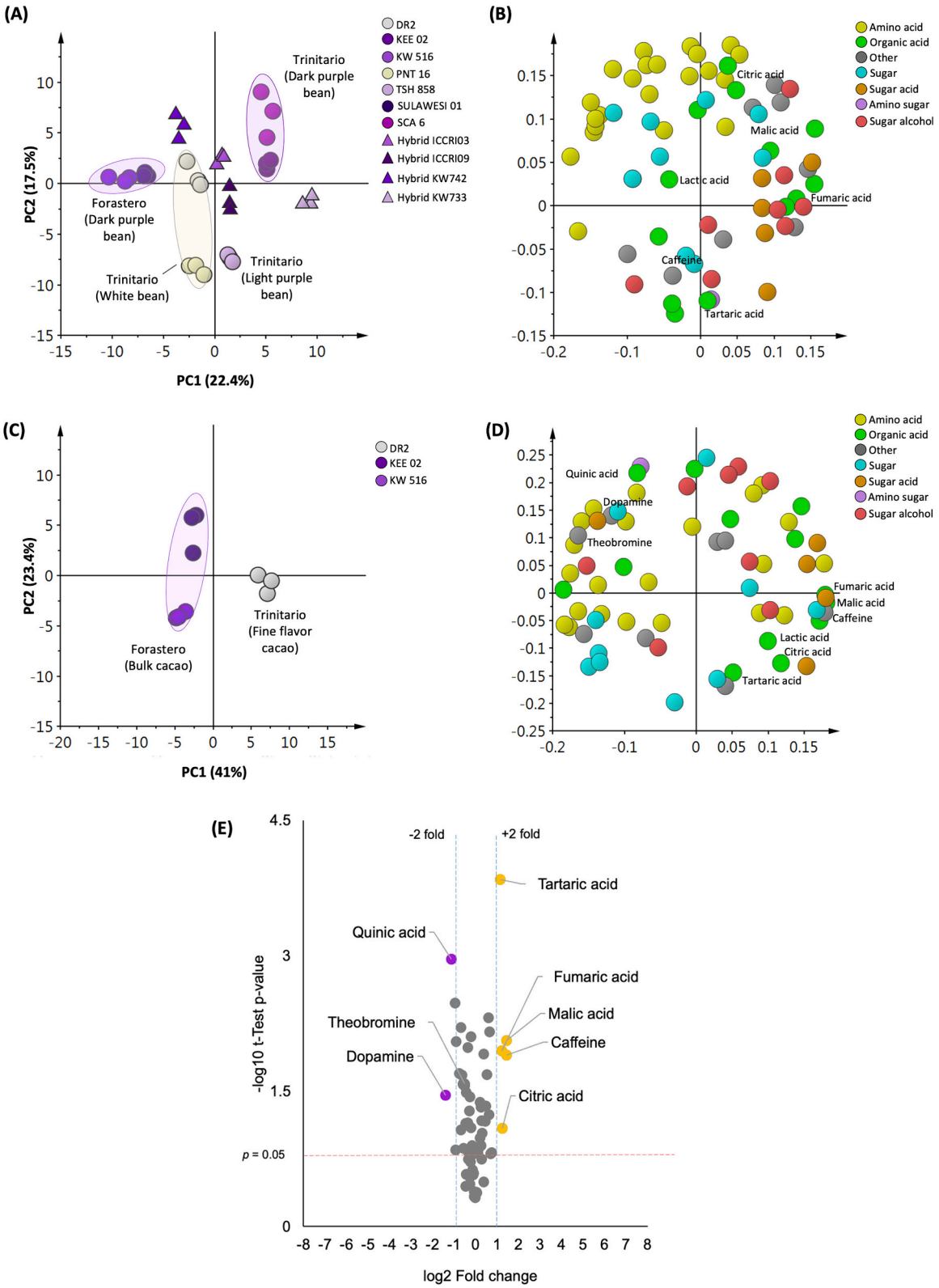


Fig. 1. The PCA score plot (A) and loading plot (B) were generated from the GC–MS-based metabolomic analysis of all samples, with auto-scaling and no transformation, using $n = 3$ replicates. The score plot (C) and loading score plot (D) illustrate the PCA comparison between fine-flavor and bulk cacao. Plot (E) is a volcano plot of fine-flavor and bulk cacao. In the score plot, circles represent cacao clones from Forastero, Trinitario with white beans, Trinitario with dark purple beans, and Trinitario with light purple beans. Triangles indicate hybrids (Trinitario × Trinitario and Trinitario × Forastero crosses). Dots in the loading plot denote metabolites associated with the observed separation, with different colors representing various metabolite classes. Highlighted metabolite names indicate influential compounds that contributed to sample clustering. In the volcano plot, yellow dots represent significantly increased metabolites (p-value <0.05) with at least a 2-fold change, while purple dots indicate significantly decreased metabolites (p-value <0.05) with at least a 2-fold change. Grey dots represent non-significant metabolites (p-value >0.05) with less than a 2-fold change. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

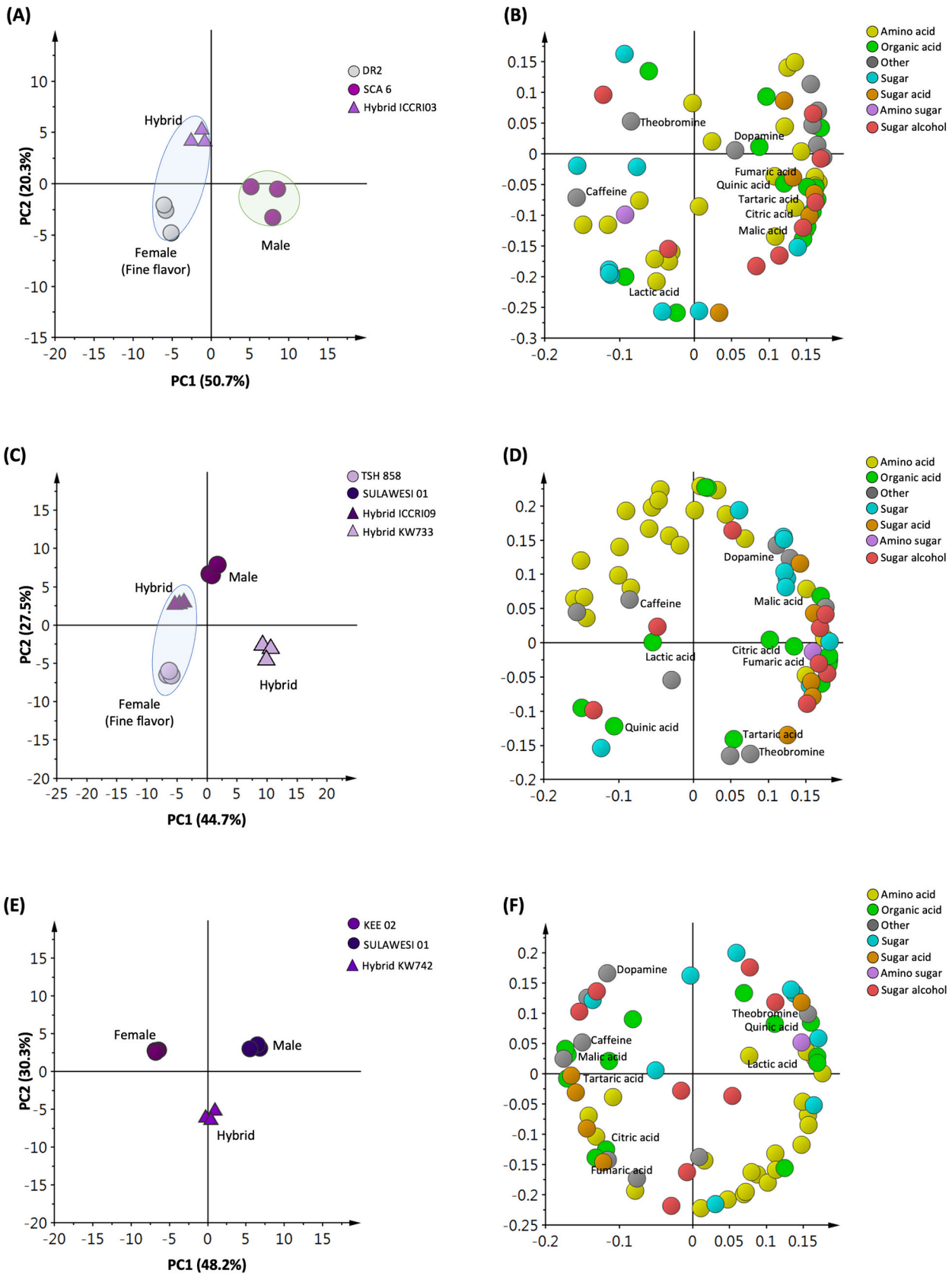


Fig. 2. The PCA score plots (A, C, E) and loading plots (B, D, F) were generated from GC–MS-based metabolomic analysis of the parent-hybrid combinations: (DR 2 × SCA 06), (TSH 858 × SULAWESI 01), and (SULAWESI 01 × KEE 02). In the score plots, circles represent the parent cacao clones, and triangles represent the hybrids. Dots in the loading plots indicate metabolites linked to the separation. The names of the highlighted metabolites are the influential compounds that clustered the samples.

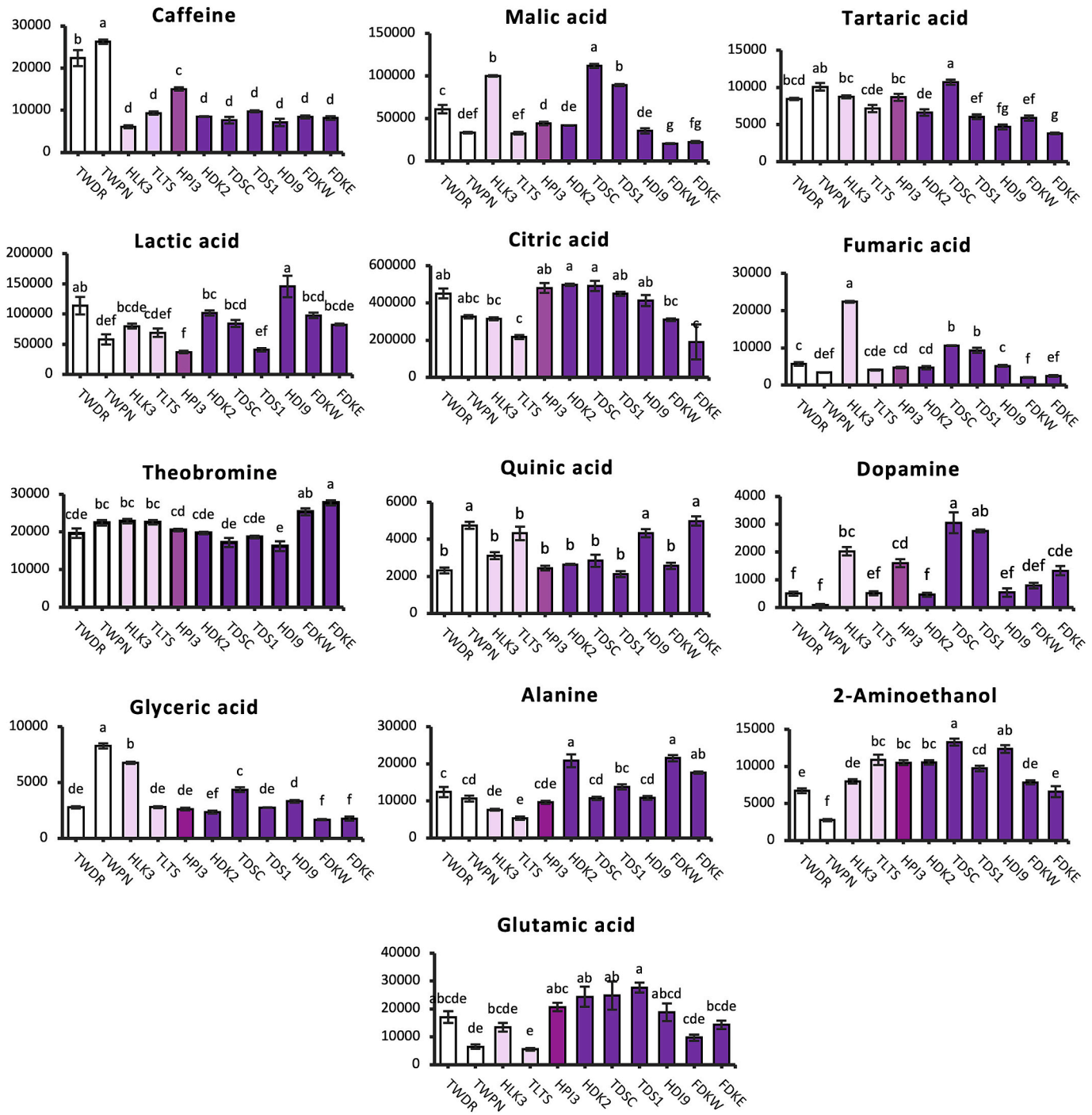


Fig. 3. The bar graphs show the relative intensity of the top higher VIP metabolites highlighted in the loading plot. Some of these graphs illustrate the relative intensity of the top three VIP metabolites listed in Tables S8-S12. The vertical axis represents the relative intensity, while the horizontal axis indicates the cacao clones. Sample codes are as follows:1st letter: variety (F for Forastero, T for Trinitario, H for Hybrid), 2nd letters: fresh bean color (W for White, L for Light purple, P for Purple, D for Dark purple). 3rd and 4th letters: clone (DR for DR2, PN for PNT16, TS for TSH, K3 for KW733, I3 for ICCRI03, K2 for KW742, SC for SCA6, S1 for SULAWESI1, I9 for ICCRI09, KW for KW516, KE for KEE02). Bars labeled with the same letters do not show a statistically significant difference according to Tukey's HSD test, with a p-value <0.05. The colors in the bar graphs represent the fresh bean color of each sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

negative side of PC1, suggesting that the metabolite profile of this hybrid is similar to that of its female parent as a fine-flavored cocoa. Another hybrid (KW 733) clustered with its male parent on the positive side of PC1. The PCA loading plot, illustrated in Fig. 2D and Table S4, revealed that caffeine, lactic acid, and amino acids accumulated on the negative side of PC1, indicating higher levels in the female parent and ICCRI 09 hybrid. The levels of malic acid, fumaric acid, citric acid, and lactic acid in the ICCRI 09 hybrid were not significantly different from those in the female parent and DR 2 (finest cocoa), indicating that the flavor characteristics of the hybrid ICCRI 09 were similar to those of the females. Based on the levels of metabolites corresponding to fine-flavor cocoa, it seems to indicate that this hybrid exhibited superior flavor characteristics compared to its sibling hybrid KW 733 but not as strong as those of ICCRI 03 and DR 2. Although hybrid KW 733 shares the same parent as

ICCRI 09, the flavor characteristics of this hybrid were significantly different from those of ICCRI 09 because of the heterozygosity of the parent. A previous study emphasized that the flavor quality of cocoa beans is influenced by the genetic composition of their parents (Seguine, 2009). The inherent heterozygosity of Trinitario contributes to the substantial variability observed in the metabolome profiles of its hybrids (Davrieux et al., n.d.).

In another parent-hybrid combination derived from SULAWESI 01 (female) and KEE 02 (male) (Fig. 2E), three distinct groups were observed. The female parent was distinguished from the male parent and clustered on the negative side of PC1, explaining 48.2 % of the variance. Hybrid KW 742 clustered separately from both males and females along the negative side of PC2, explaining 30.3 % of the variance. This indicates that the hybrid KW 742 exhibited intermediate flavor characteristics compared to its parents. The highlighted metabolites (Fig. 3) indicated that the caffeine levels in the hybrid and its parent were not significantly different from those in the Forastero variety. The relative intensities of organic acids such as citric acid, tartaric acid, and quinic acid were not significantly different between this hybrid and its females, suggesting that the flavor characteristics are similar to those of its female parent, bulk cocoa. Notably, this parent hybrid combination was derived from Trinitario and Forastero varieties. As a result, this crossbreeding may produce a hybrid with a lower flavor quality than that of ICCRI 03 and ICCRI 09. A previous study reported that Trinitario exhibits intermediate flavor quality (Luna et al., 2002) (Davrieux et al., n.d.) owing to hybridization between the Criollo and Forastero varieties. Therefore, its progenies may have reduced flavor characteristics.

3.3. Correlation between fresh bean color and metabolite profiles

Traditionally, cocoa flavor is identified by its cotyledon appearance or fresh bean color. The primary phenotypic characteristic of the finest cocoa (Criollo varieties) is a white to ivory or very light purple color resulting from the absence of anthocyanins (Lachenaud & Motamayor, 2017). Bulk cocoa from Forastero varieties has a dark purple or purple bean color due to the presence of anthocyanins. This characteristic is commonly used to differentiate between fine flavor and bulk cocoa (Kongor et al., 2016) although bean color may disappear after the fermentation process, it could potentially impact the flavor quality of cacao (Kim et al., 2023). To comprehensively understand whether this criterion correlates with the metabolites corresponding to fine-flavored cocoa, this study attempted to correlate fresh bean color with the metabolome profiles of cacao clones. Previous research has primarily focused on volatile components to distinguish between Criollo and Forastero types without quantitatively examining fresh bean color or correlating metabolite profiles with fresh bean color. For instance, Becerra et al. (2023) only correlated bean color after controlled processing with phenolic components, without addressing genotype differences.

The correlation in this study was constructed using OPLS-R, with fresh bean color as the response variable (y) and metabolome profile as the explanatory variable (x). Based on the results shown in Fig. 4, a good model was obtained from this correlation analysis, with R^2 greater than 0.6 and a Q^2 value bigger than 0.5 (34). Subsequent validation through CV-ANOVA confirmed the significance of the model with a *P-value* less

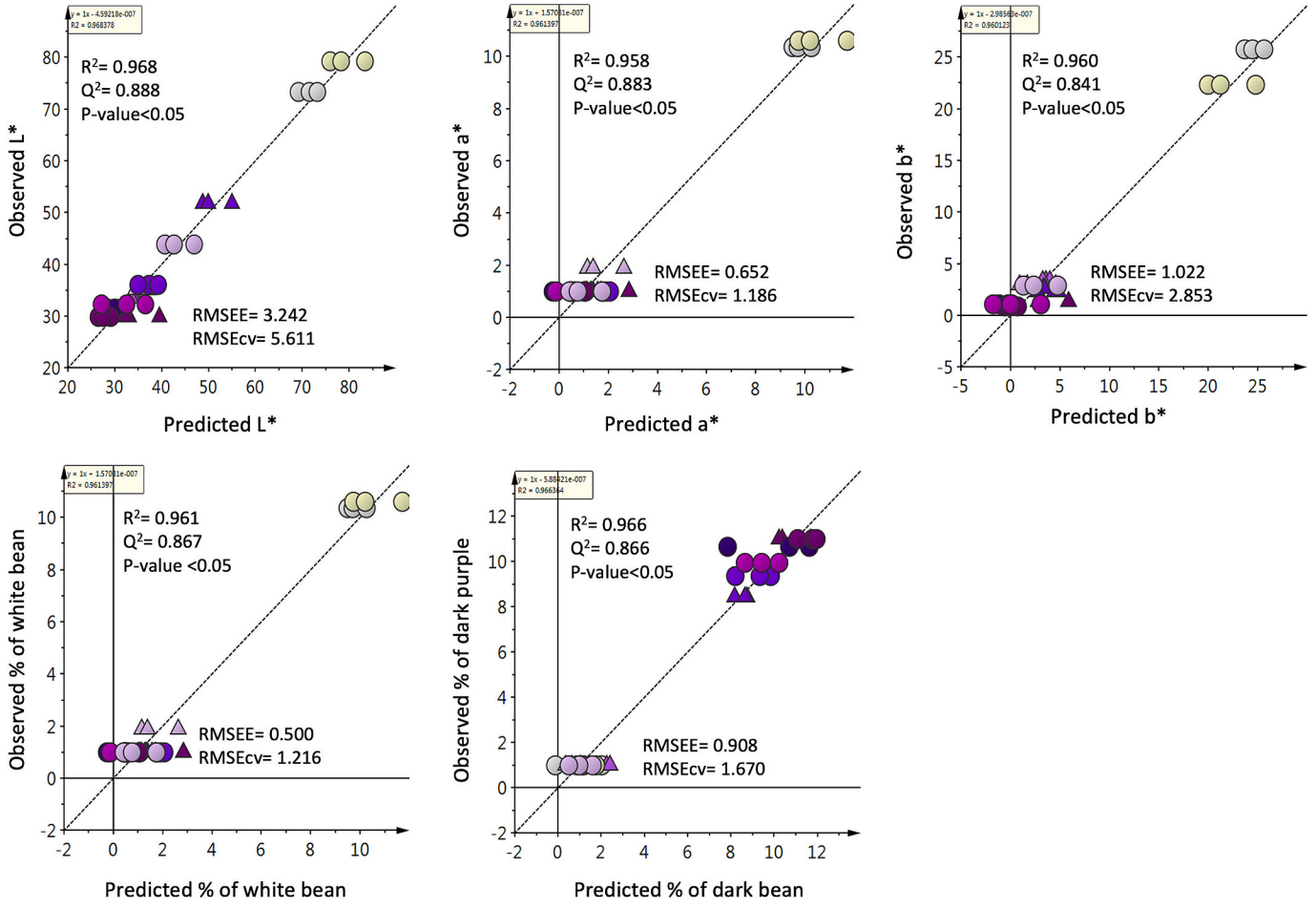


Fig. 4. Correlation models of L*a*b* value, the percentage of white bean per pod, and the percentage of dark purple bean per pod obtained from Orthogonal Projection to Latent Square Regression (OPLS-R) analysis show the correlation between metabolites and fresh bean color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

than 0.05. Detailed information regarding the correlation parameters is presented in Table S7. There was no notable trend observed in the a* value (red and green) and the percentage of the lightness bean color per pod. Therefore, the correlation analysis was limited to discussing only the lightness value, b* value (yellow to blue), the percentage of white beans per pod, and the percentage of dark purple beans per pod.

The VIP score is a key variable in this analysis and was used to identify important metabolites that are highly correlated with the response variable. Top ten VIP metabolites obtained from this analysis (Table S8-S12): caffeine, tartaric acid, and glyceric acid, exhibited strong positive correlations with the L* value (lightness), b* (blue to yellow), and percentage of white beans per pod. The relative intensities of the metabolites are shown in Fig. 3. It has been reported that the caffeine content is significantly higher in the white bean variety (Criollo type) than in the purple bean variety (Forastero variety). Previous research has revealed that caffeine content correlates with the bitter attributes of cocoa beans (Brunetto et al., 2007).

Additionally, tartaric acid and glyceric acid contribute to sourness, flowery, and fruity notes (Kim et al., 2023), with glyceric acid strongly contributing to the acidic taste and sweet flavor in coffee sample (Hanzawa et al., 2021). Another finding indicated that the percentage of dark purple beans was strongly correlated with alanine, 2-aminoethanol, and glutamic acid (Table S12). Previous studies have reported that a high flavor potential can be achieved with low acidification and reduced content of amino acids and peptides (Santander Muñoz et al., 2020). Furthermore, Forastero (purple bean) has a higher protein content than Criollo (Aprotosoaie et al., 2016). Sugars play a critical role in the development of flavor characteristics through Maillard reactions with amino acids (Aprotosoaie et al., 2016).

These results suggest that non-volatile compounds such as caffeine and organic acids, including malic acid, fumaric acid, citric acid, lactic acid, and tartaric acid, which were identified to correspond to the characteristics of fine-flavor cacao, contribute to the development of the taste and aroma of fine-flavor cocoa. While earlier studies have primarily focused on volatile components when comparing Criollo and Forastero (Qin et al., 2017; Velásquez-Reyes et al., 2023), this study underscores the importance of non-volatile compounds in differentiating the fine-flavor cacao from bulk cacao, as well as their crossing combinations. Additionally, the aroma attributes of fine-flavor cacao are also influenced by volatile compounds (Moreno-Rojas et al., 2023), suggesting that it is necessary to profile volatile compounds in these parent-hybrid combinations. A previous study (Herrera-Rocha et al., 2021) emphasized that the flavor characteristics of cocoa are determined by both volatile and non-volatile organic compounds. Therefore, future research should focus on detailed studies of the volatile profiles of these parent-hybrid combinations to fully understand their flavor attributes.

4. Conclusion

This study comprehensively characterized fine-flavor cocoa in parent-hybrid combinations resulting from a plant breeding program, using a widely targeted Gas Chromatography-Mass Spectrometry (GC–MS) metabolomics-based approach and bean phenotype analysis. The non-volatile profiles of fine-flavored cacao was characterized by high levels of caffeine and organic acids such as malic acid, fumaric acid, citric acid, lactic acid, and tartaric acid. Each type of crossbreed exhibited unique flavor profiles, with the ICCRI03-hybrid identified as a promising candidate. The insights gained from this characterization will enable plant breeders to optimize breeding schemes for further fine flavor improvement or utilization of these crossing combinations as breeding materials. Additionally, this study provides valuable information and references for farm managers to maintain cacao plantations through rejuvenation using superior clones. The identification of important metabolites in each crossbreed will be useful for selection in future studies.

CRediT authorship contribution statement

Enik Nurlaili Afifah: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Indah Anita Sari:** Writing – review & editing, Resources, Methodology. **Agung Wahyu Susilo:** Writing – review & editing, Resources. **Abdul Malik:** Writing – review & editing, Resources. **Eiichiro Fukusaki:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Conceptualization. **Sastia Prama Putri:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that may have influenced the work reported in this study.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101832.

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