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Anthocyanin Composition of Wild Bananas in Thailand

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Anthocyanins were isolated from male bracts of 10 wild species of bananas (*Musa* spp. and *Ensete* spp.) distributed in Thailand. Six major anthocyanin pigments were identified by high performance liquid chromatography (HPLC), mass spectrometry (MS), and tandem mass spectrometry (MS/MS). They are delphinidin-3-rutinoside (*m*/*z* 611.2), cyanidin-3-rutinoside (*m*/*z* 595.8), petunidin-3-rutinoside (*m*/*z* 624.9), pelargonidin-3-rutinoside (*m*/*z* 579.4), peonidin-3-rutinoside (*m*/*z* 608.7), and malvidin-3-rutinoside (*m*/*z* 638.8). On the basis of the types of pigment present, the wild bananas can be divided into 5 groups. The first group comprises *M. itinerans*, *Musa* sp. one, *Musa* sp. two, and *M. acuminata* accessions, which contain almost or all anthocyanin pigments except for pelargonidin-3-rutinoside, including both nonmethylated and methylated anthocyanins. The second group, *M. acuminata* subsp. *truncata*, contains only malvidin-3-rutinoside while the third group, *M. coccinea*, contains cyanidin-3-rutinoside and pelargonidin-3-rutinoside. The forth group, *M. acuminata* yellow bract and *E. glaucum* do not appear to contain any anthocyanin pigment. The fifth group consists of *M. balbisiana*, *M. velutina*, *M. laterita*, and *E. superbum* which contain only nonmethylated anthocyanin, delphinidin-3-rutinoside, and cyanidin-3-rutinoside. Total anthocyanin content in the analyzed bracts ranged from 0−119.70 mg/100 g bract fresh weight. The differences in the type of anthocyanin and variation in the amounts present indicate that wild bananas show biochemical diversity, which may be useful for identifying specific groups of bananas or for clarifying the evolution of flavonoid metabolism in each banana group.

KEYWORDS: wild bananas; anthocyanin; male bract; *Musa*; *Ensete*

INTRODUCTION

Bananas are an important fruit crop of the world. They are produced for local consumption and trading mostly in tropical and subtropical areas (*1*). Most cultivated bananas are believed to have originated from two wild banana species: *Musa acuminata* Colla and *M. balbisisna* Colla (*2, 3*). Simmonds (*4, 5*) proposed that wild and cultivated bananas had their origins in Southeast Asia and were later introduced to secondary centers. In many Southeast Asian locations, male buds of some wild and cultivated bananas are used as vegetables (*5*), and bract color is a potential source of food colorants (*6*). In terms of banana classification (*7*), male bract color is an important character for classifying wild bananas into species and subspecies. However, using bract color description was difficult to compare and quantify. The type of pigments in the banana bract was preliminary analyzed by acid extraction and paper chromatography (*4, 8*). Simmonds suggested from this study that the colors of bracts were due to glycoconjugated anthocyanidin pigments. However, the information of sugar type and linkage position was unattainable due to acid hydrolysis. Recently, anthocyanin in many plants has been successfully identified by high performance liquid chromatography (HPLC) and mass spectrometry (MS) (*9*−*11*) including those from strawberry (*Fragaria* × *ananassa* Duch.) (*12*), grape (*Vitis vinifera*) (*13*), blueberry (*Vaccinnium corymbosum*) (*13*), black raspberry (*Rubus occidentalis*) (*13*), red raspberry (*Rubus idaeus*) (*13*), black current (*Ribes nigrum* L.) (*14*). For bananas, anthocyanin from bracts of 59 accessions which included *Musa acuminata*, *Musa balbisiana* species and *Musa* cultivars was extracted by acid hydrolysis and analyzed by TLC and HPLC for chemotaxonomic study (*15*). The assigned pigments were 3-rutinosyl of delphinidin, cyanidin, petunidin peonidin and malvidin. Further study in *Musa* × *paradisiaca* by HPLC-MS (*6*) was also reported the same 5 anthocyanin pigments in addition with minor amount of pelargonidin-3-rutinoside, cya-

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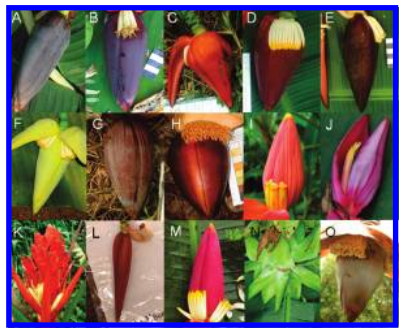

Figure 1. Male bract colors of wild bananas in Thailand. (**A**) *Musa acuminata* subsp. *siamea* 1; (**B**) *M. acuminata* subsp. *siamea* 2; (**C**) *M. acuminata* subsp. *malaccensis* 1; (**D**) *M. acuminata* subsp. *malaccensis* 2; (**E**) *M. acuminata* subsp. *truncata*; (**F**) *M. acuminata* yellow bract; (**G**) *M. balbisiana*; (**H**) *M. itinerans*; (**I**) *M. laterita*; (**J**) *M. velutina*; (**K**) *M. coccinea*; (**L**) *Musa* sp. one unknown; (**M**) *Musa* sp. two unknown; (**N**) *Ensete glaucum*; (**O**) *Ensete superbum*.

nidin-3-rhamnoside,-7-glucoside and unidentified acylated anthocyanin. However, the glycosylating group, type of anthocyanidin aglycone and metabolic relationships are still unknown in many *Musa* and *Ensete* species. In this paper, 15 samples of wild bananas species of various color types (**Figure 1**) were analyzed for their anthocyanin composition.

MATERIALS AND METHODS

Plant Materials. Wild banana specimens were collected from natural habitats in Thailand, characterized, identified and deposited in a private *ex situ* germplasm collection in Nakorn Pathom, Thailand. Voucher specimens were identified based on Simmonds (*4*) and Cheesman (*2*) and deposited at Suan Luang Rama IX Herbarium, Thailand. Details of the 15 accessions used, shown in **Table 1**, were based on Wongniam (*16*). Outermost nonopening bracts of male bud, which fully develop their colors and not yet start deterioration, were harvested from the collection on the same day as extraction.

Reagents and Standards. Standard keracyanin chloride (cyanidin-3-*O*-rutinoside chloride) was purchased from Fluka. All solvents for High Performance Liquid Chromatography and Mass Spectrometry were HPLC grade (Fisher).

Extractions. Anthocyanin pigments were extracted from fresh male bracts by grinding one gram of bract with 60% methanol containing 0.027% HCl (v/v). The slurry was then transferred into a 15 mL tube, kept overnight at 4 °C, and centrifuged at 4000 g for 20 min. The supernatant was collected and stored at −20 °C until analysis. All samples for HPLC analysis were filtered through 0.45 μm nylon membrane (Millex, Millipore) before injections. Determination of total anthocyanin was performed as described in Giusti and Wrolstad (*17, 18*) by pH differential method and total anthocyanin was calculated as equivalent grams to cyanidin-3-O-rutinoside.

High Performance Liquid Chromatography Analysis. Anthocyanins were analyzed by reversed-phase HPLC on a HPLC model 717 plus equipped with pump model 600 and photodiode array detector (PDA) model 2996 (Waters). A Nova-pak C-18 (3.9 × 300 mm, Waters) with protected guard was used. Solvent A was acetonitrile containing 0.1% trifluoroacetic acid (v/v) and solvent B was H_2O containing 0.1% trifluoroacetic acid (v/v). The program used a linear gradient from 15 to 95% solvent A for 45 min and returned to 15% solvent A for the next 15 min, with simultaneous detection at 280 and 530 nm. Injection volume was 20 μL. Anthocyanin peaks were then fractionated online with a flow rate 0.5 mL/min (1 min per fraction) using a fraction collector (Waters). Fractions were kept at −20 °C for mass spectrometric analysis.

Mass Spectrometry of Anthocyanin. Anthocyanin fractions were directly infused into a mass spectrometer (Esquire 3000 plus, Bruker) equipped with ESI-source. Anthocyanin molecular ion mass and their tandem mass spectra were detected in positive ion mode with quadrupole ion trap mass analyzer (resolution 0.1 *m*/*z*) and parameter was setup as follow: capillary 4000 V, Nebulizer 10 psi, Dry Gas 7.0 L/min, Dry temperature 250 °C, skimmer 40 V, Cap exit 121.0 V, Oct1 DC 9.0 V, Oct2 DC 1.10 V, Trap drive 40, Oct RF 100 Vpp, Lens1 −10 V, Lens2 −35 V.

RESULTS AND DISCUSSION

HPLC Chromatogram. The HPLC chromatogram of crude extract obtained from wild banana bract *M. acuminata* subsp. *siamea* 1 (**Figure 2A**) and *M. coccinea* (**Figure 2B**) revealed a total of six major anthocyanin peaks, corresponding to compounds shown in **Figure 3**. Other *Musa* and *Ensete* species exhibited profiles within 5 anthocyanin peak profiles as found in *M. acuminata* subsp. *siamea* 1, but with varying contents of each peak (**Table 3**). Each pigment elution time was similar in all samples. The elution order was from high to low pigment polarity, as follows: delphinidin-3-rutinoside to cyanidin-3-rutinoside, petunidin-3-rutinoside, pelargonidin-3-rutinoside, peonidin-3-rutinoside and malvidin-3-rutinoside, respectively.

Identification of Anthocyanins. Characterization of anthocyanins from wild banana bracts based on UV−vis spectral analysis (*18*−*20*) from HPLC and mass spectrometry data, is presented in **Table 2**. The UV−vis spectra of anthocyanin peaks 1−5 show maximum absorption in the visible region at 520−530 nm with percent ratio $A_{440}/A_{\text{vis-max}}$ of 24−44% indicating 3-glycosylation of anthocyanin (*21*), the percent ratios $A_{310}/A_{\text{vis-max}}$ of 8−32% for peaks 2−5 indicate that anthocyanins from wild bananas are simple anthocyanins, without acylation. The percent ratio $A_{310}/A_{\text{vis-max}}$ in peak 1 is 69%, which suggests a single acylation (*21, 22*), but the mass spectrometry data show no acylation of this anthocyanin. The λ_{max} obtained from our results are similar to those of previous studies in blackcurrant (*14*) and *Musa* × *paradisiaca* (*Musa* AAA group *3, 23, 24*) confirming that the glycosylation is at the position 3 of anthocyanidin core.

With regard to the mass spectrometry data, anthocyanin peaks 1−6 clearly show the molecular ions (*m*/*z*) for six major anthocyanins (**Table 2**) were as follows; 610.8, 594.7, 624.8, 579.3, 608.7, and 638.8, respectively. During MS/MS analysis, molecular ions produced fragment ions at 464.8, 448.6, 478.6, 433.3, 462.6, and 492.7, indicating that all molecular ions lost 146 *m*/*z*, corresponding to a rhamnosyl group and further loss of a glucosyl group (162 *m*/*z*) to yield the common anthocyanidin aglycone cations at 302.7 (delphinidin, Dp), 286.7 (cyanidin, Cy), 316.6 (petunidin, Pt), 271.3 (pelargonidin, Pg), 300.7 (peonidin, Pn), and 330.7 (malvidin, Mv), respectively. The fragmentation pattern of molecular ions matched with those of previous studies in black raspberry (*13, 25*) and blackcurrants (*14*). This confirmed that the rutinosyl group is attached to the anthocyanidin core as peonidin-3-rutinoside. Furthermore, the fragmentation pattern from cyanidin-3-rutinoside (peak 2) is identical to standard cyanidin-3-rutinoside chloride (data not shown).

Determination of Anthocyanin Content in Bracts of Wild Bananas from Thailand. The anthocyanin contents in the bracts, determined by HPLC peak area monitored at 530 nm, are shown in **Table 3**. Wild bananas in Thailand showed some differences in anthocyanin contents. Anthocyanin could not be detected in *M. acuminata* (yellow bract) and *E. glaucum* which possessed yellow bracts. Cyanidin-3-rutinoside was detected in all banana bracts, except in yellow bract and *M. acuminata* subsp. *truncata*. Only pelargonidin-3-rutinoside could be detected in *M. coccinea*. Five anthocyanin pigments were detected in *M. acuminata* subsp. *siamea* 1 and subsp. *siamea* 2, which their bract color did not differ greatly from each other. *M.*

Table 1. List of Bananas Used in This Paper[a]

scientific names	accession no.[b]	visual bract colors[c]	source provinces
M. acuminata subsp. *siamea* 1	96	BL	Loei
M. acuminata subsp. *siamea* 2	132	BL+PL tipYW	Nakhon Ratchasima
M. acuminata subsp. *malaccensis* 1	109	OR streak YW	Krabi
M. acuminata subsp. *malaccensis* 2	106	RP	Chumphon
M. acuminata subsp. *truncata*	206	BL	Yala
M. acuminata (yellow bract)	173	GY+YW	Nakhon Ratchasima
M. balbisiana	135	GR	Phetchaburi
M. itinerans	313	RP	Mae Hong Son
M. laterita	243	OR	Kanchanaburi
M. velutina	168	pale PL	Unknown, possibly introduced
M. coccinea	223	OR	Unknown, possibly introduced
Musa sp. one (unknown)[d]	314	RP	Mae Hong Son
Musa sp. two (unknown)[d]	246	PP	Kanchanaburi
E. glaucum	318	YW	Tak
E. superbum	319	RP	Tak

[a] Visual bract colors were defined using color chart from "Descriptor for Bananas" (*30*). Scientific names were based on Wongniam 2008 (*26*). [b] Wongniam 2008 (M.Sc. Thesis) . [c] Defined by Color chart from "Descriptor for Bananas (*30*)". BL= Blue, PL= Purple, YW= Yellow, OR= Orange red, RP= Red Purple, GY= Green Yellow, GR= Green, PP= Pink Purple. [d] Unknown species, on the confirmation.

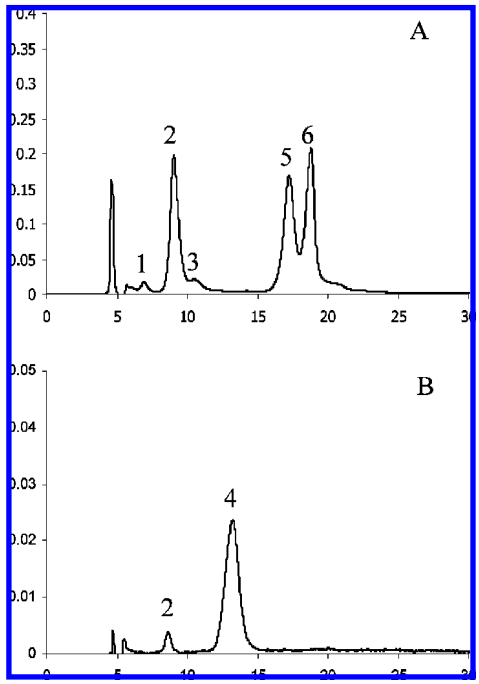

Figure 2. HPLC Chromatograms showing absorbance at 530 nm of anthocyanins obtained from *M. acuminata* subsp. *malaccensis* 1. (**A**) Peak 1, delphinidin-3-rutinoside; Peak 2, cyanidin-3-rutinoside; Peak 3, petunidin-3-rutinoside; Peak 5, peonidin-3-rutinoside; Peak 6, malvidin-3-rutinoside. and *M. coccinea*. (**B**) Peak 2, cyanidin-3-rutinoside; Peak 4, pelargonidin-3-rutinoside. (Note: The first initial peaks are unknown artifact.)

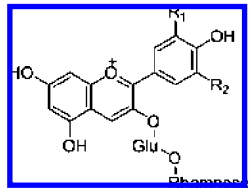

Figure 3. Chemical structure of anthocyanin pigments from bracts of wild bananas in Thailand. Delphinidin-3-rutinoside: R_1 = OH, R_2 = OH; Cyanidin-3-rutinoside: R_1 = OH, R_2 = H; Petunidin-3-rutinoside: R_1 = OCH_3, R_2 = OH; Pelargonidin-3-rutinoside: R_1 = H, R_2 = H; Peonidin: R_1 = OCH_3, R_2 = H; Malvidin-3-rutinoside: R_1 = OCH_3, R_2 = OCH_3.

itinerans had a different bract color and different proportion of anthocyanin pigments when compared to *Musa acuminata* subsp. *siamea* 1, 2. Delphinidin-3-rutinoside was found in *M. acuminata* subsp. *malaccensis* 2, but not found in *M. acuminata* subsp. *malaccensis* 1, even though they had a similar bract color. On the other hand, *M. acuminata* subsp. *malaccensis* 1 and *Musa* sp. one possessed the same anthocyanin pigments even though they showed different bract colors. In *M. acuminata* subsp. *truncata*, only malvidin-3-rutinoside was found and its bract color differed from that of *M. acuminata* subsp. *siamea* 1 and *siamea* 2. In *M. balbisiana, M. velutina*, and *E. superbum*, only two types of anthocyanin pigments are found, delphinidin-3-rutinoside and cyanidin-3-rutinoside. *M. balbisiana* contained more cyanidin-3-rutinoside than delphinidin-3-rutinoside, while *E. superbum* had more delphinidin-3-rutinoside than cyanidin-3-rutinoside, and *M. velutina* showed equal amounts of both pigments. This might help to distinguish banana species showing different contents of nonmethylated anthocyanin. *M. laterita* had orange bracts containing only cyanidin-3-rutinoside. *Musa* sp. two possessed 3 pigments: delphinidin-3-rutinoside, petunidin-3-rutinoside, and malvidin-3-rutinoside.

The range of each anthocyanin content in fresh tissue from 15 samples of wild banana samples was 0.00−66.70 mg/100 g of delphinidin-3-rutinoside, 0.00−37.52 mg/100 g of cyanidin-3-rutinoside, 0.00−11.91 mg/100 g of petunidin-3-rutinoside, 20.25 μmol/g of pelargonidin-3-rutinoside, 0.00−36.92 mg/100 g of peonidin-3-rutinoside and 0.00−70.27 mg/100 g of malvidin-3-rutinoside respectively. Total anthocyanin contents from each 15 samples ranged from 0.00 −119.70 mg/100 g.

According to the present study, anthocyanin pigment profiles can be roughly divided to 5 groups according to present pigments and possible metabolic defective steps (**Figure 4**). The **A** group, which composed of *M. acuminata* subsp. *siamea* 1 and 2, subsp. *malaccensis* 1 and 2, *M. itinerans*, *Musa* sp. one and *Musa* sp. two (all in Musa section but not sure for unknown species), contains 3 to 5 anthocyanin types which both methylated and nonmethylated anthocyanin. Though the *Musa* sp. one and the *M. acuminata* subsp. *malaccensis* 1 showed a comparable pairs of anthocyanin profiles, they possessed distinct morphology (*26*). The *Musa* sp. two and the *M. laterita* were related morphologically but their anthocyanin profiles were clearly different. The analyses, thus, indicated that these four accessions were distinct species. The **B** group, only *M. acuminata* subsp. *truncata* is in this group which presented only malvidin-3-rutinoside. It is possible to synthesize anthocyanin only via dihydromyricetin and might have so strong anthocyanin methylation that unable to detect neither delphinidin-3-rutinoside nor petunidin-3-rutinoside, which are partially methylated

Table 2. Chromatographic and Spectroscopic Characteristics of Anthocyanin Pigments from Wild Bananas

peak	retention time min.	$[M]^+$ *m/z*	fragments *m/z*	UV−vis peak nm	$A_{310}/A_{vis\ peak}$ (%)	$A_{440}/A_{vis\ peak}$ (%)	identity[a]
1	6.98	611.2	464.8, 302.9	279, 528	69	28	Dp-3-rutinoside
2	9.04	595.8	448.7, 286.7	275, 518	23	30	Cy-3-rutinoside
3	10.49	624.9	478.7, 316.7	272, 528	32	24	Pt-3-rutinoside
4	13.25	579.4	433.3, 271.2	282, 504	10	44	Pg-3-rutinoside
5	17.24	608.7	462.6, 300.7	279, 525	8	30	*Pn*3-rutinoside
6	18.88	638.8	492.7, 330.6	279, 532	17	26	Mv-3-rutinoside

[a] (Dp= delphinidin, Cy= cyanidin, Pt= petunidin, Pg= pelargonidin, Pn= peonidin, Mv= malvidin).

Table 3. Anthocyanin Contents from Bracts of Wild Bananas in Thailand

scientific names	anthocyanin content (mg/100 g bract)[a] peak 1[b]	2	3	4	5	6	total[c]
M. acuminata subsp. *siamea* 1	11.91	8.34	11.91	0.00	7.15	70.27	109.58
M. acuminata subsp. *siamea* 2	4.17	8.34	4.17	0.00	11.32	69.08	97.07
M. acuminata subsp. *malaccensis* 1	0.00	19.65	0.00	0.00	31.56	14.29	65.51
M. acuminata subsp. *malaccensis* 2	1.19	33.95	3.57	0.00	36.92	44.07	119.70
M. acuminata subsp. *truncata*	0.00	0.00	0.00	0.00	0.00	37.52	37.52
M. acuminata (yellow bract)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
M. balbisiana	6.55	32.75	0.00	0.00	0.00	0.00	39.30
M. itinerans	2.38	16.08	2.38	0.00	5.36	13.70	39.90
M. laterita	0.00	34.54	0.00	0.00	0.00	0.00	34.54
M. coccinea	0.00	1.19	0.00	20.25	0.00	0.00	21.44
M. velutina	2.98	5.36	0.00	0.00	0.00	0.00	8.34
Musa sp. one (unknown)	0.00	18.46	0.00	0.00	12.51	20.25	51.22
Musa sp. two (unknown)	5.36	7.74	4.17	0.00	7.74	15.48	40.50
E. glaucum	0.00	0.00	0.00	0.00	0.00	0.00	0.00
E. superbum	66.70	37.52	0.00	0.00	0.00	0.00	104.22

[a] As equivalent gram to cyanidin-3-rutinoside (Molecular weight 595.53) (*18*). 0.00= not detected. [b] Peak numbers as mention in **Table 2**, calculated from ratio area under each HPLC peak multiplied by total anthocyanin. [c] Calculated by pH differential method from crude bract extracts (*17*).

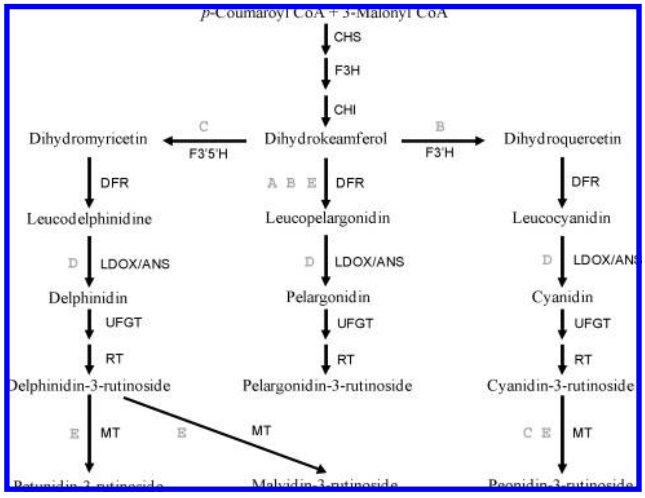

Figure 4. Possible limiting steps in anthocyanin biosynthetic pathway (modified from petunia (*29*)) from the anthocyanin profiles in each wild banana group (letter of each group in gray color at limiting steps). The enzyme names are CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; F3′H, flavanone-3′-hydroxylase; F3′5′H, flavanone 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; LDOX/ANS; leucoanthocyanidin dioxygenase/ anthocyanidin synthase; UFGT, UDP glucose:flavonoid 3-O-glucosyltransferase; RT, UDP rhamnose: anthocyanidin-3-*O*-glucoside rhamnosyltransferase; MT, methyltransferase.

anthocyanin pigments. This anthocyanin profile could be an evidence to confirm that *M. acuminata* subsp. *truncata* was a distinct subspecies from *M. acuminata* subsp. *malaccensis* (*27*). The **C** group, only *M. coccinea* (Callimusa section) presents cyanidin-3-rutinoside and pelargonidin-3-rutinoside which is unique in this study. Thus imply for *M. coccinea* that it might be able to synthesize anthocyanin mainly via dihydrokeamferol and little via dihydroquercetin and unable to do methylation on anthocyanin. In **D** group, yellow bract color group which is not detected any anthocyanin. Imply for this group that they might have problem either the step of turning leucoanthocyanidin to anthocyanidin or any prior steps in anthocyanin biosynthetic pathway. The **E** group which consists of *M. balbisiana* (Musa section), *M. laterita* (Rhodochlamys section), *M. velutina* (Rhodochlamys section) and *E. superbum* possessed 2 types of anthocyanin which are nonmethylated anthocyanin. The anthocyanin in this group could be synthesized via both dihydroquercetin and dihydromyricetin but unable to do methylation. These suspected defective metabolic steps of anthocyanin biosynthesis in bananas should be further investigated.

Our results show that only anthocyanin composition cannot directly determine visual bracts color. Other factors such as accumulation patterns, vacuolar pH and copigments in each individual cell or tissue area (*28*) also have influence. This study indicates that visual bract colors are not by themselves sufficient enough to be used as a strong character to classify bananas. Study of the anthocyanin composition or other phenotypic and molecular assessments are needed to provide more accurate classification. The present findings, together with the data from numerical morphological taxonomy (*26*) and DNA polymorphisms such as genomic DNA AFLP (*16, 27*), should be combined to validate the previous classification of wild bananas and may help to define groups or possible parental hybridizations or origins of cultivated bananas.

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