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EVALUATION OF MORPHOLOGICAL CHARACTERISTICS OF LEAF, FLOWER AND POD IN VIETNAMESE COCOA CULTIVARS

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Abstract

This study examined the morphological traits of sixty-three (63) cocoa varieties that have been imported and cultivated in Vietnam. These cocoa varieties were collected from five regions in Southern Vietnam. The morphological features were individually evaluated and analyzed, including the leaf characteristics (leaf and stem anatomy) and flower features (ligule shape, anther number, pollen, stamen and ovule, fruit, seed). The results of this study showed a large variation across all morphological characteristics of the evaluated cocoa varieties. The Vietnamese cocoa flower showed a diversity of morphological characteristics including five shapes of ligule (oval, broad, deltoid, elliptic, and sub-lanceolate) and each stamen also had dithecous anthers with the exception of tri-thecous anthers for TD11. Furthermore, the shape of pollen grains was found homogeneous in all 63 varieties. The colour of the stamens and ovules was purple and white respectively for all examined samples. Three kinds of fruit shapes were identified, namely Angoleta, Amelonado and Cundeamor, and these were of varied colours. The Vietnamese cocoa cultivars were classified into three groups based on their fruit morphology characteristics (Trinitario-Criollo, Trinitario-Forastero, and Trinitario). Additionally, an anatomical analysis on the midrib structure of the leaves from 63 varieties showed high similarities, likewise the stem structure. The colour of young leaves was observed as being green and red. This primary research scrutinizes the morphological biodiversity of Vietnamese cocoa varieties during the three-decade development of the Vietnam cocoa project. The results provide practical applications for cocoa cross breeding and botanical taxonomy.

Key words: Cocoa, Floral description, Fruit shape, *Theobroma cacao* L.

Introduction

Cocoa trees (*Theobroma cacao* L.) have recently been cultivated in some areas of Vietnam, showing themselves to be a potential crop for economic growth. Cocoa trees are well-adapted to Southern Vietnam because of the suitable climate and soil conditions, especially in the Highland areas and the Mekong Delta provinces. Vietnamese cocoa cultivation originated from two groups including the domestic group cultivars in Vietnam (TD cultivars, domestic cultivars come from ThuDuc province) and the cocoa cultivars which was imported namely CT, SCV, ICS, SCA, POUND, MAN, PA, LCTEEN, IMC, UIT, APA, AMAZ, NA, MO, SIAL, EET, IFC, and the TD cultivars from TD1 to TD14. The latter cultivar group is imported from South America and Malaysia, respectively. Dr. Pham Hong Duc Phuoc reported all Vietnamese cocoa cultivars as belonging to the Trinitario or Forastero x Forastero group based on the cocoa cultivars information when they were imported. Presently, information on these cultivars is still lacking, causing some challenges in conducting the taxonomic research. The fruit characters have played an important role in differentiating individual genotypes with regard to their position in the diversity of the species. The most notable characteristic of cocoa fruits is those of shape and colour (Bartley, 2005; Afoakwa, 2016). Floral description is an important data showing the diversity of cocoa populations (Bartley, 2005).

The economic implications as well as important contribution from the Vietnamese cocoa breeding program to the development of cocoa studies in Vietnam have been demonstrated. However, to the best of our knowledge, no studies on the morphology variation of Vietnamese *T. cacao* cultivars have been carried out. Hence, this study focused on examining the morphological features of the 63 cocoa varieties currently cultivated in southern Vietnam based on vegetative organs and reproductive organs. The morphological variability among Vietnamese cocoa cultivars was described using certain floral descriptors, leaf traits, and fruit (pod) characteristics. Consequently, this research provides vital information on the morphology collection of Vietnam which can assist the development of improved cocoa varieties.

Materials and Methods

Cocoa samples: Cocoa accessions were collected from five (5) popular cocoa regions in southern Vietnam, including Daklak province, Dong Nai province, BenTre province, Nong Lam University-ThuDuc province, and CanTho province. These 63 cocoa cultivars included 7 CT cocoa cultivars (CT population); 25 TD cultivars (TD population), and 31 other cultivars (others population). Most samples were collected with the aim to have a broadening of Vietnamese cocoa varieties with differences in morphology and genetic diversity. Among 25 TD cocoa varieties, fourteen (14) are grown in most provinces. Ten (10) out of these 14 cocoa varieties are national cocoa varieties (high quality and high yield) in southern Vietnam (Pham, 2009). Others TD hybrid varieties were collected from Nonglam University cocoa plantation (Table 1).

Table 1. Vietnamese cocoa samples.

Cocoa population	Number of cultivar	Cultivar code
AMAZ	1	AMAZ1515
APA	1	APA4
CT cultivars	7	3,5,6,7,8,9,21
EET	1	EET376
ICS	2	ICS1, ICS43
IFC	1	IFC5
IMC	3	IMC53, IMC 67, IMC105
LCTEEN	2	LCTEEN37/A, LCTEEN62/S
MA	2	MA12
MAN	1	MAN 15/2
MO	1	MO 81
NA	2	NA32, NA33, NA149
PA	8	PA24, PA70, PA88, PA120, PA127, PA137, PA156, PA169
POUND	2	POUND16/A, POUND16/B
SCA	2	SCA6, SCA9
SIAL	1	SIAL339
TD	25	1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 20, 31, 32, 42, 52, 57, 60, 70, 77
UIT	1	UIT1
Total	**63**	

Sampling collection: In Vietnam two crops of cocoa are annually harvested in March-May and October-December. In this study, the samples (flower, leaf and fruit) were collected in both of these periods. To avoid the effects of season and time of collection on the expression of descriptors, data were collected during the same season for each group of descriptors. Fruit (pod) and leaf data were collected throughout March to May (main harvest season) and flower data was collected during the flowering period, which is from October to December. Three trees were identified and were used during the harvest seasons for the sample collection (leaf, pod, and flower). The reproductive organs (flower and bub) were packed into small glass bottles, and then were put in cold boxes for further observation. The leaf and pod samples were packed in the paper bag and stored in the cold box before the examination.

Flower structure observation

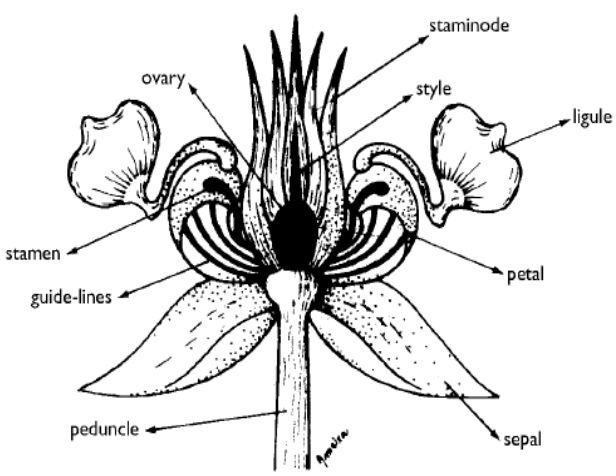

Fig. 1. Stretch of cocoa flower with name of individual parts (Bartley, 2005).

Cocoa bud (unopened): buds (5-6 cm in length and 3-4 cm in width) were randomly chosen from the trunks for the anther structure observation.

Cocoa flower: newly blooming flowers were randomly collected from trunks or branches. The particles of flowers were collected from the cocoa buds by using the forceps and needles (Fig. 1). The characteristics of flower particles (ligule shape, ovary shape, stem and midrib, staminode and stamen colour, anther structure, pollen grain shape) were observed using Motic SMZ-168 Stereo zoom Microscope equipped with a camera Olympus CX41- C5050. All measurements were conducted in triplicate per accession. The samples was distinguished based on the discrimination of ligule shape, described by Lachenaud *et al*., (1999) (Fig. 2).

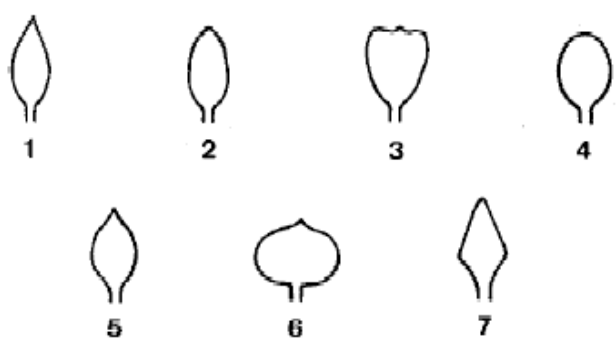

Fig. 2. Various ligule shapes: 1-lanceolate, 2-eliptic, 3-deltoid, 4-ovate, 5-sub lanceolate, 6-broad, 7-rhombic (Lachenaud *et al*., 1999).

Pollen grains were collected from the anther. The scanning electron microscopy of pollen grains was performed using a Tabletop Microscope TM-1000 (Hitachi High Technology).

Stem and midrid: the sample was sliced into sections before being stained with Carmin alune' 0.1N and Vert de Mirande 0.01N solution followed the laboratory method (Nguyen, 2007). The sections were placed on a slice, covered by a coverslip, and were observed.

Cocoa fruits: the mature and ripe fruits (yellow-green or yellow-red/purple) were randomly collected to observe the shape and colour.

Cocoa leaf: maturity leaf (length and width) were measured with a common centimetre ruler (Santos *et al*., 2012).

Seed shape and colour: Cocoa fruit was cut vertical for the examination.

Results

Vegetative organs: In this study, the vegetative organs, including stem, midrib and leaf were observed. Their anatomy structure was reported as below.

Leaf characteristics: During the observation, it was noted that the colour of the cocoa leaves was dark green in mature cocoa plants whilst the leaves from the immature blade (young) presented two different colours: red purple and green (Fig. 3). The red blade was presented in cultivars TD3, TD6, TD10, TD15, ICS1, TD31, TD17, and the rest 56 cultivars exhibited green blade leaves. The leaf shape of all accessions showed an elliptic shape.

For all 63 cocoa accessions, a cross section represents the dicots stem structure, the stem with primary growth have piths in the center, the xylems and phloems in the vascular bundles and connects together to form a continuous cylinder (Fig. 4a). The midrib in the cross section is concave-convex in structure. The vascular system of the midrib of *Theobroma cacao* L. is collateral, surrounded by a sclerenchyma system. The gland system is visible in the middle of the midrib structure (Fig. 4b).

Reproductive organs

Fruit shape: The fruit shape of 63 cocoa cultivars was classified into Amelonado, Angoleta and Cundeamor based on the description in Table 2 (Anon., 2017). The Calabacillo shape wasnot represented in the examination.

Seed shape: The 63 collected cocoa cultivars showed only one oval seed shape with purple seed in young and dark brown seed in ripe (Fig. 5).

Flower description: Ligule: The distribution of different ligule shapes was showed in Table 5 and (Fig. 6). There were five variations namely oval, broad, deltoid, elliptic and sub-lanceolate in the three represented popolations (CT, TD and others). The most common Oval and Deltoid shapes were observed in other populations, of which the percentages ranged from 50 to 36%, respectively. The other shapes were rarer (Broad, Elliptic, and Sub-lanceolate).

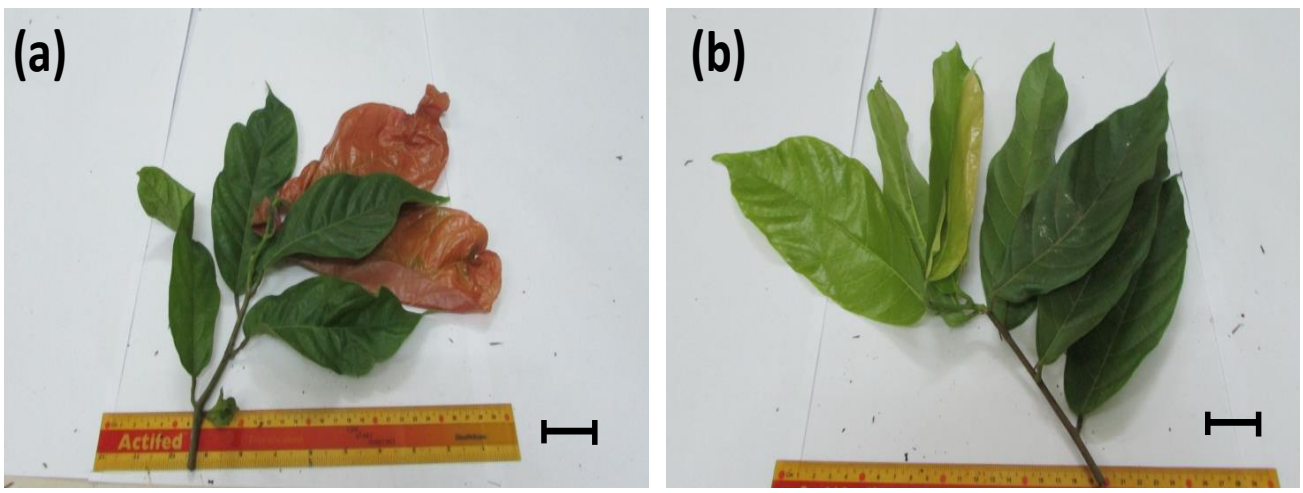

Fig. 3. Young leaf colour of Vietnamese cocoa accessions; red purple (a) and green (b). Scale bar = 3cm *Anatomy eatures* (stem and midrib).

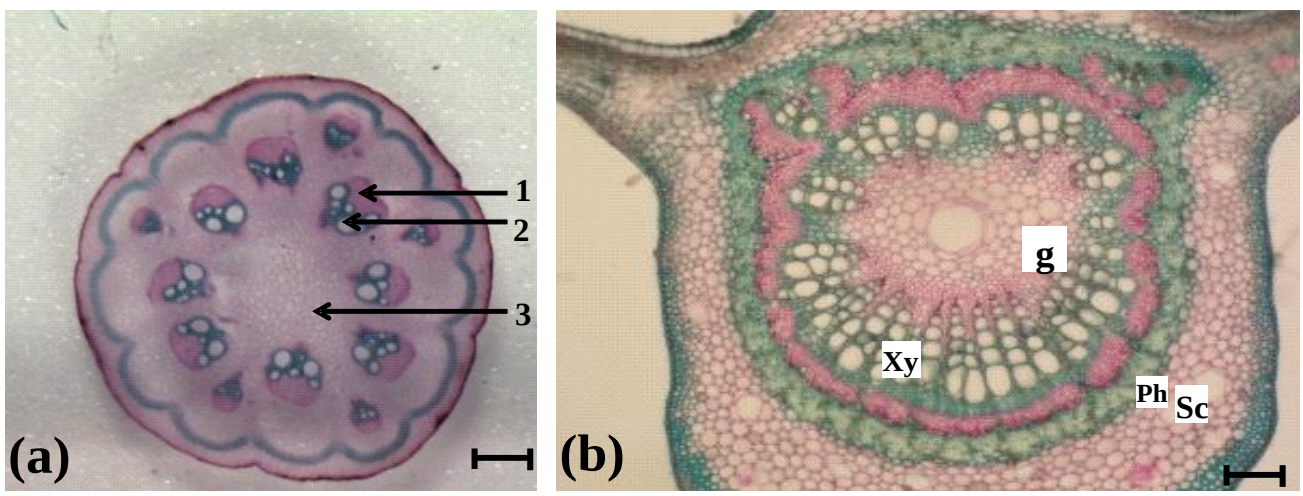

Fig. 4. Anatomical features of *Theobroma cacao* L.; (a) cross section of a stem (small trunk) of TD11 cultivar (1: Phloem, 2: Xylem, 3: Pith); (b) cross section of a midrib of CT3 cultivar (g: gland, Xy: xylem, Ph: phloem, Sc: schlerenchyma). Scale bar = 0.5mm (a), 0.267mm (b).

Table 2. Morphology characteristics of Vietnamese popular cocoa varieties

Cocoa variety	Pod characteristics	Pod color (mature to to ripe)	Seed colour	Young leaf color	
Forastero	Amelonado: spherical, smooth walled, light furrows.	Green to yellow/light green	Purple	Green-yellow	
	Calabacillo: spherical, smooth walled, without furrow.	Green to yellow/light green	Purple	Green-yellow	
Criollo	Cundeamor: elongated, rough walled, deep furrows, bottled-neck	Green-Purple to Red/yellow	White-purple	Green-purple/red	
	Angoleta: elongated, rough walled, deep furrows, no bottled-neck	Green-Purple to Red/yellow	White-purple	Green-purple/ red	
Trinitario	Hybrid cultivar between Criollo and Forastero: typically not pointed, various shape and color	Variable	Purple	Variable	

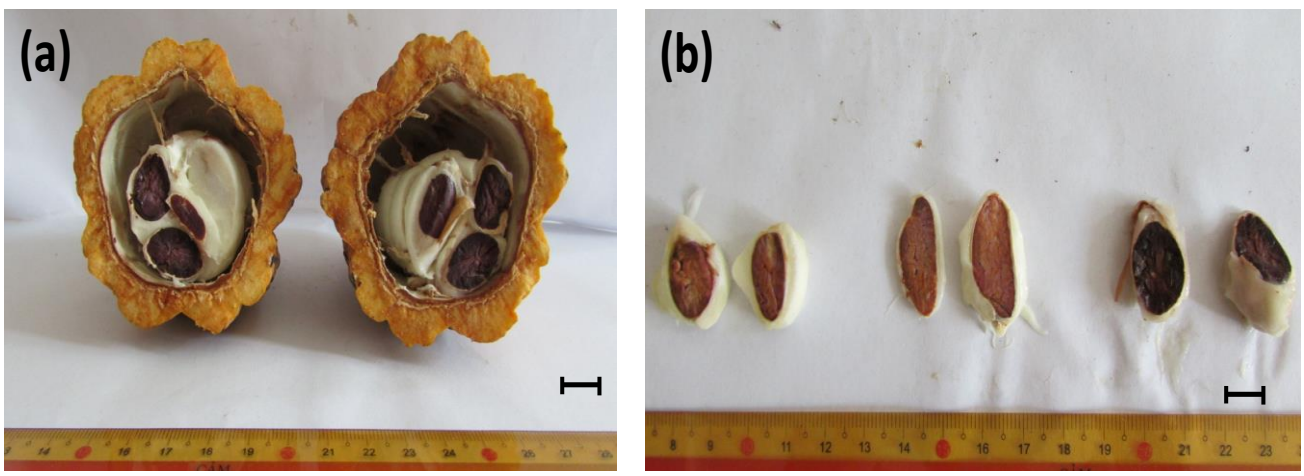

Fig. 5. The cross section of bean arrangement in ripe fruit (a), the oval and purple seed of 63 cocoa accessions (b). Scale bar = 1cm.

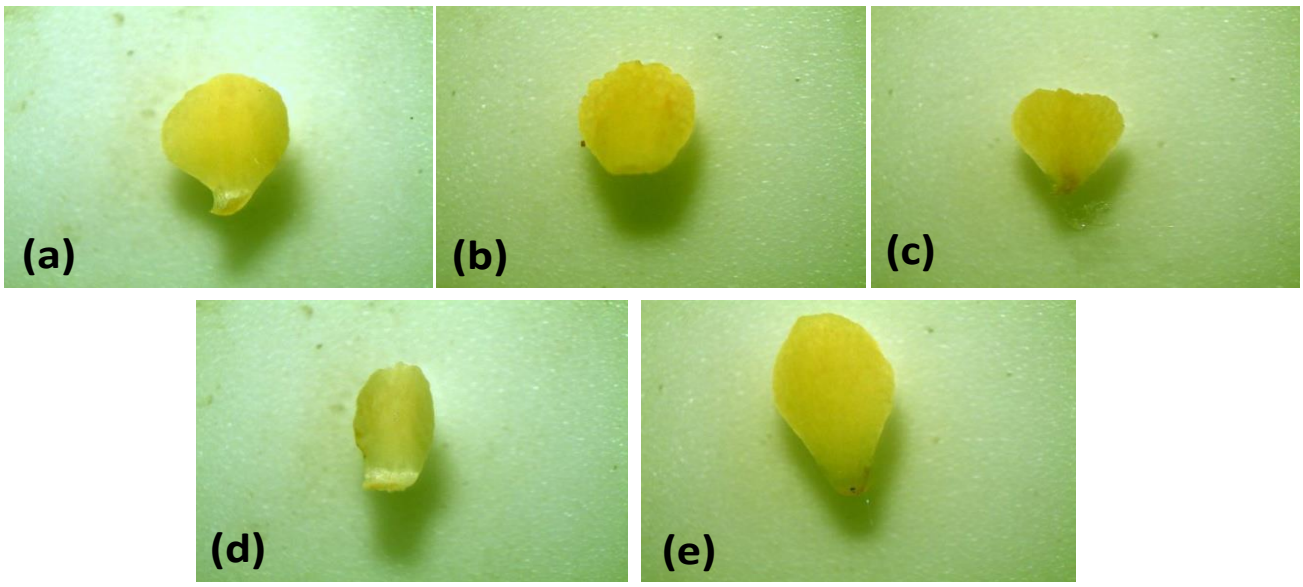

Fig. 6. Various of cocoa ligule shapes of Vietnamese collection; (a) oval, (b) broad, (c) deltoid, (d) elliptic, (e) sub-lanceolate.

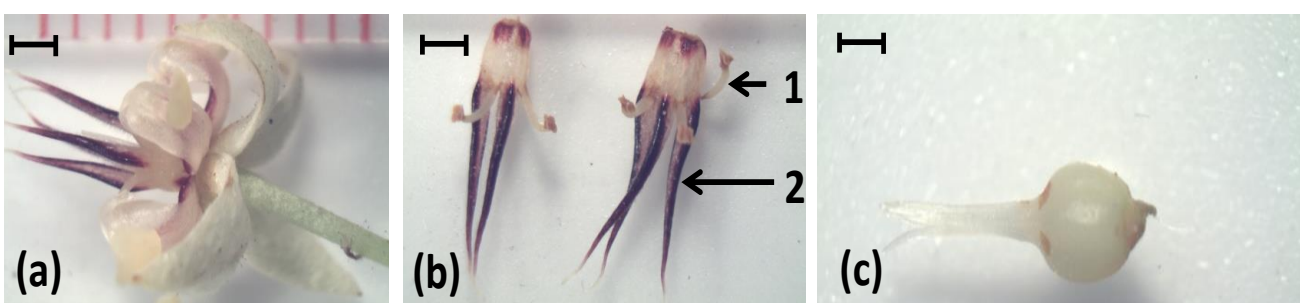

Fig. 7. Microcopy observation of flower; blossom cocoa flower (a); floral structure (b) with stamen in light pink (1) and staminode in purple (2); white ovary (c). Scale bar = 0.05cm (a) (b); = 0.5mm (c).

Staminode, stamen and ovule: The arrangement and colour of the staminode, stamen and ovule were observed. For all 63 samples observation, the staminode is purple, the stamen is light pink, and the ovary is white (Fig. 7).

Anther: Sixty-two (62) observed cocoa cultivars possessed dithecous anthers structure with two microsporangia in each lobe. In particular, the TD11 variety revealed a special arrangement with tri-thecous (tri-lobes) anthers (Fig. 8).

Pollen shape: It was observed that pollen grains, in all 63 cultivars, had a round shape (Fig. 9).

Discussion

Vegetative organs

Leaf characters: According to Bartley (2005), the colour of the fresh (young and developing) leaves is considered to be a distinctive trait for taxonomy. For example, the leaf of the Criollo group was found to have flush blade due to the presence of anthocyanin pigments (Bartley, 2005). Our result showed that the young leaf of 56 cultivars in Vietnam were identified with green shades and seven (7) cultivars represented flush leaf. Hence, these seven cultivars could be categorized into the cultivar that has the Criollo character. Soria & Enriquez (1981) indicated that there were three phenotypes of cocoa foliage including lanceolate, elliptical and oval patterns. In our analysis, 63 Vietnamese cultivars were observed to have an elliptical shape. Another foliage classification was also mentioned by Bartley (Bartley, 2005) regarding the leaf shape, namely spherical and cylindrical shapes. However, neither of the authors was able to clarify which group these foliage shapes could be classified.

Leaf colour: A previous report from Bartley (2005) revealed that the red fruit colour could be representative in some Criollo population, and a hybrid of this group has inherited this trait. Additionally, the author also agreed that the Criollo group has red leaves. The fifty six (56) cultivars indicate yellow ripe pod, which are categoried into Trinitario-Forastero. Tables 3 and 4 indicated the shape and colour characters of 14 cocoa popular cultivars. Among them, four popular cultivars (TD3, TD6, TD10, TD15) showed the red ripe fruit (character of Criollo variety). For instance, TD3 has the Criollo characteristics, which produces the decent flavoured beans (Afoakwa, 2016), but the fruit is small size (<400gr). This unexpected character could be improved by hybridization with TD9 cultivar, which is big fruit. The results showed clearly the category cocoa cultivar groups (Trinitario-Criollo and Trinitario-Forastero). Furthermore, based on

their Criollo or Forastero characters, some cocoa cultivars will be introduced as the promising cultivars. Certain projects should organize the training for farmers to recognize these varieties so that they can select and propagate new breeds with expected traits.

Table 3. Different pod shapes of the cocoa populations (in terms of numbers observed).

Population	Shape			No of varieties
	Cundeamor	Amelonado	Angoleta	
CT	3	1	3	7
TD	8	4	13	25
Others	19	8	14	31
Total	20	13	30	63

Table 4. Different pod colours of the cocoa populations (in terms of numbers observed).

Population	Colour (ripe pod)		Number of varieties
	Red/purple	Yellow	
CT	0	7	7
TD	6	18	25
Others	1	30	31
Total	7	56	63

Table 5. Different ligule shapes of the cocoa populations (in terms of numbers observed).

Population	Ligule					Number of varieties
	Ovate	Broad	Deltoid	Elliptic	Sub-lanceol	
CT	0	0	2	4	1	7
TD	9	4	3	4	5	25
Other	13	5	12	1	1	31
Total	**21**	**9**	**17**	**9**	**7**	**63**

Stem and midrib anatomy: The cross sections showed that the structure of Stem and Midrib were very similar in all 63 cultivars (Fig. 4). The same structure was reported by Garcia *et al*., (2014) in two *T. gradiflorum* and *T. speciosum* species. The reason is that all 63 samples are *T. cacao* species whose structures are similar to *T. gradiflorum* and *T. speciosum* species. These three species belong to *Theobroma* genus, hence all samples showed the same dicots structure.

Reproductive organs

Fruit shape and colour: Fruit characteristics have played an important role in differentiating individual genotypes with regard to their position in the diversity of the species (Bartley, 2005). The result of shape analysis identified three kinds of fruit shape including Amelonado, Angoleta and Cundeamor (Table 3) based on the description of Table 2. Fifty (50) cultivars showed the Cundeamor and Angoleta pod characters (elongated, rough walled, deep furrows, botted neck or without), and thirteen (13) cultivars represented the Amelonado traits (spherical, smooth walled, and ligh furrows) (Table 3). These results indicated that 63 cocoa cultivars are different in pod shape. The Vietnamese cocoa cultivars were reported to be Trinitario or Forastero x Forastero variety based on their origin information, however no publication studies on the morphology charateristics has been found. This examination of shape fruit indicated that the 50 cultivars showed the Criollo fruit characters (elongated fruit), including 20 cocoa cultivars with Cundeamor shape (elongate and bottled neck and 30 cultivars with Angoleta shape (elongated without bottled neck), and thirteen (13) Forastero spherical fruit shape (CT9, IMC105, IMC67, MO81, PA127, PA137, SCA6, SCA9, SIAL339, TD5, TD11, TD13, TD15) (Table 3).

Lachenaud *et al*., 1999 confirmed that the ICS1 cultivar is the Trinitario and IMC67 is the Forastero. The examined result demonstrated that the ICS1 cultivar which has Cundeamor shape (Criollo character). Moreover, ICS1 leaf is flush and its ripe pod is red. It can be concluded that the ICS1 which was imported from south America is Trinitario with Criollo character (Trinitario-Criollo). Phuoc (2009) reported that the TD Trinitario cultivars possessed combine characteristics of Trinitario and Forastero x Forastero cultivars. For example, TD1 cultivar is the hybrid progeny of PA35 x NA32, TD10 is the hybrid cultivar of NA31 x PA15 (Table 1). Two offsprings - PA and NA presented the Upper Amazon Forastero variety (Bekele *et al*., 2006) while Bartley (2005) confirmed PA cultivar is Criollo variety. The examined result showed that TD1 represented Angoleta shape (Criollo) and TD10 resulted the Cundeamor shape (Criollo). This can be explained that PA35, NA32, NA31, and PA15 are possible Trinitario hybrid between Criollo and Forastero varieties.

The colouration of cocoa fruit is a notable characteristic for classification. During the maturation, the green cocoa fruit turns to yellow and the purple/red fruit turns into orange/red (Soria & Enriquez, 1981; Bartley, 2005). A previous report from Bartley (2005) revealed that the red fruit colour could be representative in some Criollo population, and a hybrid of this group has inherited this trait. Additionally, Cuatrecasae (1964) and Braudeau (1969) also agreed that the Criollo group has red leaves. The work represented seven (7) cocoa cultivars with red/purple ripe pod. Hence, the study categoried these seven cultivars as Trinitario-Criollo cultivars (red pod and flush young leaf). The fifty six (56) cultivars indicate yellow ripe pod, which are categoried into Trinitario-Forastero.

Table 3 indicated the shape and colour characters of 14 cocoa popular cultivars. Among them, four popular cultivars (TD3, TD6, TD10, TD15) showed the red ripe fruit (character of Criollo variety). The selection for prominent hybrid progenies should be more effective because of the distinguishable characteristics between the Criollo and Forastero group. Hence, cocoa breeders should take into account these traits since the Criollo group is noticeably favoured because of its seed qualification (Bekele *et al*., 2006; Lachenaud *et al*., 2007). For instance, TD3 has the Criollo characteristics, which produces the decent flavoured beans (Anon., 2017b; Afoakwa, 2016), but the fruit is small size (<400gr). This unexpected character could be improved by hybridization with TD9 cultivar, which is big fruit. The results showed clearly the category cocoa cultivar groups (Trinitario-Criollo and Trinitario-Forastero). Furthermore, based on their Criollo or Forastero characters, some cocoa cultivars will be introduced as the promising cultivars. Certain projects should organize the training for farmers to recognize these varieties so that they can select and propagate new breeds with expected traits.

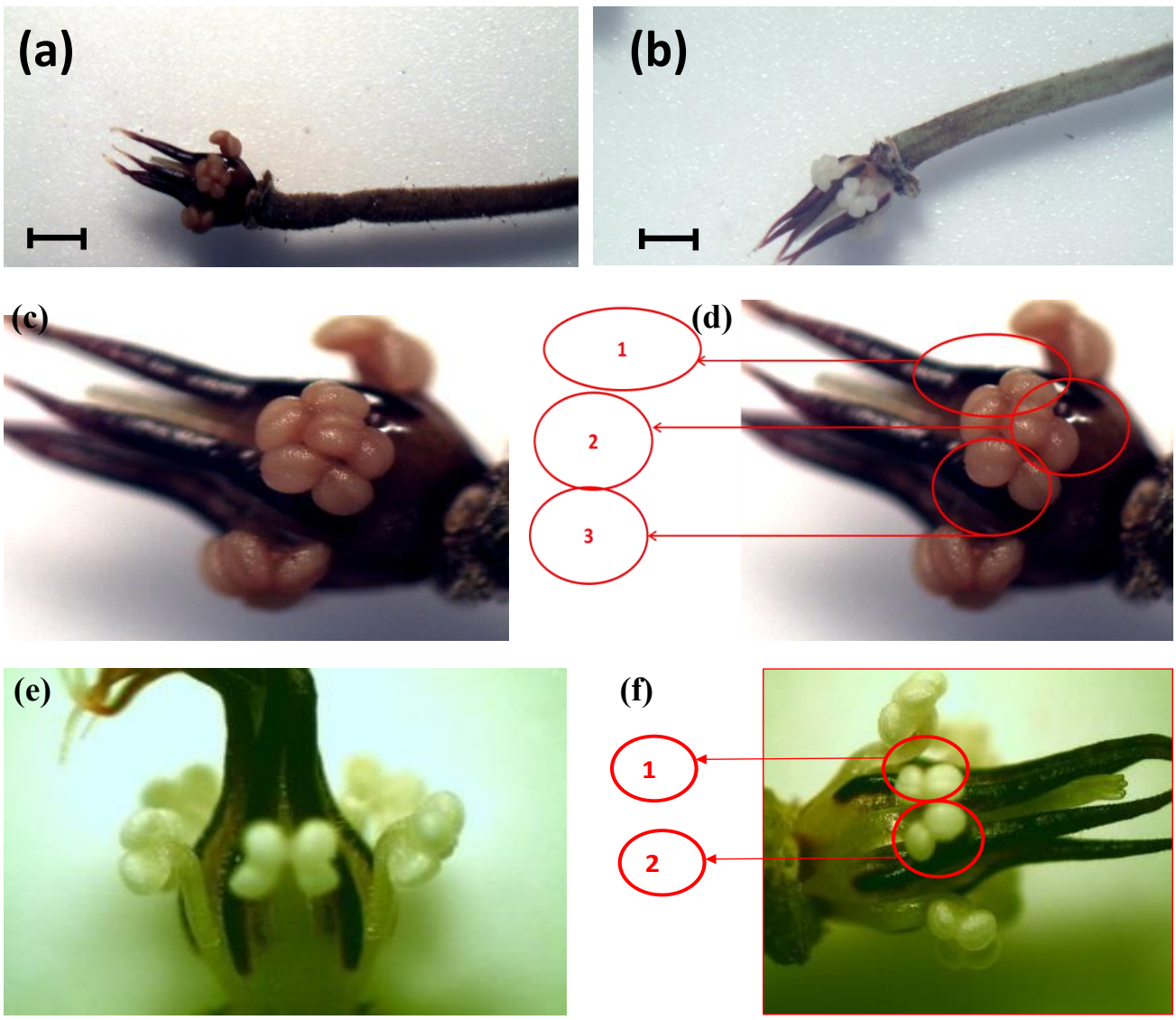

Fig. 8. The anther structure arrangement (pub observation); trilobed (tri-thecous) anthers of TD11 (a) (c) (d); and dithecous anthers (b) (e) (f). Scale bar = 0.1mm.

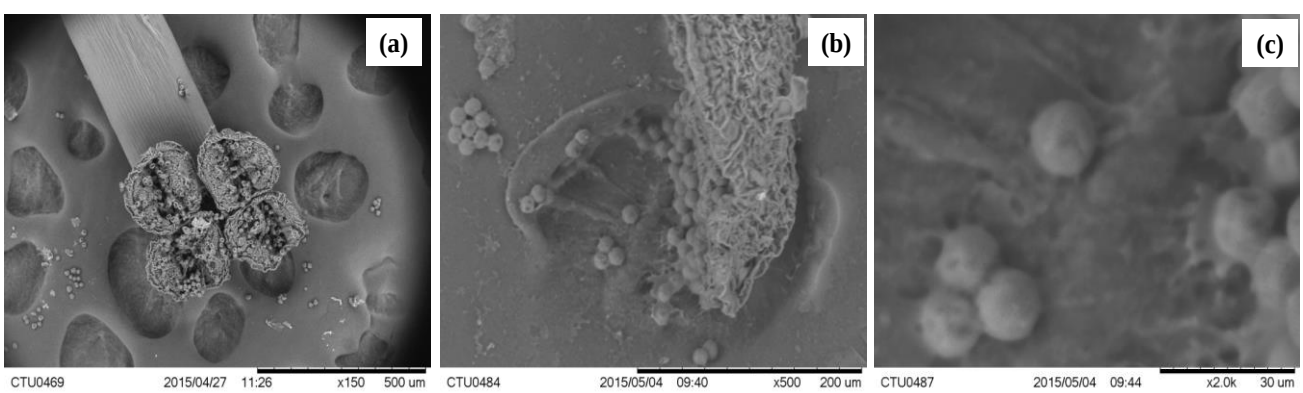

Fig. 9. Scanning electron micrographs of dithecous anther (a) scale bar = 500 µm, pollen grains x500 (b) scale bar = 200 µm, round pollen grains x 2,000 (c) scale bar = 30 µm.

Seed shape and colour: Our examination showed that the shape and colour of the seeds was not significantly different across samples. Previous studies have showed that the cocoa seed colour was white, pale purple, intermediate purple and dark purple and cocoa seed shapes varied from almost spherical to cylindrical (Soria & Enríquez, 1981; Bartley, 2005). Thus, the classification based on shape and colour is challenging in our investigation since all samples have a spherical shape and a dark purple colour.

Flower description

Ligule: Previous studies showed that cocoa ligule had a yellow hue and varied in shape (Soria & Enríquez, 1981; Lachenaud *et al*., 1999). Our result is coherent with those studies as the examined ligule of 63 cultivars diversified in shape (Fig. 6) and the colour was yellowish. The obtained results showed five shapes of ligule (oval, broad, deltoid, elliptic, and sub-lanceolate). Hence, this characteristic could be a useful feature for further classification.

Staminode, stamen and ovary: Our study showed that 63 examined cultivars of stamenoids were darkish purple while their stamens were pinkish. These results are similar to Soria and Enriquez (Soria & Enríquez, 1981) which showed that most of the floral elements acquire a pink pigment except for the ligule which has a yellow pigment. Moreover, Bartley (2005) also described the variation of colouration ranging from white to purple. The presence of anthocyanin pigments in the stamen filament is one of the most important indicators of genotypic and varietal differences. However, variations in colour patterns occur according to the variety that the genotype belongs to (Bartley, 2005). For the examination of ovary colour, all 63 samples were whitish which was also reported by previous authors (Soria & Enriquez, 1981; Bartley, 2005).

Anther: The examination of anthers indicated that the majority of cocoa accesions had an anther dithecous structure, with the exception of TD11 which revealed the tri-thecous (tri-lobed) anthers structure. In majority of angiosperms, a typical anther consists of four elongated microsporangia; usually each anther lobe consists of two microsporangia (dithecous) (Yeo *et al*., 2012; Jahan *et al*., 2014). The tri-lobed anthers structure has been observed in some species in the family of *Zingiberaceae* (*Amomum Lobulatae*) and *Commelinaceae (Murdania)* (Yeo *et al*., 2012). The increasing number of microsporangia (increasing number of pollen grains) can be offered the great potential for the pollination, which leads to an increase in the yield. This observation provides significant results for cocoa breeding program.

Pollen grains: Pollen grains are one of the most important elements in studies concerning flowers, but the results are limited. With the aim to have a detailed report about the morphology of cocoa cultivars in Vietnam, this work focused on investigating variations in pollen grain shape. The results showed that there is only one round shape of pollen grain among 63 collected cocoa samples. This study did not focus on the number of pollen grains because of the different sizes of collected cocoa bulb across the 2 cocoa seasons. Thus, an examination of the number of pollen grains needs to be further investigated.

Conclusion

Based on the morphological analysis, mainly focusing on fruit shape and colour, 63 cocoa varieties were divided into three groups namely Trinitario-Criollo (Trinitario cultivar with Criollo character), Trinitario-Forastero (Trinitario cultivar with Forastero character), and Trinitario group. Presently, Vietnamese cocoa cultivars have been characterized to belong to Trinitario or Forastero x Forastero cultivars by people who imported them from many countries. Apparently, the original Criollo and Forastero cultivars are not existent in Vietnam. There is no standard sample to compare, especially the fruit characteristics. Hence, our examination faced difficulties when comparing morphology characteristics between the original cultivars and the investigated cultivars. The obtained results are reported based on the observation of vegetative and reproductive organs of 63 cocoa cultivars.

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