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Leaf Anatomy Structure of *Codiaeum variegatum* (L.) Rumph. ex A. Juss Cultivars

Hayatul Fajri[1*], Anisyah Yuniarti[1], Ari Sunandar[2], Wolly Candramila[1]

[1] Biology Education Department, Teacher Training and Education Faculty, Tanjungpura University, Pontianak, Indonesia.
[2] Biology Education Department, Teacher Training and Education Faculty, University of Muhammadiyah, Pontianak, Indonesia.

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Corresponding Author:
Hayatul Fajri
hayatul.fajri@fkip.untan.ac.id

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Abstract: *Codiaeum variegatum* (L.) Rumph. ex A. Juss is a common ornamental plant with a high variation of leaves shape and colour. This study aimed to analyze the leaves' anatomy and the phenetic relationship between eight cultivars of *C. variegatum* which were defined by the differences in leaf morphology. The paraffin method prepared the transversal section of leaves, and anatomical characters were converted into binary data to calculate the similarity coefficient which was analyzed by Numerical Taxonomy System (NTSYS) 2.1 version. Leaves of eight cultivars of *C. variegatum* are varied in the anatomical structure of the primary vein and lamina. The eight cultivars of *C. variegatum* were clustered into four. The variations were found in 19 primary vein and lamina anatomy characteristics and the similarity coefficient is up to 0.59 between all cultivars. It can be concluded that the combination of leaf anatomical structures and midrib anatomical characters have notable taxonomic implications for future research.

Keywords: *Codiaeum variegatum*; Garden croton; Leaf anatomy; Ornamental plant

Introduction

Euphorbiaceae is the fourth-largest family of flowering plants (Webster, 1994) and consists of 5 subfamilies, 300 genera, and 6000 species (Pritchard, 2003). *Codiaeum variegatum* is one of the species belonging to *Euphorbiaceae* that show high intraspecies variation. This plant is native and distributed from Borneo, Java, to Queensland and has been introduced in several countries in Africa and Asia (POWO, 2022). *C. variegatum* has a high variation in leaves' shape and colour and is tolerant to high light exposure, so it is usually considered as an ornamental plant in the open garden. Phytochemical analysis showed that the shoot of *C. variegatum* contained alkaloids, cardiac glycosides, saponins, tannins, cardenolides, and pigmented compounds (Ogunwenmo et al., 2007). The shoot and leaves of *C. variegatum* were also reported for their ability in Pb accumulation (Sulistiana & Setijorini, 2015; Agustin & Hamidah, 2019), therefore, potential for a bioremediation process. Other reports stated that *C. variegatum* extracts are involved in the antidiarrheal activity of mice (Labu et al., 2015), screened against the influenza A virus (Forero et al., 2008), and produce the essential oil that used as insectidal activity (Lawal et al., 2018). *C. variegatum* has potential pharmacology activity to treat amoebic dysentery (Njoya et al., 2021) and have the activity of antioxidant and anti-inflammatory (Pechangou et al., 2023). The high potential of *C. variegatum* for humans and the environment implies the urgency of exploring this plant and its uses.

Intraspecies variations in *C. variegatum* indicate a high potential source of germplasm. Regarding the high demand for *C. variegatum* as an ornamental plant, an updated taxonomic study is crucial to discover the plant's potential and crossing. Studies reported the intraspecies variation of *C. variegatum* based on morphological and molecular traits. Widyaningsih (2015) classified *C. variegatum* into 54 cultivars based on morphological variation; meanwhile, Mollick et al. (2011) clustered into 29 cultivars and 4 major groups based on leaf phenotypic parameters. Moreover, AFLP analysis grouped *C. variegatum* into 44 cultivars of 7 clusters (Deng et al., 2010) and RAPD analysis showed

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106 polymorphic bands (Asniawati & Purwantoro, 2018). *C. variegatum* was classified into 24 cultivars based on the shape and color of the leaves (Faturrahman et al., 2023). The genetic variety of *C. variegatum* has reported based on ISSR and RAPD analysis (El-Tony, 2023). These reports indicated that *C. variegatum* has a high variation both in morphological and gene levels.

The anatomical structure is one of the distinguishing characteristics to determine the plant species or cultivars (Samiyarsih et al., 2019; Tripathi et al., 2023). Any modification in structure result from evolutionary change and may promotes plant's survival in a particular habitat (Hanley et al., 2007). This includes morphological modifications, histological changes in cells and tissues and physiological specialisations (Rampe et al., 2019). However, information on *C. variegatum* leaf's anatomy is still limited. Leaf shape is related to the primary function in photosynthesis and evolutionary processes as a form of environmental adaptation (Chitwood & Sinha, 2016; Holloway-Phillips, 2019; Parkhurst & Loucks, 1972) and the anatomical structure of the leaf play an important role in the light distribution through the mesophyll (Smith et al., 1997). Therefore, a high intraspecies variation in the leaf shape of *C. variegatum* calls for a better explanation to explore the evolutionary history within the species anatomically. This study aimed to analyze the anatomical structure variation of *C. variegatum* leaves and the phenetic relationship between several cultivars. This study confirms the anatomical variation in *C. variegatum* and provides a better way to identify the *C. variegatum* cultivars.

Method

Leave samples of *C. variegatum* were collected from several open gardens in Pontianak City. Eight cultivars were defined according to the differences in leaf morphology. The leaves sampled are those in the base-oriented position and relatively uniform in size, so it is assumed to have reached the final development stage. However, the three cultivars showed different leaf morphology, so basal and tip leaves were used as samples. Six morphological characteristics were described, including leaf apices or tip, leaf shape, leaf colour, leaf base, leaf edge, and variation of leaf colour (Table 1).

Fresh leave samples of eight *C. variegatum* cultivars (C1-C8) were immersed in an FAA (Formaldehydes: Glacial Acetic Acid: 70% Ethanol = 5:5:90) fixative solution for 24 hours. Slide preparation of the leaf's anatomical structure used Paraffin Method as described by Sass (1951), with some modifications in dehydration and clearing agents. We used tert-butanol for dehydration and neo-clear as clearing agents. The anatomical structure of the leaves was visualized under a camera-mounted microscope Olympus CX with 100x magnification. Tissue observation was done on the leaf's lamina and midrib parts.

The phenetic relationship between eight *C. variegatum* cultivars was analyzed by NTSYS software 2.1 version. The leave anatomy information of *C. variegatum* cultivars converted into binary data. Binary data of *C. variegatum* leaf characters were used to count the similarity coefficient by SIMQUAL using the sample matching method. The clustering of eight *C. variegatum* cultivars was analyzed using Sequential Agglomerative Hierarchical and Non-overlapping clustering (SAHN clustering) by UPGMA method. A dendrogram was built using TREE display.

Results and Discussion

Leaf samples of eight cultivars of *C. variegatum* (C1, C2, C3, C4, C5, C6, C7, and C8) showed variations in six morphological characters (Table 1). The visualization of leaf morphology for each cultivar can be seen in Figure 1. The leaf apices or tip morphology of eight *C. variegatum* cultivars is divided into acuminate, acute, and obtuse; meanwhile, the leaf base is either acute or obtuse. The leaf base could be symmetry or asymmetry, or both. The lamina coloration is divided into two groups, green-yellow and red-green-yellow. The contents of chlorophyll in the leaves of *C. variegatum* decrease with the increase of leaf age, and the coloration of the plant is mainly due to the increased red cells among the leaf epidermis. The eight cultivars of *C. variegatum* showed six types of leaf shape: linear, oval, fissus, lanceolate, lobate, and twisted. The leaf edge character is divided into integer, pinnatifid, pinnatilobus, and repandus. Some cultivars showed variation in the same character, e.g., color differences in young and mature leaves of C1, C5, and C6. Thus, the same age range of leaves is essential in cultivar type determination because the shape of each leaf changes allometrically during its development (Chitwood & Sinha, 2016).

According to Sicard et al. (2014), changes in leaf morphology result from repeated mutations in the homeobox gene and its expression in response to environmental temperature. Among them, Vlad et al. (2014) explained the role of the homeodomain reduced complexity (RCO) protein in leaflet development by suppressing the growth at their flanks. For example, RCO develops in the Brassicaceae family through gene duplication and is lost in Arabidopsis thaliana with simple leaves. Meanwhile, Piazza et al. (2010) found that non-lobed leaf morphology was proven to be caused by loss of homeobox knotted1-like gene expression Shootmeristemless (STM) in leaves and the influence of

cis-regulatory divergence. On the other hand, Mollick et al. (2012) stated that *C. variegatum* cultivars can still be identified by the leaf's dimensions, including leaf base, margin, apices, shape, coloration, and color pattern. This indicates that the designation of the eight samples as cultivars showed differences for the six morphological characters in line with Mollick et al. (2012).

Table 1. Leaf Morphological Characters to Differentiate the Eight Cultivars in This Study

Cultivars	Morphological Characters					
	LT	LB	LC	LS	LE	LCV
C1	Acuminate	Acute-Obtuse- Asymmetry	Red-Green-Yellow	Linear	Integer	Yes
C2	Obtuse	Acute-Asymmetry-	Green-Yellow	Oval	Integer	No
C3	Acute	Acute-Asymmetry- Symmetry	Green-Yellow	Fissus	Pinnatifidus	No
C4	Obtuse	Obtuse-Asymmetry-Symmetry	Green-Yellow	Lanceolate	Integer	No
C5	Acute	Acute-Asymmetry- Symmetry	Red-Green-Yellow	Lobatus	Pinnatilobus	Yes
C6	Acuminate	Obtuse-Symmetry-	Red-Green-Yellow	Lobatus	Pinnatilobus	Yes
C7	Obtuse	Acute-Asymmetry	Green-Yellow	Twisted	Repandus	No
C8	Acute	Acute-Symmetry	Green-Yellow	Lanceolate	Integer	No

LT: Leaf Tip; LB: Leaf Base; LC: Leaf Color; LS: Leaf Shape; LE: Leaf Edge; LCV: Leaf Color Variation

Figure 1. Leaf morphology for each cultivar (C1-C8) analyzed in this study

Anatomical observation of the lamina part of the leaf is shown in Figure 2. The adaxial epidermal cell shape is rectangular except that cubic in cultivar 5 (C5). The palisade cell shape in the seven cultivars is rectangular, but the rounded palisade cell shape is only found in cultivar 5 (C5). Two layers of palisade were found in cultivars 1, 2, 3, and 4 (C1-C4), but a single layer was found in cultivars 5, 6, 7, and 8 (C5-C8). The number of spongy cell layers is the most varied among other characteristics in eight cultivars of *C. variegatum*. Seven layers of the spongy cell were found in cultivar 5 and 7 (C5, C7); eight layers of the spongy cell was found in cultivar 4 and 6 (C4, C6); nine layers of the spongy cell were found only in cultivar 1 (C1), and ten layers of the spongy cell were found in cultivars 2, 3, and 8 (C2, C3, C8). Druse was found in all abaxial leaves of the eight cultivars but not in the adaxial leaves of cultivars 7 and 8 (C7, C8).

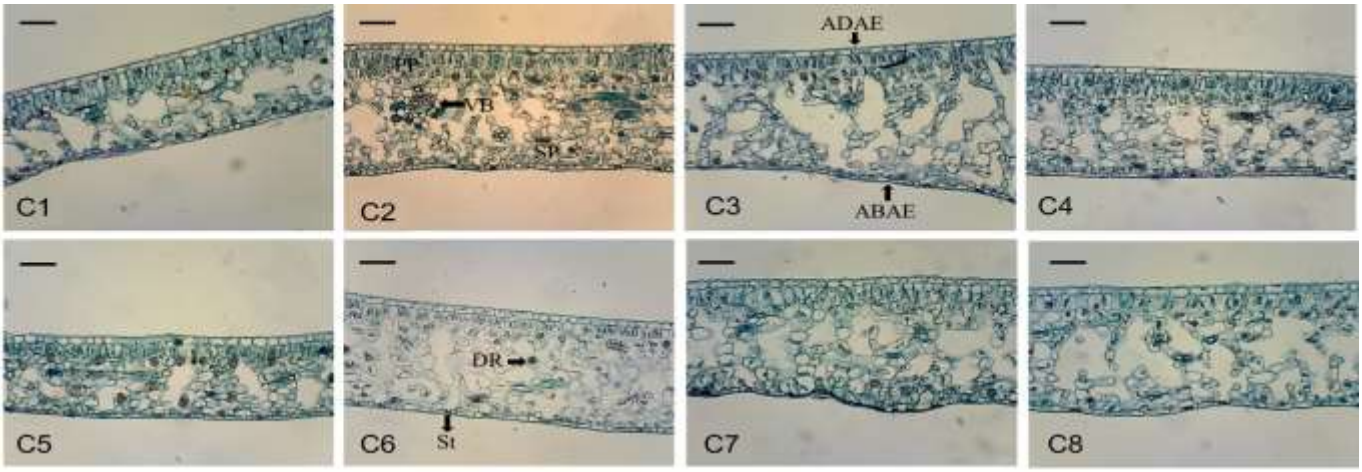

Figure 2. The cross-sections of the leaf lamina of eight cultivars *C. variegatum* (C1-C8). PP: palisade parenchyma, VB: vascular bundle, SP: spongy parenchyma, VB: vascular bundle, ADAE: adaxial epidermis, ABAE: abaxial epidermis, DR: druse. Bar scale = 100 µm

The anatomical structure of the primary vein varies between cultivars (Figure 3). The primary vein is biconvex in all *C. variegatum* cultivars (C1, C3-C8) except that uniconvex with flat adaxial in cultivar 2 (C2). Cultivars 1, 4, 5, and 6 (C1, C4-C6) showed cubic adaxial epidermis cell shape; however, rectangular adaxial epidermis cell shape was found in cultivars 2, 3, 7, and 8 (C2, C3, C7, C8). In the primary vein, the palisade was found only in cultivars 2 and 3 (C2, C3). The number of vascular bundle layers is more than three in the seven cultivars (C2-C8); however, one layer is found only in cultivar 1 (C1). The number of hypodermis cell layers in the adaxial primary vein varies; three layers were found in cultivars 3, 4, and 8 (C3, C4, C8), and four layers were found in cultivars 1, 5, 6, and 7 (C1, C5-C7). However, the hypodermis cell layer was not found in cultivar 2 (C2), which may be related to the uni convex and flat adaxial.

Among the eight cultivars, anatomical observation also showed similarities in the tissue characteristics of the leaf lamina and primary vein (Table 2). In the lamina, similarities were found in the abaxial epidermis cell shape, the presence of cuticle, spongy cell shape, and druse in the abaxial. In the primary vein, similar characteristics were found in the adaxial epidermis cell shape, the number of the palisade and spongy cell layers, palisade cell shape, and the presence of druse in the adaxial. The lamina part of *C. variegatum* leaf has a rectangular abaxial epidermis and a polygonal spongy cell shape. All cultivars have cuticles and druse in the abaxial. In the primary vein, druse was also found in all cultivars of *C. variegatum*, and the pattern of vascular bundles is open collateral.

Druse is found both in the lamina and primary vein. The presence or absence of druse crystals may represent essential taxonomic characters and can be used to understand plant species' evolutionary relationships (Franceschi & Horner, 1980; Prychid & Rudall, 1999). Many functions have been attributed to calcium oxalate crystals in plants, such as protecting plants against herbivores by their association with irritating chemicals or proteolytic toxins (Rupali et al., 2012). The mechanical effect of needle-like crystals which puncture the foraging animals is also an essential part of the plant defense (Konyar et al., 2014).

UPGMA method was used to obtain a dendrogram, as shown in Figure 4. The eight cultivars of *C. variegatum* were clustered into four. Cluster I consist of three cultivars (C1, C4, and C6) with a similarity coefficient between 0.81 and 0.82. As reflected in the anatomical structure, the same characteristics were of adaxial and abaxial epidermis cell shape in the lamina, the palisade cell shape, the presence of cuticle, the spongy cell shape, the presence of druse in abaxial and adaxial of lamina, abaxial and adaxial surface in the primary vein, adaxial epidermis cell shape of the primary vein, the pattern of vascular bundles, the presence of palisade in the primary vein, and the presence of adaxial hypodermis and druse in the primary vein. Cluster II consists of 2 cultivars (C7 and C8) with a similarity coefficient of 0.84. Similar characteristics include the adaxial and abaxial epidermis cell shape of the lamina, the number of palisade layers, palisade cell shape, the presence of cuticle, the spongy cell shape, the presence of druse in the adaxial and abaxial lamina, abaxial surface of the primary vein, adaxial epidermis cell shape of the primary vein, the pattern of vascular bundles, the presence of palisade in the primary vein, the number of vascular bundles, the presence of hypodermis in abaxial and adaxial of the primary vein, and the presence of druse in the primary vein. Cluster III comprises two cultivars (C2 and C3) with a similarity coefficient between 0.78 and 0.79.

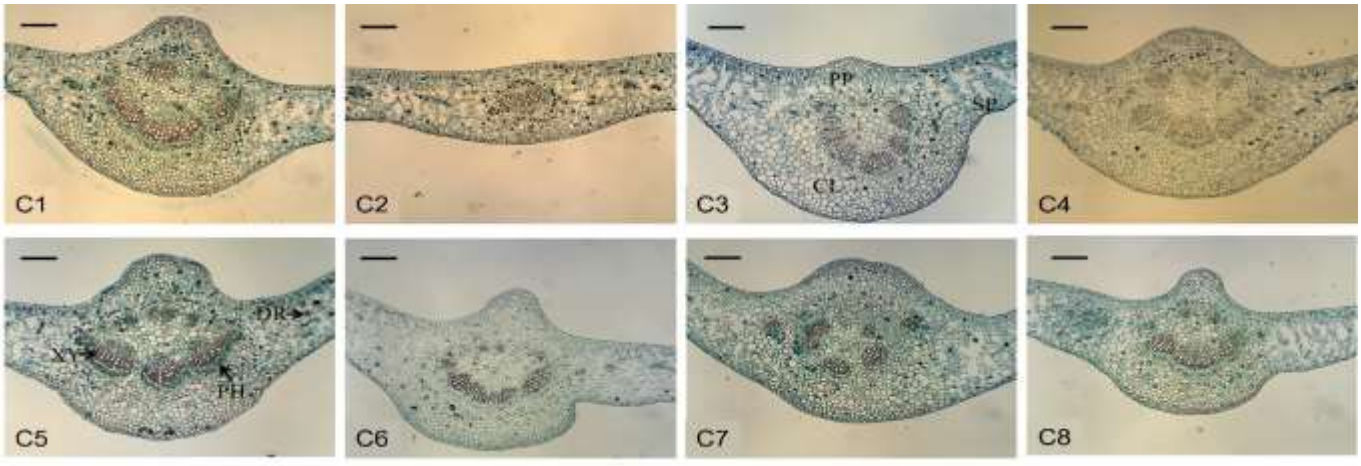

Figure 3. The cross-sections of the primary vein of the eight *C. variegatum* cultivars (C1-C8). PP: palisade parenchyma, CL: collenchyma, SP: spongy parenchyma, XY: xylem, PH: phloem, DR: druse. Bars: 100 µm

Table 2. Anatomy Character of Eight Cultivars of *C. variegatum*

No	Anatomy characters	*C. variegatum* Cultivars							
		C1	C2	C3	C4	C5	C6	C7	C8
Lamina									
1	Adaxial epidermis cell shape	Rec	Rec	Rec	Rec	Cub	Rec	Rec	Rec
2	Abaxial epidermis cell shape	Rec	Rec	Rec	Rec	Rec	Rec	Rec	Rec
3	The number of the palisade layers	2	2	2	2	1	1	1	1
4	Palisade cell shape	Rec	Rec	Rec	Rec	Rou	Rec	Rec	Rec
5	The presence of cuticle	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
6	The number of the spongy cell layers	9	10	10	8	7	8	7	10
7	Spongy cell shape	Pol	Pol	Pol	Pol	Pol	Pol	Pol	Pol
8	The presence of druse in adaxial	Yes	Yes	Yes	Yes	Yes	Yes	No	No
9	The presence of druse in abaxial	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Primary Vein									
1	Abaxial surface	Arc	Cur	Arc	Arc	V shape	Arc	Arc	Arc
2	Adaxial surface	V shape	Flat	Cur	V shape	Arc	V shape	Cur	V shape
3	Adaxial epidermis cell shape	Cub	Rec	Rec	Cub	Cub	Cub	Rec	Rec
4	The pattern of vascular bundles	OC	OC	OC	OC	OC	OC	OC	OC
5	The presence of palisade	No	Yes	Yes	No	No	No	No	No
6	The number of vascular bundles	1	>3	>3	>3	>3	>3	>3	>3
7	The presence of abaxial hypodermis	Yes	Yes	No	No	Yes	Yes	Yes	Yes
8	The presence of adaxial hypodermis	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
9	The number of hypodermis cells in adaxial	4	0	3	3	4	4	4	3
10	The presence of druse	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Rec: Rectangular; Cub: Cubic; Cur: Curved; Rou: Rounded; Pol: Polygonal; Arc: Arched; OC: Open Collateral

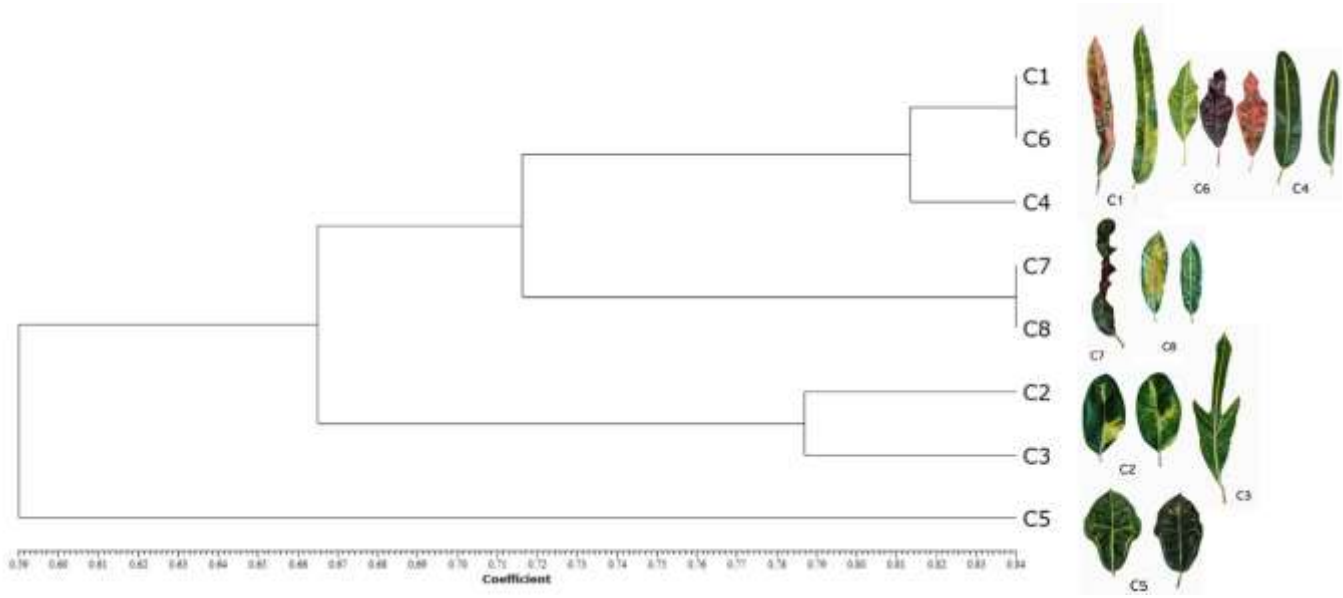

Figure 4. Dendrogram of 8 *C. variegatum* cultivars based on anatomical characteristics

Anatomical characteristics found in both cultivars include the adaxial and abaxial epidermis cell shape of the lamina, the number of palisade layers, palisade cell shape, the presence of cuticle, the number of the spongy cell layer, the spongy cell shape, the presence of druse in adaxial and abaxial of the lamina, midrib adaxial surface, adaxial epidermis cell shape of the primary vein, the pattern of vascular bundles, the presence of palisade in the primary vein, the number of vascular bundles, the presence of hypodermis in the abaxial of the primary vein, and the presence of druse in the primary vein. Cluster IV consists of only one cultivar (C5) with a similarity coefficient of only 0.59 with the other cultivars. Among cultivars, the highest similarity of 0.84 was found between C7 and C8.

Our study showed that the anatomical characters of the leaflet and primary vein could be taxonomic evidence to produce reliable diagnostic characters to identify *C. variegatum* cultivars. The same diagnostic characters were also found in the genus *Crotalaria* (Leguminosae: Papilionoideae: Crotalarieae) (Devecchi et al., 2014) and *Canthium s.l.* (Rubiaceae) (Tilney et al.,

1990). However, we must carefully determine relationships between cultivars using anatomical markers on leaves, considering the potential for environmental influences and adaptability that produce adaptive phenotypes. In the eight cultivars of *C. variegatum*, the cubic shape of the epidemic cells and the round shape of the palisade cells can be assumed to be the more primitive cell shapes. These two characteristics can be seen in the divergence between the seven cultivars and C5. The following anatomical grouping appears to align with the elongated leaf shape and more complex coloration. The same grouping for cultivars with more complex leaf colours aligns with the grouping reported by Andreastuti-K et al. (2015). Cultivars with green-yellow-red leaves were grouped into the same cluster with a genetic similarity of up to 74%. In comparison, cultivars with two color combinations (green-yellow and green-red colours) were grouped in another cluster with a genetic similarity of 69%. Interestingly, anatomical observation confirms the morphology and genotype-based dendrograms. In conclusion, the combination of leaf anatomical characters such as adaxial epidermis cell shape, palisade cell shape, the number of the palisade, the number of the spongy cell layers, the presence of druse in adaxial and midrib anatomical characters such as adaxial and abaxial surface, adaxial epidermis cell shape, the presence of palisade, the number of vascular bundles, the presence of abaxial hypodermis, and the number of hypoderms could have notable taxonomic implications.

Conclusion

Variation of *C. variegatum* were found in 19 primary vein and lamina anatomy characteristics and the similarity coefficient is up to 0.59 between all cultivars. Combination of leaf anatomical structures and midrib anatomical characters have notable taxonomic implications for future research.

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Authors Contribution
Hayatul Fajri was contributed to design, implementation of the research, analysis the results, and writing the manuscript. Anisyah Yuniarti was contributed to design and implementation of the research. Ari Sunandar and Wolly Candramila were contributed to proofreading and review the manuscript.

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Conflicts of Interest
The authors declare that there are no conflicts of interest regarding the publication of this article.

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