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# Stomata Diversification and Phylogenenetic Analysis of 13 Species of Family Euphorbiaceae *sensu lato*

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## ABSTRACT

Investigation on diversity of stomata from 13 species of family Euphorbiaceae has been carried out. Characters like type of stomata, position of stomata, presence/absence of ledge, and density both abaxial and adaxial leaf surface were examined. Stomata characters in the family were found quite diverse in this study. Monophyletic nature of Euphorbiaceae has been proved in this study on the basis of phylogenetic analysis using parsimoni method. Our data further suggested that the family can be classified into two major groups. However, surprisingly, genus *Phyllanthus* is non-monophyletic.



**Key words:** diversity, Euphorbiaceae, phylogenetic analysis, stomata.

## INTRODUCTION

Euphorbiaceae *sensu lato* is one of the most cosmopolitan plant groups within angiosperm, consisting of about 300 genera and 7500 species (Jones and Luchsinger, 1987). Many members of the family play important role in supporting tropical rain forest, horticultural value for ornamental, traditional medicine, and food resources. Although classification of the family Euphorbiaceae has been clearly defined, phylogenetic relationships among genera within the family remains unresolved due to remarkable diversification of morphological characters.

Phylogenetic analysis to classify a group of plants based upon micromorphological characters on leaf surfaces has been carried out by many researchers. Several characters, such as presence/absence of stomatal ledge, differentiation of subsidiary cells, shape of epidermal cells, morphology of trichomes can provide valuable information in taxonomic and/or phylogenetic studies (Wu and Cutler, 1985; Yukawa et al., 1992; Sudarsono et al., 2005). In many studies, these characters appear to be relatively more consistence than those of macromorphological one (e.g. Yukawa et al., 1992). Moreover, stomatal type has not only good diagnostic value for such studies, but also it can be used as indicators for naturally taxonomical similarity (Fahn, 1991; Sharma, 1993).

The diversity of stomatal shape on 13 species of Euphorbiaceae were investigated in this study. Phylogenetic analysis was also further carried out in this study to infer evolutionary relationships among samples used in the family .

## MATERIALS AND METHODS

Species examined in this study were listed in Table 1. All leaves were collected from wild plants that are grown around Bandung, West Java. Two methods, fresh dissection and parafin coated dissection were used according to Sass (1958) to investigate stomata. The former method was used to observe stomatal type, location, and density, whereas the latter method was used to observe the ledge of stomata. Mature leaves were cut into small pieces approximately 1x1 $cm^2$. These pieces were fixed in FAA (Formalin+Acetic Acid+Alcohol) 50% for minimum 24 hours. They were then dehydrated through alcohol-TBA (Tersier Butyl Alcohol) series, followed by critical point drying. They were coated with parafin and dissected using Microtom model Yamato KHKI PR-50 with 10 µm of thick. The specimens were subjected to fast green staining and examined with a light microscope with 40x10 magnification.

Density of stomata was obtained from stomatal index through calculating number of stomata that was divided by number of stomata plus number of epidermal cells, then was multiplied by 100 (Willmer, 1983). Five characters, i.e. type of stomata, position of stomata, the ledge, density of stomata both abaxial and adaxial surfaces were subjected to phylogenetic analysis using parsimony method conducted with PAUP version 4.0b10 (Swofford, 2002).

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All character states (listed in Table 1) were equally weighted and unordered (Fitch, 1971). All the data sets were analyzed by the heuristic search method with tree bisection-reconnection (TBR) branch swapping and the MULPARS option on, saving all most parsimonious trees. Evaluation of internal support of clades was conducted by the bootstrap analysis (Felsenstein, 1985) with 1,000 replicates, simple stepwise addition, TBR branch swapping, and the MULTREES option off. The number of steps, consistency indices (CI), and retention indices (RI) were calculated on one of the MPTs using the TREE SCORES.

A member of Cucurbitaceae (*Sechium edule*) was used as outgroup, because the family has been recognized as sister groups to family Euphorbiaceae *s.l.* (APG, 2003).

**Table 1.** Euphorbian species examined in this study and those data matrix for phylogenetic analysis.

| No | Species | Character states | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 1 | *Sechium edule* (Jacq.) Sw.** | 0 | 0 | 0 | 0 | 0 |
| 2 | *Phyllanthus niruri* L. (Yellow)* | 1 | 0 | 1 | 1 | 1 |
| 3 | *Phyllanthus niruri* L. (Green)* | 1 | 0 | 1 | 1 | 1 |
| 4 | *Phyllanthus acidus* (L.) Skeels | 2 | 0 | 1 | 1 | 1 |
| 5 | *Phyllanthus urinaria* L. | 2 | 1 | 1 | 0 | 2 |
| 6 | *Euphorbia pulcherrima* L. | 0 | 1 | 1 | 1 | 2 |
| 7 | *Codiaeum variegatum* (L.) A. Juss | 2 | 1 | 0 | 1 | 2 |
| 8 | *Sauropus androgynus* (L.) Merr. | 2 | 1 | 1 | 1 | 2 |
| 9 | *Manihot esculenta* Crantz | 2 | 1 | 1 | 1 | 2 |
| 10 | *Antidesma bunius* (L.) Spreng. | 2 | 1 | 0 | 0 | 2 |
| 11 | *Ricinus communis* L. | 2 | 0 | 0 | 0 | 0 |
| 12 | *Jatropha curcas* L. | 2 | 0 | 0 | 0 | 0 |
| 13 | *Claoxylon polot* (Burm.) Merr. | 2 | 0 | 0 | 1 | 0 |
| 14 | *Hevea brasiliensis* (Willd.) Muell. | 2 | 1 | 1 | 0 | 2 |

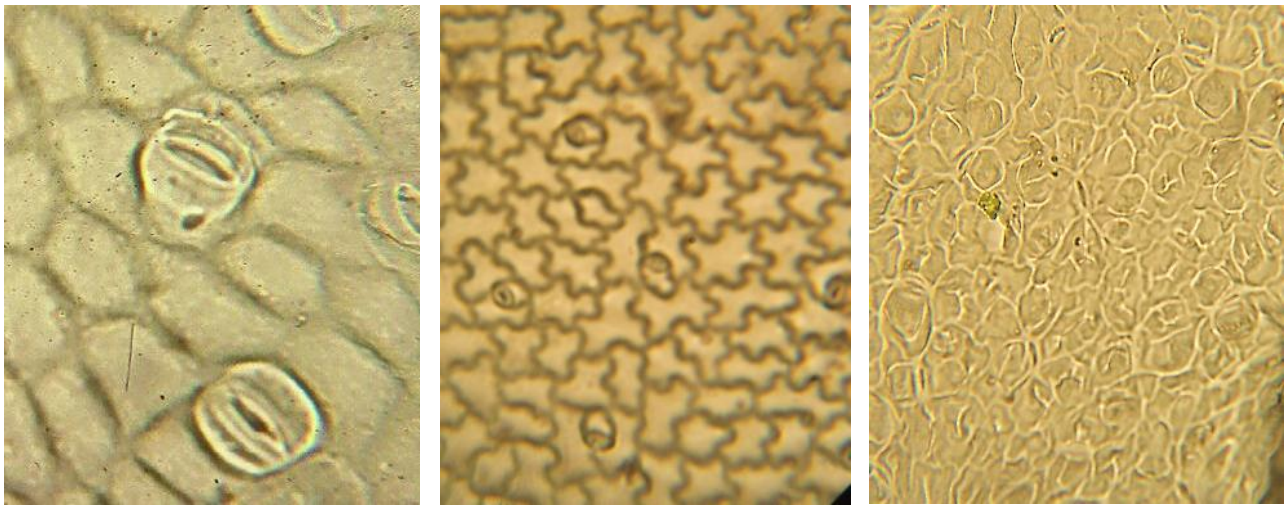
Note: * Variety of *P. niruri* that is distinguished by color of stem, yellow or green (Hadad et al., 1993). 1) Type of stomata = anomositic: 0, anisositic: 1, parasitic: 2; 2) Location of stomata = amphistomatic: 0, hypostomatic: 1; 3) Ledge = presence: 0, absence: 1; 4) Density on abaxial = ≤ 25/mm$^2$: 0, ≥25/mm$^2$: 1; 5) Density on adaxial = ≤ 10/mm$^2$: 0, ≥10/mm$^2$: 1, absence: 2. Scoring for density followed Yukawa et al. (1992). Species outgroup.

## RESULTS AND DISCUSSION

In observing the type of stomata from the studied species we found three types of stomata: parasitic, anomositic, and anisositic (Figure 1). Among them, 10 species are parasitic, one species is anomositic (*Euphorbia pulcherrima)*, and two species are anisositic (*Phyllanthus niruri* Green and *Phyllanthus niruri* Yellow). Several specimens examined have amphistomatic stomata (located at both adaxial and abaxial leaf surfaces), except *P. acidus, C. variegatum, S. androgynus, A. bunius, H. brasiliensis*, *M. esculenta*, and *E. pulcherrima*, which have hypostomatic one (located at abaxial leaf surfaces). On the basis of position of stomata relative to its epidermal cell, phaneroporic stomata is found in all species studied. Information about the density of stomata is listed in Table 2. There are eight species characterized by absence of the ledge of all specimens observed, *P. niruri* Green*, P. niruri* Yellow*, P. urinaria, P. acidus, S. androgynus, H. brasiliensis*, *M. esculenta*, and *E. pulcherrima*. In contrast, existence of the ledge are identified in the remained species.

**Table 2.** Density of euphorbian stomata.

| Species | Density (number of stomata/mm$^2$) | |
|---|---|---|
| | Abaxial | Adaxial |
| *P. niruri* Green | 48,08 | 25,74 |
| *P. niruri* Yellow | 37,08 | 12,65 |
| *P. urinaria* | 29,53 | 15,68 |
| *P. acidus* | 12,53 | - |
| *E. pulcherrima* | 36,59 | - |
| *C. variegatum* | 29,55 | - |
| *S. androgynus* | 27,35 | - |
| *M. esculenta* | 53,70 | - |
| *A. bunius* | 18,17 | - |
| *R. communis* | 20,99 | 6,08 |
| *J. curcas* | 20,26 | 5,64 |
| *C. polot* | 33,53 | 1,30 |
| *H. brasiliensis* | 18,43 | - |



**A** **B** **C**

**Figure 1.** Three types of stomata in some species of Euphorbiaceae *s.l.* A. Parasitic, B. Anisositic, C. Anomositic. Bar = 100 mm.

Phylogenetic tree obtained (Figure 2) suggests that family Euphorbiaceae is tentatively monophyletic group. We state tentative because there were only 13 species included in the phylogenetic analysis. The family is further divided into two groups. Group 1 is composed of *P*. *urinaria*, *P*. *niruri* Green, and *P*. *niruri* Yellow. With the exception of *R. communis, J. curcas,* and *C. polot*, the rest of species are housed in Group 2. As mentioned earlier, from all specimens studied there are three types of stomata, notably, parasitic, anomositic, and anisositic. This is congruent with Cronquist (1981) that these three types of stomata commonly exist in the family Euphorbiaceae. Location of stomata in Euphorbiaceae could be amphistomatic or hypostomatic. According to Susetyoadi et al. (2004) these condition are often found in mesophyte plant. In many cases, stomata is more abundant on abaxial than adaxial, even totally absence. This is one mechanism in the mesophyte plant to prevent much lost of water. The mesophyte plant is also characterized by the position of stomata that is parallel with epidermal cells (phaneropore). Unlike the mesophyte plant, hydrophyte plant is identified with prominent stomata, whereas xerophyte plant with hidden stomata; these are clearly correlated with habitat, whether plenty or poor of water respectively.

The family Euphorbiaceae is also characterized with widely variety of stomatal density (Table 2). Environmental condition and genetic factor strongly influence morphogenesis of stomata (Willmer, 1983). Environmental factors that influence the density include availability of water, intensity of light, temperature, and concentration of $CO_2$. For instance, plant with high density of stomata usually growth in condition with fully intensity of sunlight, and vice versa. The plant growth with low humidity usually has much higher density than those with high humidity. Indeed, these all factors obviously give effect for respiration and transpiration in the plant.

The ledge of stomata is presence in several genera of family Euphorbiaceae. This structure is one of the main components for opening and closing of stomata, and giving a general outer shape of stomata (Yukawa et al., 1992). The stomatal ledge will therefore determine the shape opening of stomata. In some groups of plant, the ledge is considered to be useful phylogenetic markers. In general, stomata characters of Euphorbiaceae have several advantages for use as phylogenetic markers. No shortage data, stability in shape and size, and limited collapse and shrinkage on the stomata were found during preparation.

Composition of two groups in the family as depicted in Figure 2 is generally concordant with molecular data of the family (Hidayat et al., 2008). Group 1 is inhabited by species of genus *Phyllanthus*, except *P*. *acidus* that is distant position in Group 2. Plant herb, growth erect 30-50 cm, single leave, single flower emerging at each nodus are several combination synapomorphic characters that support Group 1 (Cronquist, 1981; Ogata and Iwasaki, 1995). In contrast, Group 2 consists of genera which is characterized by plant with small to big tree, reaching more than five meters in tall, compound leaf and flower, and hypostomatic stomata. Another morphological character that support Group 2 is that they have ovary with three locus and 1-2 ovule each (Backer and Bakhuizen v.d. Brink, 1963), except *A*. *bunius* and *H*. *brasiliensis*.

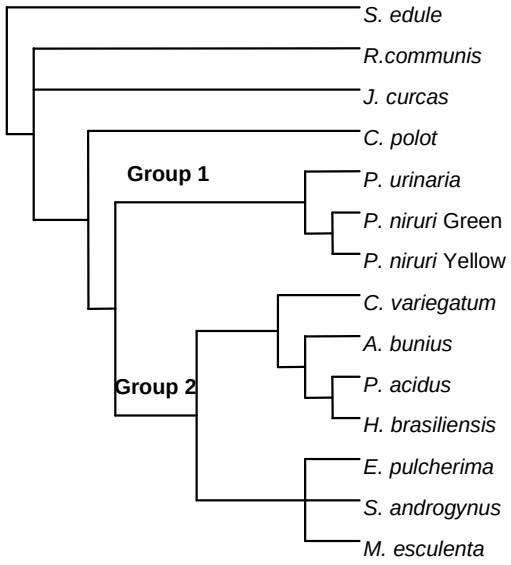



**Figure 2.** Strict consensus tree of 108 equally most parsimonious trees. Bootstrap value is not shown due to low percentages (Bootstrap Percentage/BP<50).

As we stated before, phylogenetic position of three species, namely, *R*. *communis, J*. *curcas*, and *C*. *polot* still remain unresolved. They are separated from Group 1 and 2. This indicates that character states we used do not sufficient in placing them to either Group. Surprisingly, this study revealed that *Phyllanthus* is non-monophyletic group. This result is consistent with our previous phylogenetic analysis using DNA sequences of internal transcribed spacers region (Hidayat et al., 2008). Furthermore, this information can be used as reference in attempts to perform taxonomic treatment for the genus.

This study is subjected to preliminary due to limited sampling and insufficient micromorphological information for the family. Further phylogenetic analyses with more micromorphological characters, such as trichome, and greater taxon sampling are desirable of the establishment more robust phylogenetic hypotheses for the family.

## CONCLUSION

The results of this study showed that stomatal shapes in the family Euphorboaceae *s.l.* is quite

diverse. This property is important and useful in the phylogenetic reconstruction in order to understand diversity in the family. Using characters derived from stomata, monophyletic nature of the family has tentatively been addressed: our data generally revealed two major groups in the family. However, phylogenetic status of some species studied remains unclear. Stomatal data further suggets that genus *Phyllanthus* is non-monophyletic group.

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