Leaf geometric morphometric analyses of Callicarpa and Geunsia (Lamiaceae) in the Malesian region

JENNIFER S. DANILA1,*, GRECEBIO JONATHAN D. ALEJANDRO1,2

1The Graduate School, University of Santo Tomas, España Blvd., 1015 Manila, Philippines. *email: jennifer.danila.gs@ust.edu.ph
2College of Science and Research Centre for the Natural and Applied Sciences, University of Santo Tomas, España Blvd., 1015 Manila, Philippines

Abstract. Danila JS, Alejandro GJD. 2021. Leaf geometric morphometric analyses of Callicarpa and Geunsia (Lamiaceae) in the Malesian region. Biodiversitas 22: 4379-4390. Leaves are one of the most substantial organs of plants for it serves as a basis of species identification. Leaf morphology provides distinguishing features that help in the discrimination of plant species as well as investigation of leaf features among populations. This study aimed to investigate leaf shape variations between the two genera Geunsia Blume group and its closely related taxon, Callicarpa L. (Lamiaceae) using a landmark-based geometric morphometric method. The differences in the leaf shape among former members of Geunsia, namely C. apoensis, C. basilanensis, C. flavida, C. paloensis, C. pentandra, C. ramiflora, and C. surigaensis are also evaluated. Two primary landmarks and 14 semilandmarks were assigned in all samples to represent changes around the leaf margin. The Procrustes fit was generated using MorphoJ software which displays the mean and landmark position for individual configurations. Canonical Variate Analysis (CVA) and Mahalanobis Distance (MD) were able to discriminate all samples of Geunsia species using a scatter plot. Furthermore, Procrustes ANOVA showed a significant difference ($P = 0.0082$) among the seven species of the Geunsia group. Based on the results obtained, geometric morphometrics of leaf shape is effective in interspecific discrimination within members of Geunsia. However, the result of Discriminant Analysis (DA) showed that Geunsia and Callicarpa groups made leaf shape differences inefficient in discriminating the two genera. Therefore, further morphological studies on landmark-based geometric morphometrics of leaf shape involving a larger number of samples especially in the study of intergeneric classification are suggested.

Keywords: Callicarpa, Canonical Variate Analysis, Geunsia, landmark, leaf shapes, mahalanobis distance, procrustes analysis

INTRODUCTION

The taxonomy between Callicarpa L. and Geunsia Blume was among the most controversial genera in the family Lamiaceae (Bramley 2009). Since Geunsia was described by Blume (1823), members of the group have been known as synonyms of several Callicarpa species. Currently, Geunsia is regarded as a synonym of the genus Callicarpa (Cantino 1992; Harley et al. 2004; Bramley 2009; IPNI 2021; POWO 2021) based on the examination of morphological characters (Lam and Bakhuiizen van den Brink 1921; van Steenis 1967; Govaerts et al. 2007) and molecular data (Bramley 2009). However, several rejections of previous characters (Lam 1919; Lam and Bakhuiizen van den Brink 1921; Ridley 1923; Moldenke 1982) continue to challenge the relationship of the two genera.

The most widespread, C. pentandra Roxb. together with other former members of the Geunsia group, namely C. basilanensis Merr., C. flavida Elmer, C. ramiflora Merr., C. surigaensis Merr., and C. apoensis Elmer led to a complicated taxonomic relationship between Geunsia and Callicarpa (Bramley 2011). The inclusion of several species like C. pentandra and its affinities in the genus Callicarpa led to a complex species classification whether to separate conspecific species or retain its infraspecific classification. According to Lam (1919), species of Geunsia can be distinguished through leaf arrangement, conspicuous ridge in the petioles, anthers opened by widening of the upper part of the parallel fissures, and the ovary (three-)five-celled, the cells are two seeded. However, the circumscription of the genus Geunsia remains uncertain due to overlapping characters (Lam 1919; Bramley 2009; Danila and Alejandro 2020). The members of Geunsia in the Philippines as having similar features to the three varieties of C. pentandra based on indumentum but classification seems inappropriate due to various overlapping of species (Lam and Bakhuiizen van den Brink 1921). Based on the taxonomic revision of Philippine Callicarpa reported by Bramley (2013), members of Geunsia have been classified and showed shared characters, e.g., alternate leaves, 4-merous flowers, and shape of corolla and anthers but appeared distinct based on leaf morphology and inflorescence. To avoid confusion, he also conducted a further leaf morphological study to classify species but characters like leaf shapes appeared insufficient to successfully separate species using identification key because leaf shape is very variable across its distribution.

The taxonomy of Geunsia is extremely complex due to wide geographical distribution, species overlap, high levels of variability, and phenotypic plasticity (Kawakubo 1986; Palmer 2012). These variables were often observed through their effects on the leaf structures (Vieira et al. 2014; Li et al. 2018; Morais et al. 2019). Therefore, leaf morphology is considered the most valuable answer in identifying taxa.
particularly for species belonging to the family Lamiaceae (Balant et al. 2019). Thus, leaf description has great relevance for its species discrimination along with effective diagnostic techniques and procedures. However, leaves vary in shape features depending on their leaf tips, leaf bases, and leaf margin. Currently, no studies have identified the leaf variation among Callicarpa and Geunsia groups to further separate or combine species based on leaf shapes. This study described for the first-time accessions of members of Geunsia and Callicarpa within the Malesian region through leaf geometric morphometrics, contributing to a better understanding of the species variation through leaf shapes. Furthermore, this study aimed to discriminate Geunsia and Callicarpa species and determine taxonomic characters to differentiate Geunsia and Callicarpa species. Thus, a promising approach to species characterization like geometric morphometrics (Viscosi and Cardini 2011) to assess complicated genera like Geunsia and Callicarpa is hereby introduced.

MATERIALS AND METHODS

Study area

Leaf morphology and morphometric study were carried out from selected digital herbarium samples of A, HBG, K, L, MW, NY, P, SNSB, USTH, and US (abbreviations following Index Herbariorum, Thiers) and field collections in various localities of the Philippines (Table 1). This study focuses on specimens of Geunsia and Callicarpa collected in the selected localities in the Malesian region (Figure 1). A total of 96 leaflets were sampled from 63 online accessions and 33 individual specimens from the field. Online accessions were obtained in Global Biodiversity Information Facility (GBIF) database via the web interface, while field collections were obtained from the open lowland to hill forests of the provinces of Isabela, Sorsogon, and Samar in the Philippines. Online images and descriptions were downloaded using 'Darwin Core Archive' format, which contains the URLs of the images and information in GBIF (Table S1). On the other hand, samples from the field have undergone herbarium sample collection protocol of preparation, pressing, mounting, identification, and deliberation of voucher specimen to the University of Santo Tomas Herbarium (USTH), the Philippines.

Procedures: Sampling preparation and data gathering

An initial of 96 leaflets were used to create a file for data manipulation and digitization. Based on the digital images, TPS files were created using tpsUtil32 software (Rohlf 2015). This TPS file contains the landmarks and image IDs to be used in digitizing images. In digitization, tpsDig232 software (Rohlf 2010) was used to assign landmarks on each image. Coordinates of 16 key landmarks of leaves were obtained to perform morphometric analysis (Figure 2.A). The abaxial surface of leaves was preferable because of the prominent secondary veins. Landmarks 1 and 2 were assigned, which corresponds to the junction of the petiole to the leaf tip of the primary vein (Gunz and Mitteroecker 2013).

Figure 1. Localities of selected Geunsia and Callicarpa species used in this study in the Malesian region. Philippines (A-H): A. Bangsamoro (Basilan, Cotabato, Lanao, Sulu), B. Bicol (Catanduanes, Sorsogon), C. Caraga (Agusan, Dinagat, Surigao), D. Eastern Visayas (Samar, Leyte), E. Central Visayas (Cebu), F. Davao G. Isabela, H. Zamboanga Peninsula, I. China, (J-K): Indonesia J. Kalimantan K. Sulawesi, L. Malaysia, M. Myanmar, N. Papua New Guinea, O. Vietnam, P. Singapore. (Map: www.scribblesmaps.com).
Table 1. Populations and samples of *Geunsia* and *Callicarpa* groups were used in this study

| Species     | Localities                  | Acc. no.               | Herbaria                                                                 |
|-------------|-----------------------------|------------------------|--------------------------------------------------------------------------|
| *Geunsia*   |                             |                        |                                                                          |
| *C. apoensis* Elmer | Indonesia                  | L4316447               | Naturalis Biodiversity Center (AMD)                                    |
|             | Indonesia                   | L4316456               | Naturalis Biodiversity Center (AMD)                                    |
|             | Davao, Philippines           | HBG513403              | Herbarium Hamburgense (HBG)                                             |
|             | Davao, Philippines           | P03392618              | MNHN - Museum National d'Histoire Naturelle (P)                         |
|             | Davao, Philippines           | K000194994             | Royal Botanic Gardens, Kew (K)                                         |
| *C. basilanensis* Merr. | Basilan, Philippines       | L2762581               | Naturalis Biodiversity Center (AMD)                                    |
|             | Zamboanga, Mindanao         | 714485                 | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Basilan, Philippines        | 119043                 | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Zamboanga, Philippines      | P03597180              | MNHN - Museum National d'Histoire Naturelle (P)                         |
|             | Basilan, Philippines        | P03392619              | MNHN - Museum National d'Histoire Naturelle (P)                         |
| *C. flavida* Elmer | Agusan, Philippines       | 119053                 | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Dinagat Island, Philippines | L2753770               | Naturalis Biodiversity Center (AMD)                                    |
|             | Agusan, Philippines         | 2270734                | Missouri Botanical Garden (MO)                                          |
|             | Agusan, Philippines         | 2787903                | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Agusan, Philippines         | 93872                  | Harvard University Herbaria (HUH)                                       |
|             | Agusan, Philippines         | 93873                  | Harvard University Herbaria (HUH)                                       |
|             | Agusan, Philippines         | U0000702               | Naturalis Biodiversity Center (AMD)                                    |
|             | Davao, Philippines          | 119056                 | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Davao, Philippines          | 93875                  | Harvard University Herbaria (HUH)                                       |
|             | Davao, Philippines          | L2753771               | Naturalis Biodiversity Center (AMD)                                    |
|             | Davao, Philippines          | K000194944             | Royal Botanic Gardens, Kew (K)                                         |
| *C. paloensis* Elmer | Sorsogon, Philippines     | 2787975                | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Sorsogon, Philippines       | 2787974                | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Pal, Leyte                  | 103990                 | The New York Botanical Garden (NY)                                      |
|             | Pal, Leyte                  | 93883                  | Harvard University Herbaria (HUH)                                       |
|             | Sulavesi, Indonesia         | L2757720               | Naturalis Biodiversity Center (AMD)                                    |
|             | Sulu, Philippines           | P03598409              | MNHN - Museum National d'Histoire Naturelle (P)                         |
|             | Agusan, Mindanao            | K000194946             | Royal Botanic Gardens, Kew (K)                                         |
|             | Agusan, Mindanao            | K000194949             | Royal Botanic Gardens, Kew (K)                                         |
| *C. pentandra* Roxb. | Cebu, Philippines          | K000248661             | Royal Botanic Gardens, Kew (K)                                         |
|             | Kalimantan, Indonesia       | K00094945              | Royal Botanic Gardens, Kew (K)                                         |
|             | Davao, Philippines          | K000194931             | Royal Botanic Gardens, Kew (K)                                         |
|             | Papua New Guinea            | K000194977             | Royal Botanic Gardens, Kew (K)                                         |
|             | Kalimantan, Indonesia       | K000249402             | Royal Botanic Gardens, Kew (K)                                         |
|             | Surigao, Philippines        | K000194992             | Royal Botanic Gardens, Kew (K)                                         |
|             | Davao, Philippines          | K000194930             | Royal Botanic Gardens, Kew (K)                                         |
|             | Cebu, Philippines           | K000248662             | Royal Botanic Gardens, Kew (K)                                         |
|             | Singapore                   | K001114363             | Royal Botanic Gardens, Kew (K)                                         |
|             | Samar, Philippines          | JDS008                 | University of Santo Tomas, Philippines (USTH)                          |
| *C. ramiflora* Merr. | Samar, Philippines          | K000674786             | Royal Botanic Gardens, Kew (K)                                         |
|             | Catanduanes, Philippines   | P00400805              | Naturalis Biodiversity Center (AMD)                                    |
|             | Leyte, Philippines          | 2788026                | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Leyte, Philippines          | L2762376               | Naturalis Biodiversity Center (AMD)                                    |
|             | Catanduanes, Philippines   | 2788025                | Naturalis Biodiversity Center (AMD)                                    |
|             | Leyte, Philippines          | L2754623              | Naturalis Biodiversity Center (AMD)                                    |
|             | Zamboanga, Philippines     | 119049                 | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Lanao del Sur, Philippines | 119084                 | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Lanao Lake, Mindanao        | M0111549               | Staatliche Naturwissenschaftliche Sammlungen Bayerns (SNSB)              |
|             | Catanduanes, Philippines   | P03598371              | MNHN - Museum national d'Histoire Naturelle (P)                         |
| *C. surigaensis* Merr. | Surigao, Philippines       | 2788017                | Smithsonian Institution, NMNH, Botany (US)                              |
|             | Surigao, Philippines        | 2788016                | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Surigao, Philippines        | 1269210                | Smithsonian Institution, NMNH, Botany (US)                              |
|             | Surigao, Philippines        | 1269211                | Smithsonian Institution, NMNH, Botany (US)                              |
|             | Surigao, Philippines        | 1107985                | Smithsonian Institution, NMNH, Botany (US)                              |
|             | Surigao, Philippines        | P03598359              | MNHN - Museum national d'Histoire Naturelle (P)                         |
| *Callicarpa* |                             |                        |                                                                          |
| *C. arborea* Roxb.  | Samar, Philippines          | JE8008                 | University of Santo Tomas, Philippines (USTH)                          |
| *C. eriococlona* Schauer | Sorsogon, Philippines    | SOR006                 | University of Santo Tomas, Philippines (USTH)                          |
| *C. pedunculata* R.Br. | Isabela, Philippines      | JDS001                 | University of Santo Tomas, Philippines (USTH)                          |
| *C. rubella* Lindl. | Vietnam                    | MW0757612              | Lomonosov Moscow State University (MW)                                  |
|             | Vietnam                     | 2808046                | The New York Botanical Garden (NY)                                      |
|             | Vietnam                     | P00991455              | MNHN - Museum National d'Histoire Naturelle (P)                         |
|             | Myanmar                     | 2648823                | The New York Botanical Garden (NY)                                      |
|             | China                       | 2787428                | National Museum of Natural History, Smithsonian Institution (US)        |
|             | Myanmar                     | 3231815                | The New York Botanical Garden (NY)                                      |
|             | Malaysia                    | L0534846               | Smithsonian Institution, NMNH, Botany (US)                              |
Figure 2. Leaf morphometrics. A. Abaxial surface with primary landmarks (1-2) and semilandmarks (3-16) of C. pentandra (Photo: University of Minnesota Bell Museum (2021). Bell Museum plants. Occurrence dataset https://doi.org/10.15468/bihrxd accessed via GBIF.org on 2021-08-02. https://www.gbif.org/occurrence/2265568305). B. Mean landmarks (large dots) and individual landmarks in all samples of Geunsia and Callicarpa (small dots) before elimination of outliers

Fourteen semi landmarks of secondary veins were assigned to represent changes around the leaf margin. The number of semi landmarks determined in a study depends on the complexity of curves and the position of the veins (Gunz and Mitteroecker 2013). After assigning landmarks, consensus files were generated from landmark images used in the populations of Geunsia and Callicarpa species. To create a data set, the TPS file was imported into the software package MorphoJ (Klingenberg 2011). The Procrustes fit was generated, showing the mean and landmark position for individual configurations in all samples (Figure 2.B). A preliminary transformation was conducted by eliminating outliers within the scatter plot generated during Principal Component Analysis (PCA) (Hotelling 1933). In this study, PC1 and PC2 were used to identify outliers. Potential outliers were detected by identifying data points separated from the main scatter plot ellipses (Viscosi and Cardini 2011) and cross-checked for further leaf shape deviation using MorphoJ. Outliers were deleted from the data before the computation of the mean leaf configurations of the final dataset. Outliers may contain noise that was obtained from the digital images and may affect the accuracy of the study (Du et al. 2007).

Data analysis
Multivariate analysis of outliers
In the study between Geunsia and Callicarpa groups, the analysis led to the identification of 12 outliers out of 96 data points of leaves (12.50%) observed in PC1 and PC2. Geunsia and Callicarpa have percentage values of outliers, 9.43%, and 16.28%, respectively. The new set of samples consisted of 84 leaflets from 36 and 48 leaflets of Callicarpa and Geunsia, respectively. Likewise, a separate analysis of outliers was done exclusively for the species of Geunsia, which led to the identification of 13 outliers out of 53 leaves (25%) thereby creating a new dataset of 40 leaflets. All the outliers identified through this procedure were eliminated from the data matrices of landmarks for each sample. All samples were separately extracted to create new datasets for subsequent multivariate analysis of intergeneric and interspecific classification (Figure 3.A-B).

Figure 3. Selection of outliers (encircled) among samples of: A. Geunsia and Callicarpa (black: Callicarpa; blue: Geunsia; B. combined seven species of Geunsia (black: randomly selected species of Geunsia)
Multivariate analysis of leaf shape variability

The new dataset was subjected to various statistical analyses like PCA, Canonical Variate Analysis (CVA), and Discriminant Function Analysis (DFA). PCA was used to check the total leaf shape variation of 84 individuals of combined Geunsia and Callicarpa samples and 40 individuals of the Geunsia species. PCA treats all configurations for each sample regardless of species or any form of group classification into one population. Principal component axes or shape trends were visualized through thin-plate spline reconstructions of the shapes which correspond to the leaf changes of the significant PCs. Likewise, Eigenvalues were included to measure the amount of variation explained on each PCs. In this study, data were assessed between Geunsia and Callicarpa groups and within the treatment group. CVA was used to classify samples into known groups by measuring the strength of the relationship based on the highest correlated calculation (Klingenberg 2011). The canonical variates (CVs) show which axes best discriminate between the group and within-group variation (Zelditch et al. 2004). The discrimination was best observed when using a scatterplot which allows separation of species based on treatment groups assigned. In this study, CVA was performed to discriminate samples of Geunsia based on species. Procrustes and Mahalanobis distances were also used to show patterns of morphological similarity between groups of Callicarpa and Geunsia species and among members of Geunsia through pairwise comparison. Mahalanobis Distance (MD) measures the distance between a distinct point of the specimen relative to the mean position of all points of an object. In this case, one can estimate the degree of a specimen to belong to a group whose mean is the closest (Viscosi and Cardini 2011). In comparison, Procrustes Distance (PD) refers to the measure of the absolute magnitude of shape deviation based on mean landmark changes of two samples to quantify the similarity and differences (Bookstein 1996). Likewise, a cross-validation test (permutation test with 10,000 rounds) was performed to evaluate the classification accuracy of Geunsia and Callicarpa and among members of Geunsia based on MD. The Procrustes ANOVA (Analysis of Variance) was also used for testing the shape differences among the samples of the Geunsia group if statistically significant (p ≤ 0.05). All analyses were performed using the software MorphoJ (Klingenberg 2011).

RESULTS AND DISCUSSION

Leaf geometric morphometrics between Geunsia and Callicarpa species

Significant variations from all 84 samples mostly occurred in the first four principal components (PC1, PC2, PC3, and PC4) with a cumulative variance of 77.24% (Table 2). The proportion of variance method to retain a considerable number of PCs which accounts from the highest percentage to 5-10% of the total variance was considered, whereby the proportion of variance is obtained by dividing eigenvalue for the component of interest by total eigenvalues of the correlation matrix (Jackson 1993). All Geunsia and Callicarpa species were united inside their respective ellipses (Figure 4A-B). However, the two ellipses in both diagrams showed an overlap which suggests that leaf shapes similarities exist between groups. The PC1 score (31.49%) represents changes in the width of the leaf resulting in ovate to narrowly ovate leaves (Figure 4C). The PC2 score (23.31%) represents a change in the leaf shape showing a minimal extension of the apical and distal region of the leaf. While PC3 and PC4 both constitute a low score, 14.19%, and 8.26%, respectively, but the changes became conspicuous due to asymmetric variation on the leaf shape mostly found either on one side of bases or tips of the leaves.

Comparisons between the groups were performed using the Canonical Variate Analysis (CVA), wherein the first canonical variate (CV1) was accounted for 100% of the total variance (Table 3). MD and PD were also obtained by pairwise comparison between the Geunsia and Callicarpa and showed highly significant differences (permutation 10,000 rounds in MorphoJ: P<.0001) of 1.7963 and 0.0569, respectively. A significant difference in the P-values from the permutation test for MD and PD among groups were <.0001 and 0.0024, respectively. Most of the shape changes between the groups generally accounted for the width of the leaf. Despite this, the result of Discriminant Analysis (DA) (Figure 4D, Hotelling’s T² = 66.39, P-value = 0.0708) in comparing the two taxa showed that leaf shape does not differentiate them sufficiently to allow distinction. Likewise, the cross-validation of correctly classified Callicarpa (61.11%) and Geunsia (60.42%) showed low percentage and overlap made leaf shape difference ineffective for separating genus Geunsia from Callicarpa.

Table 2. The first ten of a Principal Component Analysis (PCA) of leaf shape in 84 individuals of Callicarpa and Geunsia species

| PC | Eigenvalues | % Variance | Cumulative % |
|----|-------------|------------|--------------|
| 1  | 0.00585     | 31.488     | 31.488*      |
| 2  | 0.00433     | 23.306     | 54.795*      |
| 3  | 0.00264     | 14.185     | 68.98*       |
| 4  | 0.00154     | 8.263      | 77.243*      |
| 5  | 0.00088     | 4.748      | 81.991       |
| 6  | 0.00076     | 4.097      | 86.088       |
| 7  | 0.00057     | 3.073      | 89.16        |
| 8  | 0.00043     | 2.337      | 91.497       |
| 9  | 0.00029     | 1.544      | 93.042       |
| 10 | 0.00026     | 1.379      | 94.421       |

*Note: Only the first four are significant according to the proportion of variance with 5-10% of the total variance. Eigenvalues are derived from a matrix of 84 Procrustes-aligned configurations of 16 landmarks.
The morphometric approach confirmed leaf shape variations between Geunsia and Callicarpa (Figure 4.C). In addition, the extremely reconstructed outlines along the axes and the scatter plot (Figure 4.A-B) showed that Geunsia and Callicarpa correspond to the ovate to narrowly ovate shape pattern. This pattern is supported by collections of the field and herbarium (Bramley 2009, 2013). However, species discrimination based on CVA and DA analyses does not show a reliable pattern that distinguishes the two genera (Figure 4.D). Thus, a weak indication of leaf shape differentiation between the two taxa suggests a tendency for some samples to be less distant from the other, resulting in an overlap in the intergeneric comparison. According to Adebowale et al. (2012), an overlap in morphological characters like leaf shape in the application of geometric morphometrics could have environmental and genetic explanations. Moreover, an overlap manifested in the intergeneric assessment is possibly due to the presence of hybrid in the individual samples in species occupying similar habitat; thus similarity in leaf shape could reflect evolutionary adaptation to environmental conditions (Adebowale et al. 2012; Mirouze et al. 2012). Callicarpa has been reported to show the incidence of hybridization in several plant species (Tsukaya et al. 2003). Hence, this may affect the result of geometric morphometrics to discriminate Geunsia and Callicarpa and may underline the limitation of a single approach in solving taxonomic problems.
Leaf geometric morphometrics among members of *Geunsia* species

After eliminating the outliers, the Principal Component Analysis (PCA) from all 40 samples of the *Geunsia* group is presented in Table 4. The PCA indicated that the first five components (PC1, PC2, PC3, PC4, and PC5) were responsible for 82.61% of the significant leaf shape variation obtained from the selected samples of *Geunsia*. PC1 of 33.02% showed the highest variation representing leaf tip changes from acute to attenuate and a minimal expansion of the leaf bases. PC2 (25.87%) was responsible for width changes of leaves from narrowly ovate to ovate shape. While PC3, PC4, and PC5 of 9.42%, 8.18%, and 6.13%, respectively refer to the non-symmetric shape patterns of the leaf (Table 4, Figure 5.A). PC3 is responsible for unequal extension of the apical region with a slight curvature on its tip and a minimal contraction in the proximal part of the leaf, while PC4 and PC5 showed inconspicuous expansion in the width, distal and proximal part of the leaf.

### Table 4. The Principal Component Analysis (PCA) of leaf shape in 40 individuals of *Geunsia* species

| PC | Eigenvalues | % Variance | Cumulative % |
|----|-------------|------------|--------------|
| 1  | 0.0048015   | 33.022     | 33.022*      |
| 2  | 0.0037616   | 25.871     | 58.893*      |
| 3  | 0.0013693   | 9.417      | 68.310*      |
| 4  | 0.0011886   | 8.175      | 76.485*      |
| 5  | 0.0008905   | 6.125      | 82.610*      |
| 6  | 0.0006784   | 4.665      | 87.275       |
| 7  | 0.0003981   | 2.738      | 90.013       |
| 8  | 0.0003150   | 2.166      | 92.180       |
| 9  | 0.0002201   | 1.513      | 93.693       |
| 10 | 0.0001875   | 1.290      | 94.983       |

*Note: Only the first five are significant according to the proportion of variance with 5-10% of the total variance. Eigenvalues are derived from a matrix of 40 Procrustes-aligned configurations of 16 landmarks.*

![Figure 5](image1.png)  
**Figure 5.** Analysis in all leaf samples of 40 individuals of *Geunsia* group. A. Axis components of PC1 (33.02%), PC2 (25.87%), PC3 (9.42%), PC4 (8.18%), and PC5 (6.13%) showing the variation in leaf shape of *Geunsia* group; B. The canonical variate analysis (CVA) [C. apoensis (red), C. basilanensis (yellow), C.flavida (green), C. paloensis (light blue), C. pentandra (blue), C. ramiflora (dark blue), and C. surigaensis (purple)].

![Figure 6](image2.png)  
**Figure 6.** Transformation grids illustrate the shape changes from the overall mean shape of *C. pentandra* among other members of *Geunsia*. A. *C. apoensis*, B. *C. basilanensis*, C. *C. flavida*, D. *C. paloensis*, E. *C. ramiflora*, F. *C. surigaensis*. Dots indicate the mean shape of *C. pentandra* and the line represents the change in leaf shape relative to the position of other members of *Geunsia*.
Canonical Variate Analysis (CVA) was able to generate six variables among samples of the Geunsia species (Table 5). The scatter plot of CV1 and CV2 were accounted for 68.88% of the variation and shows that all samples (C. basilanensis, C. pentandra, C. flavida, C. ramiflora, C. paloensis, C. apoensis, and C. surigaensis) were clearly identified and successfully placed into its distinct groups (Figure 5B). Likewise, the MD scores were obtained, which range from 6.3015 (C. ramiflora and C. apoensis) to 12.6607 (C. flavida and C. basilanensis) by pairwise comparisons (Table 6). Using multivariate normal distribution, one can estimate the degree of a specimen to belong to a group whose mean is the closest (Viscosi and Cardini 2011). In this study, C. ramiflora and C. apoensis showed the nearest distance to the central point or the overall mean of data points in all samples. Furthermore, all samples used in the pairwise comparison was statistically significant in their distance relative to the centroid (Table 7). Thus, MD provided an effective and convenient way to quantitatively compare the magnitude of cluster separation in an ambiguous taxon like the Geunsia species. While PD scores ranged from 0.0684 (C. ramiflora and C. pentandra) to 0.1607 (C. ramiflora and C. flavida) also showed highly significant differences on a few samples of the Geunsia group (Table 8). Procrustes distance showed significant difference among C. ramiflora and C. flavida (<0.001), C. pentandra and C. flavida (0.0003), C. basilanensis and C. flavida (0.0048), C. flavida and C. apoensis (0.0099) and C. paloensis and C. flavida (0.0103) (Table 9). In this regard, the difference among these species could be explained by their leaf shape difference. Likewise, Procrustes ANOVA results showed significant differences among populations of Geunsia species (Table 10). Leaf shape differences among taxa were statistically significant (P = 0.0082), and strong clustering made overall leaf shape difference effective in separating members of Geunsia.

Table 5. Canonical variate analysis (CVA) of 40 samples of Geunsia species

| CV  | Eigenvalues | % Variance | Cumulative % |
|-----|-------------|------------|--------------|
| 1   | 15.920517   | 44.200     | 44.200       |
| 2   | 8.8924508   | 24.688     | 68.887       |
| 3   | 7.7232715   | 13.113     | 82.000       |
| 4   | 6.9410219   | 8.165      | 90.166       |
| 5   | 2.0241605   | 5.620      | 95.785       |
| 6   | 1.5181756   | 4.215      | 100.000      |

Table 6. Mahalanobis distances among groups showed differences in leaf shape of Geunsia species among groups based on species analyzed with Canonical Variate Analysis (CVA)

|      | BAS       | FLA       | PAL       | PEN       | RAM       |
|------|-----------|-----------|-----------|-----------|-----------|
| APO  | 10.5466   | 12.6607   | 10.025    | 7.2779    | 6.892     |
| FLA  | 8.8952    | 12.6607   | 10.025    | 7.2779    | 6.892     |
| PAL  | 10.5837   | 9.9683    | 6.4654    | 7.2779    | 6.892     |
| PEN  | 7.9616    | 9.9683    | 6.4654    | 7.2779    | 6.892     |
| RAM  | 6.3015    | 8.2473    | 9.4752    | 7.7460    | 6.3831    |
| SUR  | 8.3237    | 11.801    | 10.0538   | 6.7831    | 8.0964    |

Note: C. apoensis (APO), C. basilanensis (BAS), C. flavida (FLA), C. paloensis (PAL), C. pentandra (PEN), C. ramiflora (RAM), and C. surigaensis (SUR).

Table 7. P-values from permutation tests (10000 permutation rounds) for Mahalanobis Distances among groups

|      | BAS       | FLA       | PAL       | PEN       | RAM       |
|------|-----------|-----------|-----------|-----------|-----------|
| APO  | 0.0128    | 0.0002    | 0.0044    | 0.0001    | <0.0001   |
| FLA  | 0.0002    | 0.0036    | 0.0004    | 0.0001    | 0.0006    |
| PAL  | 0.0044    | 0.0342    | 0.0004    | 0.0001    | 0.0006    |
| PEN  | 0.0001    | 0.0014    | 0.0004    | 0.0001    | <0.0001   |
| RAM  | <0.0001   | <0.0001   | <0.0001   | 0.0029    | <0.0001   |
| SUR  | 0.0071    | <0.0001   | 0.0048    | 0.0188    | 0.0039    |

Note: All values were statistically significant. (permutation 10,000 rounds in Morpho; P < 0.0001)

Table 8. Procrustes Distances among groups showed differences in leaf shape of Geunsia species among groups based on species analyzed with canonical variate analysis (CVA)

|      | BAS       | FLA       | PAL       | PEN       | RAM       |
|------|-----------|-----------|-----------|-----------|-----------|
| APO  | 0.1223    | 0.1447    | 0.0998    | 0.0766    | 0.0684    |
| FLA  | 0.1006    | 0.1447    | 0.1202    | 0.0995    | 0.1176    |
| PAL  | 0.0805    | 0.067     | 0.1607    | 0.0955    | 0.1176    |
| PEN  | 0.095     | 0.0764    | 0.1424    | 0.0766    | 0.1149    |
| RAM  | 0.0831    | 0.1202    | 0.1607    | 0.0955    | 0.1176    |
| SUR  | 0.0842    | 0.1463    | 0.0929    | 0.0999    | 0.1176    |

Note: see the previous table (abbreviations)
Table 9. P-values from permutation tests (10000 permutation rounds) for Procrustes Distances among groups

| Source  | APO  | BAS  | FLA  | PAL  | PEN  | RAM  |
|---------|------|------|------|------|------|------|
| BAS     | 0.0547 |      |      |      |      |      |
| FLA     | 0.0099 | 0.0048 |      |      |      |      |
| PAL     | 0.3414 | 0.6587 | 0.0103 |      |      |      |
| PEN     | 0.1140 | 0.5676 | 0.0003 | 0.4064 |      |      |
| RAM     | 0.1689 | 0.0802 | <0.0001 | 0.1056 | 0.2913 |      |
| SUR     | 0.7019 | 0.308 | 0.2708 | 0.5625 | 0.2192 | 0.1526 |

Note: Statistically significant (bold) and not significant (narrow) (permutation 10,000 rounds in MorphoJ: \( P < 0.0001 \))
Note: see the previous table (abbreviations)

Table 10. Shape, procrustes ANOVA among Geunsia species

| Source    | SS      | MS      | Df | F      | P     |
|-----------|---------|---------|----|--------|-------|
| Between Treatments | 636.970461 | 106.161744 | 6  | 3.53   | 0.0082 |
| Within Treatments | 991.703317 | 30.051616  | 33 |        |       |

Note: SS = the sum of squares; MS = mean of the square; Df = degrees of freedom; F = F-value; P = P-value

According to Lam and Bakuizen van den Brink (1921 and Bramley (2013), C. pentandra shares morphological characters within the species of the Geunsia group. For example, a closely related species, C. flavida shares morphological characters like alternate leaves, 4-5-meros flowers, and the shape of corolla and anthers with C. pentandra. In a pairwise comparison of C. pentandra with other members of Geunsia, C. flavida showed the nearest distance to the centroid (MD=6.4654) relative to C. pentandra. All specimens of the two taxa were clustered and showed distinct groups. Similarly, PD (0.1424) (Figure 6.C) reveals a significant difference (\( P = 0.0003 \)) (Table 9) between C. pentandra and C. flavida. The two taxa show conspicuous leaf shape variations represented by width expansion from ovate to narrowly ovate. Thus, this study showed that leaf morphometrics has great relevance in the discrimination of species belonging to Geunsia group.

The study of seven Geunsia species using geometric morphometric analyses uncovered important leaf shape characters to discriminate species. The transformation grids’ shape changes from the overall mean shape confirmed that leaf tips, leaf bases, and width expansion are the main diagnostic characters (Figure 5.A). This also proved that symmetric variations play a major role in determining leaf shape among Geunsia species. As symmetric variations dominate overall changes in the leaf shape, asymmetrical variations were also observed among the Geunsia species showing a left-right pattern at the basal and apical ends of the leaves (Figure 5.A). Fluctuating Asymmetry (FA) (van Valen 1962) may exist in Geunsia samples, whereas they normally exist as bilaterally symmetrical on leaf shapes. FA usually occurs when species from the same population show abnormal growth resulting in unequal development, leading to asymmetrical morphological characters (Leary and Allendorf 1989). Several studies linked FA with environmental and genomic factors that may affect an organism’s overall development (Covart and Graham 1999; Mal 2002). Likewise, the result of the CVA analysis in comparing the species of Geunsia showed that leaf shape discriminated them sufficiently to allow identification. At the same time, MD and PD among groups of Geunsia species analyzed with CVA identify species that are closely related to each other based on leaf shape. It reveals that leaf shape showed to be a stable character and informative on the phylogenetic and evolutionary relationship of the Geunsia species.

In conclusion, geometric morphometric analysis of leaf shapes was highly reliable and could be used as an additional method to discriminate members of the genus Geunsia. The existence of morphological distinction in leaf variations, such as acute to attenuate leaf tips and ovate to narrowly ovate leaf shapes can be useful in grouping members of Geunsia. Leaf characters like leaf shapes and leaf tips were recognized as important diagnostic characters in the study of Geunsia species. This method also allows complicated species like C. pentandra to distinguish and identify their relationship with other members of the Geunsia group. This study can be a precursor for any future morphological, environmental and genomic analysis to investigate the relationship of the members of Geunsia with its closely related species like members of the genus Callicarpa. Future morphological studies on landmark-based geometric morphometric analysis of leaf shape involving a larger number of samples are encouraged in the study of interspecific and intergeneric classification.

ACKNOWLEDGEMENTS

The authors would like to thank the curators of A. HBG, K, L, MW, NY, P, SNSB, US, and USTH and the Global Biodiversity Information Facility (GBIF) for allowing us to access their digital collections for study. We thank the curator of the University of Santo Tomas (USTH) for giving us accessions to our specimens. Likewise, we would like to thank the Department of Environment and Natural Resources (DENR) for granting us the gratuitous permit to collect specimens in Isabela, Samar, and...
Sorsogon province. Lastly, the first author also would like to thank the Commission on Higher Education (CHED) for the Scholarship and Dissertation grant.

REFERENCES

Adebowale A, Nicholas A, Lamb J, Naidoo Y. 2012. Elliptic fourier analysis of leaf shape in southern African *Strechococcus* section *Deniflorae* (Loganiaceae). Bot J Linn Soc 170 (4): 542-553. DOI: 10.1111/j.1095-8339.2012.01308.x

Balant M, Glasnović P, Piler Pečnikar Ž, Clementi M, Surina B. 2019. In search of an identity for *Salvia bertoloni* (Lamiaceae). Phytotaxa 413 (2): 117-136. doi: 10.11646/phytotaxa.413.2.2

Blume CL. 1823. Catalogus van Eemige der Merkwaardigste Zoo in- Als Uit-Heemsche Gewassen, te Vinden in’s Lands Plantentuin te Buitenzorg, Batavia.

Bookstein FL. 1996. Combining the tools of geo- metric morphometrics. In: Marcus LF, Corti M, Loy A, Naylor GJF, Slice DE (eds) Advances in Morphometrics. Plenum, New York. DOI: 10.1007/978-1-4757-9083-2_12

Bramley G. 2009. The genus *Callicarpa* (Lamiaceae) on Borneo. Bot J Linn Soc 159 (3): 416-455. DOI: 10.1111/j.1095-8339.2009.09070.x

Bramley G. 2011. Distribution patterns in Malesean *Callicarpa* (Lamiaceae). Gard Bull Singapore 63 (1 & 2): 287-298.

Bramley G. 2013. The genus *Callicarpa* (Lamiaceae) in the Philippines. Kew Bull 68 (3): 369-418. DOI: 10.1007/s12225-013-9456-y

Cantino PD. 1992. Evidence for a polyphylectic origin of the Labiatae. Ann Missouri Bot Gard 79 (2): 361-379. DOI: 10.2307/2399774

Cowart NM, Graham JH. 1999. Within- and among individual variation in fluctuating asymmetry of leaves in the fig (*Ficus carica L.*). Int J Plant Sci 160 (1): 116-121. DOI: 10.1086/314104

Danila J, Alejandro GJD. 2020. Pollen morphology and infragenetic classification of selected *Callicarpa* species (Lamiaceae) from the Philippines and Borneo. Biodiversitas 21 (2): 5736-5746. DOI: 10.13057/biodivdata/2112134.

Du JX, Wang XF, Zhang GJ. 2007. Leaf shape-based plant species recognition. Appl Math Comput 185 (2): 883-893. DOI: 10.1016/j.amc.2006.07.072

Govaerts R, Paton AJ, Harvey Y, Navarro T. 2007. World Checklist of Lamiaceae. The Board of Trustees of the Royal Botanic Gardens, Kew. http://www.kew.org/wscp

Gunz P, Mitteroecker P. 2013. Semilandmarks: a method for quantifying curves and surfaces. Hystric Iul J Mammal 24 (1): 103-109

Harley RM, Atkins S, Budantsev AL, Cantino PD, Conn BJ, Grayer RJ, Harley MM, de Kok RPI, Krestovskaja T, Morales R, Paton AJ, Ryding O, Upson T. 2004. Labiatae. In: Kubitzki K (ed) The Families and Genera of Vascular Plants, Vol. 4. Springer-Verlag, Berlin.

Hotelling H. 1933. Analysis of a complex of statistical variables into principal components. J Educ Psychol 24 (6): 417. DOI: 10.1037/h0071325

IPNI. 2021. International Plant Names Index. Published on the Internet http://www.ipni.org, The Royal Botanic Gardens, Kew, Harvard University Herbaria & Libraries and Australian National Botanic Gardens.

Jackson DA. 1993. Stopping rules in principal components analysis: A comparison of heuristic and statistical approaches. Ecology 74 (8): 2204-2214. DOI: 10.2307/1939574

Kawakubo N. 1986. Morphological variation of three endemic species of *Callicarpa* (Verbenaceae) in the Bonin (Ogasawara) Islands. Plant Species Biol 1 (1): 59-68. DOI: 10.1111/j.1442-1984.1986.tb00015.x

Klingenberg CP. 2011. MORPHOJ: An integrated software package for geometric morphometrics. Mol Ecol Resour 11 (2): 353-357. DOI: 10.1111/j.1555-0998.2010.02924.x

Lam HJ. 1919. The Verbenaceae of the Malayian Archipelago. de Waal, Groningen.

Lam HJ. Bakhuizen van den Brink RC. 1921. Revision of the Verbenaceae of the Dutch East-Indies and surrounding countries. Bull Jard Bot Série 3 1-116 & I-III.

Leary RF, Allendorf FW. 1989. Fluctuating asymmetry as an indicator of stress: implications for conservation biology. Trends Ecol Evol 4 (7): 214-217. DOI: 10.1016/0169-5347(89)90077-3

Li Y, Liu Y, Song J, Zhang R, Yan Y, Wang Y, Du FK. 2018. Geometric morphometric analyses of leaf shapes in two sympatric Chinese *Quercus* species. Ann For Sci 75 (4): 1-12. DOI: 10.1007/s13595-018-0770-2

Mal TK, Uveges JL, Turk KW. 2002. Fluctuating asymmetry as an ecological indicator of heavy metal stress in *Lythrum salicaria*. Ecol Indic 1 (3): 189-195. DOI: 10.1016/S1470-6648(02)00004-3

Miroouze E, Staquet A, Veyz R. 2012. Morphometric and morphological analysis of leaves in two species of Araceae: *Montrichardia linfera* and *Montrichardia arborescens* among different French Guiana populations. J Ecol Fog 3: 1-15

Moldenke HN. 1982. Additional notes on the genus *Geumia*. Phytologia 50: 143-151

Morais DV de, Nunes LA, Mata VP da, Costa MAP de C, Sodré G da S, Carvalho CAL de. 2019. Leaf geometric morphometrics among populations of *Delbergia ecastaphyllum* (L.) Taub. Biosci J 35 (6): 1789-1798. DOI: 10.14393/bj-v35n6a2019-39814

Palmer CM, Bush SM, Maloof JN. 2012. Phenotypic and developmental plasticity in plants. eLS. DOI: 10.1002/9780470015902.a0002092.pub2

POWO 2021. Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet; http://www.plantsoftheworldonline.org/

 Ridley HN. 1923. The Flora of the Malay Peninsula Vol. 2. Reeves, London. DOI: 10.2307/4115416

Rohlf FJ. 2010. tpsDig. digitize landmarks and outlines, ver. 2.16. State University of New York, Stony Brook.

Rohlf FJ. 2015. tpsUtil, file utility program. version 1.61. Department of Ecology and Evolution, State University of New York, Stony Brook.

Swidnerski DL, Sheets HD, Fink WL. 2004. Geometric Morphometric Analysis for Biologists: A Primer. Elsevier Press, San Diego.
### Table S1. Authors and URLs of the digital images obtained from online herbarium used in this study

| Author | URL |
|--------|-----|
| Bijmoer R, Scherrenberg M, Creuwels J (2021). Naturalis Biodiversity Center (NL) - Botany. Naturalis Biodiversity Center. Occurrence dataset https://doi.org/10.15468/fb3ypt accessed via GBIF.org on 2021-07-16. | [Link to URLs](https://www.gbif.org/occurrence/2517003371) |
| GBIF.org (02 August 2021) GBIF Occurrence Download | [Link to URLs](https://doi.org/10.15468/dl.9bfpye) |
| Herbarium Hamburgense. Herbarium Hamburgense. Occurrence dataset https://doi.org/10.15468/31aih accessed via GBIF.org on 2021-07-16. | [Link to URLs](https://www.gbif.org/occurrence/1699806604) |
| Kennedy J (2021). Harvard University Herbaria: All Records. Harvard University Herbaria. Occurrence dataset https://doi.org/10.15468/o3pvnh accessed via GBIF.org on 2021-07-16. | [Link to URLs](https://www.gbif.org/occurrence/19999138342) |
| MNHN, Chagnoux S (2021). The vascular plants collection (P) at the Herbarium of the Muséum national d'Histoire Naturelle (MNHN - Paris). Version 69.218. MNHN - Museum national d'Histoire naturelle. Occurrence dataset https://doi.org/10.15468/ncfrxy accessed via GBIF.org on 2021-07-16. | [Link to URLs](https://www.gbif.org/occurrence/2270292394) |
| Orrell T, Informatics Office (2021). NMNH Extant Specimen Records. Version 1.45. National Museum of Natural History, Smithsonian Institution. Occurrence dataset https://doi.org/10.15468/hmnrgh3 accessed via GBIF.org on 2021-07-16. | [Link to URLs](https://www.gbif.org/occurrence/1852124824) |
| Ramirez J, Tulig M, Watson K, Thiers B (2021). The New York Botanical Garden Herbarium (NY). Version 1.35. The New York Botanical Garden. Occurrence dataset https://doi.org/10.15468/6e8mje accessed via GBIF.org on 2021-07-16. | [Link to URLs](https://www.gbif.org/occurrence/1929940947) |
| Royal Botanic Gardens, Kew (2021). Royal Botanic Gardens, Kew - Herbarium Specimens. Occurrence dataset https://doi.org/10.15468/ly60bx accessed via GBIF.org on 2021-07-16. | [Link to URLs](https://www.gbif.org/occurrence/912176866) |
| Source                                                                 | Dataset Link                                                                 |
|----------------------------------------------------------------------|------------------------------------------------------------------------------|
| Moscow University Herbarium (MW). Version 1.189.                     | https://www.gbif.org/occurrence/3004116377                                   |
| Lomonosov Moscow State University, Occurrence dataset                |                                                                              |
| Solomon J, Stimmel H (2021). Tropicos Specimen Data. Missouri Botanical Garden. Occurrence dataset | https://www.gbif.org/occurrence/1259567810                                  |
| Staatliche Naturwissenschaftliche Sammlungen Bayerns. The Vascular Plant Collection at the Botanische Staatsammlung München. Occurrence dataset | https://www.gbif.org/occurrence/2981260539                                  |
| The Herbarium Catalogue, Royal Botanic Gardens, Kew. Published on the Internet | http://specimens.kew.org/herbarium/K000194933                               |
| http://specimens.kew.org/herbarium/K000194946                         |                                                                              |
| http://specimens.kew.org/herbarium/K000194949                         |                                                                              |
| http://specimens.kew.org/herbarium/K000194954                         |                                                                              |
| http://specimens.kew.org/herbarium/K000194931                         |                                                                              |
| http://specimens.kew.org/herbarium/K000194977                         |                                                                              |
| http://specimens.kew.org/herbarium/K000249400                         |                                                                              |
| http://specimens.kew.org/herbarium/K000194902                         |                                                                              |
| http://specimens.kew.org/herbarium/K000194992                         |                                                                              |
| http://specimens.kew.org/herbarium/K000194930                         |                                                                              |
| http://specimens.kew.org/herbarium/K000194962                         |                                                                              |
| http://specimens.kew.org/herbarium/K000184363                         |                                                                              |
| University of Minnesota Bell Museum (2021). Bell Museum plants. Occurrence dataset | https://www.gbif.org/occurrence/2265568305                                  |