Quantitative Evaluation of Petal Shape and Picotee Color Pattern in Lisianthus by Image Analysis

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ABSTRACT. Petal shape and picotee colour pattern of lisianthus [Eustoma grandiflorum (Raf.) Shinn.] were qualitatively evaluated by means of personal computer-based methods. In lisianthus, many cultivars have been improved to obtain various floral characteristics. Picotee color patterns and flower shape are commercially important in this species and the availability of an objective and quantitative evaluation method is of vital importance for investigations related to the genetic and physiological aspects of these characteristics. Our objectives were to evaluate petal shape variation quantitatively and to establish a new quantitative evaluation method for picotee color patterns. We succeeded in quantitatively evaluating petal shape variation by means of elliptic Fourier descriptors and principal-components analysis, and in evaluating picotee color patterns by a newly developed procedure based on comparative marginal distribution. Petal shape variation was divided into symmetrical and asymmetrical elements of the entire shape variation. Both groups were additionally divided into several components. The variations in picotee color pattern were effectively described by the first through fourth principal components. Comparing the varietal effect of these components, nested analyses of variance showed that the differences between cultivars in picotee color pattern were smaller than those of the symmetrical shape elements. In addition, the environmental effects on picotee color formation were greater than those of symmetrical shape formation. The evaluation methods described in this study are effective for further investigations, and are applicable to other floricultural crops as well as lisianthus.

Floral characteristics such as flower shape and color pattern are important targets for improvement of floriculture crops because these characteristics are directly related to the commercial value of the products. In lisianthus, the floral characteristics have long been genetically improved to meet different consumer demands. Among various floral characteristics improved, picotee color formation has been of interest commercially. This species originated in the United States, and its commercial cultivars have been intensively developed in Japan (Haley and Kofranek, 1984). A cultivar with purple picotee color was first commercially available in 1986, ≈50 years after introduction of this species to Japan. In subsequent years, breeders and breeding companies have bred some dozens of picotee color cultivars, most of which are F₁ hybrids. Today, an important target of breeding in lisianthus is stability or uniformity within a cultivar in phenotypic expression of floral characteristics, because genetic heterogeneity exists within a cultivar and the phenotypic expression is affected by cultural conditions, especially in picotee color formation (Fukuta et al., 2005). Concurrently, development of an adequate cultivation method to increase the yield of high-quality cut flowers is expected.

In this context, many researchers and breeding companies have carried out various studies to obtain an appropriate understanding of the genetic and developmental mechanisms that govern floral characteristics. Although picotee color has been quantified by a colorimeter, other floral characteristics, such as petal shape and picotee color pattern, have been evaluated qualitatively (Fukuta and Nakayama, 2003), even if quantitative variations in such characteristics were observed. Qualitative evaluation by human visual judgement, which is often used, sometimes results in unacceptable human errors, and training and experience are required for accurate and consistent assessment. In addition, qualitative evaluation of floral characteristics is definitely inadequate to evaluate continuous variation due to genotypes and cultural conditions. Therefore, an objective and quantitative evaluation method is vital, not only for lisianthus, but for other floricultural crops as well.

Digital image analysis is one way to compensate for the weakness of the current qualitative evaluation of continuous variation in various characteristics. With recent improvements in computer performance, digital image analysis has been applied to quantitative evaluation in various agricultural and biological research, such as disease assessment (Martin and Rybicki, 1998; Niemira et al., 1999; Olmstead and Lang, 2001), measurement of various plant canopies (Adamsen et al., 1999; Ewing and Horton, 1999; Karcher and Richardson, 2003; Lukina et al., 1999; Olmstead et al., 2004; Purcell, 2000; Richardson et al., 2001), and morphological analysis (Iwata et al., 1998; Iwata and Ukai, 2002; Theobald et al., 2004; Yoshioka et al., 2004a, 2004b, 2005). In the evaluation of petal shape, a combination of elliptic Fourier descriptors and...
principal-components analysis (EF-PCA) seemed to be most effective among several methods suggested. EF-PCA describes overall petal shape mathematically by transforming coordinate information concerning its contour into elliptic Fourier coefficients (Kuhl and Giardina, 1982), and summarizes these coefficients by means of principal-components analysis (Rohlf and Archie, 1984). Meanwhile, there are few proposed methods to evaluate flower color pattern. Yoshioka et al. (2004b) suggested a simple method to evaluate the flower color pattern of *Primula sieboldii* E. Morren. However, this method is not adequate for the picotee color pattern of *lisianthus*, because it evaluates only the central part of the overall petal, and neglects information about other parts. Therefore, the development of an appropriate method for picotee color pattern evaluation is highly desirable.

In this study, we describe image-based methods to quantitatively evaluate petal shape and picotee color pattern in *lisianthus*. We first evaluated petal shape variation by means of EF-PCA, and developed a quantitative evaluation method for picotee color patterns by PCA of the data derived from comparative marginal distribution (CMD). We then evaluated the varietal contribution to the petal shape and picotee color pattern by means of nested analysis of variance (ANOVA) of the scores for each principal component (PC).

**Materials and Methods**

**Plant materials.** There are ≈25 to 30 commercial cultivars of *lisianthus* that have picotee petals in Japan. We randomly chose 15 F₁ hybrid and true-bred cultivars regardless of petal shape and picotee color pattern (numbers in parentheses are cultivar identification numbers): Candy Marine (1), Neo Swallow (2), Mira Marine (3), Kyo no Warabe (4), Clear Marine (5), Candy Dolphin (6), Neo Marine (7), Spica Marine (8), Kyono Suzu (9), Asuka no Nami (10), Excel Marine (11), Maike Sky (12), Excel Navy Ring (13), San Sarf 26 (14), and Moret Marine (15) (Fig. 1). Genetic heterogeneity among each cultivar exists because *lisianthus* is an allogamous plant. The plants were grown in a greenhouse at the National Institute of Floricultural Science (Ibaraki, Japan) from Feb. to July 2004. We randomly sampled five plants per cultivar and three flowers per plant. We used all the petals of each flower (15 cultivars × 5 plants × 3 flowers × 5 petals = 1125 petals).

**Image recording.** Before scanning, we separated the five petals from each flower with a regular kitchen knife. We obtained petal images using a digital image scanner (GT-9800F; Epson Co., Tokyo), which is suitable for obtaining plain petal images (Yoshioka et al., 2004b). We placed 15 petals (from one plant) simultaneously on the flat bed of the scanner. Since petals of most cultivars had almost two-dimensional structure, the influences of flattening these petals by flat bed cover were considered to be negligible. We fixed a sheet of red paper in the flower with a regular kitchen knife. We obtained each petal image into a binary image using a threshold method, we obtained the closed contour of the petal and chain-coded it (Freeman, 1974) using programs developed using JBuilder X (Borland Software Corp., Cupertino, Calif.). The coefficients of the elliptic Fourier descriptors (EFDs), which were normalized to avoid variations related to the size, rotation, and starting point of the contour trace, were then calculated from the chain-code data using the procedure proposed by Kuhl and Giardina (1982). By this procedure, we approximated the shape of each petal using the first 20 harmonics, and thus calculated 80 (4 × 20) standardized EFDs. The 80 coefficients could be classified into two groups related to symmetrical variations and asymmetrical variations (Iwata et al., 1998; Yoshioka et al., 2004a). We refer to the former group as the symmetrical group and to the latter as the asymmetrical group. To summarize the information contained in the coefficients in each group, we performed a PCA based on a variance-covariance matrix. To determine the effect of each PC on petal shape, we recalculated the coefficients of the EFDs, letting the score on a particular PC equal the mean ± 2 SD, while keeping the scores of the remaining components as means. A series of above processes (EF-PCA), without the chain-coding, was carried out using the program SHAPE developed by Iwata and Ukai (2002). We used the scores of the PCs as the characteristics of petal shape in the statistical analyses.

**Evaluation of petal shape using EF-PCA.** After we converted each petal image into a binary image using a threshold method, we obtained the closed contour of the petal and chain-coded it (Freeman, 1974) using programs developed using JBuilder X (Borland Software Corp., Cupertino, Calif.). The coefficients of the elliptic Fourier descriptors (EFDs), which were normalized to avoid variations related to the size, rotation, and starting point of the contour trace, were then calculated from the chain-code data using the procedure proposed by Kuhl and Giardina (1982). By this procedure, we approximated the shape of each petal using the first 20 harmonics, and thus calculated 80 (4 × 20) standardized EFDs. The 80 coefficients could be classified into two groups related to symmetrical variations and asymmetrical variations (Iwata et al., 1998; Yoshioka et al., 2004a). We refer to the former group as the symmetrical group and to the latter as the asymmetrical group. To summarize the information contained in the coefficients in each group, we performed a PCA based on a variance-covariance matrix. To determine the effect of each PC on petal shape, we recalculated the coefficients of the EFDs, letting the score on a particular PC equal the mean ± 2 SD, while keeping the scores of the remaining components as means. A series of above processes (EF-PCA), without the chain-coding, was carried out using the program SHAPE developed by Iwata and Ukai (2002). We used the scores of the PCs as the characteristics of petal shape in the statistical analyses.

**Quantitative evaluation of picotee color pattern.** We calculated the total petal area [PA (square centimeters)] based on the number of pixels occupied by the petal image. We classified
each pixel into a picotee or non-picotee category according to its RGB values, and used this information to create binary petal images. The ranges of RGB values of picotee area were determined for the classification in a pilot study. We then defined the ratio of the picotee area to the PA as the number of picotee pixels divided by the total pixel area of the petal, and used this ratio as an index of the picotee color proportion (PCP). Each petal image was rotated around a center of gravity so that the central axis of the petal corresponded approximately to a vertical line, and was resized so that the petal width corresponded to 200 pixels. That is, each petal image became an aggregate of 200 vertical lines, each one pixel wide. From each modified image, we created a comparative marginal distribution (CMD; Fig. 2): we calculated the 200 variables that represent the ratio of pigmentation pixels to petal pixels on each vertical line.

To summarize the CMD data, we performed a PCA based on the variance-covariance matrix by R 1.7.1 (R Development Core Team, 2005). In order to determine the effect of each PC on the picotee color pattern, we reconstructed picotee color patterns using the 200 variables calculated in reverse by the same method as petal shape, and drew petal images by applying recalculated CMD data to average petal shape, which was calculated by EF-PCA. The evaluation of picotee color pattern was carried out using programs developed using JBuilder X. We used the scores of the PCs as the characteristics of picotee color pattern in the statistical analyses.

**Statistical Analysis.** To examine the varietal effect on each PC score relative to variations in petal shape and picotee color pattern, we performed nested ANOVA because the samples had a hierarchical structure: cultivar, plant, flower, and petal. Nested ANOVA was performed using the following linear model:

$$
\omega_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_{ik} + \epsilon_{ijkl}
$$

where \( \omega_{ijkl} \) denotes the score of each principal component of \( i \)th cultivar, the \( j \)th plant, the \( k \)th flower and the \( l \)th petal. The \( \mu \) is the grand mean, \( \alpha_i \) is the effect of \( i \)th cultivar, \( \beta_j \) is the effect of \( j \)th plant nested within \( i \)th cultivar, \( \gamma_{ik} \) is the effect of \( k \)th flower nested within \( i,j \)th plant of \( i \)th cultivar, and \( \epsilon_{ijkl} \) is the random experimental error. In this nested ANOVA, we estimated each significant level by F-test, and calculated the proportion of the variance component in each hierarchical source. Then we regarded the proportion due to the difference between cultivars as varietal effect. We also analyzed PA and PC in a similar fashion. In addition, Pearson’s product-moment correlation analyses were also performed in order to investigate the relationships between petal shape and picotee color pattern. Correlation analyses used the mean values for each plant and flower. All statistical analyses were performed using JMP (version 4.0; SAS Institute, 2000).

**Results**

A good summary of the standardized EFDs was provided by the first four PCs of the symmetrical group and of the asymmetrical group. The symmetrical group had a larger component of overall shape variation than did the asymmetrical group (Table 1). Figure 3 shows the effect of the PCs of both groups on petal shape. These reconstructed shapes indicate that the first and second PCs of the symmetrical group are good measures of the aspect ratio and the position of the center of gravity, accounting for 67.7% and 9.2% of the overall petal shape variation, respectively (Fig. 3, Table 1). The third and fourth PCs of the symmetrical group are associated with petal curvature. Meanwhile, the PCs of the asymmetrical group express the horizontal skewness from the mean petal shape (Fig. 3). That is, the first to the fourth PCs of the asymmetrical group express the differences between the left and right parts of the bottom, sides, and top of the petal, respectively.

The eigenvalues and the contributions of the first four PCs of the picotee color pattern are shown in Table 1. The contribution of the first PC was very high and accounted for as much as 87.4% of the total variation. The cumulative contribution from the first to the fourth PC is 96.6%. Figure 4 shows the reconstructed image of each PC. These images indicate that the first PC is a good measure of the ratio of pigmentation area in the total petal. In fact, this PC was highly correlated with PCP (\( r = -0.985, N = 1200, P < 0.001 \)). Therefore, we excluded the PCP from the subsequent analysis. The second and third PCs express the patterns of pigmentation area, accounting for 6.5% and 1.6% of the total picotee pattern variation. The fourth PC seems to affect the difference in pigmentation area between the right and left parts of the petal.

Significant differences were observed in all characteristics among the 15 cultivars (Table 2). Scatterplots clearly indicate the wide variations in petal characteristics (Figs. 5 and 6). From these results, we were able to characterize the cultivars. For example, in the petal shape variation of the symmetrical group, ‘Asuka no Nami’ (10) and ‘Spica Marine’ (8) have the lowest first and second PC scores because of their narrow petals and low centers of gravity (Figs. 1 and 5). The petals of ‘Candy Marine’ (1), in contrast, are the widest and have a high center of gravity (Figs. 1 and 5). In the picotee color pattern, the petals of ‘Neo Swallow’ (2) and ‘Neo Marine’ (7), which have small picotee areas, show high scores in the first PC of CMD (Figs. 1 and 6). In contrast, ‘Mira Marine 4’ (3) and ‘San Sarf 26’ (14) show low scores in the first PC of CMD, indicating that the petals of these cultivars have large pigmentation areas (Figs. 1 and 6). ‘Mira Marine 4’ (3) shows the lowest score in the second PC and the highest score in the third PC. The specific values of ‘Mira Marine 4’ (3) are evident in Fig. 1.

The proportion of the variance component due to the varietal effect in the nested ANOVA was higher than that for any other components (plant, flower, and petal) in PA and the PCs of the symmetrical group (Table 2). In contrast, the largest proportion of the variance component was due to the differences between petals in each PC of the asymmetrical group and the fourth PC of CMD. For the remaining characteristics, the largest proportions of the variance component were due to the differences between plants in the first and second PCs of CMD, and between flow-
Table 1. Contributions of principal components in symmetrical and asymmetrical groups of elliptic Fourier descriptors (EFDs) related to petal shape variation of lisianthus, and those in comparative marginal distribution (CMD) data related to variation of picotee color patterns of lisianthus. EFDs of each petal were calculated mathematically by transforming coordinate information concerning its contours. Each group of EFDs consists of 40 coefficients. CMD data of each petal consists of 200 variables that represent the ratio of pigmentation pixels to petal pixels on each vertical line that could be drawn on the scan of its petal.

| Component | Symmetrical group (shape) | Asymmetrical group (shape) | CMD data |
|-----------|---------------------------|-----------------------------|----------|
|           | Proportion (%) of group^a | Proportion (%) of group^b   | Proportion (%)          |
| 1         | 78.5                      | 67.7                        | 87.4      |
| 2         | 10.7                      | 9.2                         | 28.0      |
| 3         | 4.0                       | 3.5                         | 11.4      |
| 4         | 2.7                       | 2.3                         | 5.6       |

^Proportion relative to the total variance of the elliptic Fourier coefficients belonging to each group.

^Proportion relative to the total variance of all coefficients of both groups.

![Diagram](https://via.placeholder.com/150)

Fig. 3. Effect of each principal component in symmetrical and asymmetrical groups of elliptic Fourier descriptors (EFDs) on petal shape of lisianthus. EFDs of each petal were calculated mathematically by transforming coordinate information concerning its contours. Each group of EFDs consists of 40 coefficients. Each shape was reconstructed from coefficients calculated by letting the score for the corresponding principal component be equal to its mean or its mean plus or minus two times the SD, and setting the scores on the remaining components at zero. Dashed line, thick solid line, and thin solid line stand for mean, mean minus two times the SD, and setting the scores on the remaining components equal to their means, respectively (Table 1). From the right, each column shows the case where the score takes +2 SD, +1 SD, Mean, 1 SD, and –2 SD, respectively.

Discussion

The visual impression of flowers directly influences consumer preference and commercial values. Petals are the main visual component of flowers, and thus directly or indirectly affect such appetites and values. Petal shape and picotee color pattern were previously measured as qualitative characteristics because of difficulties in measuring them. In this study, we successfully measured the quantitative differences between cultivars and between plants within a cultivar in lisianthus. Our quantitative measurements are more appropriate for investigating these characteristics than are the commonly used qualitative measurements. We evaluated the two-dimensional shapes and picotee color patterns of petals using several independent components, which are thought to be major morphological factors affecting the impressions given by flowers. Our approach could be used to investigate floral characteristics in many other horticultural crops.

In previous studies, EF-PCAs showed remarkable results in the evaluation of several plant organs (Furuta et al., 1995; Iwata et al., 1998, 2002; Ohsawa et al., 1998; Yoshioka et al., 2004a). We can also confirm the effectiveness of EF-PCA in the evaluation of lisianthus petals. The variations in petal shape in lisianthus can be partitioned into symmetrical and asymmetrical features. The greatest variation in petal shape is explained by the symmetrical feature, because it accounted for a larger proportion of the total variation (Table 1). The PCs in each group were independent of each other, and thus can be used as new shape characteristics of lisianthus. In addition, except for the aspect ratio (the first PC of the symmetrical group), the shape variations described by the PCs of both groups were previously difficult to measure quantitatively.

This is the first time that picotee color pattern has been quantitatively evaluated. Our newly developed procedure has three major advantages. First, this procedure can accurately detect small quantitative variations in picotee color pattern. Although the second, third, and fourth PCs accounted for 6.5%, 1.6%, and 1.1% of the total variance, respectively (Table 1), the varietal differences of these PCs were highly significant. These components are difficult to score by human visual assessment. Second, this procedure can evaluate the picotee color pattern independent of size and shape. In correlation analyses, PCs of the picotee color pattern were not correlated with PAs and PCs of symmetrical and asymmetrical petal shapes. This independence is a great advantage because human visual assessment is often deceived and mislead by size and shape factors. Third, we could visually

![Diagram](https://via.placeholder.com/150)

Fig. 4. Effect of each principal component in the comparative marginal distribution (CMD) data on picotee color pattern of lisianthus. The CMD data consists of 200 variables that represent the ratio of pigmentation pixels to petal pixels on each horizontal line that could be drawn on the scan of each petal. Each petal illustration was redrawn from CMD data calculated by the score for the corresponding principal component equal to its mean, mean plus or minus the SD, and setting the scores on the remaining components at zero. From the right, each column shows the case where the score takes +2 SD, +1 SD, mean, –1 SD, and –2 SD, respectively.
Table 2. Proportions (%) of variance components in petal area (PA) and the principal components (PC) of elliptic Fourier descriptors (EFDs) related to the petal shape (symmetrical and asymmetrical groups) and picotee color pattern (MPACD) of lisianthus derived from each of the four hierarchical sources: cultivar, plant, flower, and petal (as estimated by nested ANOVA). EFDs of each petal were calculated mathematically by transforming coordinate information concerning its contours. Each group of EFDs consists of 40 coefficients. CMD data of each petal consists of 200 variables that represent the ratio of pigmentation pixels to petal pixels on each vertical line that could be drawn on the scan of its petal.

| Source                  | PA  | PC1  | PC2  | PC3  | PC4  |
|-------------------------|-----|------|------|------|------|
| Cultivar                | 54.7** | 67.5* | 54.7** | 64.0** | 39.6** |
| Plant (cultivar)        | 4.7*   | 15.0** | 7.2**  | 0.0*  | 7.0** |
| Flower (cultivar, plant)| 37.7** | 5.6** | 8.3**  | 16.3** | 25.5** |
| Petal (cultivar, plant, flower) | 3.0 | 11.9 | 29.8 | 19.8 | 28.0 |

Principal components of the histogram data (picotee color pattern)

| Source                  | PC1  | PC2  | PC3  | PC4  |
|-------------------------|------|------|------|------|
| Cultivar                | 41.7*  | 33.0** | 9.0** | 22.6** |
| Plant (cultivar)        | 54.0** | 46.7** | 25.8** | 10.5*  |
| Flower (cultivar, plant)| 3.3*   | 2.0*  | 1.3** | 6.9** |
| Petal (cultivar, plant, flower) | 84.0 | 81.6 | 63.1 | 45.2 |

NS., *: Nonsignificant or significant at P < 0.05 or 0.01, respectively.

Table 2 shows that the first three PCs of CMD data account for most of the total variation (Table 1). Although not designed to estimate heritability, our significant varietal effects in the nested ANOVAs indicate that there are genetic components to the petal characteristics examined in this study. However, environmental factors observed in the plant and flower hierarchies are also important. These results indicate that the variations in petal shape and picotee color pattern among plants within a cultivar, and among flowers within a plant, are brought about by the external environment around each plant, or by developmental instability within a plant or flower.

Previous studies (Yoshioka et al., 2004a, 2004b) concluded that it is appropriate to consider the asymmetrical variation in petal shape and color pattern as being strongly affected by environment rather than by genotype. However, this appears to not be the case for lisianthus petals, indicating that the genetic or developmental mechanisms in the asymmetrical feature vary by the asymmetrical characteristic. Although we could observe large proportions due to the differences between petals in the first and second PCs of group B and the fourth PC of CMD data, the varietal effects in the third and fourth PCs are relatively large (Table 2). In addition, tendencies to have unidirectional asymmetrical petals were observed in several cultivars. For example, most of the petals in ‘Candy Marine’ (1) were rightward-deformed. These results indicate that the asymmetrical variations in the bottom and side parts of the petal shape and in picotee color pattern have arisen from environmental effects. In contrast, the latter asymmetrical features (PC3: asymmetrical variation in the top part of petal, PC4: the effect cannot be clearly understood) would be strongly controlled by genotype.

Fukuta and Nakayama (2003) reported that formation of the picotee color pattern is subject to environmental effects, especially temperature change. In this study, we reared all plants under the same cultural conditions, which is thought to be most suitable for developing stable picotee color patterns in cut flowers (Fukuta et al., 2005). Nonetheless, differences in picotee color formation were observed between plants and between flowers within a cultivar. As we expected, the variance components are largest in the differences between plants for the first and second PCs of
In conclusion, we have described computer-aided procedures to evaluate variations in petal shape and picotee color pattern; these procedures could also reveal some relationships between cultivars in the symmetrical formation of picotee color pattern, because the varietal effects of picotee color pattern are smaller than those of the symmetrical shape variations (Table 2). Therefore, the stability or uniformity of picotee color pattern within a cultivar is likely to be less than that of petal shape.

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