

# Diallel Analysis of Leaf Shape Variations of Citrus Varieties Based on Elliptic Fourier Descriptors

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The inheritance of citrus leaf shape was investigated by analyzing a diallel set of crosses of five citrus varieties/selections. Leaf contours were extracted by image processing, and described by elliptic Fourier descriptors. Mathematically independent shape characteristics were then identified by principal component analysis of the descriptors. The 1st, 2nd, 3rd and 4th components accounted for 51%, 20%, 10% and 6% of the total shape variation, and were good measures of the ratio of length to width, the position of the centroid, the curvature and the leaf wing size, respectively. Diallel analyses indicated that these components differed substantially in their mode of inheritance. The broad sense heritability was fairly high ( $> 0.90$ ) in the 2nd and 4th components and high ( $> 0.70$ ) in the 1st component, whereas the narrow sense heritability was high only in the 2nd component. There was no significant genetic effect in the 3rd component. The 1st, 2nd and 4th components showed over-, incomplete- and complete- dominance, respectively. The results indicate that elliptic Fourier descriptors can be successfully applied to the quantitative genetic analysis of citrus leaf shape.

**Key Words:** citrus, leaf shape, diallel analysis, elliptic Fourier descriptors, image analysis.

## Introduction

Leaf shape has wide variations in genus *Citrus* and has been used as a key taxonomic character in classifying citrus species and varieties (Swingle 1967, Tanaka 1969, Handa and Oogaki 1985). Polyembryony, the production of seed containing both sexual and asexual embryos, is a major problem in citrus breeding. Several attempts have been made on using the leaf shape as a morphological marker in differentiating between zygotic seedlings, which are useful in

breeding, and nucellar seedlings, which are useless in a progeny test (Teich and Spiegel-Roy 1972, Hearn 1977, Chikaizumi and Matsumoto 1978). Moreover, the leaf morphology, i.e., shape and size, is an important target in citrus breeding because it relates directly to the easiness of treatment in cultivation and may relate to the fruit quality and quantity, for example, through the ability of photosynthesis. In spite of its importance in taxonomy and breeding, the inheritance of citrus leaf shape has been hardly determined.

The lack of genetic studies of citrus leaf shape is partly due to the difficulty in quantification of the variations in leaf shape. The variations are generally continuous, and quantitative measurements are essential for their genetic analysis. The leaf shape of citrus, however, has been measured mainly through graded classifications (e.g. Chikaizumi and Matsumoto 1978, Handa and Oogaki 1985). For example, in the registration of citrus varieties in Japan, the length to width ratio of the leaf blade is measured, and leaves are further graded into four or five representative classes with respect to the shape of the overall leaf, the apex, the base of the blade and the wing. While the first of these measurements is suitable for genetic analyses, the other characteristics measured in graded classifications are inappropriate because they take discrete values though they primarily have continuous variations. Moreover, the graded classifications often lead to considerable artificial error owing to their dependence on the subjective judgement of the observers.

Quantitative measurement based on elliptic Fourier descriptors (Kuhl and Giardina 1982) has been successfully applied to the analysis of organ shape of several plant species: such as *Betula* leaf (White *et al.* 1988), *Begonia* leaf (McLellan 1993), soybean leaflet (Furuta *et al.* 1995), buckwheat kernel (Ohsawa *et al.* 1998) and radish root (Iwata *et al.* 1998). The elliptic Fourier descriptors can delineate any type of shape with a closed two-dimensional contour and will be generally used as a method for measuring a biological shape. Iwata *et al.* (2000) used the descriptors in a diallel analysis of radish root shape, and elucidated the mode of inheritance of root shape characteristics, which are important factors to consider in the genetic improvement of Japanese radish. The inheritance of leaf shape of plant species, however, has not been well investigated by the descriptors.

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Previously we showed that the key characteristics of citrus leaf shape that are mathematically independent of each other, can be efficiently identified and measured by analyzing the principal components of the descriptors (Iwata *et al.* submitted). Analysis of genotype by environment interactions affecting the leaf shape of citrus varieties showed that the variation due to genotypes was much larger than those due to either environmental factors or the interaction. That is, factors affecting leaf shape in citrus varieties appeared to be highly heritable. However, these analyses were not based on the data from crossing experiments, and could not give information on genetic parameters, such as heritability, the degree of dominance and so on.

The objectives of this study were to assess whether the elliptic Fourier descriptors is efficient in the investigation of the inheritance of citrus leaf shape through determining the inherited characteristics of the leaf shape and clarifying the mode of inheritance of the characteristics. For this purpose we used a diallel set of crosses among five citrus varieties/selections, which have diverse genetic backgrounds and large variations in their leaf shape, and conducted a diallel analysis of leaf shape using the principal component scores of elliptic Fourier descriptors as shape characteristics.

## Materials and Methods

As parents of diallel crosses, we used five citrus varieties/selections; 'Hayasaki' (*Citrus grandis* Osbeck  $\times$  *C. grandis* Osbeck), 'Hyuganatu' (*C. tamurana* hort. ex Tanaka), 'A-255' [*C. unshiu* Marc.  $\times$  *C. hassaku* hort. ex Tanaka]  $\times$  *C. sinensis* Osbeck], 'Hirakishu' (*C. kinokuni* hort. ex Tanaka) and 'Clementine-Vita' (*C. clementina* hort. ex Tanaka). These varieties/selections have diverse genetic backgrounds, and have specific characteristics not only in the leaf morphology but also in the morphology of other organs such as fruit. In order to eliminate the effects of nucellar seedlings, we chose all the parents from monoembryonic varieties/selections.

In 1989 and 1990, the five parents were crossed in all possible combinations including reciprocal crosses and selfing (except 'Hayasaki' ♀ and 'A-255' ♂). The seedlings of 19 F<sub>1</sub>s and five selfing progenies were grown for one or two years in a greenhouse and resulting plants were transplanted to an experimental field of the National Institute of Fruit Tree Science in Okitsu (Shimizu, Shizuoka) in 1992. The plants were spaced 20 cm apart in two rows spaced 60 cm apart in ridges spaced 2 m apart. Plots, each of which consisted of plants from a progeny, were randomly arranged in the field.

In 1994, 100 plants (one plant is one offspring) of each F<sub>1</sub> or selfing progeny, and five branches from each plant were randomly sampled. A single leaf was taken from the middle of the leaf-bearing zone of each branch, because they are less variable here than at lower and higher positions (Iwata *et al.* 1994).

The image of sampled leaves was acquired as R (red),

G (green) and B (blue) digital images, with a 512  $\times$  512 spatial resolution and 256 gray levels for each color, using a video camera (PHV-A7, SONY, Tokyo) and a real time A/D converter (FA-310, For-A Corp, Tokyo). The closed contours of the leaves were obtained through binary images that were converted from R images with appropriate thresholds, and were described by a chain-code (Freeman 1975), with a color image processor (HRU-TAICHI IV80, Ezel Sharp, Tokyo). The coefficients of elliptic Fourier descriptors that were normalized to avoid variations related to the size, rotation and starting point of the contour traces, were then calculated from the chain-code through the procedure based on the ellipse of the first harmonic (Kuhl and Giardina 1982). By this procedure, the leaf shape was approximated by the first 20 harmonics, which correspond to the 77 coefficients of normalized elliptic Fourier descriptors.

To summarize the information contained in the coefficients of the Fourier descriptors, we performed principal component analysis based on a variance-covariance matrix of the coefficients, and the scores of the components were used in subsequent analysis as leaf shape characteristics. The variation in shape accounted for by each component was visualized using inverse Fourier transformation (Rohlf and Archie 1984, Furuta *et al.* 1995).

Diallel analyses based on the scores of these components were performed according to the method of Hayman (1954a, b), using the computer program "DIALL" developed by Ukai (1989). Prior to the analyses, the principal component scores were averaged over branches and plants within a cross. The data for a cross between 'Hayasaki' ♀ and 'A-255' ♂, which was missing in this study, were replaced by data from the reciprocal cross. Because there was no replication in the present data, error variance was estimated from the variance due to differences between reciprocal crosses, assuming that the reciprocal crosses were not genetically different. From the diallel tables, we also estimated the broad and narrow sense heritabilities after Mather and Jinks (1971).

## Results

Independent shape characteristics were identified by principal component analysis of the Fourier descriptors. The contribution of the 1st principal component was high (51%), and the first four components, each of which contributed

**Table 1.** Eigenvalues and contribution of principal components.

Component	Eigenvalue <sup>1)</sup>	Proportion (%)	Cumulative (%)
1	3248	51.24	51.24
2	1250	19.72	70.95
3	638	10.06	81.02
4	362	5.71	86.73
5	147	2.32	89.05
Total Variance	6339		

<sup>1)</sup> Eigenvalues are scaled by 10<sup>-6</sup>

more than 5%, cumulatively accounted for over 85% of the total variation (Table 1). The reconstructed contours indicated that the 1st, 2nd, 3rd and 4th components are good measures of the length to width ratio, the position of the centroid of the leaf along its midrib, the curvature and the relative size of the leaf wing, respectively (Fig. 1). The scores of these components were used as shape characteristics in the following analyses.

Analyses of variance of diallel tables for these components showed that the additive effect (a) was significant in the 1st, 2nd and 4th components (Table 2). The *F* ratio for (a) was largest in the 2nd component. The mean dominance deviation ( $b_1$ ) was significant only in the 4th, and the additional dominance deviation due to parents ( $b_2$ ) and the residual deviation ( $b_3$ ) were significant at the 1% probability level in the 4th and at the 5% probability level in the 1st and 2nd components. Because no significant genetic effect was observed in the 3rd component, we excluded it from the further analyses.

In the 1st component, the broad and narrow sense heritabilities were 0.785 and 0.464, respectively (Table 3). The average degree of the dominance was above unity (1.446) and the line of regression of *Wr* on *Vr* with unit slope intersected the *Wr*-axis below the origin (Fig. 2a), indicating over-dominance. The average direction of dominance was positive, showing that the alleles for broad leaf are dominant over those for narrow leaf. 'Hirakishu' and 'Clementine-Vita' were located at a position far from the origin in the (*Vr*, *Wr*) graph, showing that most of the alleles carried by these parents are recessive, while 'Hayasaki', 'Hyuganatu' and 'A-255' were located at a position near the origin, indicating that these parents harbor dominant genes at most of the segregating loci.

In the 2nd component, both broad and narrow sense heritabilities were high (Table 3). The average degree of dominance was 0.718 and the regression line with unit slope intersected the *Wr*-axis above the origin (Fig. 2b), indicating incomplete dominance. The average direction of dominance was negative, indicating that leaf shapes where the centroid is near the base are associated with dominant alleles. The

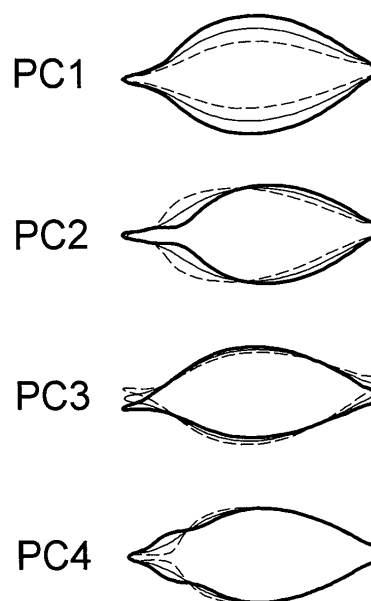


Fig. 1. Amount of variation in leaf shape that can be accounted for by each principal component. Each shape was reconstructed from coefficients calculated by letting the score for the corresponding principal component be equal to its mean or its mean plus or minus two times the standard deviation, and setting the scores on the remaining components at zero. Broken line, thin solid line, and thick solid line represent mean  $-2$  S.D., mean, and mean  $+2$  S.D., respectively.

(*Vr*, *Wr*) graph indicated that 'Hirakishu' and 'Clementine-Vita' have mostly dominant alleles, and 'Hyuganatu' and 'A-255' mostly recessive alleles.

In the 4th component, broad sense heritability was high (0.940), while narrow sense heritability was low (0.321) (Table 3). The average degree of dominance was fairly close to unity and the line of regression of *Wr* on *Vr* with unit slope intersected the *Wr*-axis at a position fairly near the origin (Fig. 2c), indicating complete dominance. The proportion of dominant genes was high. The average direction of dominance was negative, indicating that the alleles for narrow wing leaf are dominant. The (*Vr*, *Wr*) graph indicated that 'Hayasaki' has mostly recessive alleles for this trait, while the other varieties have mostly dominant alleles.

Table 2. Analysis of variance of diallel tables of principal component scores.

Source <sup>1)</sup>	<i>df</i>	PC1		PC2		PC3		PC4	
		MS <sup>2)</sup>	<i>F</i>	MS <sup>2)</sup>	<i>F</i>	MS <sup>2)</sup>	<i>F</i>	MS <sup>2)</sup>	<i>F</i>
a	4	106523	11.98**	216263	77.36**	947	2.40ns	34248	27.95**
b	10	35024	3.94	13701	4.90	801	2.03	26443	21.58
$b_1$	1	6258	0.70ns	83	0.03ns	75	0.19ns	59579	48.63**
$b_2$	4	42406	4.77*	17908	6.41*	1200	3.04ns	37708	30.78**
$b_3$	5	34872	3.92*	13058	4.67*	627	1.59ns	10805	8.82**
Error	9	8893		2795		395		1225	

ns, \*, \*\*: Not significant and significant at the 5% and 1% probability levels, respectively

<sup>1)</sup> a, Additive effect; b, dominance effect;  $b_1$ , mean dominance deviation;  $b_2$ , dominance deviation due to parents;  $b_3$ , residual deviations due to cross combinations

PC1-4, 1st to 4th principal components

<sup>2)</sup> Mean squares (MS) are scaled by  $10^{-8}$

**Table 3.** Genetic parameters estimated for each principal component

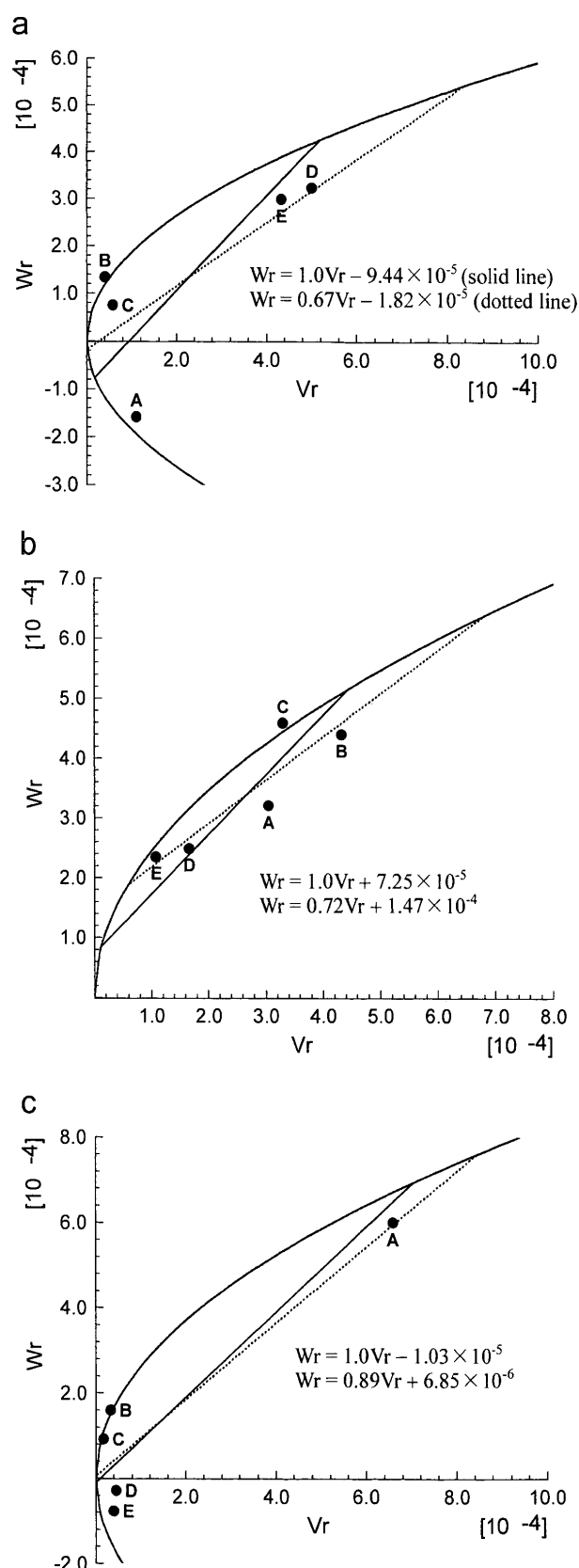
	PC1	PC2	PC4
Average Degree of Dominance	1.446	0.718	1.030
Proportion of Dominant Genes	0.581	0.408	0.773
Average Direction of Dominance (Sign)	+	-	-
Heritability (Broad)	0.785	0.945	0.940
Heritability (Narrow)	0.464	0.837	0.321

PC1, 2, 4; 1st, 2nd and 4th principal components, respectively

## Discussion

Diallel analysis after Hayman (1954b) assumes that parents employed in a diallel set of crosses are homozygous at all the loci controlling the quantitative trait to be examined. However, there may possibly be heterozygous loci harbored by the parents employed in allogamous species. The application of the analysis after Hayman (1954b) to allogamous species may be justified by the following reasons: (1) Heterozygosity in allogamous species will not be so large that most of the loci involved in a trait become heterozygous. For example, according to the literature, the mean of gene diversity, i.e., expected heterozygosity, estimated on the allozyme variation in allogamous plant species is less than 0.17 (Table 2 and 3 in Hamrick and Godt 1990). (2) Loci involved in an agronomically important trait generally have a tendency to be homozygous under natural and/or artificial selection, unless the loci are relevant to heterosis. (3) A simulation study indicated that existence of heterozygous parents in a diallel table do not largely influence the results of analysis if the proportion of heterozygous parents is not high (Ukai, in press). (4) If highly heterozygous parents are included in the analysis, they can be detected from the locations of the parents plotted on a ( $V_r$ ,  $W_r$ ) graph (Dickinson and Jinks 1956). So far, the analysis after Hayman (1954b) has been applied to allogamous plants such as apple (Bessyo *et al.* 1989), alfalfa (Tamimi and Rumbaugh 1963) and orchard grass (Parker 1968), and animals such as rat (Jinks and Broadhurst 1963), silkworm (Gamou *et al.* 1985a, 1985b) and *Drosophila* (Hill 1964, Ruiz-Dubreuil and Del-Solar 1993). However, it should be noted that the average degree of dominance is underestimated if heterozygotes are included in the analysis (Dickinson and Jinks 1956).

As mentioned above, the heterozygosity of parents can be inferred from the locations of the parents plotted on a ( $V_r$ ,  $W_r$ ) graph. That is, it is expected that highly heterozygous parents may be located at a position above the regression line and near the limiting parabola. In this study, most points for the parents appeared close to the regression lines for the analyzed component, suggesting that the parents employed in this study are highly homozygous with respect to the loci controlling leaf shape variations observed here. However, some parents, 'A-255' and 'Hyuganatu' in the 1st and 3rd principal components and 'A-255' in the 2nd component, showed a position above the regression line and near the limiting parabola, indicating partial heterozygosity. Because



**Fig. 2.** ( $V_r$ ,  $W_r$ ) graphs of the (a) first, (b) second and (c) fourth principal components. Parent varieties/selections A to E correspond to 'Hayasaki', 'Hyuganatu', 'A-255', 'Hirakishu' and 'Clementine-Vita', respectively.  $V_r$  and  $W_r$  were derived from the array variance and covariance minus the estimated contribution of environmental variance, respectively. The broken line represents the best fitting regression of  $W_r$  on  $V_r$ , and solid line the regression with the unit slope.

'A-255' is a hybrid selection between species, it is possibly heterozygous at most of the segregating loci.

Diallel analysis after Hayman (1954b) also assumes the absence of epistasis. The lines of the regressions of  $W_r$  on  $V_r$  in the ( $V_r$ ,  $W_r$ ) graph (Fig. 2) had slopes that were not significantly different from unity in the three components analyzed, suggesting that the assumption of the absence of epistasis in the diallel analysis is satisfied.

The results of the diallel analyses indicated that the shape characteristics, identified by the principal component analysis of elliptic Fourier descriptors, are heritable in general, but the magnitude of heritability differed among them. In the 3rd component, which is a representation of leaf curvature, a significant genetic effect was not observed, indicating that curvature is not heritable and the main causes of its variation are micro-environmental factors. In the 2nd component, both narrow and broad sense heritabilities were fairly high, suggesting the position of the leaf centroid may be an important taxonomic trait in the classification of citrus varieties.

The results also suggested that the modes of inheritance differed substantially among the identified characteristics, i.e., over-, incomplete- and complete-dominance was found for the ratio of leaf length to width, the position of the centroid and the leaf wing size, respectively. The results showed that decomposing the leaf shape variation into independent characteristics by principal component analysis is an indispensable step for meaningful genetic analysis. Without this decomposition, each shape characteristic would be so confounded with the others that its mode of inheritance could not be clarified.

In conclusion, we were able to determine the inheritance of leaf shape variations of the five citrus varieties/selections using elliptic Fourier descriptors. This indicates the descriptors can be successfully applied to quantitative genetic analysis of citrus leaf shape. The number of parents used in this study, however, was not sufficient to acquire the general information about the inheritance of citrus leaf shape. In order to comprehend the inheritance of leaf shape variations observed in citrus, it is necessary to analyze more diverse genetic variations using the method presented here.

In this study, we evaluated the inheritance of quantitative variations of citrus leaf shape using diallel analysis. Although diallel analysis is suitable to grasp the mode of inheritance of quantitative traits, it is not useful to clarify the effect of each locus controlling the traits. In citrus, QTL analysis has been performed several quantitative traits with the aid of molecular marker linkage maps (Tozlu *et al.* 1999a, 1999b, Garcia *et al.* 1999, Garcia *et al.* 2000, Ling *et al.* 2000). The loci responsible for leaf shape variations among citrus varieties and species will also be able to be determined through QTL analysis of shape characteristics based on the elliptic Fourier descriptors.

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