Quantitative Study of Fruit Flavonoids in *Citrus* Hybrids of King (*C. nobilis)* and Mukaku Kishu (*C. kinokuni*)

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Twenty-four *Citrus* hybrids of King (*C. nobilis*) and Mukaku Kishu (*C. kinokuni*) were examined for their flavonoid profiles of the edible part by reversed-phase HPLC analysis. Two hybrids (G-155 and G-156) contained higher amounts of natsudaidain than their parents, whereas the remainder of the hybrids had a character intermediate between those of King and Mukaku Kishu on the basis of polymethoxylated flavone composition. Principal component analysis revealed the distribution of the hybrids by quantifying 23 flavonoid contents.

Keywords: *Citrus nobilis; Citrus kinokuni; flavonoids; HPLC; multivariate analysis*

INTRODUCTION

Citrus species are a rich source of flavonoids, and determination of *Citrus* fruits with high concentrations of individual flavonoids is desirable in order to study their biological properties. In a recent literature review on *Citrus* flavonoids, a broad spectrum of biological activity, including anticarcinogenic and antitumor activities, was discussed (*1*, *2*). It is commonly accepted that cancer formation can be prevented by the consumption of certain foods (*3*), and flavonoids in *Citrus* fruits and juices are some of the most prominent potential cancer-preventing agents.

In previous research, we found strong differentiation-inducing activity toward HL-60 leukemic cells from the readily extractable fraction of King (*C. nobilis*) juice. The active principles were isolated and identified as four polymethoxylated flavones, namely, nobiletin, 3,3′,4′,5,6,7,8-heptamethoxyflavone, natsudaidain, and tangeretin (*4*). Among these polymethoxylated flavones natsudaidain demonstrated the most potent activity (*5*). Natsudaidain also possesses strong antiproliferative activity toward several cancer cell lines (*6*). HPLC analysis of major flavonoids in the edible part of various *Citrus* and near-*Citrus* relatives has revealed that King had a peculiar flavonoid profile; King contains higher concentrations of 3,3′,4′,5,6,7,8-heptamethoxyflavone and natsudaidain than of nobiletin and tangeretin, whereas most of other species showed the reversed profiles of polymethoxylated flavones. The characteristic profile of polymethoxylated flavones seemed to be responsible for the biological activity of the King juice.

Composition and inheritance of rutinosyl-flavonoid and neohesperidosyl-flavonoid alleles in *Citrus* plants are used in *Citrus* taxonomy and breeding (*7*, *8*). Few studies, however, have been conducted to establish similarity among *Citrus* cultivars by composition of not only flavonoid glycosides but also polymethoxylated flavones. Polymethoxylated flavones are the characteristic feature of *Citrus* plants, and they are the reported to have many important biological activities.

To examine the effect of hybridization on the inheritance of the polymethoxylated flavone profile, we surveyed 24 hybrids of King and Mukaku Kishu (*C. kinokuni*), which have a reversed profile of polymethoxylated flavone content. Mukaku Kishu is the one of the highest nobiletin-containing species, but it lacks natsudaidain (*9*). The influence of hybridization on flavonoid composition was totally evaluated by multivariate analysis of the composition of 23 flavonoids.

MATERIALS AND METHODS

Flavonoids. The flavonoids (Figure 1) used in the present study are as follows: apigenin, diosmin, eriocitrin, hesperidin, isorhoifolin, kaempferol, naringenin, naringin, narirutin, neodiosmin, neoeriocitrin, neohesperidin, neoponcirin, poncirin, rhoifolin, and rutin were purchased from Funakoshi (Tokyo, Japan), and luteolin, quercetin, and taxifolin were from Sigma-Aldrich (Tokyo, Japan). 3,3′,4′,5,6,7,8-Heptamethoxyflavone, natsudaidain, nobiletin, and tangeretin were isolated from King juice (*C. nobilis*) (*4*).

Fruit Samples. King was crossed as a seed parent with Mukaku Kishu as a pollen parent. The cross was made in 1986, and most of the hybrids that produced fruits were top-grafted and maintained on Hayashi (*C. unshiu*) in 1988. All samples were harvested in the same field and year and, therefore, were produced under the same conditions of climate and soil. Fruits were collected at random from trees at the National Institute of Fruit Tree Science, Okitsu, Shimizu, Shizuoka, Japan in December 1997. The sample consisted of at least three fruits and was freeze-dried. The freeze-dried fruits were divided into peel and edible parts. The edible parts, which consisted of juice sac and segment epidermis, were ground, and 100 mg of the sample was extracted with 1 mL of MeOH/DMSO (1:1) three times. The extracts were combined and made up to 5 mL by MeOH. The flavonoid recovery by MeOH/DMSO (1:1) solvent extraction was determined as 82.6 ± 8.7% ($n = 3$) for naringin as the representative of flavonoid glycosides, added to the edible part of King, which does not contain naringin. Similarly, recovery of polymethoxylated flavone was determined as 75.1

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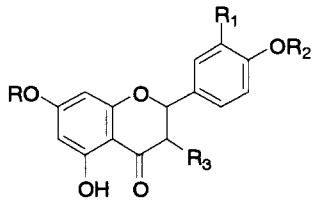
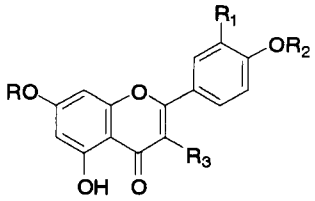
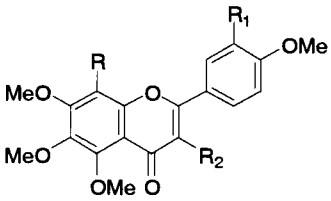


Eriocitrin: R=rutinosyl, R_1=OH, R_2=R_3=H (**1**)
Hesperidin: R=rutinosyl, R_1=OH, R_2=Me, R_3=H (**2**)
Neoeriocitrin: R=neohesperidosyl, R_1=OH, R_2=R_3=H (**3**)
Naringenin: R=R_1=R_2=R_3=H (**4**)
Naringin: R=neohesperidosyl, R_1=R_2=R_3=H (**5**)
Neohesperidin: R=neohesperidosyl, R_1=OH, R_2=Me, R_3=H (**6**)
Neoponcirin: R=rutinosyl, R_1=H, R_2=Me, R_3=H (**7**)
Narirutin: R=rutinosyl, R_1=R_2=R_3=H (**8**)
Poncirin: R=neohesperidosyl, R_1=H, R_2=Me, R_3=H (**9**)
Taxifolin: R=H, R_1=OH, R_2=H, R_3=OH (**10**)



Apigenin: R=R_1=R_2=R_3=H (**11**)
Diosmin: R=rutinosyl, R_1=OH, R_2=Me, R_3=H (**12**)
Isorhoifolin: R=rutinosyl, R_1=R_2=R_3=H (**13**)
Kaempferol: R=R_1=R_2=H, R_3=OH (**14**)
Luteolin: R=H, R_1=OH, R_2=R_3=H (**15**)
Neodiosmin: R=neohesperidosyl, R_1=OH, R_2=Me, R_3=H (**16**)
Rhoifolin: R=neohesperidosyl, R_1=R_2=R_3=H (**17**)
Rutin: R=H, R_1=OH, R_2=H, R_3=O-rutinose (**18**)
Quercetin: R=H, R_1=OH, R_2=H, R_3=OH (**19**)



3,3',4',5,6,7,8-Heptamethoxyflavone: R=R_1=R_2=OMe (**20**)
Nobiletin: R=R_1=OMe, R_2=H (**21**)
Natsudaidain: R=R_1=OMe, R_2=OH (**22**)
Tangeretin: R=OMe, R_1=R_2=H(**23**)

Figure 1. Structures of flavonoids studied.

± 11.3% for tangeretin, added to the edible part of *Poncirus trifoliate*, which is a near-*Citrus* relative and does not contain tangeretin (*9*). Twenty microliters of the combined extracts was injected to HPLC.

HPLC Analysis. HPLC analysis of flavonoids was done primarily according to the method described by Vandercook and Tisserat (*10*). Analytical conditions were as follows: Hypersil RP-18 column, particle size = 5 μm, 10 cm × 4.6 mm i.d. (Hewlett-Packard, Wilmington, DE); gradient elution consisting of an initial 2 min of 80% 0.01 M H_3PO_4 and 20% MeOH followed by a linear gradient to 55% 0.01 M H_3PO_4 and 45% MeOH in 43 min, used for the analysis of taxifolin, eriocitrin, neoeriocitrin, rutin, narirutin, naringin, hesperidin, isorhoifolin, neohesperidin, rhoifolin, diosmin, neodiosmin, kaempferol, and apigenin. For the analysis of polymethoxylated flavones, namely, nobiletin, natsudaidain, 3,3′,4′,5,6,7,8-heptamethoxyflavone, and tangeretin, isocratic elution (mobile phase = 50% 0.01 M H_3PO_4 and 50% MeOH) was done to obtain better peak separation. A different isocratic elution

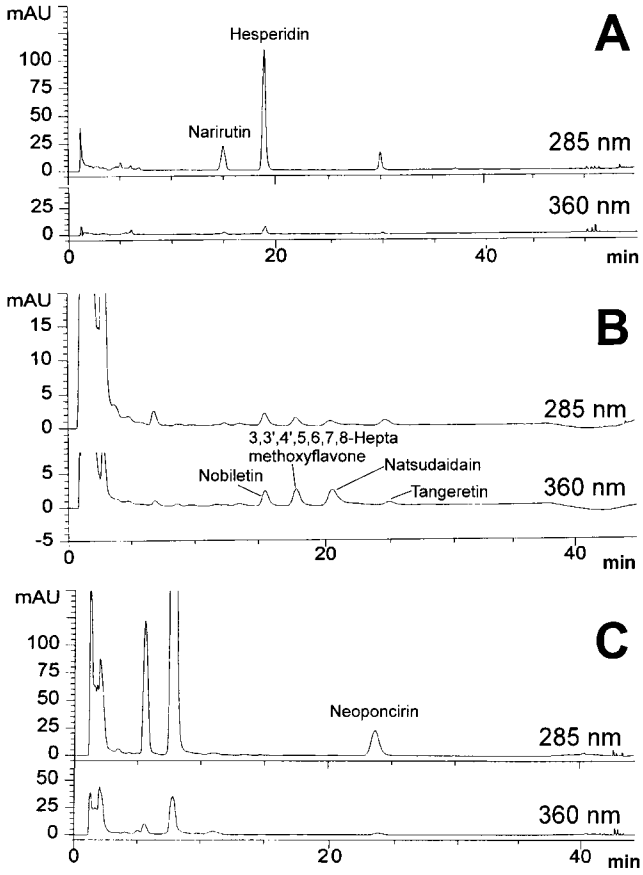


Figure 2. Typical HPLC chromatograms of (A) linear gradient elution from 80% 0.01 M H_3PO_4/20% MeOH to 55% 0.01 M H_3PO_4/45% MeOH, (B) isocratic elution of 50% 0.01 M H_3PO_4/50% MeOH, and (C) isocratic elution of 70% 0.01 M H_3PO_4/30% MeOH.

(mobile phase = 70% 0.01 M H_3PO_4 and 30% MeOH) was used for quercetin, naringenin, luteolin, neoponcirin, and poncirin analysis. Under these conditions, the peaks were separated sufficiently to integrate peak area (Figure 2). Assignment of flavonoids was done by cochromatography with authentic standards. Concentrations of the compounds were determined by an absolute calibration method with two data points, and quantitation of flavonoids was done using linear region of the calibration. The UV diode array detector was set to measure spectra from 200 to 400 nm and monitor the eluent at 285 nm for flavanones and at 360 nm for flavones and polymethoxylated flavones.

Statistical Analysis. A quantitative data set, which was composed of values taken from HPLC analysis of the edible parts of the hybrids and their parents, was used for multivariate analysis. The statistical analysis program JMP (SAS Institute Inc., Cary, NC) was used to calculate the data, and the graph software Kaleida Graph (Synergy Software, Reading, PA) was used to plot results from principal component analysis (PCA). Each principal component was calculated by taking a linear combination of an eigenvector of the correlation matrix with a standardized original variable.

RESULTS AND DISCUSSION

The quantitative determination of the major flavonoids for 24 hybrids and their parents is given Table 1. Hybridization between King and Mukaku Kishu, which have a reversed profile of polymethoxylated flavone content relative to each other, gave several characteristic hybrids. The highest amount of natsudaidain was found in G-156 (6.9 μg/100 mg of dried sample), followed by G-155 (5.4 μg/100 mg of dried

Table 1. Flavonoid Contents in the Edible Part of Hybrids of King and Mukaku Kishu[a]

hybrid	eriocitrin	marirutin	hesperidin	isorhoifolin	diosmin	neoponcirin	nobiletin	heptameth-oxyflavone	natsu-daidain	tangeretin
King	1.6	319.3	1172	nd	nd	45.9	1.4	4.0	2.5	2.0
Mukaku Kishu	4.6	129.0	1319	9.9	25.9	7.7	12.5	0.3	nd	6.0
G-149	nd	30.2	176.6	nd	nd	22.7	5.1	0.3	nd	3.0
G-150	nd	8.1	557.9	nd	nd	12.8	2.3	2.3	0.7	2.1
G-151	nd	28.8	419.6	nd	nd	27.9	2.4	0.4	nd	1.7
G-152	3.9	60.1	536.7	nd	nd	22.1	1.9	1.8	0.9	2.3
G-153	4.1	67.2	897.6	nd	nd	48.9	1.3	1.0	0.3	0.8
G-154	11.4	1077	571.3	nd	nd	168.3	6.4	0.2	nd	1.9
G-155	5.0	71.2	521.4	nd	nd	40.2	6.4	8.7	5.4	4.1
G-156	nd	39.7	620.3	nd	nd	43.0	3.5	4.7	6.9	2.5
G-157	nd	23.0	96.4	nd	nd	10.9	0.7	0.4	0.1	0.5
G-158	nd	48.4	849.8	nd	nd	10.1	4.1	3.0	0.7	2.7
G-159	nd	78.7	1196	nd	10.0	52.8	3.9	3.0	1.1	2.5
G-160	nd	178.0	321.6	nd	nd	39.6	5.1	nd	nd	5.0
G-161	nd	85.9	705.9	nd	nd	26.6	9.0	0.8	nd	4.4
G-162	nd	11.6	449.0	nd	nd	5.4	1.7	1.1	2.2	2.2
G-163	3.5	507.8	610.5	nd	nd	52.3	1.6	0.9	1.9	2.1
G-165	nd	241.9	605.8	nd	nd	40.3	3.1	0.3	nd	3.5
G-166	nd	102.6	398.3	nd	nd	38.2	4.0	nd	nd	1.8
G-167	nd	251.4	1005	nd	nd	45.1	9.4	0.3	nd	6.2
G-168	nd	50.0	500.3	nd	nd	29.1	3.0	1.2	0.7	3.1
G-169	7.2	572.6	915.1	nd	nd	56.5	17.3	0.6	nd	5.2
G-170	nd	58.0	394.8	nd	nd	11.4	7.7	0.3	nd	1.9
G-171	nd	202.8	1174	nd	11.5	30.6	1.6	0.8	0.5	1.4
G-172	nd	157.6	855.5	nd	nd	38.7	10.1	0.8	nd	2.6
G-173	nd	56.5	595.6	nd	nd	7.2	1.3	1.0	1.2	1.9

[a] All values are given in μg/100 mg of dried sample. Hybrids are listed in increasing retention order on an RP-18 HPLC coulumn. nd, not detected. The flavonoids taxifolin, neoeriocitrin, rutin, naringin, neohesperidin, rhoifolin, neodiosmin, quercetin, naringenin, luteolin, poncirin, kaempferol, and apigenin were not detected in King, Mukaku Kishu, or any of the hybrids.

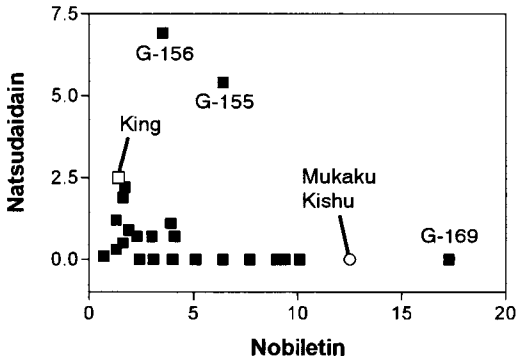


Figure 3. Relationship between nobiletin and natsudaidain: (□) King; (○) Mukaku Kishu; (■) hybrids of King and Mukaku Kishu.

Table 2. Correlation Coefficient Matrix for the Flavonoids and Principal Components

	PC1	PC2	PC3	PC4	PC5
eigenvalue	3.17	2.48	1.85	1.12	0.64
%	31.72	24.75	18.50	11.21	6.36
cum %	31.72	56.47	74.97	86.18	92.54
eigenvectors					
eriocitrin	0.36	0.37	0.15	−0.11	0.38
narirutin	0.34	0.47	−0.04	−0.08	−0.06
hesperidin	0.33	−0.18	0.23	−0.28	−0.76
isorhoifolin	0.36	−0.38	0.04	−0.22	0.48
diosmin	0.34	−0.38	0.07	−0.42	0.11
neoponcirin	0.25	0.52	0.06	−0.12	−0.09
nobiletin	0.42	−0.06	−0.03	0.53	−0.03
heptamethoxyflavone	−0.12	0.01	0.69	0.09	−0.02
natsudaidain	−0.16	0.04	0.66	0.05	0.16
tangeretin	0.34	−0.20	0.07	0.61	−0.05

sample). These hybrids contained higher amounts of natsudaidain than King (2.5 μg/100 mg of dried sample) and seemed to strongly inherit the polymethoxylated flavone profile of King, as they contained higher amounts of 3,3′,4′,5,6,7,8-heptamethoxyflavone and natsudaidain than of nobiletin and tangeretin.

Figure 3 shows the scatter diagram for nobiletin and natsudaidain. Although most of the hybrids have a feature intermediate between those of King and Mukaku Kishu, G-155 and G-156 had the predominant feature of King; that is, these hybrids contained relatively higher amounts of 3,3′,4′,5,6,7,8-heptamethoxyflavone and natsudaidain. On the other hand, G-169 contained a higher amount of nobiletin than Mukaku Kishu. Previously we reported that King demonstrated a strong differentiation-inducing activity toward HL-60 cells as well as the characteristic pattern of polymethoxylated flavones (*4*). King contains higher concentrations of 3,3′,4′,5,6,7,8-heptamethoxyflavone and natsudaidain than of nobiletin and tangeretin, whereas other species show reversed profiles of polymethoxylated flavone content. The characteristic profile of polymethoxylated flavones seems to be responsible for the biological activity of King. We reported the existence of significant correlation between nobiletin and tangeretin (coefficient of correlation = 0.9075) and between 3,3′,4′,5,6,7,8-heptamethoxyflavone and natsudaidain (coefficient of correlation = 0.4810) based on the results of quantitative analysis of major flavonoid contents of taxonomically and/or economically representative *Citrus* species. Most of the *Citrus* species could be classified into high-nobiletin−tangeretin species or high-3,3′,4′,5,6,7,8-heptamethoxyflavone−natsudaidain species (*10*).

The data for the 10 flavonoid contents were used for the PCA. The eigenvalues obtained from the correlation matrix are 3.17, 2.48, 1.85, 1.12, and 0.64 (Table 2). Choosing only eigenvalues >1 led to the reduction of 10 variables to 4 principal components (PC), comprising 86.2% of the total variability. The percentages of variance for the four PCs are 31.7% for the first one, 24.8% for the second, 18.5% for the third, and 11.2% for the last.

Factor loadings indicate the relative extent to which each original variable contributes to the variance con-

Table 3. Factor Loadings and Communality Values

	factor loading				
	PC1	PC2	PC3	PC4	communality
eriocitrin	−0.29	−0.09	−0.04	−0.16	0.81
narirutin	−0.32	0.00	0.09	−0.15	0.93
hesperidin	−0.02	−0.36	−0.06	−0.10	0.61
isorhoifolin	0.05	−0.37	0.05	0.05	0.82
diosmin	0.10	−0.48	0.08	−0.10	0.94
neoponcirin	−0.30	0.01	0.03	−0.23	0.90
nobiletin	−0.24	0.14	−0.10	0.40	0.89
heptamethoxyflavone	−0.01	−0.03	−0.51	−0.07	0.93
natsudaidain	0.01	−0.03	−0.48	−0.11	0.86
tangeretin	−0.17	0.15	−0.20	0.54	0.89

tained in each PC. The correlation between each PC and each original variable is given in Table 3. The five flavonoids that contributed most to the first principal component (PC1) are neoeriocitrin, narirutin, neoponcirin, nobiletin, and tangeretin. These flavonoids were negatively correlated with PC1. With the second principal component (PC2), nobiletin and tangeretin were positively correlated, and hesperidin, isorhoifolin, and diosmin were negatively correlated. 3,3′,4′,5,6,7,8-Heptamethoxyflavone and natsudaidain were strongly correlated with the third principal component (PC3).

Scatter plots of PCA factor loadings are shown in Figure 4. Four associations appeared, namely, A (3,3′,4′,5,6,7,8-heptamethoxyflavone and natsudaidain), B (nobiletin and tangeretin), C (narirutin, neoponcirin, and eriocitrin), and D (hesperidin, isorhoifolin, and diosmin) on the basis of factor loadings for PC1 and PC2. These possible correlations between natsudaidain and 3,3′,4′,5,6,7,8-heptamethoxyflavone and between nobiletin and tangeretin seemed to reflect their structural similarities. 3,3′,4′,5,6,7,8-Heptamethoxyflavone is a 3-OH methylated derivative of natsudaidain. Tangeretin has a structure similar to that of nobiletin, but it lacks the C-3′ methoxyl group of nobiletin. There was a tendency that the contents of flavanone and flavone differentiated these compounds from one another. All flavonoids in the third association (C) were flavanone, and two of three flavonoids in the fourth association (D) were flavone.

The scores for PC1 versus PC2 (A) and PC3 versus PC4 (B) are plotted as scatter diagrams in Figure 5. For PC1 and PC2, which shared 56.5% of the total variability, hybrids were more like King than Mukaku Kishu (Figure 5A). The similarity of hybrids to King was due to PC1, which was contributed by eriocitrin, narirutin, neoponcirin, nobiletin, and tangeretin, and PC2, contributed by hesperidin, isorhoifolin, and diosmin. Although most of the hybrids were more like Mukaku Kishu, some hybrids (G-155 and G-156) showed a peculiar feature on the basis of PC3, which strongly reflects natsudaidain and 3,3′,4′,5,6,7,8-heptamethoxyflavone content as mentioned above (Figure 5B). PCA also revealed the characteristic feature of high-3,3′,4′,5,6,7,8-heptamethoxyflavone−natsudaidain-containing hybrids, namely, G-155 and G-156.

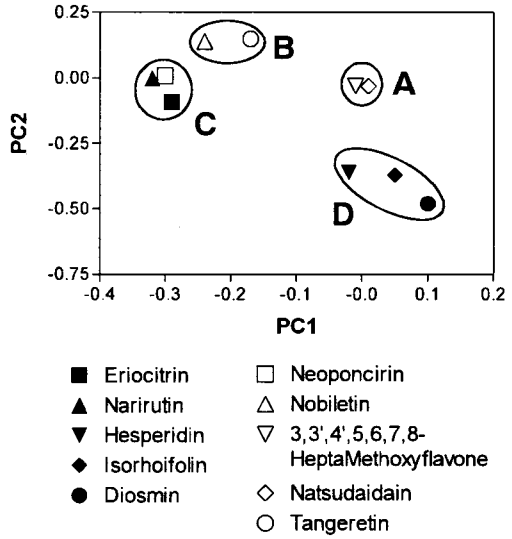


Figure 4. Scatter diagram of factor loadings of each flavonoid to PC1 and PC2.

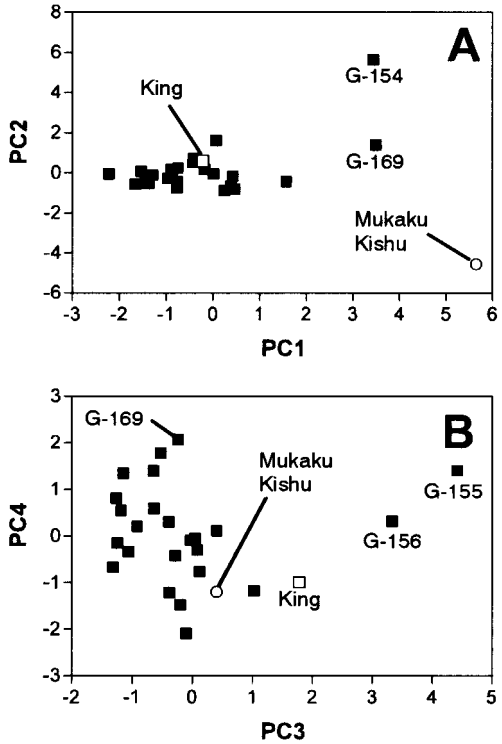


Figure 5. Scatter diagram from a PCA: (A) PC1 vs PC2; (B) PC3 vs PC4; (□) King; (○) Mukaku Kishu; (■) hybrids of King and Mukaku Kishu.

It is well established that the nature of a hybrid can be demonstrated by the presence of parent-specific compounds in the hybrid. Flavonoids are important markers to detect hybridization of *Citrus* plants (*11*). *Citrus* species are known to be heterozygous and to produce sexual offspring of varying character, some of which may be outside the parental range (*12*, *13*). One of the important factors that affect and alter the expected inheritance in a sexually reproducing organism is structural hybridity developing from within, due to cryptic chromosomal rearrangements (*14*). However, the flavonoid profile of hybrids from the crossing of King and Mukaku Kishu followed parental characteristics. Taxifolin, neoeriocitrin, rutin, naringin, neohesperidin, rhoifolin, neodiosmin, quercetin, naringenin, luteolin, poncirin, kaempferol, and apigenin, which were not present in either parent, did not appear in the hybrids. Hesperidin, which is the most abundant flavonoid, was detected in all hybrids, but the amounts were significantly reduced in almost all hybrids. The amount of narirutin was also decreased in many hybrids, but a greatly increased narirutin amount was found in several hybrids (G-154, G-163, and G-169). Isorhoifolin and diosmin, which were detected only in one parent, appeared in few or no hybrids.

In conclusion, we have characterized 24 hybrids of King and Mukaku Kishu by using flavonoid profiles, which are a key feature to detect hybridization of *Citrus* plants. The goal of this study was to identify hybrids that contain high amounts of polymethoxylated flavones. This information will be used to select varieties, via horticultural breeding programs, rich in potential anticancer substances.

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