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Multivariate Analysis by Measurement of Peroxidase and Essential Oil Components in *Citrus* Flavedo†

Masayoshi SAWAMURA, Xiao Hong ZHENG, Yoshitaka OOTANI, Hiroyuki UKEDA, and Hirozo KUSUNOSE

Department of Bioresources Science, Faculty of Agriculture, Kochi University, B-200 Monobe, Nankoku-shi, Kochi 783, Japan
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Cluster analysis was done of the oxygenated compounds in cold-pressed oil (CPO) prepared from the flavedo of 45 *Citrus* cultivars. The compounds were classified into six main clusters. Pummelos formed two clusters containing mostly medium or larger sizes. Most of the sour *Citrus* fruits were placed in the pummelo clusters, but each of *C. limon* and *C. sudachi* formed a cluster that contained no other members. Isozymic analysis for peroxidase showed a band pattern of not more than three bands (P_1, P_2, and P_3) for the 42 *Citrus* cultivars tested. P_1 was the key band of the pummelo species. The confusion between pummelos and other fruits such as sour *Citrus* fruits in cluster analysis based on CPO components only was resolved on the basis of the zymograms of peroxidase followed by cluster analysis.

The genus *Citrus* grows from the tropical to the temperate zones. In recent years, production of either grapes or *Citrus* fruits has been the most abundant for fruit worldwide. Researchers aiming at the preservation of gene resources in plants have collected more kinds of fruit trees in this genus than in any other. *Citrus* can be propagated and new varieties can be produced sexually, by asexual nucellar or chance seedlings, by crossing, and by mutation. In addition to these natural forms of propagation, *Citrus* breeders have created many artificially crossed new cultivars. The classification in this enlarging family is complex and is becoming confused. The best known taxonomies of the genus *Citrus* are those of Swingle[1] and of Tanaka.[2] Their taxonomies differ widely in the number of species admitted, as Swingle and Tanaka define 16 and 159 species, respectively. Their arrangements of the species by taxonomy, however, are similar.

Flavor components also are a means of classifying species, as isozymes are inherited. Multivariate analysis of essential oil components has been applied in the judgment of *Citrus* product quality[3,4] and for chemotaxonomy of *Citrus* fruits.[5-8] We[9] have reported multivariate analysis of components of cold-pressed oil (CPO) for 37 *Citrus* cultivars. Taxonomical research by analysis of isozymes has been undertaken by many other workers.[8,10-13] We examined some analytical conditions for peroxidase and esterase from *Citrus* flavedo.[14] This paper describes revised multivariate analysis based on the essential oils of 45 *Citrus* cultivars, and introduces a combined taxonomy based on both chemical components and zymograms of peroxidase from *Citrus* flavedo.

Materials and Methods

Materials. The *Citrus* samples used, besides those reported previously,[9] are listed in Table I. ITO and MAP were obtained from the Okitsu Branch of the Fruit Tree Research Station, Ministry of Agriculture, Forestry, and Fisheries, in Shizuoka Prefecture. Scientific names of zabon 1 (ZA1), 2 (ZA2), and 3 (ZA3) are unidentified. ZA1, called bontan, was purchased at a market in Kagoshima City and weighed 1.5 kg. ZA2 and ZA3 were obtained in Malaysia (in August 1992), and weighed 2.6 and 1.6 kg, respectively. There were no seeds in ZA2 but ZA3 had many tiny seeds. The flesh color was pink in ZA2 and slightly pink in ZA3. Other fruits were obtained from the Kochi Fruit Tree Experiment Station. NAS is grown locally in Kochi Prefecture and has not been officially named yet. It seems to resemble *C. inflata* Hort ex Tanaka (mochiyuzu).

All samples were obtained in December 1992 except for ZA2 and ZA3. *Citrus* common names and their abbreviations follow here: TOS, Tosa-buntan; SUI, suisho-buntan, AKA, akano-buntan; BAO, banokan; HAS, hassaku; KAW, Kawachibankan; NAT, natsudaidai, KAN, kawanonatsudaidai; DUN, dunkan; GRA, grapefruit; KAB, kabosu; NAO, naoshichi; BAP, banpeiyu; UCH, uchimurasaki; ANS, anseikan; HIR, Hirado-buntan; EGA, egami-buntan; HON, honda-buntan; MAT, mato-buntan; VIE, Vietnam-buntan; DAI, daidai; TOK, tokosu; MOC, mochiyuzu; HYU, Hyuganatsu; SAN, sanbokan; IYO, Iyokan; MIN, mineola; PON, ponkan; ORL, orland; NOV, nova; OTA, ootachibana; UNS, unshu mikan; TAC, tachibana; YUZ, yuzu; UJU, ujukitsu; SUD,

Table I. List of *Citrus* Samples

No.	Abbr.	Common name	Botanical name
1	ITO	Itoshima-bankan	*Citrus grandis* Osbeck forma Itoshima-bankan
2	MAP	Mato-peiyu	*C. grandis* Osbeck forma Mato-hakuyu
3	KOT	Kotokan	*C. kotokan* Hayata
4	YNA	Yoshida navel	*C. sinensis* Osbeck forma Yoshida navel
5	FUK	Fukuhara orange	*C. sinensis* Osbeck forma Fukuhara
6	CLE	Clementine	*C. clementina* Hort. ex Tanaka
7	KAR	Kara	*C. unshiu* Marcov. × *C. nobilis* Loureiro
8	TAN	Tankan	*C. tankan* Hayata
9	MUR	Murcott	*C. reticulata* Blanco × *C. sinensis* Osbeck
10	KIY	Kiyomi	*C. unshiu* Marcov. × *C. sinensis* Osbeck
11	NAS	Narukawa-sumikan	Unknown
12	OYU	Ooyu	Unknown
13	LE1	Lemon	*C. limon* Burm. f. cv. Lisbon
14	LE2	Lemon	*C. limon* Burm. f. cv. Eureka
15	ZA1	Zabon or Bontan	*C. grandis* Osbeck
16	ZA2	Zabon or Bontan	*C. grandis* Osbeck
17	ZA3	Zabon or Bontan	*C. grandis* Osbeck

† Taxonomy of the *Citrus* Genus. Part II.

sudachi. Botanical names of *Citrus* fruits described above can be seen in the previous paper.[9]

Sample preparation for measurement of CPO and peroxidase. The preparation of CPO from *Citrus* flavedo[15] and its assay by GC[16] were done as previously reported. The preparation of peroxidase from the flavedo as an acetone powder and the method of polyacrylamide gel electrophoresis was as described before.[14] The bands on zymograms were analyzed by a Shimadzu densitometer (CS-9000) with monitoring at 436 nm. Cluster analysis was done as in a previous paper,[9] and the dendrograms for hierarchial clustering were obtained by application of Ward's method to the Euclidian distances. Forty-five *Citrus* samples were analyzed for CPOs, and 39 samples were analyzed for peroxidase.

Results and Discussion

Cluster analysis by CPO components

The revised classification by cluster analysis based on the oxygenated compounds in CPOs of 45 cultivars is summarized in Table II, with results similar to those obtained previously.[9] There were six main clusters. Cluster 1 included pummelos and their medium-sized relatives (about 500 g): HAS, KAW, NAT, and KAN. Cluster 2 included large pummelos (1 kg or more) except for ANS and ITO. It is reasonable that ZA1, ZA2, and ZA3, which weigh more than 1 kg were grouped in this cluster. Nootkatone, the key compound in pummelos,[16,17] was detected in their CPOs. These unidentified *Citrus* fruits are therefore understandably thought of as being pummelos. There are several sour cultivars, such as KAB and DAI in these two clusters. OYU smells faintly like YUZ, but it was in cluster 2. LE1, at present, is the only member of cluster 3, although it was in a group containing BAP, ANS, and others before.[9] It is assumed that LEM is a hybrid of citron and lime. If we can add data on citron and lime to the results shown here, the hereditary characteristics of LEM would be known.

Cluster 4 includes sweet oranges and their relatives such as HYU, IYO, and YNA, but other species, such as mandarins and tangelos, are included. MIN and ORL are tangelos crossed with tangerine and pummelo. These hybrids are not be classified by Swingle or Tanaka. OTA, which is a pummelo, had an Euclidian distance of 15.7 from cluster 1 in cluster 4. The inclusion of OTA into cluster 4 for sweet oranges may arise by its large amount of decanal. UNS and TAC in cluster 5 are classified as mandarins by Tanaka.

SUD was the only member of a cluster much further from other clusters; the Euclidian distance between cluster 1 and cluster 6 was 52.96. According to Tanaka, most sour *Citrus* fruits belong to the YUZ category. Our results suggest that the relationship between YUZ and other sour *Citrus* fruits is more distant than that.

The classification of YUZ has been difficult. Swingle stated that YUZ is a hybrid of *C. ichangensis* belonging to the Papeda subgenus together with *C. reticulata* var. Austera. Tanaka, on the other hand, thought it to be independent and close to the mandarin species. Our results show that YUZ and the mandarins are in cluster 5, and that YUZ and sour *Citrus* fruits are in different clusters. The relationship between YUZ and sour *Citrus* fruits will be further discussed in Fig. 3.

Classification by a combination of several methods is more reliable than classification by one method. We used isozyme analysis in addition to essential oil analysis.

Table II. Classification by Cluster Analysis Based on the Percentages of Oxygenated Compounds in Cold-pressed Oils

Cluster No.	Euclidian distance	*Citrus* cultivars
1		TOS, SUI, AKA, BAO, HAS, KAW, NAT, KAN, DUN, GRA, KAB*, NAO*
2	7.52 (1–2)[a]	BAP, UCH, ANS, HIR, EGA, HON, MAT, VIE, ZA1, ZA2, ZA3, ITO, MAP, DAI*, TOK*, MOC*, OYU*, NAS*
3	6.78 (2–3)	LE1
4	15.74 (1–4)	HYU, SAN, IYO, YNA, MIN*, PON*, ORL*, NOV*, OTA*
5	5.18 (4–5)	UNS, TAC, YUZ*, UJU*
6	52.96 (1–6)	SUD

[a] Between these two clusters.

Asterisks mark cultivars are different from this cluster judging from the taxonomy of Swingle or Tanaka.

Table III. Band Patterns on Zymograms of Isoperoxidase from *Citrus* Flavedo

Band pattern[a]	*Citrus* cultivars
P1+P2	HIR, UCH, BAP, ZA2
P1+P2+P3	OTA, KAN, GRA, TOS, BAO, SUI, HAS, KOT, ANS, DAI
P2	PON, YUZ, TAC, SUD
P3	MIN, ORL, NOV, YNA, KAB, NAO, TOK, MOC, NAS, OYU, SAN, IYO, UNS, KIY, LE1, LE2, FUK, CLE, KAR, TAN, MUR, SAN
P2+P3	HYU, UJU

[a] The electrophoretic zymograms of peroxidase from *Citrus* flavedo have no more than three bands.

Peroxidase analysis

Table III shows the band patterns of peroxidase from 42 kinds of *Citrus*. A total of any three bands of peroxidase have been detected in 9 species of *Citrus* flavedo.[14] Pummelos and their relatives have two or three bands (some combination of P_1, P_2, and P_3), and non-pummelos have either P_2 or P_3 but lack P_1. These simple patterns are useful in discriminating pummelos from others. DAI is one exception, with three bands (P_1, a very week P_2, and P_3), but it seem not to be a pummelo.

Cluster analysis by densitograms of peroxidase

A densitogram of a peroxidase zymogram from TOS is shown in Fig. 1. The maximum resolution was 0.05 mm with this instrument. Cluster analysis on the basis of the peak area was done for 13 cultivars of pummelos and their relatives, which had both P_1 and P_2 or else all three bands, P_1, P_2, and P_3. The dendrogram (Fig. 2) shows that the large and medium-sized pummelos were separated by the analysis. The Euclidian distance between the cluster containing large pummelos such as HIR, UCH, BAP, and ZA2 and the cluster containing medium-sized such as OTA and KAN was 164. This clear-cut discrimination arose because of the absence of a P_3 band in the large pummelos, so that quantity is taken to be zero when

the densitogram was drawn. There was a small distance, 4, between the two clusters for the medium-sized pummelos with all three bands, P_1, P_2, and P_3. The contributory factor to such a difference in clustering seemed to be as follows. The ratio of the percentage of the area of the P_2 band to that of the P_3 band was less than 1 in OTA, KAN, GRA, and TOS, and 1 or more in BAO, SUI, HAS, KOT, and ANS. Jang *et al.*[18] tried to classify *Pyrus* species by cluster analysis based on the measurement of peroxidase zymograms, and discussed genetic relationships among the cultivars. Our quantitative results suggest that the relationships of TOS and OTA, and of SUI and BAO are close.

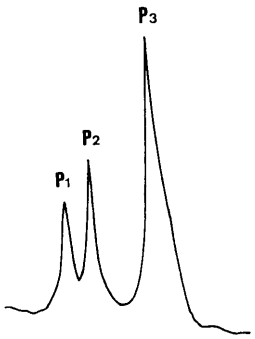

Fig. 1. Densitogram of Peroxidase from the Flavedo of *Citrus grandis* Osbeck forma Tosa-buntan.

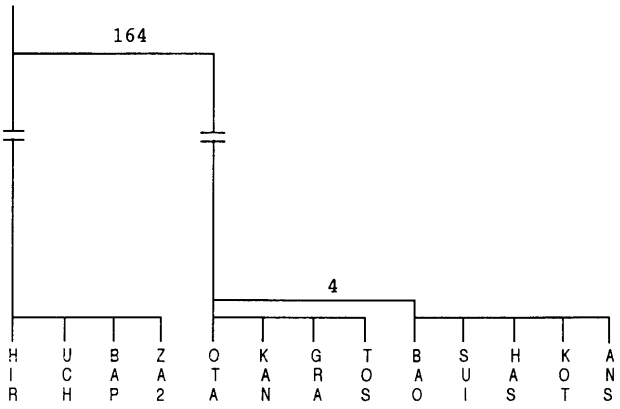

Fig. 2. Dendrogram from Cluster Analysis Based on Densitometric Amounts of Peroxidase from Pummelos.

All samples selected had a P_1 band on the zymogram of their peroxidase. The numerals on the dendrogram give the Euclidian distances.

Consequently, the peroxidase pattern is one means of dicriminating pummelos from *Citrus* fruits in general (Table III). Statistical analysis based on the results of measurement of peroxidase zymograms of pummelos relates fruit size to hereditary characteristics such as isozymes.

Cluster analysis among Citrus fruits without P_1 band

Classifications based on chemical components and isozyme analysis were different. Taxonomy involving chemical components may depend on the scale of the population; reliability is greater as the scale increases. This kind of chemotaxonomy is generally based on both the qualities and quantities of components. The presence or absence of bands on the zymograms designates the genotype, so the results based on this quality only are not variable or dependent on the sample number. *Citrus* species cannot always be classified only by isozyme analysis, but the band pattern of peroxidase clearly discriminates pummelos from other species. With clusters 1 and 2, pummelos are grouped with several sour fruits because of the similarity of their CPOs. If we here ignore the results of clustering for pummelos and their relatives, the relative Euclidian difference among the fruits would be further magnified, and additional groupings would occur.

Twenty-one cultivars without a P_1 band of peroxidase were selected for repeated cluster analysis based on the oxygenated compounds in their CPOs. The new cluster I includes mandarins and tangelos such as MIN, ORL, and NOV (Fig. 3). The tangelos are similar to mandarins or tangerines such as UNS and PON in their morphology. The single P_3 band in MIN, ORL, and NOV may be an

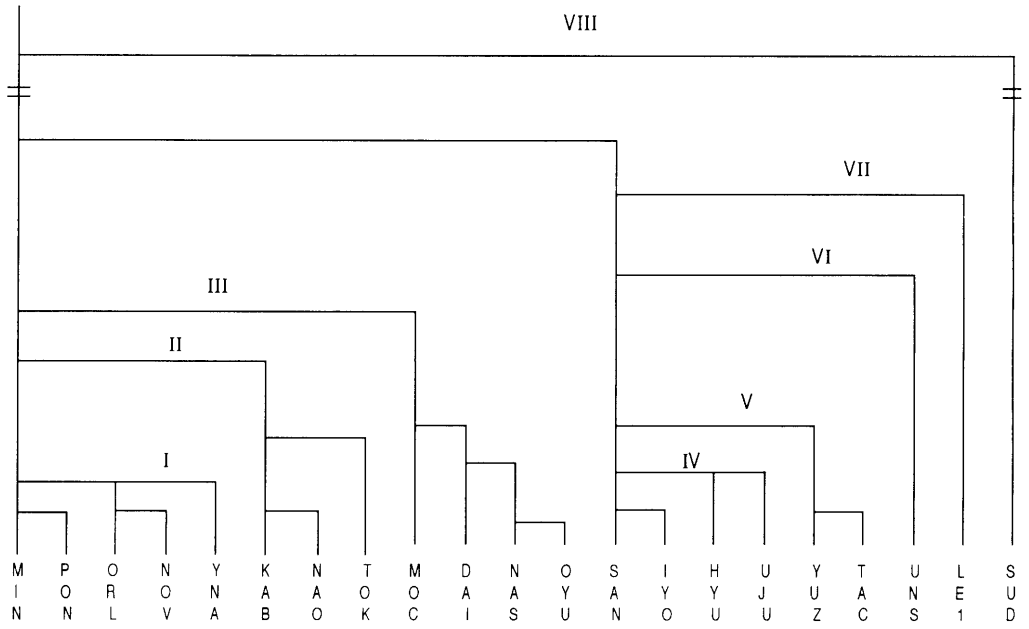

Fig. 3. Dendrogram from Cluster Analysis Based on the Oxygenated Compounds of Cold-pressed Oils.

All samples selected had no P_1 band on the zymogram of their peroxidase.

inheritance from mandarins.

Most sour *Citrus* fruits were grouped into the YUZ species by Tanaka,[2] but our data led us to different conclusions, as was seen in Table II. Clusters II and III, as shown in Fig. 3, seem to be represented by KAB and MOC, respectively, and SUD was in cluster VIII. KAB, MOC, and SUD are, therefore, distant from YUZ in cluster V on the dendrogram. Ichangensin, a limonoid, is found in YUZ,[19] SUD,[20] KAB,[21] and sour orange (*C. aurantium*).[22] Chemotaxonomy done with regard to limonoids suggests that these sour *Citrus* fruits may be of the same species because ichangensin is abundant in all of them, but is found in few in any other *Citrus* fruits.[20] There might be a relationship between *C. ichangensis* and YUZ because of the presence of ichangensin in the former.[19] The genotypes of these fruits are not identical, however.[23] The results shown in Fig. 3 suggest that YUZ is not closely related to other sour *Citrus* fruits such as KAB, MOC, and SUD.

UNS and LE1 each form single clusters. Swingle and Tanaka grouped UNS and PON into the mandarin species, but they are separated by a long distance on the dendrogram. The difference in the P_3 band for UNS and the P_2 band for PON (Table III) is in agreement with results from leaf isozymes.[23]

Barrett and Rhodes[24] recognized only three basic species: *C. grandis* Osbeck, as in the pummelo; *C. medica* L., as in the citron, a sour fruit; and *C. reticulata* Blanco, as in the highly variable mandarin group. Three separate bands of peroxidase in *Citrus* flavedo may be helpful in simplifying the complex *Citrus* classification.

We concluded that analyses of essential oil components and peroxidases are useful for establishment of the taxonomy of the genus *Citrus*. Knowledge about peroxidases from *Citrus* flavedo will make possible discrimination between pummelos and other species. It would be best to carry out multivariate analysis based on CPO components of *Citrus* samples already selected by their peroxidase pattern. When we deal with unknown *Citrus* in the future, these procedures will provide information with which we may find which species the fruit belongs to.

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References

1) W. T. Swingle, in "The Citrus Industry," Vol. 1, ed. by H. J. Webber and L. D. Batchelor, University of California Press, Berkeley, 1943, pp. 129–474.
2) T. Tanaka, *Bull. Univ. Osaka Prefect., Ser. B*, **21**, 133–138 (1969).
3) J. A. Pino, R. G. Torricella, F. Orsi, and L. Figueras, *J. Food Qual.*, **9**, 205–216 (1986).
4) C. Vélez, E. Costell, L. Orlando, M. I. Nadal, J. M. Sendra, and L. Izquierdo, *J. Sci. Food Agric.*, **61**, 41–46 (1993).
5) R. W. Scora and S. Torrisi, *Proc. Am. Soc. Hort. Sci.*, **88**, 262–271 (1966).
6) R. W. Scora, J. W. Cameron, and A. B. England, *Proc. Am. Soc. Hort. Sci.*, **92**, 221–226 (1968).
7) J. M. Ortiz, J. Kumamoto, and R. W. Scora, *International Flavours and Food Additives*, **7**, 224–226 (1978).
8) Y. Gogorcena and J. M. Ortiz, *J. Sci. Food Agric.*, **48**, 275–284 (1989).
9) M. Sawamura, K. Shichiri, Y. Ootani, and X. H. Zheng, *Agric. Biol. Chem.*, **55**, 2571–2578 (1991).
10) I. Ueno, *Bull. Fruit Tree Res. Stn. B (Okitsu)*, **3**, 9–24 (1976).
11) A. Esen and R. Soost, *J. Hered.*, **67**, 199–203 (1976).
12) E. E. Protopapadakis, *J. Hort. Sci.*, **62**, 413–419 (1987).
13) T. Matsuyama, R. Motohashi, T. Akiyama, and M. Omura, *Jpn. J. Breed.*, **42**, 155–159 (1992).
14) X. H. Zheng, Y. Ootani, and M. Sawamura, *Biosci. Biotech. Biochem.*, **57**, 1800–1802 (1993).
15) M. Sawamura and T. Kuriyama, *J. Agric. Food Chem.*, **36**, 567–569 (1988).
16) M. Sawamura, T. Tsuji, and S. Kuwahara, *Agric. Biol. Chem.*, **53**, 243–246 (1989).
17) M. Sawamura, S. Kuwahara, K. Shichiri, and T. Aoki, *Agric. Biol. Chem.*, **54**, 803–805 (1990).
18) J. T. Jang, K. Tanabe, F. Tamura, and K. Banno, *J. Jpn. Soc. Hort. Sci.*, **61**, 273–286 (1992).
19) F. Hashinaga, Z. Herman, and S. Hasegawa, *Nippon Shokuhin Kogyo Gakkaishi*, **37**, 380–382 (1990).
20) F. Hashinaga and S. Hasegawa, *J. Jpn. Soc. Hort. Sci.*, **58**, 227–229 (1989).
21) M. Miyake, "Studies on Limonoids," Ph.D. Thesis, Kyushu University, Fukuoka, 1993, pp. 21–25.
22) M. Miyake, S. Ayano, Y. Ozaki, Z. Herman, and S. Hasegawa, *Nippon Nōgeikagaku Kaishi*, **66**, 31–34 (1992).
23) M. Hirai and I. Kajiura, *Jpn. J. Breed.*, **37**, 377–388 (1987).
24) H. C. Barrett and A. M. Rhodes, *Syst. Bot.*, **1**, 105–136 (1976).