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Cluster Analysis by Measurement of Peroxidase and Esterase from *Citrus* Flavedo[†]

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The zymograms of peroxidase (POD) and esterase (EST) from seventy-two kinds of *Citrus* fruits and the fruit of both *Poncirus* and *Fortunella* were obtained by polyacrylamide gel electrophoresis. It was found that there was little variance in the band pattern of POD and EST of the fruits harvested after September. There were at least three bands of POD and approximately ten of EST in each cultivar. The density of each band for EST was measured with a densitometer. There was a characteristic band for POD identifying pummelos. The band patterns of EST were also characteristic among each species or cultivar. Two kinds of ootachibana (*Citrus otachibana* Hort. ex Tanaka) were shown to be different cultivars from each other according to their EST zymograms and peel oil analysis. Cluster analysis based on EST was done and discussed together with POD.

Key words: peroxidase; esterase; isozyme; taxonomy; *Citrus* genus

The *Citrus* genus has produced many varieties and species because of easy crossbreeding. Swingle[1] and Tanaka[2] published taxonomies of *Citrus* genus according to morphology. There are, however, many varieties of *Citrus* fruits that have not been widely known and classified properly, while a number of new cultivars have been bred every year. Recently chemical and biochemical techniques using essential oil components,[3–5] isozymes,[4,6–12] and DNA[10,11,13,14] have been introduced in the classification of the *Citrus* genus, supplementing the traditional morphological technique.

We previously reported on chemotaxonomy using multivariate analysis of essential oil compositions and isozyme patterns.[5,15,16] This paper deals with the classification by POD and EST, of seventy-two kinds of *Citrus* fruits, including some newly bred cultivars, some unclassified cultivars, and a few other genera.

Materials and Methods

Materials. All the fruits were obtained between October and December of 1993 and 1994. They were provided by the following stations: Kochi Fruit Tree Experiment Station; Shizuoka Fruit Tree Experiment Station: Izu Branch, Shizuoka Fruit Tree Experiment Station; Kagoshima Fruit Tree Experiment Station; Okitsu Branch, Fruit Tree Research Station, Ministry of Agriculture, Forestry, and Fisheries, Shizuoka Prefecture. The samples and their abbreviations are listed in Table I.

Isozyme analysis. The preparation of POD and EST from flavedo as an acetone powder, and the method of polyacrylamide gel electrophoresis were done as described in our previous paper.[15]

Cluster analysis for esterase. The stained gel was dried and the bands on the zymograms were analyzed with a Shimadzu densitometer (CS-9000), monitoring at 450 nm. The dendrograms for hierachial clustering were obtained by application of Ward's method to the Euclidean distance according to the cluster analysis described previously.[5]

Gas chromatography of peel oils. The cold-pressed oils were prepared by the following procedure: the flavedo was bent to crack the oil glands; the discharged crude oil was centrifuged after being saturated with sodium chloride. The supernatant, dehydrated with anhydrous sodium sulfate, was stored overnight at 5°C and then filtered. The filtrate was used for analysis of the peel oil composition. The analytical conditions of gas chromatography were the same as those described previously.[17]

Results and Discussion

Seasonal changes of the patterns of POD and EST

Both POD and EST patterns from June to December were examined in four varieties of pummelos, Tosa-buntan (TOS), anseikan (ANS), hassaku (HAS), and Hirado-buntan (HIR).[18] The pictures of the patterns are shown in Fig. 1. As deseribed before,[16] the characteristic POD band patterns of TOS, ANS, and HAS showing the three bands, and that of HIR showing the two bands were found throughout all the stages up to December. The pictures show that little variance occurs in the band patterns of both POD and EST from the *Citrus* flavedo after September. We suppose this tendency will be applicable to other *Citrus* fruits.

POD analysis

Typical zymograms for POD of the *Citrus* genus and other genera including trifoliate orange (TRI) and kumquat (KUM) are shown in Fig. 2. There were at most three bands in all the *Citrus* fruits as in P1, P2, and P3 in the order of rate of mobility. KUM showed a single band, P0, which was higher than band P1. TRI showed two bands, P3 and P4. The bands P0 and P4 have not been observed previously.

Table II shows the peroxidase genotype of seventy-two *Citrus* fruits and fruits of two other genera. There weren't more than three bands on the POD zymograms in each of the *Citrus* fruits. The band patterns were classified into eight

† Taxonomy of the *Citrus* Genus. Part III.
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Table I. List of *Citrus* Samples

No.	Abbr.	Common name	Botanical name
1	ANS	Anseikan	*Citrus grandis* Osbeck forma var. Anseikan Hort. ex. Tanaka
2	BAO	Banokan	*C. grandis* Osbeck forma Banokan Hort. ex Tanaka
3	BAP	Banpeiyu	*C. grandis* Osbeck forma Banhakuyu
4	BER	Bergamot	*C. bergamia* Risso et Point
5	BEY	Beniyu	Unknown
6	BUS	Fingered citron	*C. medica* Linn. var. sarcodactylis (Noot.) Swingle
7	CLE	Clementine	*C. clementina* Hort. ex Tanaka
8	CTP	Citrumelo	*C. paradisi* Macfadyen × *Poncirus trifoliata* Raf.
9	CTR	Troyer orange	*Poncirus trifoliata* Raf. × *C. sinensis* Osbeck
10	DAI	Daidai	*C. aurantium* Linn var. Cyathifera Y. Tanaka
11	DAT	Daito	*C. sinograndis* Hort. ex Tanaka
12	FUK	Fukuhara orange	*C. sinensis* Osbeck forma Fukuhara
13	GRA	Grapefruit	*C. paradisi* Macfadyen
14	HAN	Hanayu	*C. hanaju* Hort. ex Shirai
15	HAS	Hassaku	*C. hassaku* Hort. ex Y. Tanaka
16	HAY	Hayasaki	*C. grandis* Osbeck forma Hirado × *C. grandis* Osbeck forma Mato
17	HEB	Hebezu	*C.* sp.
18	HIR	Hirado-buntan	*C. grandis* Osbeck forma Hirado
19	ICL	Ichang lemon	*C. Wilsonii* Tanaka
20	ISU	Imamura × Suisyo	*C.unshiu* Marcov. forma Imamura × *C. grandis* Osbeck forma Suisyo
21	IYO	Iyokan	*C. iyo* Hort. ex Tanaka
22	KAB	Kabosu	*C. sphaerocarpa* Tanaka
23	KAN	Kawanonatsudaidai	*C. aurantium* L.
24	KAO	Kao phuang	*C. grandis* Osbeck forma Kao Phuang
25	KAR	Kara	*C. unshiu* Marcov. × *C. nobils* Loureiro
26	KAW	Kawachibankan	*C. kawachinensis* Hort. ex. Y. Tanaka
27	KIK	Kinukawa	*C. glaberrima* Hort. ex Tanaka
28	KIN	Kinkoji	*C. obovoidea* Hort. ex Tanaka
29	KIY	Kiyomi	*C. unshiu* Marcov. × *C. sinensis* Osbeck
30	KOT	Kotokan	*C. kotokan* Hayata
31	KUM	Kumquat	*Fortunella japonica* Swingle
32	KUN	Kunenbo	*C. nobilis* Lour. var. Kunep Tanaka
33	LE1	Lemon	*C. limon* Burm. f. cv. Lisbon
34	LE2	Lemon	*C. limon* Burm. f. cv. Eureka
35	LIM	Lime	*C. aurantifolia* Swingle
36	MAS	Marsh grapefruit	*C. paradisi* Macf. × *C. clementina* Hort. ex Tanaka
37	MIN	Minneola	*C. paradisi* Macfadyen × *C. tangerina* Tanaka
38	MKI	Mukaku-kishu	*C. kinokuni* Hort. ex Tanaka
39	MOC	Mochiyuzu	*C. inflata* Hort. ex Tanaka
40	MUR	Murcott	*C. reticulata* Blanco × *C. sinensis* Osbeck
41	NAN	Nanko	*C. unshiu* Marcov. forma Sanpo-wase × Clementine
42	NAO	Naoshichi	*C. taguma-sudachi* Hort. ex Tanaka
43	NAS	Narukawa-sumikan	Unknown
44	NAT	Natsumikan	*C. natsudaidai* Hayata
45	4NA	Natsumikan	*C. natsudaidai* Hayata (tetraploid)
46	NOV	Nova	*C. paradisi* Macfadyen × *C. tangerina Tanaka*
47	NTK	Naruto	*C. medioglobosa* Hort. ex Tanaka
48	OHY	Hyuganatsu	*C. tamurana* Hort. ex Takahashi
49	ORL	Orland	*C. paradisi* Macfadyen × *C. tangerina* Tanaka
50	OTA	Ootachibana	*C. otachibana* Hort. ex Tanaka (from Shizuoka)
51	OTB	Ootachibana	*C. otachibana* Hort. ex Tanaka (from Kagoshima)
52	OZU	Oozu	*C. ozu* Hort. ex Y. Tanaka
53	PON	Ponkan	*C. reticulata* Blanco. cv. F-2426
54	SAN	Sanbokan	*C. sulcata* Hort. ex Takahashi
55	SEI	Seiho	Kiyomi × Minneola
56	SEK	Sekitoyu	*C. grandis* Osbeck forma Sekitoyu
57	SHI	Shiikuwasha	*C. depressa* Hayata
58	SUD	Sudachi	*C. sudachi* Hort. ex Shirai
59	TAC	Tachibana	*C. tachibana* Tanaka
60	TAN	Tankan	*C. tankan* Hayata
61	TAR	Tarocco	*C. sinensis* Osbeck var. sanguinea Tanaka forma Tarocco
62	TNI	Tanikawa-buntan	*C. grandis* Osbeck forma Mato × *C. sulcata* Hort. ex Takahashi
63	TOK	Tokosu	*C. aurantium* L.
64	TOS	Tosa-buntan	*C. grandis* Osbeck forma Tosa buntan
65	TOU	Tosa-ujukitsu	*C. tosa-ujukitsu* Hort. ex Y. Tanaka
66	TRI	Trifoliate	*Poncirus trifoliata* Raf.
67	TSU	Tsunoka	Kiyomi × *C. unshiu* Marcov. var. Praecox Tanaka cv. Okitsu-wase
68	UCH	Uchimurasaki	*C. grandis* Osbeck forma Benikawa
69	UJU	Ujukitsu	*C. ujukitsu* Hort. ex Shirai
70	UNS	Unshiu mikan	*C. unshiu* Marcov.
71	VAL	Valencia orange	*C. sinensis* Osbeck forma Valencia
72	YNA	Yoshida navel	*C. sinensis* Osbeck forma Yoshida navel
73	YUK	Yuko	*C. yuko* Hort. ex Tanaka
74	YUZ	Yuzu	*C. junos* Sieb. ex Tanaka

groups, though five groups were shown in our previous paper.[16] Ueno[6] detected several bands, more than three from *Citrus* leaves, but there were at most three bands in the leaf according to Hirai and Kajiura,[8] as well as in our data from the flavedo. The disagreement could arise from differences in analytical conditions, such as polyacrylamide and starch gels.

Most genotypes coincided with those reported by Hirai and Kajiura.[8] The genotype of *BB* showing a set of two bands (P1 and P2) consisted of larger-sized pummelos whose weight is more than 1 kg. Banpeiyu (BAP), uchimurasaki (UCH), and hayasaki (HAY) also showed the same genotype as that of Hirado-buntan (HIR), Kao phuang (KAO), and sekitoyu (SEK) earlier reported.[8] It may be reasonable that a new variety, HAY, carries the morphological heredity of the parent, being larger-sized pummelos. There are, however, a few exceptions such as oozu (OZU) and bergamot (BER), which are not thought to be pummelos. Cold-pressed oil of BER contained 0.03% nootkatone, which is a characteristic compound of the

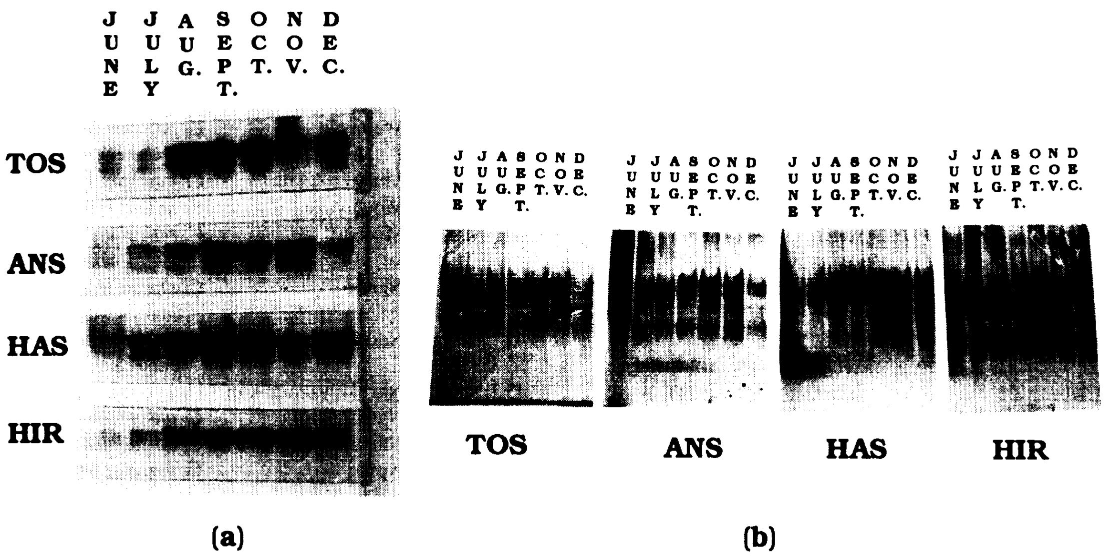

Fig. 1. Seasonal Changes of the Band Patterns of Peroxidase and Esterase from Some Kinds of *Citrus* Flavedo.
(a) Peroxidase, (b) Esterase. The *Citrus* samples were monthly examined from June to December.

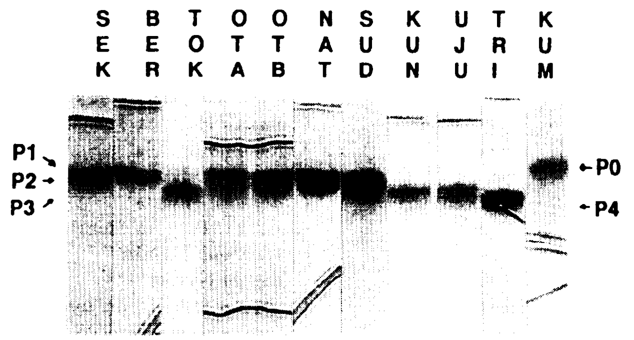

Fig. 2. Zymograms Showing Patterns of Peroxidase from *Citrus* Flavedo.

pummelo. Nootkatone was not detected in OZU. Thus, BER seems to have been originally related to the pummelo, but the genetic relationship of OZU still remains unclear.

Most fruits consisting the genotype of *BD* are medium-sized pummelos and their relatives weighing 400 to 600 g. Additional pummelos such as Marsh grapefruit (MAS), Tanikawa-buntan (TNI), Kawachibankan (KAW), and tetraploid natsudaidai (4NA) also showed this genotype. Daidai (DAI) was also in the group. It was suggested that pummelos had possibly acted as a maternal parent when sweet orange and sour orange originated.[3,7,8] Our data supported the suggestion by Yamamoto,[14] since the genotype of *BD* containing P2 and P3 bands in addition to a P1 band (Table II and Fig. 2). It has been a question why DAI is classified in the group of pummelos.[16] If we can consider DAI as a hybrid of sweet orange and pummelo on the basis of DNA analysis, there would be no wonder why DAI is in the same group as the pummelo.

The *Citrus* fruit showing the genotype of *CC* coincided with the previous data,[8] except for yuzu (YUZ). This group includes fingered citron (BUS), Mexican lime (LIM), tachibana (TAC), and ponkan (PON). YUZ, hanayu (HAN), and sudachi (SUD) are also in this genotype. Swingle considered YUZ as a hybrid of *C. ichangensis* and *C. reticulata.* The genotype of *AC* reported by Hirai and Kajiura[8] seems to be reasonable, because *C. ichangensis*

Table II. Genotype of *Citrus* Species and Cultivars Based on Peroxidase from Flavedo

Genotype	Band pattern[a]	*Citrus* cultivars
BB	P1 + P2	HIR, UCH, BAP, HAY, KAO, SEK, OZU, BER
BD	P1 + P2 + P3	OTA, OTB, KAN, GRA, TOS, MAS, BAO, HAS, KOT, ANS, TNI, KAW, KIK, NTK, KIN, NAT, 4NA, DAI
CC	P2	PON, YUZ, TAC, SUD, LIM, BUS, HAN
DD	P3	MIN, ORL, NOV, YNA, KAB, NAO, NAS, SAN, DAT, IYO, UNS, KIY, LE1, LE2, OHY, CLE, KAR, TAN, FUK, MUR, VAL, TAR, KUN, MKI, TSU, SEI, ISU, CTR, NAN
AD	P1 + P3	TOK, HEB, BEY, SHI, ICL, CTP
CD	P2 + P3	UJU, TOU, MOC, YUK
DP	P3 + P4	TRI
FF	P0	KUM

[a] R_f values are as follows: P0, 0.23; P1, 0.24; P2, 0.25; P3, 0.28; P4, 0.29.

has the same genotype as YUZ. It has, however, been reported that YUZ showed a complex fragment pattern in mitochondria DNA analysis, and there was no identical pattern in other *Citrus* cultivars.[14] Our result differed from that of Hirai and Kajiura[8] since YUZ showed a single band pattern as in P2. The question will be further studied.

The genotype of *DD* included most mandarins, oranges, and their relatives, which were Minneola (MIN), Orland (ORL), Nova (NOV), kiyomi (KIY), murcott (MUR), nanko (NAN), tsunoka (TSU), and seiho (SEI). Troyer orange (CTR) was also in this group. The results were in agreement with the report[9] concerning 16 isozymes analysis of mandarin hybrids. These data suggest that the relationship between mandarins and sweet oranges would be close. Scora and Torrisi[3] pointed out the possibility that the sweet orange had occurred as an hybrid of mandarin and pummelo. Thus, it may be reasonable that mandarins and oranges are concentrated in this group. Lisbon (LE1) and

Eureka (LE2) lemons were also the *DD* genotype. There is a report that the genotype of lemon is *CD*.[8] Torres *et al.*,[7] on the other hand, demonstrated that *C. limon* showed the same pattern as *C. sinensis* in the analysis of phosphoglucose isomerase and phosphoglucose mutase.

Shiikuwasha (SHI) had two bands shown by the genotype *AD*, as reported previously.[8] Tokosu (TOK), hebezu (HEB), and beniyu (BEY) are not described botanically. The genotype of Ujukitsu (UJU), Tosa-ujukitsu (TOU), mochiyuzu (MOC), and yuko (YUK) were *CD*. TRI and KUM are different genera of *Citrus* in the *Rutaceae* family. The bands P4 and P0 were characteristic of the two genera, respectively.

EST analysis

Figure 3 shows an example of zymograms of EST stained with α-naphthylacetate. There were roughly ten bands in most species and cultivars, but both the number and band pattern were different in each. The range of bands was separated into two zones, as shown previously,[15] where one zone (E1) was defined as a group of bands for $R_f \leq 0.38$ and the other (E2) was done as that for $R_f > 0.38$. We can distinguish two types of band patterns among the pummelos, as shown in Fig. 3. Two strongly stained bands occurred in E2 of KAO, SEK, TOS, and OTB, but no band in TNI and OTA. TRI, CTP, and CTR showed a similar band pattern in E2, as they are trifoliate or their relatives. The band of KUM was unique. The pattern of *Poncirus* is distinct from those of *Citrus* and *Fortunella*, but *Fortunella* cv. oval kumquat could not be distinguished from *Citrus* since it was grouped together with LIM and YUZ according to DNA analysis.[14] POD analysis showed the clear difference among the three genera (Table II). EST zymograms (Fig. 3) also showed different band patterns among *Citrus*, *Poncirus*, and *Fortunella*.

Cluster analysis of Citrus genus by EST pattern and POD analysis

We have seen various genotypes of POD from flavedo in Table II. It seems to be too complex to define genotypes of EST at present, because there were more than ten bands (Fig. 3). The number and intensity of EST bands are characteristic in each cultivar or species. Thus, we tried multivariate analysis based on the intensities of the EST bands as used for *Pyrus*.[19] An example of a densitogram of Valencia orange (VAL) is given in Fig. 4. Each peak area was measured mechanically by the perpendicular division method. The relative peak area was recalculated as the ratio of each peak to the largest.

Seventy *Citrus* fruits were classified by the cluster analysis. The clusters mainly fell into four groups, and the results are summarized in Table III. There were large-sized pummelos having a *BB* genotype and Iyokan (IYO) in the cluster 1 (C-1) and 3 (C-3). As IYO has been thought of as a tangor (tangerines × oranges), it appears to be incorrectly placed. One recent report assumed that IYO might have descended from the pummelo.[20] The major cluster, C-2, is mostly composed of tangerines and their hybrids, and is further divided into five. Subcluster 1 in C-2 (C-2-1) included the pummelos such as banokan (BAO), kotokan (KOT), kinukawa (KIK), kinkoji (KIN), and grapefruit (GRA), and the tangerines and their hybrids, such as clementine (CLE), kunenbo (KUN), nanko (NAN), TSU, MIN, ORL, and SEI.

TOK, HEB, and OZU have a sour taste as well as DAI, but their classification has not been described. It is suggested that those fruits might be closely related to DAI.

LIM was classified in C-2-3. BUS, BER, LE1, and LE2, which were classified in the subgenus *archicitrus*. Tanaka reported that YUZ, HAN, SUD, MOC, YUK, NAO, ICL, and KAB are very close to each other, and classified them in Osmocitrus-Euosmocitrus in subgenus *metacitrus*.[2]

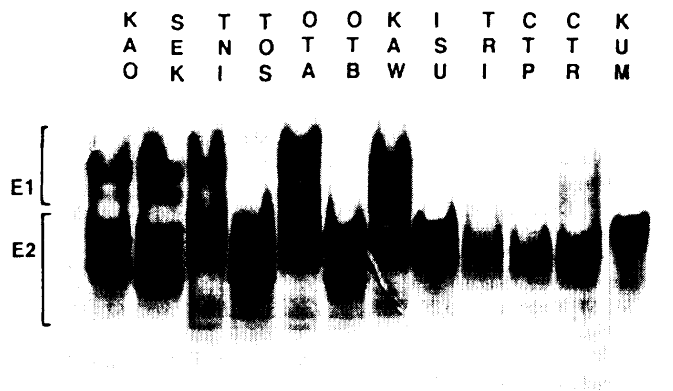

Fig. 3. Zymograms Showing Patterns of Esterase from *Citrus* Flavedo.

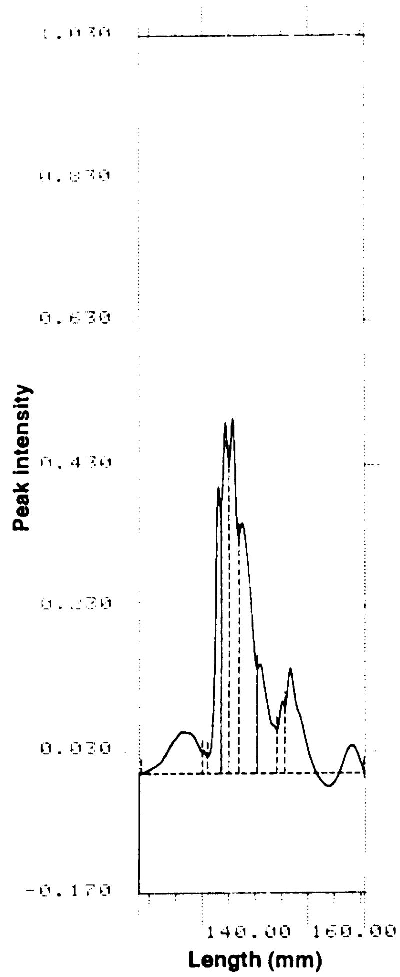

Fig. 4. A Densitogram of Esterase from Valencia Orange.

Each peak area was calculated mechanically according to the perpendicular division method.

Table III. Classification by Cluster Analysis Based on Esterase from *Citrus* Flavedo

Cluster No.	Euclidian distance	Subcluster No.	*Citrus* cultivars
1			HIR, BAP, MAS, KAW, HAY, TNI, OTA, IYO
2	484 (1–2)[a]	1	BAO, KOT, SEI, MIN, OZU, ORL, ICL, KIK, NAN, TSU, CLE, TOK, HEB, DAI, KIN, GRA, KUN
		2	OHY, NOV, KAR, PON, UNS, KIY
		3	LIM
		4	BUS, LE1, LE2, YUZ, SUD, BER, HAN
		5	ISU, KAB, MOC, NAO, YUK
3	770 (1–3)	1	UCH, SEK, KAO, TOU, UJU, ANS, HAS, TAC, MKI, TOS, OTB
		2	KAN, DAT, SHI, NAT, MUR, 4NA, NTK, NAS, BEY, SAN
4	868 (1–4)		FUK, TAN, YNA, VAL, TAR

[a] Between these two clusters.

Table IV. Comparison of Preodominant Compositions of Essential Oils from OTA, OTB, and TOS

Component	OTA	OTB	TOS
Sabinene	0.33[a]	0.26	0.23
Limonene	85.87	88.82	89.60
γ-Terpinene	7.68	5.54	4.96
p-Cymene	0.11	0.04	0.04
Terpinolene	0.33	0.21	0.21
Octanal	0.08	0.14	0.13
Linalol	0.76	0.08	0.06
Nootkatone	0.05	0.12	0.11

[a] % of peak area.

Those fruits were, however, different patterns in both POD and EST. ICL was classified in C-2-1, showing a band of P1+P3. YUZ, SUD, and HAN were in C-2-4, showing a band of P2. There were KAB and NAO with a band of P3, and MOC and YUK with a band of P2+P3 in C-2-5. This study suggests that YUZ and SUD are very close to each other, and KAB, NAO, MOC, and YUK are not closely related to YUZ and SUD. We reported previously that YUZ differed from NAO and KAB on the basis of cold-pressed oil analysis.[5] These results did not coincide with Tanaka's classification. The pummelos, in addition to C-1, also clustered in C-3-1. In C-3-2, different kinds of *Citrus* fruits, including Narukawa-sumikan (NAS) and BEY seem to be confused. C-4 was segregated far from the other clusters and all the cultivars in the cluster were sweet oranges.

The difference between OTA and OTB

There are instances in which some fruits are different botanically from each other, but their names are the same. In this regard, *C. otachibana* may be just the case. The OTA used in this experiment was obtained from the Okitsu Branch of Fruit Tree Research Station in Shizuoka Prefecture. OTB, on the other hand, was from the Kagoshima Fruit Tree Experiment Station in Kagoshima Prefecture. The shape of the two fruits are a little different: OTA is shaped like a pear, while OTB is round, like TOS. Though there were no differences between the two cultivars in POD analysis (Fig. 2), the EST zymogram showed a great difference between OTA and OTB. The OTB showed two strong bands in the E2 zone, while the OTA band pattern was different from that of OTB, as shown in Fig. 3. Moreover, the pattern of OTB is quite similar to that of TOS. Further proof of differences between OTA and OTB is given in Table IV, where the predominant compositions of cold-pressed oils from OTA, OTB, and TOS are shown. The compositions of OTB and TOS oils are similar, and different from that of OTA. The level of linalol in OTA was about ten times as much as in OTB and TOS. On the other hand, the amount of nootkatone in the former was half that in the latters.

It has been said that the progenitor of TOS is likely to be OTB. As shown in Table III, OTA is classified in C-1, while OTB and TOS are in C-3-1, being located far from the C-1. It is concluded that OTA and OTB are different cultivars, in spite of the name. TOS could be the same cultivar as OTB. This conclusion is mainly derived from chemotaxonomy. A comprehensive morphological classification of properties will be required.

We used two isozymes, POD and EST, as a means of classification. Investigation of many other enzymes may give us more detailed information.[9,12] A combination of chemical and biochemical procedures will reveal some potential factors which have not been shown by morphological methodology in the *Citrus* taxonomy.

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