Phylogenetic Relationships of Citrus Revealed by RFLP Analysis of Mitochondrial and Chloroplast DNA

Masashi YAMAMOTO¹⁾, Shozo KOBAYASHI²⁾, Yuri NAKAMURA²⁾ and
Yoshio YAMADA¹⁾

¹⁾ Kuchinotsu Branch, Fruit Tree Research Station, Kuchinotsu,
Nagasaki, 859-25

²⁾ Akitsu Branch, Fruit Tree Research Station, Akitsu, Hiroshima,
729-24

Restriction fragment length polymorphisms (RFLPs) among 28 species and cultivars in the genus *Citrus*, *Poncirus* and *Fortunella* were studied using eight mitochondrial and one chloroplast DNA probe/restriction enzyme combinations. According to mitochondrial DNA analysis, *Citrus* was divided into three major groups; citron, pummelo and mandarin. Furthermore, the pummelo group was divided into two sub-groups; pummelo and yuzu. The pummelo sub-group contained pummelos (*C. grandis*), grapefruit (*C. paradisi*), lemon (*C. limon*), sour orange (*C. aurantium*), sweet orange (*C. sinensis*), Kunenbo (*C. reticulata*), Natsudaikai, Hassaku, Sudachi, Hyuganatsu and Iyo. The yuzu sub-group contained Yuzu (*C. ichangensis* hybrid) and lime (*C. aurantifolia*). The mandarin group consisted of mandarin (*C. reticulata*) and tangor (hybrids of mandarin and sweet orange). *Poncirus* was distinct from *Citrus* and *Fortunella*. However, *Fortunella* could not be distinguished from *Citrus* since the mitochondrial genome of *Fortunella* was similar to that of Yuzu and lime. In the chloroplast DNA analysis, the fragment pattern was divided into two types. *Poncirus*, *Fortunella*, citron, Yuzu, Calamondin and the cultivars contained in the mandarin group showed the same fragment pattern. The cultivars contained in the pummelo sub-group and lime showed the other fragment pattern.

KEY WORDS: *Citrus*, *Fortunella*, *Poncirus*, mitochondrial DNA, chloroplast DNA, RFLPs, taxonomy.

Introduction

Although citrus is one of the major fruit crops in the world, the taxonomy of the genus *Citrus* is complex. For instance, SWINGLE (1967) identified 16 species, while TANAKA (1977) proposed 162 species. The difficulty in the analysis of the *Citrus* taxonomy is due to the asexual seed reproduction (nucellar embryony) and wide cross ability of the member of the genus.

In recent years, numerical (BARRETT and RHODES 1976, POTVIN *et al.* 1983, HANDA and OOGAKI 1985) and biochemical (UENO 1976, TORRES *et al.* 1978, HANDA *et al.* 1986, HIRAI and KAJIURA 1987, ZHU 1988, NIEDZ *et al.* 1991) analyses have been carried out for the classification of the genus *Citrus*. BARRETT and RHODES (1976), POTVIN *et al.* (1983), HANDA and OOGAKI (1985) and HANDA *et al.* (1986) recognized three basic species; citron (*C. medica*), pummelo (*C. grandis*) and mandarin (*C. reticulata*).

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The analysis of the restriction fragment length polymorphisms (RFLPs) has been found to be useful for determining the phylogenetic relationships among various economically important species (LOFTUS *et al.* 1988, SONG *et al.* 1988, DEBENER *et al.* 1990, FURNIER *et al.* 1990, MILLER and TANKSLEY 1990, GAWEL and JARRET 1991, MITA *et al.* 1991, YAMAMOTO *et al.* 1991). In citrus, RFLPs also appear effective for the classification and for genetic studies (GREEN *et al.* 1986, ROOSE 1988, DURHAM *et al.* 1992, JARRELL *et al.* 1992).

In this paper, we examined RFLPs in mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) to clarify phylogenetic relationships in the genus *Citrus*.

Materials and Methods

Citrus species and cultivars and its related genera preserved in Akitsu Branch, Fruit Tree Research Station were used. Some were obtained from Kuchinotsu Branch, Fruit Tree Research Station. In this study, we analyzed 26 *Citrus*, 1 *Fortunella*, 1 *Poncirus* species and cultivars (Table 1).

Total DNA was extracted from mature leaves according to the method of ROGERS and BENDICH (1985). DNA from each species and cultivar was digested with *Hind*III, *Eco*RI and *Pst*I for 7h at 37°C. Fragments were separated on 0.8% agarose gels, transferred to nylon membranes according to SOUTHERN (1975), and baked in vacuo for 2h at 80°C.

DNA fragments prepared from recombinant plasmids (mtDNA library and pTBa1) were used as probes. MtDNA library and pTBa1 contained *Pst*I or *Sal*I fragments of *Brassica campestris* mtDNA (PALMER and SHIELDS 1984) and a 19.6-kb *Bam*HI fragment of *Nicotiana tabacum* cpDNA (SUGIURA *et al.* 1986), respectively. These mtDNA clones and pTBa1 were kindly provided by Drs. J. D. PALMER and M. SUGIURA, respectively.

Labelling of probe DNA and visualization of probe-target DNA hybrid were carried out with the DIG-AMPPD system (Boehringer mannheim) except for one case (*Hind*III, probe P5.7) for which the ECL method (Amersham) was used.

In this paper, we represented species and cultivars according to the SWINGLE systematics (1967).

Results and Discussion

For the mtDNA analysis, two restriction enzymes (*Hind*III, *Eco*RI) and six mtDNA probes (P4.8, P5.7, P9.7, P12.4, S8.3, S11.8 which were scattered throughout the mitochondrial genome of *B. campestris*) were used. Polymorphism in the fragment patterns was observed in eight probe/restriction enzyme combinations (Fig. 1, Table 2).

For all the probe/restriction enzyme combinations, the fragment pattern of *Poncirus* differed from that of *Citrus* and *Fortunella*. Citron had a unique fragment pattern for four out of eight combinations. The fragment patterns of pummelos, Natsudaidai, Hassaku, grapefruit and Sudachi were identical for all the probe/restriction enzyme combinations. They were identical in lemon and sour orange and in sweet orange and Kunenbo. The identical fragment pattern was always observed among 'Murcott', King, 'Encore', satsuma mandarin, Kinokuni, Clementine and Ponkan.

According to NEI (1975), phylogenetic similarity was estimated based on the percentage of

common fragments between paired samples (Table 3). A dendrogram was constructed by applying unweighted pair grouping method (Fig. 2).

The dendrogram showed that *Poncirus* was distinct from *Citrus* and *Fortunella*. *Citrus* was

Table 1. List of species and cultivars used in this study

No.	Common name	Latin name	
		Swingle's system	Tanaka's system
1	Trifoliolate orange	<i>Poncirus trifoliata</i>	<i>P. trifoliata</i>
	Kumquat and its relatives		
2	Oval kumquat	<i>Fortunella margarita</i>	<i>F. margarita</i>
3	Calamondin		<i>C. madurensis</i>
	Citron		
4	Fingered	<i>Citrus medica</i>	<i>C. medica</i>
	Lemon		
5	Villafranca	<i>C. limon</i>	<i>C. limon</i>
	Lime		
6	Mexican	<i>C. aurantifolia</i>	<i>C. aurantifolia</i>
	Pummelo		
7	Hirado buntan	<i>C. grandis</i>	<i>C. grandis</i>
8	Matou buntan	<i>C. grandis</i>	<i>C. grandis</i>
9	Banpeiyu	<i>C. grandis</i>	<i>C. grandis</i>
	Pummelo relatives		
10	Hassaku		<i>C. hassaku</i>
11	Natsudaïdai cv. Kawano natsudaïdai		<i>C. natsudaïdai</i>
12	Marsh grapefruit	<i>C. paradisi</i>	<i>C. paradisi</i>
	Yuzu and its relatives		
13	Yuzu	<i>C. ichangensis</i> hybrid	<i>C. junos</i>
14	Sudachi Sour orange		<i>C. sudachi</i>
15	Kaiseito	<i>C. aurantium</i>	<i>C. aurantium</i>
	Sweet orange		
16	Trovita	<i>C. sinensis</i>	<i>C. sinensis</i>
	Miscellaneous		
17	Hyuganatsu		<i>C. tamurana</i>
18	Iyo cv. Miyauchi iyokan		<i>C. iyo</i>
	Tangor		
19	Tankan		<i>C. tankan</i>
20	Murcott	parentage unknown, possibly tangor	
	Mandarin		
21	Kunendo	<i>C. reticulata</i>	<i>C. nobilis</i>
22	King	<i>C. reticulata</i>	<i>C. nobilis</i>
23	Encore	King × <i>C. reticulata</i>	
24	Satsuma mandarin cv. Juman	<i>C. reticulata</i>	<i>C. unshiu</i>
25	Tachibana	<i>C. tachibana</i>	<i>C. tachibana</i>
26	Kinokuni	<i>C. reticulata</i>	<i>C. kinokuni</i>
27	Ponkan cv. F2428	<i>C. reticulata</i>	<i>C. reticulata</i>
28	Clementine	<i>C. reticulata</i>	<i>C. clementina</i>

divided into three major groups; citron, pummelo and mandarin. Furthermore, the pummelo group was divided into two sub-groups; pummelo and yuzu. *Fortunella* cv. Oval kumquat could not be distinguished from *Citrus* since it was grouped with Calamondin, lime and Yuzu which were included in yuzu sub-group. Hyuganatsu, Iyo, sweet orange, sour orange, lemon and Kunenbo were closely related to pummelo, and they should be belonged to pummelo sub-group.

For the cpDNA analysis, *Pst*I and pTBaI were used as the restriction enzyme and probe, respectively. The fragment pattern was divided into two types (Fig. 3). One of the fragment patterns was observed and recognized as identical among trifoliate orange, kumquat, Calamondin, citron, Yuzu, Tankan, 'Murcott' and mandarin cultivars except Kunenbo. Pummelos, Natsudaidai, Hassaku, grapefruit, Sudachi, lemon, lime, sweet orange, sour orange, Hyuganatsu, Iyo and Kunenbo showed another fragment pattern. The latter included pummelos and all the cultivars which were closely related to pummelo (pummelo sub-group) as revealed by the above RFLPs of mtDNA.

The present results for the most part were in agreement with those obtained in numerical taxonomic studies (BARRETT and RHODES 1976, POTVIN *et al.* 1983, HANDA and OOGAKI 1985) and biochemical studies (HANDA *et al.* 1986). *Poncirus* was distinct from *Citrus* and

Fig. 1. Southern blot hybridization of restriction endonuclease digests of total DNA to labelled mtDNA fragments. A: *Hind*III digested, probe P12.4 B: *Hind*III digested, probe S8.3. C: *Hind*III digested, probe S11.8. D: *Eco*RI digested, probe S8.3 1: Trifoliate orange, 2: Oval kumquat, 3: Calamondin, 4: Fingerd citron, 5: Villafranca lemon, 6: Mexican lime, 7: Hirado buntan, 8: Matou buntan, 9: Banpeiyu, 10: Hassaku, 11: Natsudaidai cv. Kawano natsudaidai, 12: Marsh grapefruit, 13: Yuzu, 14: Sudachi, 15: Sour orange cv. Kaiseito, 16: Sweet orange cv. Trovita, 17: Hyuganatsu, 18: Iyo cv. Miyauchi iyokan, 19: Tankan, 20: Murcott, 21: Kunenbo, 22: King, 23: Encore, 24: Satsuma mandarin cv. Juman, 25: Tachibana, 26: Kinokuni, 27: Ponkan cv. F2482, 28: Clementine.

Fortunella, and *Citrus* could be classified into three major groups; citron (*C. medica*), pummelo (*C. grandis*) and mandarin (*C. reticulata*) groups.

Based on the cpDNA analysis performed by GREEN *et al.* (1986), the *Citrus* species could be grouped according to their plastome. The cpDNA fragment pattern of pummelo was similar to that of lemon, sweet orange, sour orange and grapefruit. The second type consisted of mandarin and the third type of citron. These results were in close agreement with the present results of mtDNA analysis.

Table 2. Type of mtDNA fragment pattern of citrus

No.	cultivar	Type of fragment pattern ¹⁾							
		<i>Hind</i> III ²⁾						<i>Eco</i> RI	
		P4.8 ³⁾	P5.7	P9.7	P12.4	S8.3	S11.8	P9.7	S8.3
1	Trifoliate orange	a	a	a	a	a	a	a	a
2	Oval kumquat	b	b	b	b	b	b	b	b
3	Calamondin	c	b	b	b	c	b	c	b
4	Fingered citron	d	b	c	b	c	c	d	c
5	Villafranca lemon	e	c	d	c	c	b	c	b
6	Mexican lime	f	b	b	b	c	d	e	b
7	Hirado buntan	g	c	e	c	b	b	f	b
8	Matou buntan	g	c	e	c	b	b	f	b
9	Banpeiyu	g	c	e	c	b	b	f	b
10	Hassaku	g	c	e	c	b	b	f	b
11	Natsudaidai	g	c	e	c	b	b	f	b
	cv. Kawano natsudaidai								
12	Marsh grapefruit	g	c	e	c	b	b	f	b
13	Yuzu	e	d	b	b	c	d	g	b
14	Sudachi	g	c	e	c	b	b	f	b
15	Sour orange cv. Kaiseito	e	c	d	c	c	b	c	b
16	Sweet orange cv. Trovita	e	c	e	c	c	b	c	b
17	Hyuganatsu	g	c	e	c	b	b	c	b
18	Iyo cv. Miyauchi iyokan	h	c	e	c	d	b	f	b
19	Tankan	b	b	f	d	b	e	h	b
20	Murcott	b	b	g	e	b	e	i	b
21	Kunenbo	e	c	e	c	c	b	c	b
22	King	b	b	g	e	b	e	i	b
23	Encore	b	b	g	e	b	e	i	b
24	Satsuma mandarin cv. Juman	b	b	g	e	b	e	i	b
25	Tachibana	b	b	f	e	b	e	i	b
26	Kinokuni	b	b	g	e	b	e	i	b
27	Ponkan cv. F2428	b	b	g	e	b	e	i	b
28	Clementine	b	b	g	e	b	e	i	b

¹⁾ Same letter indicates same fragment pattern.

²⁾ Restriction enzyme.

³⁾ Probe.

Table 3. Percentage of common fragments of citrus based on mtDNA RFLP data

Code No. ¹⁾	1	2	3	4	5,15	6	A ²⁾	13	16,21	17	18	19	B ³⁾
2	56.0												
3	56.8	93.0											
4	56.0	75.0	78.9										
5,15	51.4	81.7	88.6	70.4									
6	53.8	90.7	94.6	77.3	83.8								
A	50.7	86.1	81.7	66.7	90.1	77.3							
13	54.1	84.5	91.4	76.1	88.6	89.2	81.7						
16,21	51.4	81.7	88.6	70.4	97.1	83.8	93.0	88.6					
17	51.4	87.3	82.9	64.8	91.4	78.4	98.6	82.9	94.3				
18	49.4	83.8	84.9	70.3	93.2	80.5	97.3	84.9	95.9	95.9			
19	56.8	81.7	77.1	64.8	68.6	70.3	73.2	74.3	68.6	74.3	71.2		
B	58.3	84.1	79.4	69.6	70.6	75.0	75.4	76.5	70.6	76.5	73.2	94.1	
25	57.5	82.9	78.3	68.6	69.6	74.0	74.3	75.4	69.6	75.4	72.2	95.7	98.5

¹⁾ 1: Trifoliate orange, 2: Oval kumquat, 3: Calamondin, 4: Fingered citron, 5: Villafranca lemon, 6: Mexican lime, 7: Hirado buntan, 8: Matou buntan, 9: Banpeiyu, 10: Hassaku, 11: Natsudaikai cv. Kawano natsudaikai, 12: Marsh grapefruit, 13: Yuzu, 14: Sudachi, 15: Sour orange cv. Kaiseito, 16: Sweet orange cv. Trovita, 17: Hyuganatsu, 18: Iyo cv. Miyauchi iyokan, 19: Tankan, 20: Murcott, 21: Kunenbo, 22: King, 23: Encore, 24: Satsuma mandarin cv. Juman, 25: Tachibana, 26: Kinokuni, 27: Ponkan cv. F2428, 28: Clementine.

²⁾ A: 7-12, 14.

³⁾ B: 20, 22-24, 26-28.

Citron (*C. medica*) is considered to be a species of the original type in *Citrus*, which differentiated from other species (HANDA *et al.* 1985). Lemon and lime are assumed to be hybrids derived from citron (BARRETT and RHODES 1976). However, both the mtDNA and cpDNA fragment pattern of lemon and lime differed from that of citron. Citron may thus not be the maternal parent of lemon and lime. The mtDNA and cpDNA fragment pattern of lemon and sour orange was identical and similar to that of pummelo. These results suggest that pummelo was the maternal parent of lemon and citron played as a paternal parent.

Grapefruit, Natsudaikai and Hassaku may be hybrids derived from pummelo (BARRETT and RHODES 1976, TORRES *et al.* 1978, HANDA and OOGAKI 1985, GREEN *et al.* 1986, HANDA *et al.* 1986). This assumption is supported by the analysis of the RFLPs of mtDNA and cpDNA. The mtDNA and cpDNA fragment pattern of these cultivars was identical with that of pummelo, indicating that pummelo was the maternal parent of these cultivars.

Iyo has been considered to be a tangor, *i.e.*, a hybrid of mandarin and sweet orange (HODGESON 1967). However, isozyme analysis (HIRAI *et al.* 1986) suggested that Iyo had genes from pummelo. HANDA and OOGAKI (1985) reported that Hyuganatsu was more closely related to sweet orange than to mandarin. However, based on isozyme analysis (HIRAI *et al.* 1986, HIRAI and KAJIURA 1987), Hyuganatsu was closely related to Japanese mandarin. Sudachi is assumed to be a hybrid between Yuzu and mandarin (IWAMASA 1976, HIRAI *et al.* 1986). In the mtDNA and cpDNA fragment pattern, a resemblance was found between Iyo, Hyuganatsu, Sudachi and pummelo. Considering the morphological and biochemical charac-

Fig. 2. Dendrogram of citrus based on mtDNA RFLP data.

1: Trifoliate orange, 2: Oval kumquat, 3: Calamondin, 4: Fingerd citron, 5: Villafranca lemon, 6: Mexican line, 7: Hirado buntan, 8: Matou buntan, 9: Banpeiyu, 10: Hassaku, 11: Natsudaikai cv. Kawano natsudaikai, 12: Marsh grapefruit, 13: Yuzu, 14: Sudachi, 15: Sour orange cv. Kaiseito, 16: Sweet orange cv. Trovita, 17: Hyuganatsu, 18: Iyo cv. Miyauchi iyokan, 19: Tankan, 20: Murcott, 21: Kunenbo, 22: King, 23: Encore, 24: Satsuma mandarin cv. Juman, 25: Tachibana, 26: Kinokuni, 27: Ponkan cv. F2428, 28: Clementine.

teristics, these three cultivars are not recognized as hybrids directly derived from cross of pummelo with the other cultivar. There had been complicated and frequent crosses when they were born. It is evident, however, that cultivars with the cytoplasm of pummelo has behaved as a maternal parent to Iyo, Hyuganatsu and Sudachi.

SCORA (1975) considers that sour orange is a hybrid of pummelo and mandarin. Though sweet orange is closely related to Chinese mandarin, genetic effects from pummelo have been detected (HIRAI and KAJIURA 1987). In this study, the mtDNA fragment pattern of sweet orange and sour orange was not identical with that of pummelo, but similar to that of pummelo. Neither the mtDNA nor cpDNA fragment pattern of sweet orange and sour orange was similar to that of most of the mandarins. Pummelo had possibly played as a maternal parent when sweet orange and sour orange originated.

Numerical and biochemical taxonomic studies have indicated the presence of variation in mandarins within a species (TORRES et al. 1978, HANDA and OOGAKI 1985). The results of isozyme analysis (HIRAI and KAJIURA 1987) suggested that mandarin cultivars had originated from three genetic sources; Indian, Chinese, and Japanese. In this study, Tachibana, a wild mandarin native to Japan, was distinct from other mandarins based on the mtDNA analysis. However, no differences were detected between Chinese mandarin such as Kinokuni and Indian mandarin such as Ponkan, as the mtDNA and cpDNA of most of them showed an identical pattern.

Fig. 3. Southern blot hybridization of restriction endonuclease digests of total DNA to labelled cpDNA fragments. *Pst*I digested, probe pTBa1. 1: Trifoliate orange, 2: Oval kumquat, 3: Calamondin, 4: Fingerd citron, 5: Villafranca lemon, 6: Mexican lime, 7: Hirado buntan, 8: Matou buntan, 9: Banpeiyu, 10: Hassaku, 11: Natsudaikai cv. Kawano natsudaikai, 12: Marsh grapefruit, 13: Yuzu, 14: Sudachi, 15: Sour orange cv. Kaiseito, 16: Sweet orange cv. Trovita, 17: Hyuganatsu, 18: Iyo cv. Miyauchi iyokan, 19: Tankan, 20: Murcott, 21: Kunenbo, 22: King, 23: Encore, 24: Satsuma mandarin cv. Juman, 25: Tachibana, 26: Kinokuni, 27: Ponkan cv. F2428, 28: Clementine.

'Murcott' and Tankan are considered to be tangor (HODGESON 1967). Their mtDNA and cpDNA fragment patterns were identical with or similar to those of mandarins. Thus, mandarin is considered to be the maternal parent of both cultivars.

Kunenbo originated in Indo-China, and Kunenbo and King may belong to the same species (HODGESON 1967). By isozyme analysis (TORRES *et al.* 1978, HIRAI *et al.* 1986, HIRAI and KAJIURA 1987), Kunenbo and King could not be distinguished from mandarin, but HODGESON (1967) assumed that they belonged to tangor. In our results, the mtDNA and cpDNA fragment patterns of Kunenbo were identical with those of sweet orange, but not with those of King and other mandarins. These results indicate that Kunenbo is a tangor which has sweet orange's cytoplasm. Because there is different cytoplasm between Kunenbo and King, the classification of Kunenbo and King which belong to the same species should be revised.

Calamondin is assumed to be a hybrid of kumquat and mandarin (SWINGLE 1943, UENO 1976, HANDA and OOGAKI 1985). The mtDNA fragment pattern of Calamondin was more similar to that of kumquat than to that of mandarin, indicating that the maternal parent of Calamondin was kumquat. The fragment patterns of the mtDNA of Calamondin and Oval kumquat were not identical. As kumquat (*Fortunella*) consists of many cultivars (SWINGLE 1943), mtDNA analysis using many cultivars of kumquat should be conducted to identify the parental cultivar of Calamondin.

The analysis of the RFLPs of mtDNA and cpDNA was useful in this study for estimating the maternal parents and for determining the phylogenetic relationships among *Citrus* and related genera. However, several questions still remain unanswered. For instance, lime was found to belong to the yuzu sub-group by the analysis of mtDNA, although its cpDNA fragment pattern differed from that of yuzu and was identical with that of the pummelo sub-group. Further studies using a larger number of cultivars and probe/restriction enzyme combinations should be conducted to clarify the above points and elucidate the phylogenetic relationships among *Citrus* and related genera.

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ミトコンドリアDNA及び葉緑体DNAのRFLPからみたカンキツの類縁関係

山本雅史¹⁾・小林省藏²⁾・中村ゆり²⁾・山田彬雄¹⁾¹⁾果樹試験場口之津支場, 長崎県南高来郡口之津町, 〒 859-25²⁾果樹試験場安芸津支場, 広島県豊田郡安芸津町, 〒 729-24)

カンキツ類の類縁関係については, 化学分類及び数量分類の適用により様々な成果が得られているが, まだ不明確な点も多い. そこで, ミトコンドリアDNA (mtDNA) 及び葉緑体DNA (cpDNA) のRFLPを利用してカンキツの類縁関係について検討した.

カンキツ属, カラタチ属及びキンカン属の28種・品種を供試した (Table 1). CTAB法により葉から全DNAを抽出し, *Hind*III, *Eco*RI 及び *Pst*I で切断してアガロース電気泳動を行った. カブの mtDNA 断片 (P 4.8, P 5.7, P 9.7, P 12.4, S 8.3, S 11.8, ミシガン大 J. D. Palmer 氏より分譲) 及びタバコの cpDNA 断片 (pTBal, 名古屋大 杉浦昌弘氏より分譲) をプローブとしてサザンハイブリダイゼーションを行った. DNA の検出には原則として DIG-AMPPD システムを用いたが, 一部 ECL 法を用いた.

mtDNA については, 2種類の制限酵素 (*Hind*III, *Eco*RI) と6種類の mtDNA 断片を用い8組合せで多型を得た (Fig. 1, Table 2). 明瞭に認められたバンドについて, それぞれのバンドの有無を相互に比較し, 各種類毎の共有バンド率を求めた (Table 3). この共有バンド率に基づいて UPG 法により系統樹を作成した (Fig. 2).

その結果, カラタチは他のカンキツ類とは類縁性が低く, キンカンはカンキツ属と近縁であった. カンキツ属はシトロン群, プンタン群及びマンダリン群の三群に大別できた. 更に, プンタン群は, 樹めてプンタンに近縁な群 (プンタン亜群) とややプンタンとの差異が認められる群 (ユズ亜群) に区別できた. プンタン亜群には, プンタン, グレープフルーツ, レモン, サワーオレンジ, スイートオレンジ, スダチ, クネンボ, ハッサク, ナツミカン, ヒュウガナツ及びイヨガ, ユズ亜群にはユズ, ライム, シキキツが含まれた. また, マンダリン群にはほとんどのマンダリンとタンゴールであるタンカン, 'マーコット' が属した.

次に, cpDNA について RFLP を検出したところ, 供試した種は2群に分類できた (Fig. 3). 一方には, プンタン亜群に属するものとライムが含まれ, もう一方には, カラタチ, キンカン, シトロン, マンダリン, タンゴール, ユズ及びシキキツが含まれた.

これら, mtDNA 及び cpDNA の RFLP で得られた結果は, 従来の化学分類及び数量分類とよく一致した. すなわち, カンキツ類はシトロン, プンタン, マンダリンの三群に大別でき, カラタチはキンカンに比べてカンキツ属との類縁性が低かった. また, mtDNA 及び cpDNA を用いることにより, 雑種起源とされているものについては種子親の推定も可能となった.

今後, 更に多数の種・品種及び制限酵素と mtDNA 及び cpDNA のプローブの組合せを用いることによって, カンキツ分類上の種々の問題点が解決できることであろう.