Molecular-based analysis of genetic diversity and classification of Japanese melon breeding lines

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For the breeding of Japanese netted melon, various types of foreign cultivars have been utilized for improving adaptability, disease and pest resistance, fruit quality and so on. However, little is known about their genetic diversity and relationships, since most cultivars derived from crosses between various horticultural groups. To figure out the genetic structure of Japanese melon, in this study, 57 melon accessions from three horticultural groups were examined using 55 RAPD markers produced by 24 RAPD primers. Genetic diversity of the Japanese netted melon was as high as those of cultivar groups of Groups Cantalupensis and Inodorus, while it was low in Group Conomon irrespective of large variations in fruit traits. Cluster analysis and PCO analysis based on genetic distance showed that Group Conomon was distantly related to other melon accessions. Among the latter, European cantaloupe (non-netted) and American open-field type (netted) proved to be genetically close, while England glasshouse melon (netted) including 'Earl's Favourite' is distantly related to these two groups and closely related with Group Inodorus. It was therefore suggested that England glasshouse type was established from hybrids between European cantaloupe and Group Inodorus. Japanese netted melon was most closely related with England glasshouse type, irrespective of the fact that various kinds of melon accessions have been crossed to improve adaptability, disease resistance and so on. In contrast, pure line cultivars of the Japanese netted melon bred by pure line selection from 'Earl's Favourite' or by crossing 'Earl's Favourite' with 'British Queen' were confirmed to be mostly homogenous, and it was difficult to establish RAPD markers to discriminate each cultivar. Group Conomon var. *makuwa* and var. *conomon*, which have been cultivated and utilized as different crops, proved to be genetically indistinguishable and were considered to share the same gene pool.

Key words : breeding, classification, genetic diversity, melon, RAPD

Introduction

Melon is an important horticultural crop in Japan, and has a long history of utilization and cultivation dating back to the end of the first millennium BCE[1, 2]. The traditional melon belongs to Groups Conomon and Agrestis, among which the latter is a weedy, feral or free-living melon having a bitter taste[3]. Archeological evidence clearly indicates that Group Conomon has been cultivated and utilized at the Shikata site, Okayama Prefecture, at least from 180 CE[4]. This type of melon has a smooth skin, soft epicarp and poor shelf life, and both ripe and young fruits are consumed as dessert and vegetable[2, 3, 5]. Subsequently, melon of Group Momordica was also introduced to Japan, and became popular from ca. 800 CE[2]. The sweet and sour types of Group Conomon are called "*makuwa*"and "*shirouri*", and are consumed as dessert and vegetable, respectively, and various kinds of local landraces have been established in each area[6].

Another type of melon introduced after ca. 1900 is netted, sweet melon of Groups Cantalupensis[7]. Among the cultivars introduced, the English cultivars showed rather good adaptability to Japanese conditions, and one cultivar 'Earl's Favourite' introduced in 1925 became the founder of the Japanese netted melon. Various kinds of pure line cultivars have been bred through pure line selection from 'Earl's Favourite' and crossed with 'British Queen', and contributed to the production of high quality fruits, mainly in Shizuoka Prefecture[8]. For large-scale production of good quality melon, 'Earl's Favourite' and pure line cultivars have been crossed to various kinds of melon genetic resources to improve adaptabil-

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ity and resistance to diseases and pests[9]. Such cultivars and breeding lines are now used as parents of F_1 hybrid cultivars of the Japanese netted melon. These facts clearly indicate that Japanese netted melon has been established by utilizing various genetic resources of domestic and foreign origins, as indicated by Nakata et al[10]. Kato et al.[11] analyzed peroxidase isozyme of the Japanese melon cultivars including commercial F_1 hybrids, and, based on isozyme polymorphism, they classified the cultivars into three types ; Px_{2A} type mainly including breeding lines with disease resistance, Px_{2B} type mainly consisting of pure line derivatives of 'Earl's Favourite', and Hetero type comprised of commercial F_1 hybrids. However, little is known about the details of their genetic structure.

In the present study, therefore, we analyzed the genetic diversity and relationships among melon cultivars, many of which have been utilized in breeding programs, by RAPD (random amplified polymorphic DNA) analysis which has been widely used for diversity analysis of melon[e.g. 12, 13]. Further, we made an attempt at figuring out the genetic structure of the Japanese netted melon.

Materials and Methods

Plant materials

Two sets of melon accessions were used in this study. The details of melon accessions are summarized below and indicated in Table 1. The first set was comprised of 47 accessions of melon (*Cucumis melo* L.) from four horticultural groups. Twenty-two accessions of Group Cantalupensis (European cantaloupe : 6, netted melon (England glasshouse type : 5, American open-field type : 7, Japanese breeding line : 4)), 15 accessions of Group Inodorus (Honeydew type : 4, Chinese Hami-melon : 6, winter melon from Spain and Russia : 5), 10 accessions of Group Conomon (var. *makuwa* : 5, var. *conomon* : 5). The second set consisted of 10 pure line cultivars of the Japanese netted melon among which 'Natsu 1', 'Natsu 4' and 'Natsu 7' were bred by crossing 'Earl's Favourite' with 'British Queen' and the rest by pure line selection from 'Earl's Favourite'. Two plants were examined for all accessions. Seeds of these accessions were provided by NARO Institute of Vegetable and Tea Science (NIVTS), Japan.

DNA extraction

Seeds of each plant were sown on wet filter paper in a Petri dish and germinated in an incubator maintained at 26℃ with a 16 h light and 8 h dark cycle at a light

Table 1 List of the first set of melon accessions analyzed in the present study

Horticultural group or cultivar group	Abbreviation	Accession
Group Cantalupensis		
European cantaloupe	C	#. 58-21, Carosello Scopatizzo Barese, Cantaloup de Bellegarde, Charentais, Melon Cantalupo di Charentais, Ogen (780045),
American open-field type	RA	Georgia 47, Hales Best, Homegarden, Rio Gold, Rocky Ford, SC108, Spicy
England glasshouse type	RE	Barnet Hill Favourite, Blenheim Orange, British Queen, Earl's Favourite, Hero of Lockinge
Japanese breeding line	RJ	Kurume 2, Melon Parental Breeding Line 1, Melon Parental Breeding Line 2, Paru
Group Inodorus		
Honey dew type	I	Honey Dew, Honey Dew (610002), Honey Dew (600011), Honey Dew (650013)
Chinese Hami melon	IC	Chinese Honey Dew, Hamiuri, Hamiuri 2, Hamiuri 6, Hamiuri H, Hamiuri J
Russian melon	IR	Ak-Urug, Kokand, Mirzuchulskaja
Spanish melon	IS	Spain Noboru 3 gou, Tendral o Invernale a Buccia Verde
Group Conomon		
makuwa	Ma	Kanro, Kinpyo, Mi-tang-ting, Nanbukin, Seikan
conomon	Co	Hyogo-aoshima-uri, Karimori, Nagasaki-tsuke-uri, Takada-shiro-uri, Tokyo-wase-shiro-uri

intensity of 46.5 $\mu M\ s^{-1}\ m^{-2}$. After 2 weeks, cotyledons from two seedlings of each accession were ground individually in liquid nitrogen. Total DNA was extracted using the cetyl-trimethyl-ammonium bromide method[14] with minor modifications. The quality and quantity of each DNA sample were determined with a spectrophotometer.

RAPD analysis

A total of 176 random primers (12 mer, Bex, Japan) were tested by using five cultivars of melon : Group Cantalupensis cv. 'Earl's Favourite' (netted), Group Cantalupensis cv. 'Rocky Ford' (netted), Group Cantalupensis cv. 'Charentais', Group Conomon var. *makuwa* cv. 'Kinpyo', and Group Conomon var. *conomon* cv. 'Takada-shiro-uri'. Twenty-four random primers selected for their ability to detect polymorphism and for the stability of PCR amplification were used for RAPD analysis (Table 2). PCR amplification was done in a 10 μL mixture containing 50 ng genomic DNA, 1 μLPCR buffer (Sigma-Aldrich®, USA : 10 mM Tris-HCl (pH 8.3), 50 mM KCl), 2.5 mM $MgCl_2$, 0.25 U *Taq* polymerase (Pharmacia, USA), 0.1 mM dNTP and 0.5 μM primer by using PC-700 (ASTEC, Japan). An initial denaturing step at 95℃ for 3 mins., 40 PCR cycles at 94℃ for 1 min., 40℃ for 2 mins. and 72℃ for 2 mins was done, with a final extension at 72℃ for 5 mins. After the amplification, samples underwent electrophoresis on 1.5% agarose gel (L03, Takara, Japan) at constant voltage 100 V (Mupid-2, Cosmo Bio, Japan). Then the PCR products were visualized with ethidium bromide staining and their polymorphisms were evaluated.

Data analysis

The RAPD marker band was scored as 1 for present and 0 for absent. From these data, the polymorphic index content (PIC) was calculated according to Anderson et al[15]. Genetic similarity (GS) among accessions was calculated as described by Apostol et al.[16], and their genetic distance (GD) was calculated with the formula GD = 1-GS. A dendrogram was constructed by the PHYLIP program using the unweighted pair group method with arithmetic mean (UPGMA) method. Principal co-ordinate analysis (PCO)[17] based on the genetic similarity matrix was done to show multiple dimensions of each group and melon accessions on a scatter-plot.

Table 2 Twenty-four RAPD primers analyzed in this study with sizes of polymorphic fragments

Primer No.	Sequence (5'→3')	Polymorphic fragment				
		No.	Size (bp)			
A07	GATGGATTTGGG	2	1353,	872		
A20	TTGCCGGGACCA	4	1500,	1400,	1100,	900
A22	TCCAAGCTACCA	3	1520,	1000,	970	
A23	AAGTGGTGGTAT	3	1860,	700,	670	
A26	GGTGAGGATTCA	3	1700,	1500,	1400	
A31	GGTGGTGGTATC	1	800			
A39	CCTGAGGTAACT	2	2027,	872		
A41	TGGTACGGTATA	3	1353,	1020,	930	
A53	GACGCCCATTAT	2	1860,	900		
A57	ATCATTGGCGAA	3	1353,	1078,	800	
B13	GGTCACCGATCC	3	1078,	970,	780	
B15	CCTTGGCATCGG	3	1800,	1300,	600	
B29	GATGGTCCGTTT	3	1600,	1400,	600	
B32	ATCATCGTACGT	1	940			
B37	AGGGCTCTAGGC	1	1600			
B39	GAGCTCCCGACA	1	2000			
B55	TGGCTTCATCAC	3	1700,	1500,	1400	
B68	CACACTCGTCAT	1	1078			
B71	GGACCTCCATCG	1	1020			
B84	CTTATGGATCCG	3	700,	600,	550	
B86	ATCGAGCGAACG	2	1400,	1350		
B96	GTGAAGACTATG	3	2000,	850,	750	
B99	TTCTGCTCGAAA	3	1600,	1500,	1400	
C00	GAGTTGTATGCG	1	1350			

Result

RAPD primers amplified multiple bands with different sizes as shown in Fig. 1. In case of primer B13, a 1050 bp band was absent in lanes 4, 9, and 10, while a 1000 bp band was amplified in lanes 6, 9, and 10. In addition, a 750 bp band was present only in lane 1. In this way, three marker bands were produced by a single

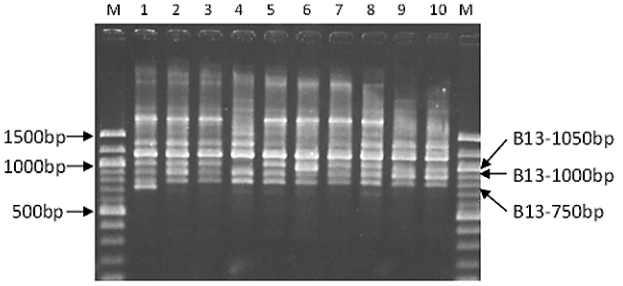

Fig. 1 Varietal variation of RAPD profiles obtained with primer B13. Three polymorphic fragments are shown by arrows on the right. Lane M represents 100bp DNA Ladder and the size was shown on the left. Lane 1 : Earl's Favourite (RE), Lane 2 : Rocky Ford (RA), Lane 3 : Paru (RJ), Lane 4 : Charentais (C), Lane 5 : Honey Dew (I), Lane 6 : Hamiuri (IC), Lane 7 : Kokand (IR), Lane 8 : Tendral o Invernale a Buccia Verde (IS), Lane 9 : Mi-tang-ting (Ma), Lane 10 : Takada-shiro-uri (Co).

primer B13. RAPD analysis of 47 melon accessions successfully detected 55 marker bands which are polymorphic among accessions and stable in PCR amplification (Table 2). The number of marker bands ranged from one to four per primer, and the average was 2.29. The most polymorphic markers are A26–1400 bp, B71–1020 bp and B84–600 bp, which were amplified from 24, 23, 24 accessions, respectively. The least polymorphic markers are A20–1400 bp, B13–780 bp, B15–1300 bp and B86–1400 bp, which were amplified from 2, 1, 45, 46 accessions, respectively. A marker band A53–900 bp was amplified from 10 accessions of Group Conomon, but not from the other 37 accessions. Similarly, two marker bands A20–1100 bp and C00–1350 bp were not amplified in 10 accessions of Group Conomon or 'Ano 3' of the Japanese breeding line, but amplified from the other 36 accessions. These three markers could be used to distinguish the Asian melon Group Conomon from other Groups, since 'Ano 3' was bred by the cross with Group Conomon cv. 'Mi-tang-ting'.

Genetic diversity within each cultivar group was evaluated based on PIC which is summarized in Table 3, along with the number of polymorphic markers. The PIC value was 0.175 in 47 accessions, and was over 0.070 in cultivar Groups Cantalupensis and Inodorus except of Honeydew type. In contrast, it was smaller than 0.054 in Group Conomon. The number of polymorphic markers was also higher in Groups Cantalupensis and Inodorus than that of Group Conomon.

GD among the 47 accessions ranged from 0 between 'Honey Dew (610002)' and 'Honey Dew (650013)' to 0.709 between 'Kokand (Russia)' and 'Mi-tang-ting'. Genetic relationship among the 47 accessions was visualized by UPGMA cluster analysis based on GD. As shown in Fig. 2, the 47 accessions were classified into six clusters. The most divergent was cluster I which consists of 10 accessions of Group Conomon. The second divergent was cluster VI which includes 11 accessions of Group Cantalupensis (European cantaloupe : 5, American open-field type : 6). All of the England glasshouse melons were grouped into cluster IV, along with two Spanish accessions of Group Inodorus. Furthermore, other accessions of Group Inodorus appeared in clusters III and V both of which were closely related with cluster IV. These results indicate genetic differentiation within Group Cantalupensis, and may suggested genetic introgression from Group Inodorus for the establishment of the England glasshouse melon. The Japanese breeding lines belonged to clusters II and IV. Among them, 'Pearl', known for good fruit quality, was grouped together with 'Earl's Favourite' in cluster IV, while the rest bred by introducing disease resistant genes from

Table 3 Polymorphic index and number of polymorphic markers in each variety or cultivar group

Horticultural group or cultivar group	Abbreviation	No. of accessions	PIC	No. of markers[1)]
Group Cantalupensis	C			
European cantaloupe		6	0.108	31
American open-field type	RA	7	0.104	29
England glasshouse type	RE	5	0.070	20
Japanese breeding line	RJ	4	0.092	31
Group Inodorus				
Honey dew type	I	4	0.014	4
Chinese Hami melon	IC	6	0.092	26
Russian melon	IR	3	0.071[2)]	19[2)]
Spanish melon	IS	2		
Group Conomon				
makuwa	Ma	5	0.054	16
conomon	Co	5	0.047	13

1) Number of polymorphic markers
2) Values were calculated for five cultivars of IR and IS.

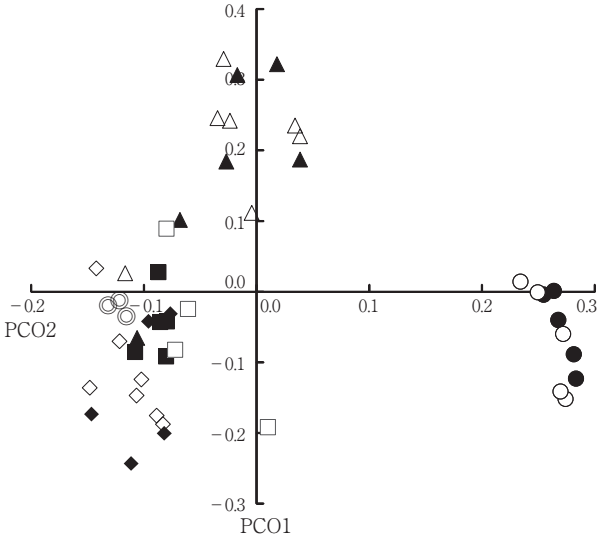

Fig. 3 Distribution on the first two principal co-ordinates of 47 accessions of melon.
Symbols represent group/type of each accession. △ : American open-field type, ■ : England glasshouse type, □ : Japanese breeding line, ▲ : European cantaloupe, ◎ : Honey Dew, ◇ : Chinese Hami melon, ◆ : Russian and Spanish melon, ● : var. *makuwa*, ○ : var. *conomon*.

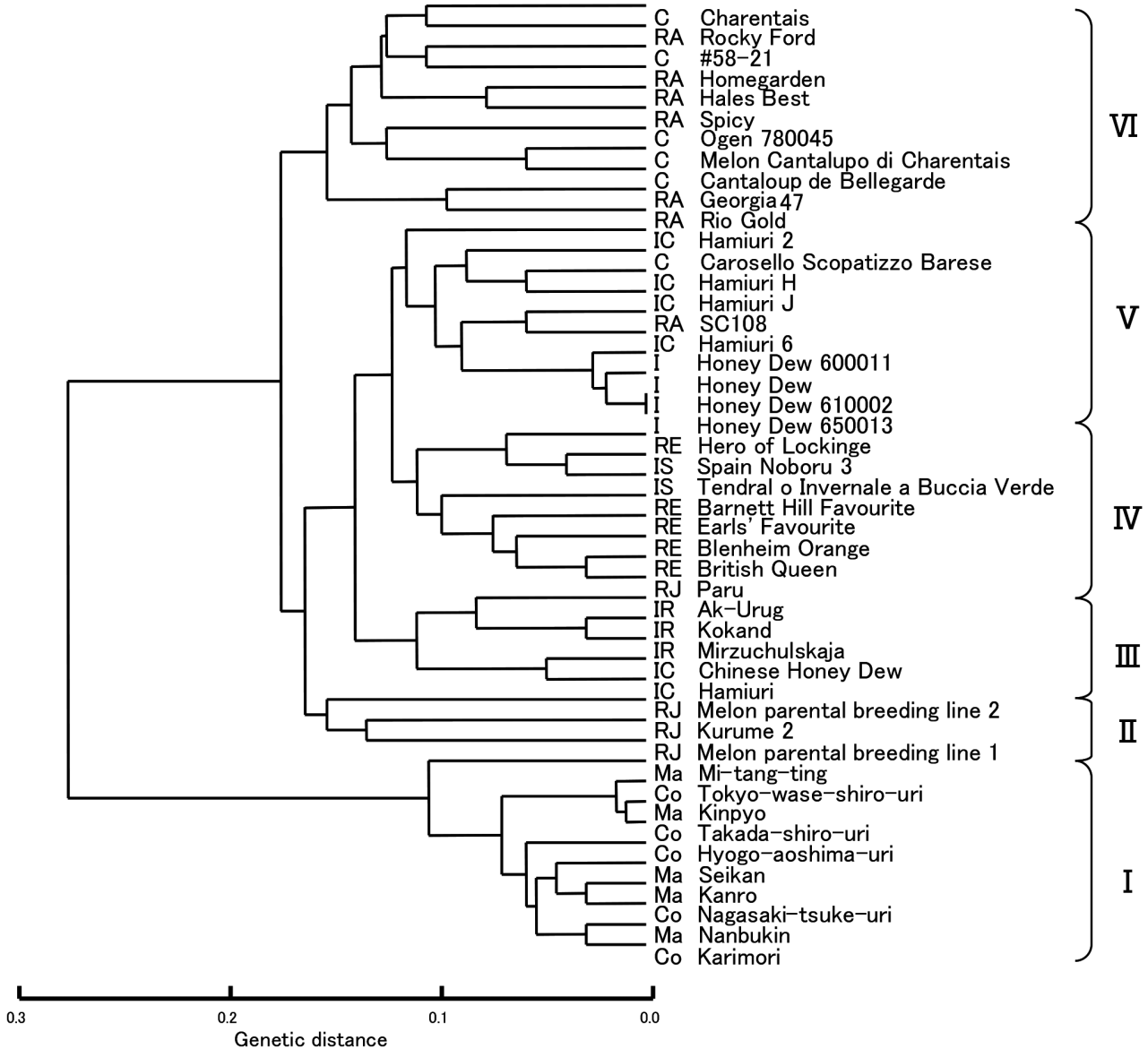

Fig. 2 Genetic relationship between 47 accessions of melon, revealed by UPGMA cluster analysis based on GD. RA : American open-field type, RE : England glasshouse type, RJ : Japanese breeding line, C : European cantaloupe, I : Honey Dew, IC : Chinese Hami melon, IR : Russian melon, IS : Spanish melon, Ma : var. *makuwa*, Co : var. *conomon*.

genetic resources formed cluster II.

Genetic relationships among melon accessions demonstrated on dendrogram was also reproduced on PCO plot (Fig. 3). PCO1 explaining 34.9% of total variation clearly separated Group Conomon from other groups. The distant relationship of European cantaloupe and American open-field type with England glasshouse melon became obvious on PCO1 and PCO2 plot, of which the latter explained 8.9% of total variation. The England glasshouse melon including 'Earl's Favourite' was surrounded by Group Inodorus accessions, as also shown in Fig. 2.

The second set of melon accessions was also analyzed by RAPD. However, most of the accessions showed RAPD profile identical with that of 'Earl's Favourite', and polymorphism among 10 accessions was rarely detected. Among 24 primers, two primers successfully detected polymorphism. As shown in Table 4, A07

Table 4 RAPD polymorphism among pure line cultivars derived from 'Earl's Favourite'

Cultivar	RAPD marker[1)]	
	A07 740bp	A53 2000bp
Haru 3	−	−
Haru 3B	−	−
Natsu 1	−	−
Natsu 4	−	−
Natsu 7	−	−
Aki 1	+	−
Fuyu 1	−	+
Fuyu 1A	−	−
Fuyu 3	−	+
Fuyu 4	−	+

1) +, − ; Presence or absence of RAPD marker band.

primer amplified a 740 bp band only in 'Aki 1', while A53 primer produced a 2000 bp band in three accessions, 'Fuyu 1', 'Fuyu 3', and 'Fuyu 4'. Three cultivars, 'Natsu 1', 'Natsu 4', and 'Natsu 7', were bred by crossing 'Earl's Favourite' with 'British Queen'[8)], and were thus expected to show some difference from others. However, no difference was detected by RAPD analysis using 24 primers. These results indicated that pure line cultivars selected from 'Earl's Favourite' or derived from the cross with 'British Queen' are mostly homogenous.

Discussion

Although the Japanese netted melon has a shorter history compared with those of the traditional Japanese non-netted melon and European and American netted melon, various kinds of melon accessions have been introduced and utilized for improving adaptability, disease resistance, fruit quality and so on[8, 9, 18, 19, 20, 21, 22)]. In the present study, genetic diversity and relationships among these melon accessions were surveyed by RAPD analysis, in order to understand the genetic basis of the Japanese netted melon. Although the number of melon accessions was only four, the genetic diversity, shown by PIC, in the Japanese netted breeding lines was 0.092 and equivalent to those of other cultivar groups of Group Cantalupensis (Table 3). Close genetic relationship was also recognized in other Japanese netted melons[10)]. Use of various types of melon in a short period of breeding history was evidenced by the results of this study. Further, GD among the Japanese netted melon and other groups of melon was compared. The average GD within the Japanese netted melon accessions was 0.255. In contrast, GD among the Japanese netted melon and four cultivar groups, that is, England glasshouse type, European cantaloupe, American open-field type, and Group Conomon, were 0.244, 0.358, 0.342, and 0.513, respectively. Such a genetic relationship was clearly demonstrated in two Japanese netted melon lines, 'Ano 1' and 'Ano 3'. Although Group Conomon cv. 'Mi-tang-ting' was used as one of the cross parents to introduce resistant genes for CMV, Gummy stem blight, and Fusarium wilt[19)], they are closely related with 'Earl's Favourite' (GD from 0.236 to 0.309) compared with 'Mi-tang-ting' (from 0.455 to 0.527). According to Tanaka et al.[23)], England glasshouse type shares the same chloroplast genome type as Japanese netted melon. Based on these results, it was clearly indicated that Japanese netted melon is most closely related with England glasshouse type, irrespective of the fact that various melon accessions have been crossed to improve adaptability, disease resistance and so on.

England glasshouse type and American open-field type commonly have net on fruit rind, and were classified as var. *reticulatus* according to Pitrat[3)]. However, only a rather distant relationship among these two types of netted melon was indicated in this study (Fig. 2). Instead, a much closer relationship among American open-field type and European cantaloupe, which was formerly classified as var. *cantalupensis*, was clearly shown in Fig. 2, as also shown by the analysis of chloroplast genome[23)]. These results support the establishment of a new classification system in which all three types are to be classified as Group Cantalupensis[24)].

Fig. 2 also highlighted the close relationship between England glasshouse type and Group Inodorus. The average GD within England glasshouse type was 0.175. Contrasting with this, GD among England glasshouse type and three cultivar groups, that is, European cantaloupe, American open-field type, and Group Inodorus, were 0.286, 0.303, and 0.236, respectively. Furthermore, accessions of Group Inodorus were clustered together with England glasshouse type accessions (Fig. 2) and surrounded them on PCO plot (Fig. 3). Taken all together, the present results indicated a close relationship between England glasshouse type and Group Inodorus. One of the traits by which 'Earl's Favourite' successfully adapted to Japanese conditions is considered to be the shelf life of fruit which is clearly longer than accessions of European cantaloupe[9)]. Group Inodorus is well known for its very long shelf life, and

rightly called "winter melon" because it can be stored until winter[3, 20, 25]. The fruit characteristics presented us with a clear picture that England glasshouse type was established from hybrids between European cantaloupe and Group Inodorus, as suggested by Aierkin et al[25].

As mentioned above, Group Conomon is often used as a cross parent with important genetic resources for disease resistance, such as fusarium wilt, gummy stem blight and cucumber mosaic virus[19, 26]. It was formerly classified as var. *makuwa* and var. *conomon*, both of which have a long history of cultivation and utilization in East Asia[5]. The var. *makuwa* is characterized by sweet fruits and utilized as a dessert, while the var. *conomon* has sour fruits and is mainly utilized as a vegetable[3]. Although these two varieties have been recognized as different crops in China and Japan, the detail of their origin and genetic relationship has not been analyzed. In the present study, it was clearly indicated that genetic diversity was small in Group Conomon compared with other groups of melon (Table 3), which is in good accordance with the previous studies[27, 28, 29]. As shown in Fig. 2, it was clearly demonstrated that Group Conomon endemic to East Asia was distantly related with Groups Cantalupensis and Inodorus as also shown by previous studies[10, 12, 29]. Fig. 2 also demonstrated that accessions of vars. *makuwa* and *conomon* are not differentiated at all, since they were intermixed in cluster I. Interestingly, six accessions located at the bottom of cluster I, which formed a subcluster, have green color of fruit rind, while others have white or yellow rind. It was therefore suggested that vars. *makuwa* and *conomon* share the same gene pool through intercrossing in the field mediated by bees, and this supported a new classification system in which both varieties are classified together as Group Conomon. Non-netted melon grown in the eastern part of India is considered as the prototype of this group, that is, both var. *makuwa* and var. *conomon*, and introduced to East Asia through Southeast Asia[28].

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分子遺伝学的手法を用いたわが国メロン品種の多様性と分類

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わが国のネットメロン育種では栽培適性，病虫害抵抗性，果実品質改良などのために国内外の多様なメロン品種が交配に用いられてきた．しかしながら，これらの交配母本自体が様々な品種群間での交配により育成されているので，その遺伝的多様性や類縁関係についてはほとんど明らかにされていない．そこで本研究では，国内外のメロン57系統を供試して，24種類の RAPD プライマーを用いて解析した．その結果，日本のネットメロン育成系統が欧州キャンタロープ，米系露地メロン，フユメロンなどと同程度に多様であるのに対し，マクワ・シロウリは果実特性が多様にもかかわらず遺伝的多様性は小さかった．品種間での遺伝的距離を用いたクラスター分析及び PCO 解析の結果，欧州キャンタロープと米系露地メロンが近縁であることが判明した．一方，英国温室メロンは両群とはやや遠縁であり，むしろフユメロンと近縁なことが示された．この結果より，英国温室メロンが欧州キャンタロープとフユメロンの交雑により成立したことが示唆された．日本のネットメロンは，予想通りに英国温室メロンと最も近縁なことが確認された．なお，本研究では，‘Earl’s Favourite’ から純系選抜された，あるいは ‘Earl’s Favourite’ と ‘British Queen’ との交雑により育成された純系10品種の多様性も解析したが，RAPD 多型が検出されたのは一部の品種だけであり，遺伝的に極めて均質な品種群であることが確認された．最後に，マクワ・シロウリについては，それぞれデザート用，漬物用として別々の作物として栽培・利用されてきたにもかかわらず，遺伝的にはまったく区別することができず，両者が自然交配などによってジーンプールを共有しながら別々の作物として利用されてきたことが明らかになった．

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