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Diversification and genetic differentiation of cultivated melon inferred from sequence polymorphism in the chloroplast genome

Katsunori Tanaka[1)], Yukari Akashi[2)], Kenji Fukunaga[3)], Tatsuya Yamamoto[4)], Yasheng Aierken[5)], Hidetaka Nishida[2)], Chun Lin Long[6)], Hiromichi Yoshino[6)], Yo-Ichiro Sato[7)] and Kenji Kato*[2)]

[1)] *Faculty of Humanities, Hirosaki University*, 1 Bunkyo, Hirosaki, Aomori 036-8560, Japan
[2)] *Graduate School of Environmental and Life Science, Okayama University*, 1-1-1 Tsushima-Naka, Kita, Okayama, Okayama 700-8530, Japan
[3)] *Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima*, 562 Nanatsuka, Shoubara, Hiroshima 727-0023, Japan
[4)] *Hagihara Farm Co. Ltd.*, 984 Hokanji, Tawaramoto, Shiki, Nara 636-0222, Japan
[5)] *Hami Melon Research Center, Xinjiang Academy of Agricultural Science*, Urumuqi, 830000, PRC
[6)] *Kunming Institute of Botany*, CAS, Heilongtan, Kunming, Yunnan, PRC
[7)] *Research Institute for Humanity and Nature*, 457-4 Motoyama, Kamigamo, Kita, Kyoto, Kyoto 603-8047, Japan

Molecular analysis encouraged discovery of genetic diversity and relationships of cultivated melon (*Cucumis melo* L.). We sequenced nine inter- and intra-genic regions of the chloroplast genome, about 5500 bp, using 60 melon accessions and six reference accessions of wild species of *Cucumis* to show intra-specific variation of the chloroplast genome. Sequence polymorphisms were detected among melon accessions and other *Cucumis* species, indicating intra-specific diversification of the chloroplast genome. Melon accessions were classified into three subclusters by cytoplasm type and then into 12 subgroups. Geographical origin and seed size also differed between the three subclusters. Subcluster Ia contained small-seed melon from Southern Africa and South and East Asia and subcluster Ib mainly consisted of large-seed melon from northern Africa, Europe and USA. Melon accessions of subcluster Ic were only found in West, Central and Southern Africa. Our results indicated that European melon groups and Asian melon groups diversified independently and shared the same maternal lineage with northern African large-seed melon and Southern African small-seed melon, respectively. Cultivated melon of subcluster Ic may have been domesticated independently in Africa. The presence of 11 cytoplasm types in Africa strongly supported African origin of cultivated melon and indicated the importance of germplasm from Africa.

Key Words: chloroplast genome, *Cucumis*, genetic differentiation, genetic diversity, maternal line, melon, polyphyletic origin.

Introduction

Classification for the Cucurbitaceae has been focused by several studies (e.g., Jeffrey 1980). The Cucurbitaceae comprises 15 tribes, and includes 942–978 species of 95 genera (Schaefer and Renner 2011). Genus *Cucumis* belongs to the tribe Benincaseae and comprises 65 species, most of which grow wild in Africa and includes two important horticultural crops, melon (*Cucumis melo* L.) and cucumber (*Cucumis sativus* L.). Melon is divided into weedy melon (Group Agrestis) and cultivated melon. The latter is further classified into Groups Cantalupensis, Inodorus, Dudaim, Conomon, Flexuosus and Momordica (Robinson and Decker-Walters 1997). Groups Cantalupensis and Inodorus are cultivated mainly in Western countries, such as USA, European countries and Russia and have sweet flesh and some fruits are netted. The other groups are distributed in Asia, except Group Agrestis, which is distributed worldwide, and are characterized by low sugar content and smooth skin. These Asian melons are clearly separated from US and European melon by analysis of 35 plant and fruit traits (Liu *et al.* 2004).

Seed length (Akashi *et al.* 2002, Fujishita 1983) and complimentary genes causing bitterness of the young fruit placenta of inter-varietal F_1 hybrids (Fujishita *et al.* 1993) also reflect genetic and geographical differentiation among diversified melon groups. Based on the seed length, melon is classified into large-seed type (≥9.0 mm) and small-seed type (<9.0 mm). Melon accessions of Groups Cantalupensis and Inodorus are mostly classified as large-seed type and those of Groups Agrestis and Conomon as small-seed type.

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*Corresponding author (e-mail: kenkato@cc.okayama-u.ac.jp)

In contrast, both seed types are found in Groups Flexuosus, Dudaim and Momordica collected from South to Southeast Asia (Akashi *et al.* 2002). Genetic differentiation between melon groups of different geographical origins detected by analysis of the above-mentioned complimentary genes separated Asian melon into a South-Southeast Asian melon group and an East Asian melon group (Fujishita *et al.* 1993). DNA markers were used to classify melon, and genetic differentiation was shown between Groups Cantalupensis and Inodorus and Group Conomon (Monforte *et al.* 2003, Stepansky *et al.* 1999). Phylogenetic relationships between melon groups from USA, Europe, Africa and Asia were analyzed using random amplified polymorphic DNA (RAPD) markers (Garcia *et al.* 1998, López-Sesé *et al.* 2003, Mliki *et al.* 2001, Nakata *et al.* 2005). Although molecular markers of the nuclear genome are highly polymorphic and are suitable to detect polymorphism among melon accessions, a DNA marker specific to each Group has not been established. Therefore, molecular markers whose sequences are relatively conserved compared with nuclear genome markers should be developed to explain the phylogenetic relationship of melon groups of different geographical origins.

The chloroplast genome generally shows maternal inheritance in higher plants (Reboud and Zeyl 1994) and has a similar gene arrangement among plant species and a low rate of base substitution (Clegg *et al.* 1994, Hiratsuka *et al.* 1989, Palmer 1985, Perl-Treves and Galun 1985, Provan *et al.* 1999). Nakamura *et al.* (1997) detected sequence polymorphism in the linker sequence between *Rpl16* and *Rpl14* genes and designated it as a plastid subtype ID (PS-ID) sequence. Polymorphism in the PS-ID sequence was detected between subspecies *japonica* and *indica* of rice (Nakamura *et al.* 1997) and the maternal lineage of Japanese rice cultivars was investigated by PS-ID analysis (Ishikawa *et al.* 2002a, 2002b). Chung and Staub (2003) developed 23 consensus chloroplast simple sequence repeat (ccSSR) markers for Cucurbitaceae crops based on the complete chloroplast genome sequence of *Nicotiana tabacum* (NC_001879). Analysis of ccSSR markers using 18 accessions of *Cucumis* species showed that cucumber and melon are distinct from African wild species of *Cucumis* (Chung *et al.* 2006). Renner *et al.* (2007) and Sebastian *et al.* (2010) compared the DNA sequence of five regions of the chloroplast genome as well as the nuclear internal transcribed spacer (ITS) region and showed that cucumber and melon are related closely to Asian and Australian wild species *Cucumis hystrix* Chakr. and *Cucumis picrocarpus* F. Muell., and rather closely to African wild species *Cucumis sagittatus* Peyr. and *Cucumis metuliferus* E. Mey. These results clearly showed that sequence polymorphism of the chloroplast genome can explain phylogenetic relationships within *Cucumis* and suggest its applicability to phylogenetic analysis of melon.

In this study, we determined the nucleotide sequences of nine regions of the chloroplast genome to determine the plastid type of 66 accessions of *Cucumis* species. Genetic diversity among 60 accessions of melon and six accessions of *Cucumis* wild species is shown by sequence comparison and their phylogenetic relationships are discussed based on their plastid types.

Materials and Methods

Plant materials

Sixty-six accessions of *Cucumis*, which included six accessions of four *Cucumis* wild species and 60 accessions of melon (*C. melo* L.), were analyzed (Table 1). The six accessions of wild species consisted of one accession each of *Cucumis anguria* L. from Zimbabwe, *C. metuliferus* from South Africa and *C. sagittatus* from Namibia and three accessions of *C. hystrix* from China. Of the 60 accessions, 18 accessions were selected from six horticultural groups: three accessions of Group Cantalupensis (European cantaloupe: 1, netted melon: 2 [England glasshouse type: 1, American field type: 1]), three accessions of Group Inodorus (Chinese Honeydew: 1, Spanish winter melon: 1, Chinese Hami melon: 1), one accession each of Group Flexuosus (India), Group Momordica (India) and Group Conomon var. *makuwa* (Japan) and nine accessions of Group Agrestis (Sudan: 2, Cameroon: 1, Senegal: 4, Ghana: 1, Korea: 1). The remaining 42 accessions, consisting of unclassified landraces, were: 21 accessions from Africa (Egypt: 2, Morocco: 1, Ethiopia: 1, Sudan: 1, Chad: 1, Mali: 1, Senegal: 1, Sierra Leone: 1, Cameroon: 1, Zambia: 4, Zimbabwe: 7), 13 accessions from West and Central Asia (Turkey: 5, Syria: 2, Iraq: 1, Iran: 1, Afghanistan: 4) and eight accessions from South Asia (Maldives: 1, India: 7). Because sequence polymorphism was detected between two plants in each of the following three accessions, the two plants of each accession were treated as different accessions (Zambia: PI 505599-1 and -2; PI 505602-1 and -2 ; Turkey: PI 169379-1 and -2) (Table 1). The complete chloroplast genome sequence of cucumber (DQ865976) registered in the GenBank, was also included for data analysis.

Seeds of these accessions were mostly provided by the National Institute of Vegetable and Tea Science (NIVTS), Japan, the North Central Regional Plant Introduction Station, Iowa State University (USDA-ARS), USA (Table 1) and they were cultivated in the field or in a glasshouse of Okayama University, Japan.

Fruit characteristics, such as height and diameter of fruit and soluble solid content (Brix grade) were measured for two fruits of 52 melon accessions. Length and width of the representative seeds were measured for all accessions, which were then classified into a large-seed type (≥9.0 mm) and a small-seed type (<9.0 mm), according to Akashi *et al.* (2002) (Table 1). As shown in Table 1, of the 60 melon accessions examined here, 49 were used in our previous RAPD analysis (Akashi *et al.* 2006), and 55 were used for dCAPS analysis of the linker sequence between *Rpl16* and *Rpl14* (Tanaka *et al.* 2006).

DNA extraction

Seeds were sown on a filter paper and were grown at

Table 1. Details of 66 accessions of *Cucumis* species analyzed in this study

Name of cultivar/ Accession No.	Group/Species name[b]	Country	Market class/Area	Seed source[c]	Seed[d]		Fruit size (cm)		Brix (°)	Sample studied by[e]		Cluster No[f]
					Type	Length (mm)	Height	Diameter		Akashi	Tanaka	
Rio Gold	Cantalupensis	America		1	L	9.5	11.6	10.9	14.4	–	+	Ib-2
Earl's Favourite	Cantalupensis	England		1	L	9.6	16.0	15.5	14.0	–	+	Ib-2
Ogen (780045)	Cantalupensis	France		1	L	10.1	13.0	14.0	–	–	+	Ib-1
Hami-gua 6	Inodorus	America	Chinese Honeydew	1	L	14.2	18.5	16.5	12.8	–	+	Ib-3
Tendral[a]	Inodorus	Spain		1	L	12.5	18.0	15.0	8.6	–	+	Ib-3
Hami-gua	Inodorus	China		1	L	11.7	25.5	12.0	–	–	+	Ib-2
PI 614576	Flexuosus	India (Center)	Madhya Pradesh	2	L	9.0	65.0	7.5	2.0	+	+	Ia-1
PI 182952	Momordica	India (West)	Gujarat	2	L	10.0	12.5	10.0	2.8	+	+	Ib-1
Kinpyo	Conomon	Japan		1	S	6.8	9.5	7.0	13.8	+	+	Ia-1
940111	Agrestis	Sudan		1	S	6.0	7.0	4.5	5.0	+	+	Ic-5
sud-4	Agrestis	Sudan		1	S	4.9	–	–	–	–	–	Ic-5
940065	Agrestis	Cameroon		1	S	6.0	–	–	–	+	+	Ic-5
940108	Agrestis	Senegal		1	S	5.5	4.0	2.0	9.6	+	+	Ic-3
PI 436532	Agrestis	Senegal		2	S	6.5	10.3	5.7	6.6	+	+	Ic-3
PI 436534	Agrestis	Senegal		2	S	4.8	–	–	–	+	+	Ic-3
940112	Agrestis	Senegal		1	S	5.0	4.0	3.0	5.0	+	+	Ic-4
PI 185111	Agrestis	Ghana		2	S	6.4	–	–	–	+	+	Ic-1
Weedy melon	Agrestis	Korea		3	S	6.0	4.0	3.0	5.0	+	+	Ia-3
PI 525111	–	Egypt		2	L	13.0	14.0	7.5	5.0	+	+	Ib-2
PI 525105	–	Egypt		2	L	14.0	16.0	11.5	4.0	+	+	Ib-1
PI 207661	–	Morocco		2	L	12.0	11.3	9.2	5.6	+	+	Ib-3
PI 193495	–	Ethiopia		2	S	7.0	–	–	–	–	–	Ib-1
119	–	Sudan		1	L	11.5	7.9	5.6	4.8	–	–	Ib-1
940281	–	Chad		1	S	6.0	9.0	6.5	4.0	+	+	Ic-5
PI 490388	–	Mali		2	L	13.0	–	–	–	+	+	Ic-3
PI 436533	–	Senegal		2	S	5.8	8.6	6.5	8.0	+	+	Ic-2
PI 320993	–	Sierra Leone		2	L	11.5	14.0	11.3	6.2	+	+	Ia-1
Cam-84-3	–	Cameroon		1	S	7.0	12.0	7.0	5.4	+	+	Ic-5
PI 505599-1	–	Zambia		2	S	5.7	14.5	9.5	4.0	+	+	Ia-1
PI 505599-2	–	Zambia		2	S	7.6	14.5	9.5	4.0	+	+	Ic-6
PI 505602-1	–	Zambia		2	S	7.9	13.5	7.5	3.0	+	+	Ia-1
PI 505602-2	–	Zambia		2	S	7.9	13.5	7.5	3.0	+	+	Ic-6
PI 482411	–	Zimbabwe		2	S	7.0	12.0	7.0	4.0	+	+	Ia-1
PI 482424	–	Zimbabwe		2	S	7.0	11.0	8.0	4.0	+	+	Ia-3
PI 482429	–	Zimbabwe		2	S	8.0	15.0	9.0	4.0	+	+	Ia-3
PI 482398	–	Zimbabwe		2	S	6.0	8.0	5.0	5.0	+	+	Ic-6
PI 482413	–	Zimbabwe		2	S	6.0	6.0	5.5	5.0	+	+	Ic-6
PI 482422	–	Zimbabwe		2	S	8.6	7.8	5.6	5.5	–	–	Ic-6
PI 482396	–	Zimbabwe		2	S	7.0	9.5	5.2	6.2	–	–	Ic-6
PI 169379-1	–	Turkey		2	L	10.0	31.0	11.5	5.0	+	+	Ib-1
PI 169379-2	–	Turkey		2	L	10.0	31.0	11.5	5.0	+	+	Ia-1
PI 172818	–	Turkey		2	L	11.0	17.0	12.0	10.0	+	+	Ia-3
Karasu melon	–	Turkey		1	L	9.0	9.0	9.5	8.0	+	+	Ib-2
PI 176928	–	Turkey		2	L	10.0	15.0	10.0	9.4	+	+	Ib-2
PI 534605	–	Syria		2	L	12.0	12.7	10.4	3.0	+	+	Ib-1
PI 181872	–	Syria		2	L	11.0	14.8	14.5	4.4	+	+	Ib-2
PI 435290	–	Iraq		2	L	13.5	6.5	4.7	4.0	+	+	Ib-2
PI 143231	–	Iran		2	L	14.0	22.0	14.0	7.0	+	+	Ib-1
PI 125931	–	Afghanistan		2	L	12.0	13.5	11.5	12.6	+	+	Ib-1
PI 125961	–	Afghanistan		2	L	14.0	–	–	–	+	+	Ib-1
PI 126047	–	Afghanistan		2	L	15.0	–	–	–	+	+	Ib-2
PI 126054	–	Afghanistan		2	L	12.0	10.5	10.0	9.5	+	+	Ia-3
PI 536480	–	Maldives		2	S	8.0	22.0	9.7	4.6	+	+	Ia-1
PI 116666	–	India (West)	Punjab	2	L	9.0	18.5	10.0	3.2	+	+	Ia-1
PI 116738	–	India (West)	Punjab	2	S	7.0	11.0	7.0	4.5	+	+	Ib-2

Table 1. (continued)

Name of cultivar/ Accession No.	Group/Species name[b]	Country	Market class/Area	Seed source[c]	Seed[d]		Fruit size (cm)		Brix (°)	Sample studied by[e]		Cluster No[f]
					Type	Length (mm)	Height	Diameter		Akashi	Tanaka	
PI 614588	–	India (Center)	Madhya Pradesh	2	L	8.5	7.0	5.0	3.4	+	+	Ia-2
PI 124435	–	India (Center)	Madhya Pradesh	2	L	11.0	45.0	25.0	5.5	+	+	Ib-2
PI 614542	–	India (Center)	Madhya Pradesh	2	S	7.0	14.0	9.5	6.8	+	+	Ia-1
PI 164585	–	India (South)	Tamil Nadu	2	L	10.0	12.3	9.0	5.2	+	+	Ib-2
PI 124096	–	India (South)	Andra Pradesh	2	L	10.0	16.0	9.5	6.3	+	+	Ib-1
PI 292190	*C. metuliferus*	South Africa		2	–	6.0	–	–	–	–	–	III
PI 482383	*C. anguria*	Zimbabwe		2	–	5.0	–	–	–	–	–	III
PI 282441	*C. sagittatus*	Namibia		2	–	5.0	–	–	–	–	–	III
CYS68-1	*C. hystrix*	China		4	–	3.6	–	–	–	–	–	II
CYS68-2	*C. hystrix*	China		4	–	3.6	–	–	–	–	–	II
Sm021-3	*C. hystrix*	China		4	–	3.4	–	–	–	–	–	II

[a] Tendral o Invernale a Buccia Verde.

[b] –: Local landraces whose horticultural group or variety was not specified as shown in the text.

[c] 1: National Institute of Vegetable and Tea Science (NIVTS), Japan. 2: North Central Regional Plant Introduction Station, Iowa State University (USDA-ARS), USA. 3: Okayama University, Japan. 4: Kunming Institute of Botany, PRC.

[d] S: small-seed melon, L: large-seed melon.

[e] Plants also analyzed by Akashi *et al.* (2002) and Tanaka *et al.* (2006) are indicated by ''+''.

[f] Cluster number refers to Fig. 1.

26°C in a 16 h light-8 h dark cycle at light intensity 46.5 $\mu Ms^{-1} m^{-2}$. Ten-day-old seedlings were individually ground in liquid nitrogen, and total DNA was extracted by using the procedure of Murray and Thompson (1980) with minor modifications.

Primer design

The seven target regions of the chloroplast genome were: inter-genic regions *TrnK-MatK* (Cmcp1), *PsbK-PsbI* (Cmcp3), *AtpH-AtpI* (Cmcp5), *PsaI-Ycf4* (Cmcp15) and *NdhF-Rpl32* (Cmcp6), and intron 1 of *Rps16* (Cmcp2) and *NdhA* (Cmcp11), among which Cmcp1 included 127 bp of *maturase K* sequence (Table 2). The primer pair was designed to amplify these regions based on the chloroplast sequences of tobacco (*N. tabacum*: NC_001879) and cucumber (*C. sativus*: DQ119058, DQ865975, DQ865976, AJ970307) registered in the GenBank. For Cmcp5 and Cmcp11, additional primers, F2 and R2, were designed for complete sequencing. Another two target regions of the chloroplast genome regions were inter-genic regions of *Rpl16-Rpl14* (PS-ID) and *PsbC-TrnS* (ccSSR7), for which the specific primer pairs were developed by Nakamura *et al.* (1997) and Chung and Staub (2003), respectively. Among these regions, Cmcp6 and Cmcp11 are in the small single-copy region of the melon chloroplast genome (JF412791) and the remaining seven regions are in the large single-copy region.

PCR amplification and sequencing

PCR amplification was done in a 40 µl mixture (100 ng total DNA, 1 × Ex*Taq*™ buffer [10 mM Tris-HCl at pH 8.3, 50 mM KCl, 20 mM $MgCl_2$], 0.25 U Ex*Taq*™ polymerase [TaKaRa, Japan], 0.1 mM dNTPs and 0.25 µM of each primer) by using an i-Cycler (Bio-Rad, USA). The PCR cycle was: initial denaturing at 95°C for 3 mins with hot start, 35 PCR cycles at 95°C for 30 secs, 57°C for 30 secs and 72°C for 30 secs. The final extension step was at 72°C for 3 mins. For Cmcp11, the annealing reaction was modified to 62°C.

For direct sequencing, the PCR products were purified by using a Wizard^R SV Gel and a PCR Clean-UP System (Promega, USA). The sequencing reaction was run by using BigDye™Cycle Sequence Ready Reaction DNA (Applied Biosystems, USA) and a specific primer for each region. The nucleotide sequence was determined for both strands by using an ABI PRISM^R 3730 DNA Analyzer (Applied Biosystems, USA), except for the PS-ID region whose sequence for a reverse strand could not be determined because of two SSRs near the annealing site of the reverse primer.

Classification of large- and small-seed types based on seed weight

To determine the worldwide distribution patterns of large- and small-seed melon, seed size type of 7,396 melon accessions were estimated based on 100 seed weight data of the GRIN database (USDA-ARS, http://www.ars-grin.gov/npgs/). Since seed weight apparently depends on the condition and degree of ripening, it is difficult to accurately classify into large- and small-seed melon just based on 100 seed weight. However, a preliminary analysis of seed length and 100 seed weight, using 99 melon accessions, indicated that melon accessions with 100 seed weight below or over 2.5 g could be classified as small- or large-seed melon, respectively, with an accuracy of 87.9%. Therefore, in this study,

Table 2. Primer sequence and number of polymorphic site of nine chloroplast genome regions of 67 accessions of *Cucumis* species

Region[a]	Gene name	Marker name		Forward (F), Reverse (R) primer (5′ to 3′)[b]	Marker position (bp)		Aligned length (bp)[c]	Gap (bp)[c]	Total no. of site (bp)[c]	SNP[c]	
					Position	Length				No.	%
LSC	*TrnK-MatK*	Cmcp1	F	AACCCGGAACTAGTCGGATGG	1,638–1,658	388	390	3	387	21	5.4
			R	TCGGAATTATTGGAAGAATTCTT	2,069–2,047		388	–	388	3	0.8
	Rps16 intron1	Cmcp2	F	GAAGTTTTCTCCTCGTACGGC	5,231–5,251	609	617	14	603	30	5.0
			R	GATGGAGCTCGAGTAGAAAGTAT	5,883–5,861		612	4	608	3	0.5
	PsbK-PsbI	Cmcp3	F	TGTTTGGCAAGCTGCTGTAAG	7,996–8,016	377	387	24	363	32	8.8
			R	GAGAGTAAGCATTACACAATCTCCAAG	8,420–8,394		373	5	368	2	0.5
	AtpH-AtpI	Cmcp5	F1	AATAACGGAAGCGGCAGAAAT	14,556–14,576	976	946	53	893	49	5.5
			R1	AGCTCTTATTTTTGCAACTTTAGC	15,576–15,553		920	–	920	6	0.7
			F2	GGTTGTCTTATAGCAATTCGATTC	15,086–15,109						
			R2	CATGAACTTAGATTAATAAAATATTG	15,288–15,263						
	PsbC-TrnS	ccSSR 7	F	CGGGAAGGGCTCGKGCAG	37,185–37,202	297	276	12	264	14	5.3
			R	GTTCGAATCCCTCTCTCTCCTTTT	37,523–37,500		276	5	271	1	0.4
	PsaI-Ycf4	Cmcp15	F	TCGAGGTATCCATTCTATGAC	61,339–61,359	534	569	44	525	37	7.0
			R	ATCCATATACGTTCTGATCGC	61,914–61,894		557	25	532	1	0.2
	Rpl16-Rpl14	PS-ID	A	AAAGATCTAGATTTCGTAAACAACATAGAGGAAGAA	83,600–83,565	504	474	14	460	13	2.8
			B	ATCTGCAGCATTTAAAAGGGTCTGAGGTTGAATCAT	83,025–83,060		474	4	470	2	0.4
SSC	*NdhF-Rpl32*	Cmcp6	F	GAGATGAACGGTATGATCCATGA	114,328–114,350	679	720	258	462	29	6.3
			R	TCCGTTTTTTTGATATAGAAGTGCG	115,049–115,025		676	3	673	6	0.9
	NdhA intron1	Cmcp11	F1	TATAGGTTGACGCCACAAATTC	122,070–122,091	1,213	1,254	88	1,166	59	5.1
			R1	ATCAATATCTCTACGTGTGATTCG	123,328–123,305		1,215	33	1,182	6	0.5
			F2	TTGTAATTGGATTCTTTCCATT	122,650–122,671						
			R2	GATATTTTTTATTGTTTCTTATCTCC	122,634–122,609						
Total						5,577	5,633	510	5,123	284	5.5
							5,491	79	5,412	30	0.6

[a] LSC and SSC indicate large- and small-single copy region in the chloroplast genome, respectively. Gene name, maker position and marker length refer to the melon chloroplast genome (accession No. JF412791).

[b] K = T or G

[c] The upper side and the lower side value of each region were calculated for 67 accessions of *Cucumis* and 60 melon accessions, respectively.

Total number of site = (Aligned length) – (Gap).

7,396 melon accessions were roughly classified as large- and small-seed melon by these criteria.

Data analysis

Sequences of nine chloroplast regions were aligned using CLUSTAL W (Thompson *et al.* 1994). For phylogenetic analyses, all gaps and inversions were removed from the sequences, because the mutational process of chloroplast SSRs is not clear (Provan *et al.* 2001). Phylogenetic relationships were determined by using the neighbor joining (NJ) method with MEGA 5 (Tamura *et al.* 2011). Genetic distance (GD) was calculated by using the maximum composite likelihood method (Tamura *et al.* 2004) and a phylogenetic tree was constructed by using the NJ method with a bootstrapping of 1000 replications. The phylogenetic relationships were also analyzed by using a maximum parsimony (MP) method with PAUP* version 4.0b10 (Swofford 2002). The MP method was run as described by Yamane and Kawahara (2005).

Results

Sequence analysis

The total length of determined nucleotide sequence differed between accessions, and 5633 nucleotides were aligned with nucleotide gaps (510 bp total), comprising nine regions of the chloroplast genome: *TrnK-MatK* (Cmcp1), *Rps16* intron 1 (Cmcp2), *PsbK-PsbI* (Cmcp3), *AtpH-AtpI* (CmCp5), *PsbC-TrnS* (ccSSR7), *PsaI-Ycf4* (Cmcp15), *Rpl16-Rpl14* (PS-ID), *NdhF-Rpl32* (Cmcp6) and *NdhA* intron 1 (Cmcp11) (Table 2 and Supplemental Table 1). Alignment of sequences between *C. melo* cv. 'Earl's Favourite', *Arabidopsis thaliana* (NC_000932), *N. tabacum* (NC_001879) and *C. sativus* (DQ865976) showed 68% to 98% identities across nine regions (Table 3), confirming amplification of the chloroplast genome in *Cucumis* accessions. Within the genus *Cucumis*, 67 accessions, including *C. sativus* (DQ865976), shared an identity of over 93% (Supplemental Table 2). The sequence homology between 'Earl's Favourite' and *C. sativus* was over 96% (Table 3), and single nucleotide polymorphisms (SNP) were detected at 284 sites (5.5%) among 68 accessions of *Cucumis* (Table 2). The most polymorphic region was Cmcp3 where the number of SNPs was observed at 32 sites (8.8%).

Among the 60 melon accessions, the total length of sequence determined in nine regions was from 5433 to 5479 bp (Table 4) and their homology was over 99% (Supplemental Table 2). Intra-specific SNPs were detected at 30 sites (0.6%) (Table 2), among which six sites were detected in Cmcp6, which was the most polymorphic region at frequency 0.9%. The frequency of SNPs did not differ greatly between the large single-copy region and the small single-copy region, at one polymorphism every 0.5% and 0.6%, respectively. And five insertions and/or deletions (InDel1-5) and 12 simple sequence repeats (SSR1-12) were also detected in addition to 30 SNPs (SNP1-30) (Table 4). SNP2 (C or A) and SNP3 (G or C) were detected in the coding sequence at 55 bp and 114 bp downstream from the transcription start site of *maturase K*, respectively. The former was a nonsynonymous substitution, and the latter was a synonymous substitution.

Table 3. Sequence homology (%) of of nine chloroplast genome regions between four plant species

No.	Name of species	Name of cultivar/ Accession No.[a]	No.			
			1	2	3	4
1	*Cucumis melo*	Earl's Favourite	–			
2	*Cucumis sativus*	DQ865976	96–98	–		
3	*Nicotiana tabacum*	NC_000932	72–86	71–87	–	
4	*Arabidopsis thaliana*	NC_001879	68–87	68–87	64–84	–

[a] Accession numbers are of DDBJ and NCBI.

Phylogenetic relationships between Cucumis species

Sixty-seven accessions of *Cucumis*, including the reference sequence of *C. sativus* (DQ865976), were divided into three clusters in the NJ tree constructed with the GD based on 284 SNPs (Fig. 1). Cluster I comprised all melon accessions. Cluster II comprised *C. hystrix* and *C. sativus*, even though these two species have different chromosome numbers, $2n = 14$ and $2n = 24$, respectively. Cluster III comprised African wild species *C. anguria*, *C. metuliferus* and *C. sagittatus*. Cluster I was slightly closer to cluster II at GD 0.01692 to 0.01950 than to cluster III at GD 0.02205 to 0.02440 (Fig. 1 and Table 5). The GD between *C. hystrix* and *C. sativus* was 0.00974 and was significantly shorter than the average distance between clusters II and III (GD = 0.02400). The GD between African wild species of cluster III was 0.01935 to 0.02213 (Table 5). Among them, *C. sagittatus* related most closely to *C. melo* accessions of cluster I (GD = 0.02205–0.02253) and then to *C. metuliferus* (GD = 0.02311–0.02387).

Melon accessions of cluster I were divided into three subclusters (Fig. 1 and Table 4). Subclusters Ia, Ib and Ic comprised 16, 26 and 18 accessions, respectively. The GD between subclusters Ia and Ic and between subclusters Ib and Ic were 0.00426 and 0.00446, respectively (Table 5). In contrast, the GD between subclusters Ia and Ib was 0.00132, indicating that subcluster Ic was rather distinct from subclusters Ia and Ib. The alignment of their sequences showed that melon accessions of subcluster Ic were uniquely characterized by eight SNPs (SNP3, 10, 12, 14, 16, 20, 27, 28) and subclusters Ia and Ib were unique by seven SNPs (SNP5, 6, 12, 13, 17, 26, 30) (Table 4). Differentiation between subcluster Ic and subclusters Ia and Ib was also detected by seven additional polymorphisms (InDel5; SSR1, 3, 5, 10, 11, 12). The uniqueness of subclusters Ia and Ib were represented by three sites (SNP18, 19; SSR4) and five sites (SNP2, 8, 9, 11; SSR7), respectively.

Genetic composition of melon groups

The eighteen melon accessions of subcluster Ic were all landraces of African origin (Fig. 2) and were divided into six

Table 4. Sequence polymorphism at 47 sites of nine chloroplast genome regions polymorphic among 60 accessions of melon

Species[a]	Cluster No.[b]		No. of accessions	Sequence length (bp)[c]	Cmcp1			Cmcp2			Cmcp3		Cmcp5						ccSSR7	Cmcp15	PS-ID		Cmcp6					
					SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10	SNP11	SNP12	SNP13	SNP14	SNP15	SNP16	SNP17	SNP18	SNP19	SNP20	SNP21	SNP22	SNP23	SNP24
C. melo	I	a-1	10	5457	C	C	G	C	T	C	C	G	C	T	G	T	T	G	T	A	A	A	A	C	C	C	A	G
C. melo	I	a-2	1	5457	C	C	G	C	T	C	C	G	C	T	G	T	T	G	T	A	A	A	A	C	C	C	A	G
C. melo	I	a-3	5	5456	C	C	G	C	T	C	C	G	C	T	G	T	T	G	T	A	A	A	A	C	C	C	A	G
C. melo	I	b-1	11	5479	C	A	G	C	T	C	C	A	A	T	A	T	T	G	T	A	A	T	G	C	C	C	A	G
C. melo	I	b-2	12	5478	C	A	G	C	T	C	C	A	A	T	A	T	T	G	T	A	A	T	G	C	C	C	A	G
C. melo	I	b-3	3	5474	C	A	G	C	T	C	C	A	A	T	A	T	T	G	T	A	A	T	G	C	C	C	A	G
C. melo	I	c-1	1	5433	T	C	C	A	G	A	C	G	C	C	G	A	C	T	T	C	C	T	G	A	C	C	A	G
C. melo	I	c-2	1	5472	T	C	C	C	G	A	A	G	C	C	G	A	C	T	T	C	C	T	G	A	C	C	A	G
C. melo	I	c-3	4	5471	T	C	C	C	G	A	A	G	C	C	G	A	C	T	T	C	C	T	G	A	C	C	A	A
C. melo	I	c-4	1	5472	T	C	C	C	G	A	A	G	C	C	G	A	C	T	T	C	C	T	G	A	A	C	A	A
C. melo	I	c-5	5	5448	C	C	C	C	G	A	C	G	C	C	G	A	C	T	C	C	C	T	G	A	C	G	G	G
C. melo	I	c-6	6	5455	C	C	C	C	G	A	C	G	C	C	G	A	C	T	C	C	C	T	G	A	C	G	G	G
C. hystrix	II		2	5468	C	C	G	A	G	A	A	T	C	T	G	C	C	G	T	A	C	T	G	C	A	C	A	G
C. hystrix	II		1	5468	C	C	G	A	G	A	A	T	C	T	G	C	A	G	T	A	C	T	G	C	A	C	A	G
C. sativus	II		1	5460	C	C	G	–	G	A	C	T	C	T	G	C	C	G	T	A	C	T	G	C	A	C	A	G
C. metuliferus	III		1	5460	C	C	G	C	G	A	A	G	C	T	G	C	C	G	T	A	C	T	G	C	A	C	A	A
C. sagittatus	III		1	5239	C	C	G	C	G	A	C	G	C	T	G	C	C	G	G	A	C	T	–	–	A	C	A	G
C. anguria	III		1	5436	C	C	G	–	G	A	C	G	C	T	G	C	C	G	G	A	A	T	G	C	A	–	A	G

Species[a]	Cluster No.[b]		No. of accessions	Sequence length (bp)[c]	Cmcp11[d]						ccSSR7	Cmcp15	Cmcp11			Cmcp2			Cmcp3	Cmcp15	PS-ID		Cmcp6		Cmcp11		
					SNP25	SNP26	SNP27	SNP28	SNP29	SNP30	InDel1	InDel2	InDel3	InDel4	InDel5	SSR1	SSR2	SSR3	SSR4	SSR5	SSR6	SSR7	SSR8	SSR9	SSR10	SSR11	SSR12
C. melo	I	a-1	10	5457	G	T	G	T	G	T	0	0	+18	+4	−5	(A)5	(T)7	(A)10	(A)17	(T)11	(T)11	(T)13	(A)2	(T)2	(A)9	(T)8	(T)8
C. melo	I	a-2	1	5457	C	T	G	T	T	T	0	0	+18	+4	−5	(A)5	(T)7	(A)10	(A)17	(T)11	(T)11	(T)13	(A)2	(T)2	(A)9	(T)8	(T)8
C. melo	I	a-3	5	5456	C	T	G	T	T	T	0	0	+18	+4	−5	(A)5	(T)7	(A)10	(A)16	(T)11	(T)11	(T)13	(A)2	(T)2	(A)9	(T)8	(T)8
C. melo	I	b-1	11	5479	C	T	G	T	T	T	0	+23	+18	+4	−5	(A)5	(T)7	(A)10	(A)15	(T)11	(T)11	(T)14	(A)2	(T)2	(A)9	(T)8	(T)8
C. melo	I	b-2	12	5478	C	T	G	T	T	T	0	+23	+18	+4	−5	(A)5	(T)7	(A)10	(A)15	(T)11	(T)10	(T)14	(A)2	(T)2	(A)9	(T)8	(T)8
C. melo	I	b-3	3	5474	C	T	G	T	T	T	−5	+23	+18	+4	−5	(A)5	(T)7	(A)10	(A)15	(T)11	(T)11	(T)14	(A)2	(T)2	(A)9	(T)8	(T)8
C. melo	I	c-1	1	5433	–	C	A	G	T	C	0	0	0	+4	−6	(A)4	(T)8	(A)13	(A)13	(T)9	(T)11	(T)13	(A)3	(T)3	(A)10	(T)2	(T)9
C. melo	I	c-2	1	5472	C	C	A	G	T	C	0	+23	+18	+4	−6	(A)4	(T)7	(A)12	(A)15	(T)9	(T)11	(T)13	(A)2	(T)2	(A)10	(T)2	(T)9
C. melo	I	c-3	4	5471	C	C	A	G	T	C	0	+23	+18	+4	−6	(A)4	(T)7	(A)12	(A)14	(T)9	(T)11	(T)13	(A)1	(T)3	(A)10	(T)2	(T)9
C. melo	I	c-4	1	5472	C	C	A	G	T	C	0	+23	+18	+4	−6	(A)4	(T)7	(A)12	(A)14	(T)9	(T)11	(T)13	(A)1	(T)4	(A)10	(T)2	(T)9
C. melo	I	c-5	5	5448	C	C	A	G	T	C	0	0	+18	−1	−6	(A)4	(T)8	(A)13	(A)12	(T)9	(T)12	(T)15	(A)2	(T)2	(A)10	(T)3	(T)10
C. melo	I	c-6	6	5455	C	C	A	G	T	C	0	+7	+18	−1	−6	(A)4	(T)8	(A)13	(A)12	(T)9	(T)12	(T)15	(A)2	(T)2	(A)10	(T)3	(T)10
C. hystrix	II		2	5468	–	C	G	T	T	C	0	0	0	0	−5	(A)3	(T)5	(A)9	(A)13	(T)9	(T)11	(T)12	(A)2	(T)5	(A)13	(T)2	(T)9
C. hystrix	II		1	5468	–	C	G	T	T	C	0	0	0	0	−5	(A)3	(T)5	(A)9	(A)13	(T)9	(T)11	(T)12	(A)2	(T)5	(A)13	(T)2	(T)9
C. sativus	II		1	5460	–	C	G	T	T	C	0	0	0	0	−5	(A)3	(T)6	(A)9	(A)14	(T)9	(T)11	(T)11	(A)2	(T)5	(A)15	(T)2	(T)8
C. metuliferus	III		1	5460	–	C	G	T	T	C	0	0	0	−1	0	(A)3	(T)6	(A)18	(A)14	(T)12	(T)5	(T)9	(A)2	(T)4	(A)11	(T)2	(T)10
C. sagittatus	III		1	5239	–	C	G	T	T	C	0	0	0	0	0	(A)3	(T)6	(A)17	(A)11	(T)15	(T)8	(T)9	–	–	(A)12	(T)2	(T)12
C. anguria	III		1	5436	–	C	G	T	T	C	0	−7	0	0	0	(A)3	(T)6	(A)14	(A)14	(T)13	(T)11	(T)8	(A)2	(T)4	(A)10	(T)2	(T)11

[a] Single nucleotide polymorphism, insertion or deletion and simple sequence repeat are represented by "SNP", "In/Del" and "SSR", respectively. SNPs data of wild species was also indicated. Absence of sequence at SNP and SSR sites was indicated by "–".
[b] Cluster number refers to Fig. 1.
[c] Sequence length indicating total number of nucleotides of nine chloroplast genome regions.
[d] SNP25 was located in the motif sequence of In/Del3.

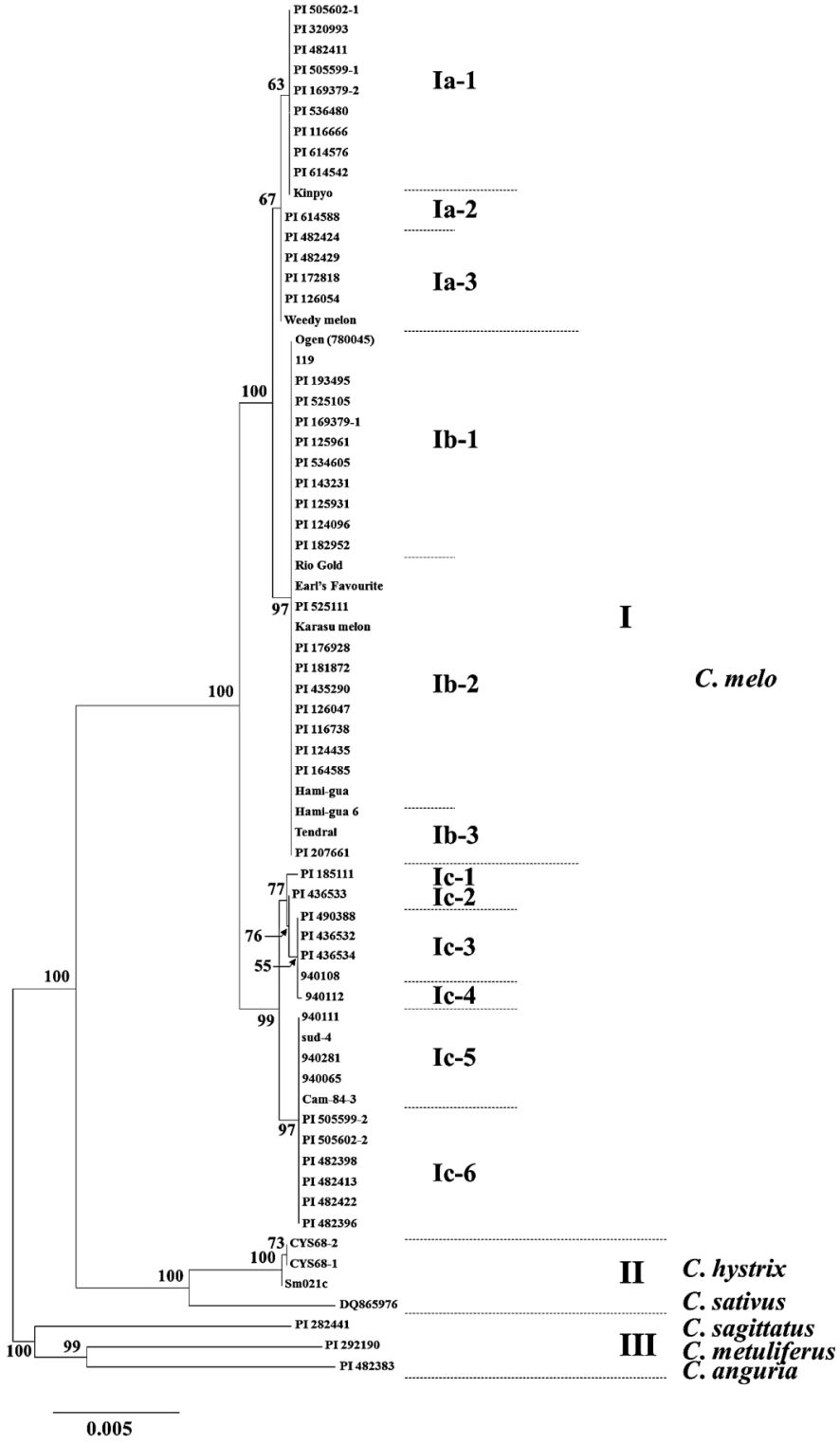

Fig. 1. Neighbor-joining tree showing relationships of 67 accessions of *Cucumis* species from SNPs in nine chloroplast genome regions. Bootstrap values over 50% for 1000 replicates are shown beside the branch. Tendral: Tendral o Invernale a Buccia Verde.

subgroups (Ic-1, -2, -3, -4, -5, -6) based on the polymorphism at 20 sites (Table 4). Subgroups Ic-5 and Ic-6, which differed at InDel2, were separated from the other subgroups by four SNPs (SNP1, 15, 22, 23), one indel (InDel4) and five SSRs (SSR4, 6, 7, 11, 12). Subgroups Ic-1, Ic-2, Ic-3 and Ic-4 were classified by four SNPs (SNP4, 7, 21, 24), two indels (InDel2, 3) and five SSRs (SSR2, 3, 4, 8, 9). The GD within subcluster Ic, corresponding to nucleotide diversity (π)

Table 5. Genetic distance (GD) between eight groups of *Cucumis* accessions, calculated from the frequency of nucleotide in 284 SNPs

No.	Name of species	Cluster No.	No. of accession		No.								GD within group[a]
					1	2	3	4	5	6	7	8	
1	*C. melo*	I	a	16	–								0.00019
2			b	26	0.00132	–							0
3			c	18	0.00426	0.00446	–						0.00084
4	*C. hystrix*	II		3	0.01692	0.01701	0.01721	–					0.00012
5	*C. sativus*			1	0.01888	0.01896	0.01950	0.00974	–				nc
6	*C. sagittatus*	III		1	0.02205	0.02253	0.02233	0.02145	0.02350	–			nc
7	*C. metuliferus*			1	0.02311	0.02358	0.02387	0.02330	0.02566	0.02200	–		nc
8	*C. anguria*			1	0.02380	0.02407	0.02440	0.02534	0.02636	0.02213	0.01935	–	nc

[a] GD not calculated within a group is indicated by "nc".

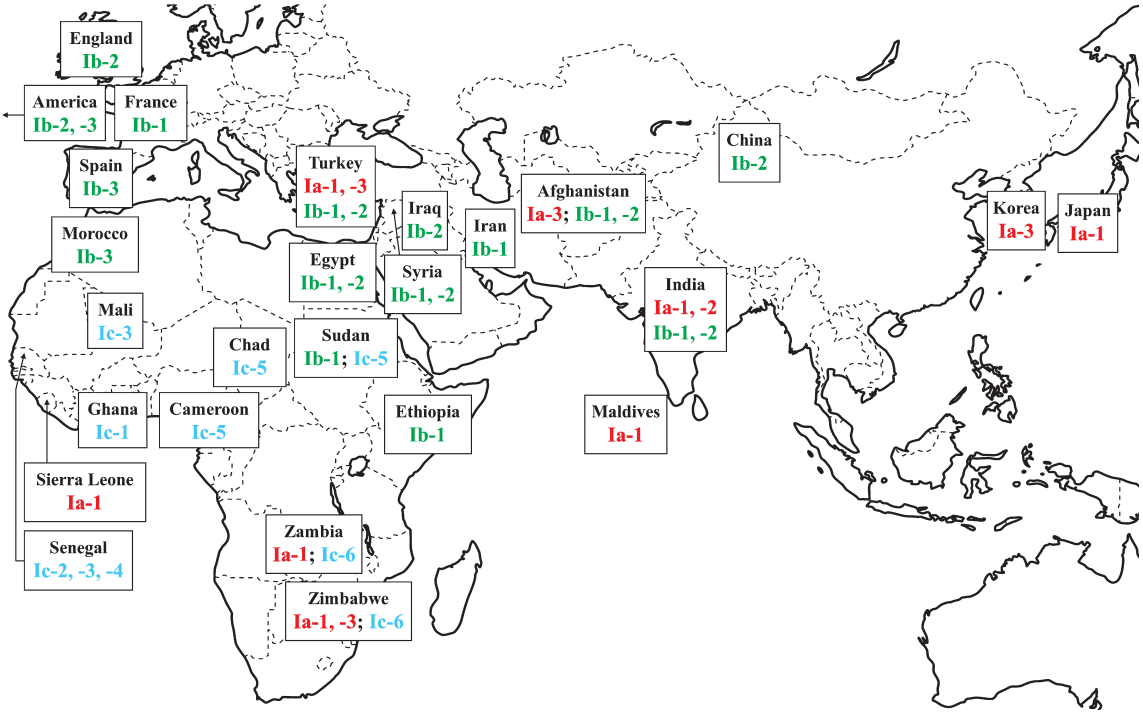

Fig. 2. Geographical distribution of cytoplasm types in melon landraces. Cluster number refers to Fig. 1.

according to Nei and Li (1979), was 0.00084 (Table 5).

Twenty-six melon accessions of subcluster Ib comprised accessions from USA and Europe (UK, France, Spain), northern Africa (Sudan, Ethiopia, Morocco, Egypt), West and Central Asia (Turkey, Syria, Iraq, Iran, Afghanistan), South Asia (India) and East Asia (China, Fig. 2). The chloroplast genome sequence of these melon accessions was identical for over 5000 bp, except polymorphic sites SSR6 and Indel1, by which subcluster Ib was divided into subgroups Ib-1, -2 and -3 (Fig. 1 and Table 4). Twelve accessions of subgroup Ib-2 were unique in the repeat number of SSR motif (T) at SSR6, and three accessions of subgroup Ib-3 had a deletion of 5 bp (ATATT) at Indel1.

Subcluster Ia comprised 16 melon landraces from West and Southern Africa (Sierra Leone, Zambia, Zimbabwe), West and Central Asia (Turkey, Afghanistan), South Asia (Maldives, India) and East Asia (Korea, Japan) (Fig. 2). These melon accessions were divided into subgroup Ia-1 and subgroups Ia-2 and Ia-3 by SNP25 and 29 in Cmcp11 and the latter two subgroups differed by SSR4 in Cmcp3 (Table 4). The GD within subcluster Ia was 0.00019 (Table 5).

By combining the sequences of the nine regions, four parsimony trees were retained based on 133 parsimony informative sites; consistency index and retention index showed higher values which was 0.9290 and 0.9712, respectively. These four trees were the same as the NJ tree on the diagram between melon and other *Cucumis* species and among subcluster Ia, Ib and Ic (Figs. 1, 3).

Worldwide distribution patterns of large- and small-seed types

Melon accessions (7,396) in the GRIN database were divided into large- and small-seed melon based on the 100

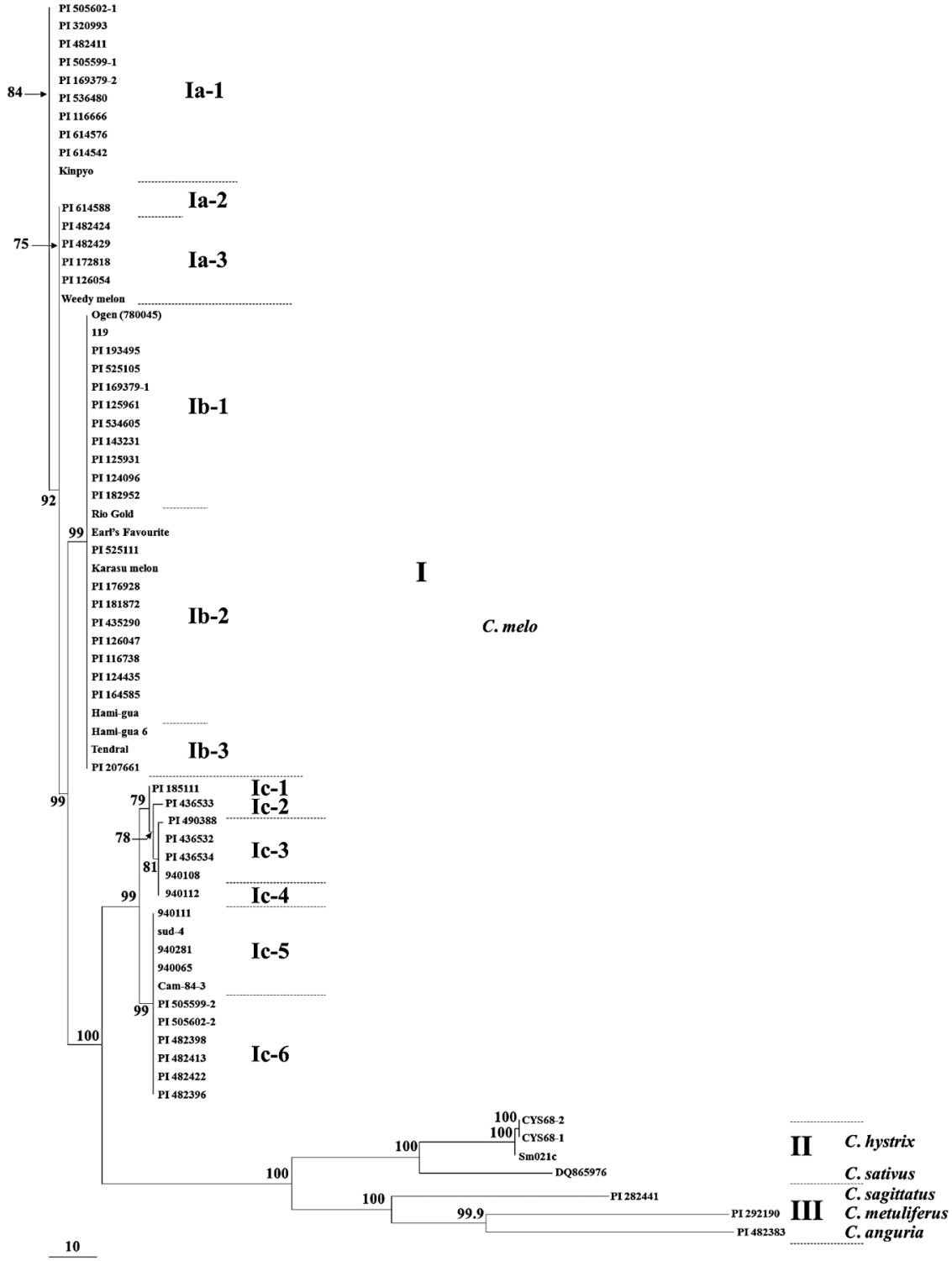

Fig. 3. Parsimony tree showing relationships of 67 accessions of *Cucumis* species, from SNPs of nine chloroplast genome regions. Length = 330 steps; consistency index = 0.9290; retention index = 0.9712. Bootstrap values for 1000 replicates are shown beside the branch; values less than 50% are not shown. Tendral: Tendral o Invernale a Buccia Verde.

seed weight (Table 6). The frequency of large-seed melon was 93.7% (1,782/1,901 accessions) in Europe, USA and Russia and 96.9% (2,538/2,619 accessions) in West and Central Asia. The frequency of small-seed melon increased from West Asia towards the east from 51.4% (1,092/2,123 accessions) in South Asia to 62.2% (194/312 accessions) in East Asia. In Africa, the frequency of large-seed melon differed among areas at 98.6% in northern Africa (275/279

Table 6. Varietal and geographical variation of 100 seeds weight among 7,396 accessions of melon

Area	Country	No. of accessions	100 seeds weight (g)[a]																			
			0–	0.5–	1.0–	1.5–	2.0–	2.5–	3.0–	3.5–	4.0–	4.5–	5.0–	5.5–	6.0–	6.5–	7.0–	7.5–	8.0–	8.5–	9.0–	9.5–
			Small-seed type					Large-seed type														
North America		224	–	40	8	7	14	35	59	43	10	5	1	–	–	–	1	–	–	–	1	–
Central America		40	–	2	7	4	2	5	5	1	6	5	1	1	–	–	–	1	–	–	–	–
South America		39	–	2	1	–	–	–	6	9	7	12	1	1	–	–	–	–	–	–	–	–
Europe		1,520	1	–	1	7	19	51	125	213	294	271	235	144	88	37	21	8	3	1	1	–
Russia/Former Soviet Union		78	–	–	1	–	3	9	12	14	19	14	3	–	2	1	–	–	–	–	–	–
West and	Turkey	660	–	–	1	5	15	37	78	133	157	110	72	32	11	4	4	–	1	–	–	–
Central Asia	Afghanistan	962	–	2	2	1	9	30	59	98	157	205	170	135	61	19	5	5	2	–	1	1
	Other	997	–	7	1	10	28	44	83	130	157	205	134	107	49	22	12	6	1	1	–	–
Africa	North	279	–	–	1	–	3	4	9	34	49	71	52	33	21	1	1	–	–	–	–	–
	West and Center	23	–	–	5	3	–	–	1	5	4	3	2	–	–	–	–	–	–	–	–	–
	South	121	–	1	14	39	35	23	5	2	2	–	–	–	–	–	–	–	–	–	–	–
South Asia	Pakistan	73	–	–	2	–	7	15	21	8	10	4	1	2	2	–	1	–	–	–	–	–
	India	1,999	1	57	202	291	501	378	269	142	101	44	10	3	–	–	–	–	–	–	–	–
	Maldives	33	–	19	2	1	1	5	4	1	–	–	–	–	–	–	–	–	–	–	–	–
	Myanmar	18	–	–	6	2	–	1	5	3	1	–	–	–	–	–	–	–	–	–	–	–
Southeast Asia		18	–	2	2	1	3	2	2	2	4	–	–	–	–	–	–	–	–	–	–	–
East Asia	China	202	–	–	23	55	19	7	19	18	21	17	9	10	2	2	–	–	–	–	–	–
	Korea	28	–	–	12	12	2	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–
	Japan	82	–	2	20	42	7	7	1	3	–	–	–	–	–	–	–	–	–	–	–	–
		7,396	2	134	311	480	668	654	764	859	999	966	691	468	236	86	45	20	7	2	3	1

[a] The data of 100 seeds weight was obtained from the GRIN database of USDA-ARS (http://www.ars-grin.gov/npgs/).

accessions) and 26.4% in southern Africa (32/121 accessions), respectively. These findings demonstrate geographical differences in seed size: large-seed melon is mainly cultivated in USA, Europe, West and Central Asia and northern Africa and small-seed melon is frequent in southern Africa, South and East Asia. Indian melon was diversified, and both large- and small-seed melon showed similar frequencies.

Discussion

The nucleotide sequence of nine inter- and intra-genic regions of the chloroplast genome, in total 5239 bp to 5479 bp, was compared for 66 *Cucumis* accessions, including wild species of *C. sagittatus*, *C. anguria*, *C. metuliferus* and *C. hystrix* with one reference accession of cucumber (*C. sativus*). Sequence polymorphism was successfully detected among the melon accessions (*C. melo*) (Table 4). Sixty melon accessions, including weedy melon, were separated into three subclusters of cytoplasm (types Ia, Ib and Ic) based on polymorphism at 47 sites (SNPs: 30, InDel: 5, SSR: 12). This is the first report to show intra-specific diversification of cytoplasm type within *C. melo*, while interspecific variation among 113 accessions including 17 melon accessions, was successfully shown by Sebastian *et al.* (2010). The NJ and parsimony trees showed the three cytoplasm types differentiated in the direction of (Ic, (Ib, Ia)) (Figs. 1, 3). These cytoplasm types were classified further into 12 cytoplasm types Ia (-1, -2, -3), Ib (-1, -2, -3) and Ic (-1, -2, -3, -4, -5, -6), by 3, 2 and 20 polymorphic sites, respectively (Table 4). The assumption is reasonable that these polymorphisms accumulated in the chloroplast genome after the differentiation of the three types in melon.

Groups Cantalupensis and Inodorus from Greece (Staub *et al.* 2004), Spain (López-Sesé *et al.* 2003) and Hungary (Szabó *et al.* 2005) have genetic affinities among themselves. Six accessions of Groups Cantalupensis and Inodorus, used in this study, were also classified together as the subcluster Ib which consists mainly of large-seed melon (Table 1). However, subcluster Ib was further divided into three subgroups, Ib-1, Ib-2 and Ib-3. Subgroups Ib-2 and Ib-3 were differentiated from subgroup Ib-1, independently, because of a sequence slippage ([T]11 → [T]10) at SSR6 (PS-ID region, *Rpl16-Rpl14*) in Ib-2 and a deletion of the motif sequence (ATATT) at InDel1 (*PsbC-TrnS*, ccSSR7) in Ib-3, respectively (Table 4). Melon accessions of subgroups Ib-1 and Ib-2 were distributed in diverse areas, such as northern Africa, West Asian countries and India (Fig. 2), suggesting that the cytoplasmic diversification in large-seed melon should have occurred before the spread of melon from Africa. Subgroup Ib-2 contained Groups Cantalupensis and Inodorus accessions; 'Rio Gold' (netted melon, USA) and 'Earl's Favourite' (netted melon, UK) of Group Cantalupensis and 'Hami-gua' (China) of Group Inodorus. In contrast, Group Inodorus was not in subgroup Ib-1 and Group Cantalupensis was not in subgroup Ib-3. Although the number of melon accessions analyzed was limited, these results indicate that Groups Cantalupensis and Inodorus were established in independent maternal lines.

Group Conomon, cultivated in East and South Asia, was classified into subcluster Ia together with Group Agrestis from Asia (Table 1). Although only one accession of each group was tested in this study, Nhi *et al.* (2010) confirmed that 24 accessions of Groups Conomon and Agrestis from Vietnam and 14 accessions of Group Conomon from China uniformly had "A" at SNP18, which was a diagnostic sequence of subcluster Ia (Table 4). In contrast, all accessions of Group Agrestis from Africa were classified as subcluster Ic together with 10 accessions of African cultivated melon (Fig. 1 and Table 1). These results indicated that Group Agrestis, a companion weed in a crop field, is diversified in its maternal line and shares the same maternal line with cultivated melon in each area. Several studies on diversity in the nuclear genome have provided evidence that Group Agrestis shares a common gene pool with small-seed melon or landraces in each area (Akashi *et al.* 2002, McCreight *et al.* 2004, Nakata *et al.* 2005). Therefore, these results suggested that Group Agrestis was not an ancestral type of cultivated melon, but was established independently as an escape from cultivated melon in each area.

According to Pitrat (2008), Groups Conomon and Agrestis from Asia and Groups Cantalupensis and Inodorus from Europe and USA were classified as different subspecies, that is, subsp. *agrestis* and subsp. *melo*, respectively. Genetic differentiation between Groups Conomon and Agrestis from Asia and Groups Cantalupensis and Inodorus from Europe and USA has been clearly shown by analysis of nuclear genome markers (Aierken *et al.* 2011, Monforte *et al.* 2003, Stepansky *et al.* 1999). In this study, Asian melon with small seeds was classified as subcluster Ia by six SNPs (SNP 2, 8, 9, 11, 18, 19) and was genetically differentiated from European and US melon with large seeds in subcluster Ib (Fig. 1 and Table 4). Such a differentiation clearly indicated that Asian melon with small seed and US and European melon with large seed were established independently in different maternal lines. However, melon accessions of subcluster Ia also included large-seed melon from Turkey (PI 169379-2, PI 172818), Afghanistan (PI 126054) and India (PI 116666, PI 614588). Based on the results summarized in Table 6, North American, European, North African and West and Central Asian melon mainly consisted of large-seed type (≥2.5 g) and East Asian melon included small-seed type (<2.5 g). However in India, both large- and small-seed melons were at similar frequencies, as also reported by Tanaka *et al.* (2007). In our previous study, using dCAPS marker of SNP18, 71 melon accessions from South Asia were found to be a mixture comprising not only large-seed melon of subgroup Ib and small-seed melon of subgroup Ia but also large-seed melon of subgroup Ia and small-seed melon of subgroup Ib (Tanaka *et al.* 2006). In the present study, among the Indian melon accessions, large-seed accessions PI 116666 and PI 614588 were classified as subcluster Ia and small-seed accession PI 116738 was classified as subcluster Ib (Fig. 1). These results suggested that spontaneous hybridization between large- and small-seed types occurred in India and resulted in establishing large-seed melon of subcluster Ia and small-seed melon of subcluster Ib.

Africa is considered the center of evolution of *Cucumis* species, and archaeological remains and historical records hypothetically infer the origin of cultivated melon in Africa (Bates and Robinson 1995, Robinson and Decker-Walters 1997). Twenty-nine accessions of African melon were diversified and were assigned to 11 cytoplasm types, among which five cytoplasm types (subclusters Ia and Ib) were commonly found also in other continents and the other six cytoplasm types (subcluster Ic) were unique to Africa (Fig. 2). Although the number and area coverage of African melon landraces examined is limited, the geographical distribution pattern clearly differed between the cytoplasm types. Melon accessions of subcluster Ib, which included large-seed melon from Europe and USA, were only in northern Africa, such as Egypt, Morocco, Ethiopia and Sudan. In contrast, melon accessions of subcluster Ia, which included Asian small-seed melon, were mainly in Southern Africa, such as Zambia and Zimbabwe. Noteworthy is that the African accessions included five subgroups of subclusters Ia and Ib. Melon accessions of subcluster Ic were endemic to Western, Central and Southern Africa, and those of subgroup Ic-6 were from Southern Africa (Zambia and Zimbabwe). Similar geographical variation was observed in seed size. Three of four melon accessions (75%) were large-seed melon from northern Africa, and 78.6% and 100% of melon accessions were small-seed melon from West and Central Africa and Southern Africa, respectively (Table 1). These results may suggest that large-seed melon Groups Cantalupensis and Inodorus were established from northern African melon landraces in maternal line Ib.

Groups Conomon and Agrestis, small-seed melon commonly found in Asia, share the same maternal line with Southern African small-seed melon, even though these two regions are geographically distant. RAPD polymorphism indicated genetic differentiation between melon landraces from northern and Southern Africa, which are related closely to European and US melon and East Asian melon, respectively (Mliki *et al.* 2001). Nakata *et al.* (2005) also indicated a genetic relationship between South African melon and East Asian melon. The third cytoplasm type, Ic, differed distinctly from cytoplasm types Ia (GD = 0.00426) and Ib (GD = 0.00445) (Table 5), which suggests that the cultivated melon accessions of subcluster Ic were established independently from those of subclusters Ia and Ib. Further studies using more accessions of African melon are required to validate these hypotheses on the multiple origin of cultivated melon.

In this study, we analyzed one reference accession of *C. sativus* and six accessions of wild species *C. hystrix*, *C. anguria*, *C. sagittatus* and *C. metuliferu*s (Table 1). These species are genetically close to *C. melo* from analysis of chromosome pairing (Dane *et al.* 1980), pollen-pistil interaction (Kho *et al.* 1980), self-incompatibility (Deakin *et al.* 1971, Den Nijis and Visser 1985) and seed fertility of F_1

hybrids (Norton and Granberry 1980, Singh and Yadava 1984). Isozyme analysis (Perl-Treves *et al.* 1985), RFLP analysis (Perl-Treves and Galun 1985), southern blotting analysis of satellite DNA (Helm and Hemleben 1997) and sequence analysis of the ITS region (Garcia-Mas *et al.* 2004) indicate that *C. melo* is genetically related to *C. sagittatus* and *C. metuliferus*, rather than *C. anguria*. However, analysis of the chloroplast genome indicated that *C. melo* is more closely related to Asian species of *Cucumis*, such as *C. sativus* and *C. hystrix*, compared with African wild species, even though the chromosome number differs between *C. melo* and *C. sativus* (Chung *et al.* 2006, Renner *et al.* 2007). In this study, cluster I of only melon accessions was related more closely to cluster II of *C. sativus* and *C. hystrix* and then to cluster III of *C. sagittatus*, *C. metuliferus* and *C. anguria* (Figs. 1, 3 and Table 5). These phylogenetic relationships supported the previous results of Chung *et al.* (2006) and Renner *et al.* (2007) and provided an approximate relationship between *Cucumis* species. However, the genetic distance was not small, even between *C. melo* and *C. hystrix* (Table 5) and thus identifying the direct wild ancestor of cultivated melon was difficult. *C. picrocarpus* from Australia is related more closely to melon than to *C. hystrix* (Sebastian *et al.* 2010). Intra-specific variation of the chloroplast genome sequence, detected for *C. melo*, *C. hystrix* (Table 4) and *C. metuliferus* (Rennar *et al.* 2007), may provide important clues, and thus intra-specific variation of the chloroplast genome should be surveyed for each *Cucumis* species.

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