Analysis of genetic diversity and population structure in Cambodian melon landraces using molecular markers

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Chapter 1

General introduction

Cucumis melo L. (melon) which belongs to genus *Cucumis* is one of the important horticultural crops and grown worldwide to be consumed as dessert and vegetable. However, intensive cultivation of modern melon cultivars with narrow genetic basis indirectly caused explosive increase of disease and pest damages, and thus the breeding of resistant cultivars is an urgent task. Wild species of *Cucumis* are considered as important genetic resources for resistance, but it's still difficult to produce interspecific hybrid between cultivated melon and wild *Cucumis* species. Therefore, practical, and efficient approach is to utilize the genetic resources of landraces which are also considered to have resistant genes or tolerant genes.

Cucumis genus belongs to the family Cucurbitaceae, sub-family Cucurbitoideae and placed in the tribe Benincaseae. According to Kirkbride (1993), the genus *Cucumis* is represented by 32 species. Recently, based on molecular phylogenetic studies, the genus *Cucumis* added the species of 5 genera *Cucumella, Dioelospermum, Mukia, Mymecosicuos* and *Oreosyce*, contains 52 species. Among which only two species of *Cucumis, C. melo* and *C. sativus*, are cultivated plants. *Cucumis* species were traditionally assumed to have an African origin and posed to Asia, only *Cucumis hystrix* is in Asia, however, some new phylogenetic studies show that both melon and cucumber are of Asian origin and have numerous previously overlooked species-level relatives in Australia and around the Indian Ocean (Sebastian *et al*. 2010). Sequences of the Himalayan entities *Cucumis trigonus* and *Cucumis callosus* are nearly identical to those of *C. melo* and likely represent the wild progenitor of cultivated melon as these forms are fully crossable with *C. melo*. The sister species to *C. melo* populations is the Australian *Cucumis picrocarpus,* which had been synonymized under *C. melo*, but is genetically and morphologically highly distinct. Therefore,

studying the phylogenetic to know the closet relatives and natural composition of the *Cucumis* species is important because this will supply not only evidence to resolving the question related to origin, evolution, phylogenetic relationship with melon and cucumber but will also provide several desirable sources of disease and pest resistance genes to melon and cucumber breeding from wild relatives through interspecific hybridization.

Melon (*Cucumis melo* L.) is considered one of the most morphologically diverse species among the major cucurbit crops, even among vegetables (Pitrat, 2008). According to the ovary's hairiness, melon is divided into two subspecies: *ssp.melo* with long hairs and *ssp.agrestis* with short hairs (Jeffrey, 1980). Various intraspecific classifications based on fruit characters and cultivation regions have been proposed. Naudin *et al*. (1895) first attempted to define nine tribes of cultivated melon and one wild form. Up to now, classification of Pitrat *et al*. (2008) with fifteen botanical groups of melon including five botanical groups in *ssp. agrestis* (*conomon, momordica, chinensis, makuwa and acidulus*) and ten botanical groups in *ssp. melo* (*chate, flexuosus, tibish, adana, ameri, cantalupensis, chandalak, reticulatus, inodorus, dudaim*) are widely accepted and seemed to be steady until recent discoveries of the African and Asian melons which are not composed in this taxonomy made it become limited within cultivated melons. Therefore, the question of geographic origin and region of domestication of melon one time has been raised and the requirement to be thoroughly uncovered is getting urgent.

Relied on the fact that the numerous wild melon species have been found in Africa as well as the identical chromosome number of melon and African *Cucumis*, many authors supposed Africa as origin center of melon. The hypothesis is supported by several phylogenetic research (Kerje and Grum, 2000; Pitrat *et al*., 2008; Tanaka *et al.,* 2013). Especially, Tanaka *et al*. (2013) demonstrated that large-seed melon groups (*cantalupensis*

and *inodorous*) and small-seed melon groups (*conomon* and *agrestis*) shared the same maternal lineage with Northern African melon and Southern African melon, respectively. Parallel to the above hypothesis, recent molecular phylogenies affirmed that melon originated from Asia had numerous species-level relatives in Australia and around the Indian Ocean (Renner *et al.,* 2007; Sebastian *et al*., 2010). Nonetheless, this viewpoint was firstly suggested from nineteenth century (Naudin, 1895), but at that time they had not enough evidence to prove. Furthermore, many studies observed the large genetic diversity in Indian melon germplasm which can be considered one more evidence. The most update research (Malik *et al*., 2014) showed the close affinity in terms of genetic variability of Indian landraces dividing in to three groups (*momordica*, *cantalupensis, reticulatus*) and USA *reticulatus*. Two explanations for this conclusion are either Indian melon moved to USA through central Asia and Europe, or it is the result of intercrossing of Indian landraces with USA derived cultivars (Malik *et al*., 2014). Although the domestication history of melon is still unclear, several studies agree that Asia is one of the domestication centers of melon (Mallick *et al*., 1986; Serres-Giardi *et al*., 2010; Sebastian *et al*., 2010, Endl *et al*., 2018). The most recent research (Endl *et al*., 2018) showed that melon was domesticated at least twice in Africa and in Asia and found a close wild relative of melon (drought tolerant species) in India (*Cucumis trigonus* Roxb.) which strongly supported the idea of an Asia domestication center. The most update research proves that India is the primary center of cultivated melons (Gonzalo *et al*., 2019).

Obviously, the original center and domestication of melon have remained intransparent. Both of two hypotheses (Asian origin/ African origin) are being acceptable simultaneously. Studies of phylogenetic relationship between melon landraces of each region and wild melons collected from Asia/ Africa have contributed to clear these viewpoints. Nonetheless, the limited number of wild accessions as well as the geography-bias sampling in most of research

might be the reason causing to multiple hypotheses. In short, soon or late, the complete picture of melon domestication and diversification will be brought to light.

Diverse melon germplasms are essential for pursuing successful breeding efforts, as they increase genetic diversity in breeding lines, particularly for preserving unique traits or rare alleles of interest and aid the development of new melon cultivars with desirable traits. During recent years markers that detect diversity directly on the DNA level have become more and more important and have replaced morphological markers and the formerly widely utilized isozyme marker systems almost completely. Unlike morphological markers, genetic markers are unaffected by the conditions in which the plants are grown and usually show a larger number of loci and a higher level of polymorphisms than isozyme markers. Polymorphisms in the nucleotide sequence can be revealed by different techniques. For the assessment of diversity in plant populations different marker systems have been established on morphological, physiological and DNA levels. Each of the various marker systems utilized for the characterization of similarities and differences between individuals has its specific strengths and constraints, e.g., regarding the number of available markers, the polymorphism per marker, the mode of inheritance or the genomic location of the markers. Hence, to obtain unbiased estimates of the genetic diversity within a population, attention must be paid to the choice of the marker system utilized as well as of the statistical methods applied once an appropriate data set is obtained.

Identification and characterization of melon germplasm serve this purpose well, helping to define the lineage relationships among the genetic materials and eliminate the phenomenon of homonyms as well as aiding identification of breeding lines bearing genes of interest by means of molecular markers. Genetic diversity is an important feature of crop genetic resources and can be determined by using morphological and molecular markers. Molecular

markers based on DNA sequence polymorphism are independent of environmental conditions and show higher levels of polymorphism. In the last decade molecular markers have been frequently used to analyze genetic diversity. Several types of molecular markers have been used to evaluate the genetic diversity of melon: restriction fragment length polymorphisms (RFLP; Neuhausen, 1992), isozymes (Akashi *et al.* 2002; McCreight *et al.* 2004), amplified fragment length polymorphism (AFLP; Garcia-Mas *et al.* 2000), random amplified polymorphic DNA (RAPD; Mliki *et al.* 2001; Lopez-Sese *et al.* 2003; Staub *et al.* 2004; Nakata *et al*. 2005; Dhillon *et al*. 2007; Tanaka *et al.* 2007; Yi *et al*. 2009; Nhi *et al*. 2010), simple sequence repeats (SSRs; Monforte *et al*. 2003; Dhillon *et al*. 2007; Nhi *et al*. 2010; Fukino *et al*. 2007; Tanaka *et al*. 2013; Raghami *et al.* 2014;). For establishing genetic relationships between melon genotypes all these markers have been equally informative. Akashi *et al*. (2002), McCreight *et al.* (2004), Tanaka *et al*. (2007) and Nhi *et al*. (2010) evaluated genetic variation in East and South Asian melon by analysis of isozyme, RAPD and SSRs polymorphism; they reported that genetic diversity of Indian melon was rich compared to East Asian melon. Markers based on polymerase chain reaction (PCR) especially RAPD and SSR have received much attention because both require a small amount of DNA, yield high level of polymorphisms and are cost-efficient. However, RAPD markers are incapable to detect allelic differences in heterozygotes and difficult to reproduce between different laboratories due to their lack of specificity, whereas SSRs often require high resolution and laboratory intensive techniques (Jiang *et al*., 2013). Furthermore, the combination of different types of markers was basically deployed to increase the confidence of phylogenic tree (for examples Nhi *et al*., 2010 used both RAPD and SSR markers; Malik *et al*., 2014 combined biochemical analysis with SSR markers).

Asia is one of the diversity centers of *Cucumis melo* L., especially the subspecies *agrestis* which consists of five botanical groups (*Momordica, Conomon, Chinensis, Makuwa* and

Acidulus) (Pitrat *et al*., 2008). Melons in East, Far East and South Asia have been much paid attention. Several studies focused on their diversity and phylogenetic relationship (McCrieght J.D, 2004; Tanaka *et al*., 2007; Tanaka *et al*., 2013; Malik *et al*., 2013). Screening of resistant genes in the germplasm resources from South and East Asia was also the main topic for many researches. Group *Momordica* mainly cultivated in India was shown to be highly resistant to downy mildew, resistant to CGMMV (Cucumber green mottle mosaic virus) and medium resistant to Fusarium wilt (Pan *et al*., 1996). Partial resistance to Fusarium *race 1.2* was found in oriental melon of Far East such as Ogon 9 and Kogane Nashi Makuwa (Risser and Rode, 1973; Perchepied and Pitrat, 2004). Melon landraces from Far East and India also showed high resistance to downy mildew (Olczak-Woltman *et al*., 2011), whereas less knowledge has been reported for melon landraces from the Southeast Asia.

Cambodia is one of the Southeast Asian countries so that melon landraces here might share the same characters with the melons in South and East Asia, especially also containing resistant genes as those from South and East Asia. As expected, fruit characters of melon landraces from Eastern part of Cambodia proved to be similar with those from East Asian var. *conomon* and var. *makuwa*. In contrast, the average seed length of Cambodian melon was 7.8 mm which is shorter than that of East Asian melon and equivalent to that of var. *momordica* grown in India (Tanaka *et al.,* 2016). Field survey conducted on melon landraces in four regions of Cambodia (East, North, West, and South) showed that they were morphologically different from those of the neighboring countries. The typical fruit shape in Cambodia is elongated; meanwhile, Vietnamese melon fruit varies from oblong to elongated; Lao and Yunnan melons are oblong. However, there is the lack of information about the genetic resources of melon in Myanmar and Vietnam both of which are geographically close to Cambodia. Basically, South Asia and East Asia is connected through Cambodia.

Therefore, the aim of the present study was to evaluate morphological traits and the genetic diversity of melon landraces collected from Cambodia at the molecular level, by using RAPD and SSR markers and to uncover their relationship with melon in neighboring regions. At the same time, I tried to develop interspecific hybrids by crossing *Cucumis melo* with its related wild species that can be used for genetic improvement of melon.

Chapter 2

Morphological trait evaluation of Cambodian melon landraces

2.1 Introduction

Melon (*Cucumis melo* L.) which belongs to genus *Cucumis* is one of the most important horticultural crops and grown worldwide to be consumed as dessert and vegetable. However, intensive cultivation of modern melon cultivars with narrow genetic basis indirectly caused explosive increase of disease and pest damages, and thus the breeding of resistant cultivars is an urgent task. One of the solutions is to explore melon germplasm from Asia and Africa. *Cucumis melo* L. is the most variable species in the genus *Cucumis*. According to Boualem et al. (2008) cultivated melon predominantly belong to andromonoecious (bisexual flowers and male flowers in the same plant) or monoecious (male flower and female flower in the same plant) sex type. Gynoecious (bear only female flower) and hermaphrodite (bear only bisexual flowers) more two types of sex expression also distinguished in melon plants. Monforte et al. (2014) reported that fruit morphology, the size varies from very small (less than 100 g) to very large (more than 4 kg, up to 10 kg), and fruit shape varies from slightly flat, ellipsoid, obovoid, round, and long to extremely long. Other fruit traits such as rind color, flesh content and color, flesh thickness, flesh texture, flesh firmness, sweetness, sourness, aromatic compounds also show highly polymorphic. Based on the morphology, Pitrat et al., (2008) divided C. *melo* into two ssp. *agrestis* with 5 groups (var. *conomon*, var. *makuwa*, var. *chinensis*, var. *momordica* and var. *acidulus*) and ssp. *melo* with 10 groups (var. *chate*, var. *flexuosus*, var. *tibish*, var. *adana*, var. *ameri*, var. *cantalupensis*, var. *chandalak*, var. *reticulatus*, var. *inodorus*, var. *dudiam*). The differentiation in seed size is one of the taxonomy keys showed the correspondence with the difference in geographical distribution. Large seed type (seed length more than or equal 9 mm) is mainly distributed in the north,

west and central of Asia; north, central and west of India and western of China with typical groups including Dudaim, Flexuosus, Chandalak, Ameri and Inodorus; whereas, the small seed type (seed length less than 9mm) is distributed in the south and east of India, Southeast Asia and Far-east Asia with typical groups consisting of Momordica, Chinensis, Conomon, Makuwa and Acidulus (Stepansky et al., 1999; Akashi et al., 2002, Yashiro et al., 2005, Tanaka et al., 2007, Pitrat et al., 2008; Tanaka et al., 2013).

Morphological characterization is the most frequently used method for primary evaluation of genetic diversity of melon. When molecular markers were introduced as a convenient tool to assess the genetic diversity of a population, the molecular marker becomes more popular. Stepansky et al. (1999) and Jung et al. (2020) reported that due to phenotypic variation of melon, until now, the molecular markers could not explain all case of genetic diversity and remain different in genetic diversity estimated by two approaches. For the analysis of melon diversity still now morphological characters are widely used to supporting information in conjunction with molecular marker (Jung et al. 2020). International Plant Genetic Resources Institute, Rome, Italy (IPGRI 2003) documented well in variations of melon morphological features, distribution patterns, and adaptive and agronomic characters. Costa et al. (2016) said that geographically distinct populations can differ in their levels of genetic diversity or in the spatial distribution of that diversity. The study of diversity among different varieties from different geographical regions expand the relationships among the cultivated melon for further conservation and breeding program.

In Asia, India is rich in genetic diversity of melon as well as cucumber and considered as the primary center of cultivated melon (Gonzalo et al. 2019). Seed length is also diverse and both large-seed type (≥ 9.0 mm) and small-seed type (< 9.0 mm) are grown frequently (Akashi et al. 2002). In contrast, East Asian melon classified as two varieties conomon and makuwa is of small-seed type and was suggested to be originated from Indian small-seed type

melon (Tanaka et al. 2007). However, the detail was not known because of the unavailability of melon landraces in Southeast Asian countries which locate between India and East Asia. Thereafter, melon landraces have been successfully collected from Southeast Asian countries by several times of field expeditions, and genetic diversity and genetic structure were uncovered for melon landraces from Myanmar and Vietnam. Melon from Myanmar and mountainous areas of Vietnam proved to relate closely with Indian small-seed type melon, while melon from plain areas of Vietnam was classified as two varieties conomon and makuwa (Nhi et al.2010 and Duong et al. 2021). These results obtained by previous studies revealed the presence of varieties conomon and makuwa in Vietnam as well as China, Korea, and Japan, and suggested the possibility that varieties conomon and makuwa had been diversified and established in/around Vietnam.

Cambodia has a border with Vietnam and a long history of cultural interchange including crop seeds. However, genetic resources of Cambodian melon landraces were not available at all outside Cambodia. The Japanese expedition team including Okayama University started field research in Cambodia from 2014, collaborating with the Cambodian Agricultural Research and Development Institute (CARDI), and successfully introduced melon seeds into Japan with the Standard Material Transfer Agreement (SMTA). the field survey was conducted in four regions to cover whole area of Cambodia (West in 2014, East in 2015, Center and North in 2016, and South in 2017). Therefore, the aim of the study was to analyze the morphological traits of the plant, fruit, seed, and inflorescence of Cambodian melon landraces collected from four regions of Cambodia.

2.2 Materials and methods

Plant materials

60 accessions of cultivated melon and two accessions of weedy melon were selected and analyzed in this study from the field survey was conducted in four regions to cover whole area of Cambodia (West in 2014, East in 2015, Center and North in 2016, and South in 2017) (Fig 2.1). The experiment consists of 62 (Table 2.2) samples of Cambodian melon along with two Japanese cultivars were grown and evaluated in the open field of Cambodian Agricultural Research and Development Institute (CARDI) and standard cultural practice was applied to protect germplasm in the growing season. For each accession, five plants were grown and a total of 37 horticultural traits were evaluated (data not shown). Artificial pollination (self-pollination) was done for all the samples to get the fruit for seeds and observation. Some of them were failed to get fruit setting, in this case, open pollinated fruits were kept for evaluation. Japanese cultivar, Kinpyu produced 5 fruits while Harukei 3 Gou did not give any mature fruits. Those two Japanese cultivars performed well at vegetative stage, but abnormal and poor growth was observed affected by Aphid. The performance of Harukei 3 Gou was poor than Kinpyu cultivar which were seriously damaged by fruit fly since the young fruit stage, though it produced some fruits.

Morphological characterization

The trial was conducted in the open field of Cambodian Agricultural Research and Development Institute (CARDI) during 2014-2017 and standard cultural practice was applied to protect germplasm in the growing season. For each accession, five plants were grown and data were recorded from the germplasm based on 14 characteristics, namely plant type, internode length, leaf blade size length, sex of flower, female flower bearing ratio, fruit shape, ground color of the skin, warts of the fruit, strength of attachment peduncle at maturity, fruit grooves, crease of fruit surface, density of cork formation of fruit, fruit weight and seed length. In addition, 12 characteristics were also evaluated. Morphological trait evaluation of Cambodian melon landraces was carried out using different selected descriptors for plant type,

inflorescence, fruit, and seed mainly established by International Plant Genetic Resources Institute (IPGRI) and European Cooperative Program for Plant Genetic Resources (ECPGR) (Table 2.1). Seed length of melon generally differed between groups and seed length was a good character for a rough classification into each group of *C. melo* (Akashi et al, 2002). Most of Cambodian melon have not been systematically classified into varieties, they were classified into large-seed type (≥ 9.0 mm) and small-seed type (< 9.0 mm) based on seed length (Table 2.2).

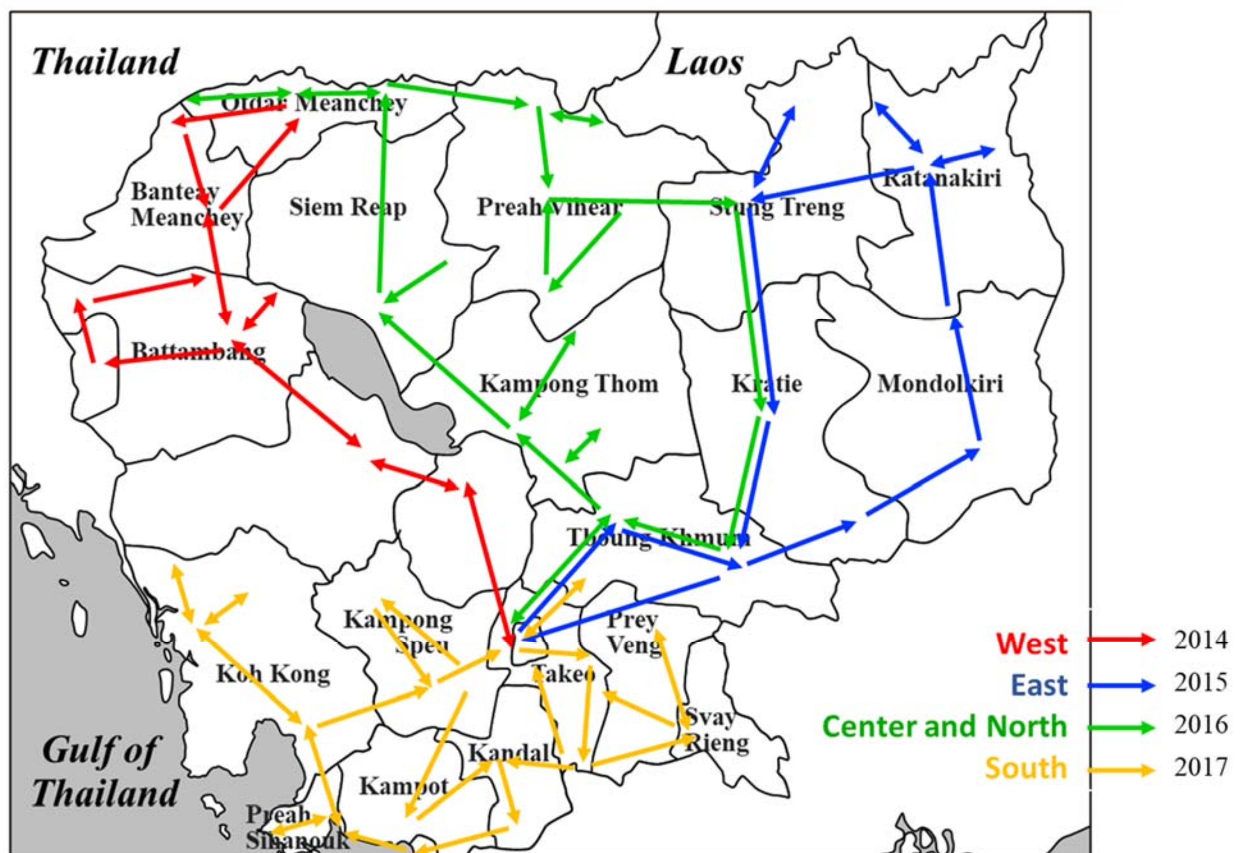


Fig. 2.1 Route map of Cambodia where melon landraces were collected

Statistical analyses

Data of different morphological characters were subjected to ANOVA, principal component analysis (PCA) and clustering analysis using R software (R Development Core Team 2012). We compared the seed lengths of the accessions from four geographical regions in Cambodia by using one way ANOVA test. ANOVA were carried out to determine the relative variance among regions and among melon types for each trait. PCA was performed to identify landraces groups and to determine the axes and the characters significantly contributing to the variation. In this procedure, the similarity matrix was used to generate eigenvalues and scores for the accessions. The first two principal components, which accounted for the highest variation, were then used to plot two-dimensional scatter plots. The Gower's algorithm was used to determine the distance matrix of quantitative and qualitative descriptors (Gower 1971). From the dissimilarity matrix, the clustering of accessions was performed by average linkage method of *hclust* function in R software (R Development Core Team 2012).

Table 2.1 Plant, inflorescence, fruit, and seed characteristic used for characterization Cambodian melon landraces.

Descriptor	State/Unit
Plant type	2- Self topping, 3-Bus, 4-Intermediate, 5-Normal
Sex of flower	1-Adroecious, 2-Monoecious, 3-Trimonoecious, 4-Andromonoecious, 5-Gynomonoecious, 6-Gynoecious, 7-Hermaphroditic
Internode length	Cm
Leaf blade length	cm
Leaf blade width	cm
Fruit shape	1-Ovate, 2- Medium elliptic, 3-Broad elliptic, 4-Circular, 5-Quadrangular, 6-Oblate, 7-Obviate, 8-Elongated
Ground color of the skin	1-white, 2-yellow, 3-Green, 4-Gray
Fruit groove	0- Absent, 2-Very weakly express, 3-Strongly express
Fruit length	cm
Fruit width	cm
Fruit shape index	Fruit length/ Fruit width
Fruit weight	kg
Brix	Degree
Flesh Thickness	cm
Flesh Texture	3-Mealy, 5-Fragile, 7-None-mealy, 9-Melting
Flesh Firmness	1-Extremely soft, 2-Very soft, 3-soft, 4-slightly soft, 5-Intermediat, 6-Slightly firm, 7-Firm, 8-Very firm, 9-Extremely firm
Seed length	cm
Seed width	cm

Fig. 2.2. Photographs of Cambodian melon landraces collected from five regions of Cambodia.

2.3 Results

Cambodia has a border with Vietnam and a long history of cultural interchange including crop seeds. However, genetic resources of Cambodian melon landraces were not available at all outside Cambodia. The Japanese expedition team including Okayama University started field research in Cambodia from 2014, collaborating with the Cambodian Agricultural Research and Development Institute (CARDI), and successfully introduced melon seeds into Japan with the Standard Material Transfer Agreement (SMTA). Field survey was conducted in four regions to cover whole area of Cambodia (West in 2014, East in 2015, Center and North in 2016, and South in 2017). From the samples collected, 60 accessions of cultivated melon and two accessions of weedy melon were selected and analyzed in this study (Table 2.2). Among them the morphological characteristics of the plant, inflorescence, fruit, and seed measured in 45 accessions were presented in Table 2.3 and summarized in table 2.4 (average performance ANOVA). Photographs of typical fruits are shown for each region in Figure 2.2. All the Cambodian melon landraces had normal plant type. Internode length, leaf blade length and leaf blade width ranges from 25.6cm-41.2cm, 10.3cm-18.4cm and 11.0cm-20.1cm respectively. Variation observed in fruit and seed characters in case of fruit traits. Fruit shape and fruit groove ranges from 2-8 (Medium elliptic to Elongated), 0-2 (very weakly express) respectively. Ground color of the skin ranges from white to gray. Fruit length, fruit width and fruit shape index ranges from 3.9 to 43cm, 3.5 to 17.3cm and 1.0-3.8 respectively. Fruit weight ranged from 0.43 to 2.613 kg among accessions. The Brix degree ranged from 3.4 to 8.8. The highest value was observed in accession number CAs65. Flesh thickness, flesh texture and flesh firmness varied from 0.6 to 3.4 cm, 3.0 to 9.0 cm and 2-6 (Very soft to Slightly firm) respectively. All the landraces had smooth skin and were classified as small-seed type (shorter than 9mm in length). In seed size, seed length showed variation ranges from 4.6 to 8.7 mm and seed width were ranges from 2.1-5.9mm. In general,

Cambodian melon landraces show monoecious type of sex expression and Brix value below 6.0, have oblong shape of fruits with smooth skin and seeds shorter than 9 mm in length (small-seed type). Melon landraces showing these traits were reported in Myanmar and mountainous areas of Vietnam ("Dua thom" and "Montok"), showing the distribution of similar type of melon in these areas. Geographical variation of horticultural traits was also found in Cambodia. For example, fruit height and seed length were shorter in landraces from eastern region compared to those from western region (Table 2.4), and the difference was statistically significant ($p<0.01$). Similarly, fruit weight was also different ($p<0.05$). These results might indicate the selection by local farmers. Although fruit morphology of Cambodian melon was similar with that of varieties *conomon* and *makuwa*, sex expression type was different.

Table 2.4. The average performance of four regions of Cambodian melon landraces.

Name of the region	Number of accessions	Fruit length (cm)	Fruit weight (kg)	Seed length (cm)	Sex expression
West	6	33.28a	1.727a	8.31a	M
East	13	25.75b	1.315b	7.71b	M
North	11	29.97ab	1.695b	7.87b	M
South	11	26.55ab	1.227b	7.66b	M

Mean value with the same letter indicate non – significant differences at 0.01 level (fruit length and seed length) and 0.05 level (fruit weight) by One-way ANOVA test.
Sex expression is indicated by: M- Monoecious

Principal component analysis (PCA) and a dendrogram were performed to determine relationships among populations collected from different regions and to obtain information on the usefulness of those characters for the description of cultivars based on morphological data. The principal component analysis revealed that internode length, leaf blade length, leaf blade width, fruit shape, ground color of the skin, fruit grooves, fruit length, fruit width, fruit

shape index, fruit weight, flesh sugar content by brix, flesh thickness, flesh texture, flesh firmness, seed length; and seed width were varied. Two components PCO1(41.71%) and PCO2 (11.24%) explained up to 52.95% of total variation (Fig 2.3). The second principal axis (PCO2) divided sixteen morphological characters into two parts. PCO1 was related to internode length, leaf blade length, leaf blade width, fruit shape, ground color of the skin, fruit grooves, fruit length, fruit width, fruit shape index, fruit weight, flesh thickness, flesh texture, seed length; and seed width, although PCO2 was related to flesh firmness and brix. About 62 accessions, 43 accessions collected from four regions were used for PCA analysis, using 16 morphological characters, they varied in morphological traits, although no obvious differences were detected among the four

geographical areas by PCO1 and PCO2 (Fig. 2.4).

The generated UPGMA dendrogram has discriminated the collected accessions into four main clusters (cluster I, II, III and IV) (Fig. 2.5). Cluster I, II, III comprised of 18, 16 and 9 accessions respectively from cultivated melon. The cluster IV contained two accessions from southern part of Cambodia (CA61 and CA65) which are weedy melons with seed length arranging from 4.6 mm to 4.7 mm (Table 2.2). Fruit varies from Obviate to elongated in case of cluster I while Medium elliptic to elongated in case of cluster II and III. Cluster IV comprised only circular shape of fruit. Brix value is below 6 incase cluster I, II, and III but higher than 7 in cluster IV. Average seed length of cluster I, II, and III 8.08, 7.78, and 7.44 respectively.

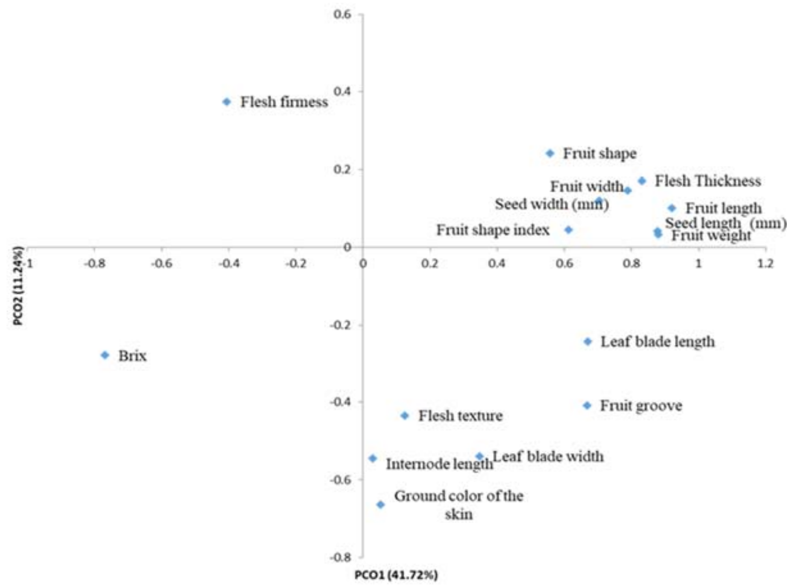


Fig. 2.3 Distribution on the first two principal coordinates of 43 melon accession from four regions in Cambodia. Principal component score of each accession was calculated in PCO analysis by 16 morphological characters.

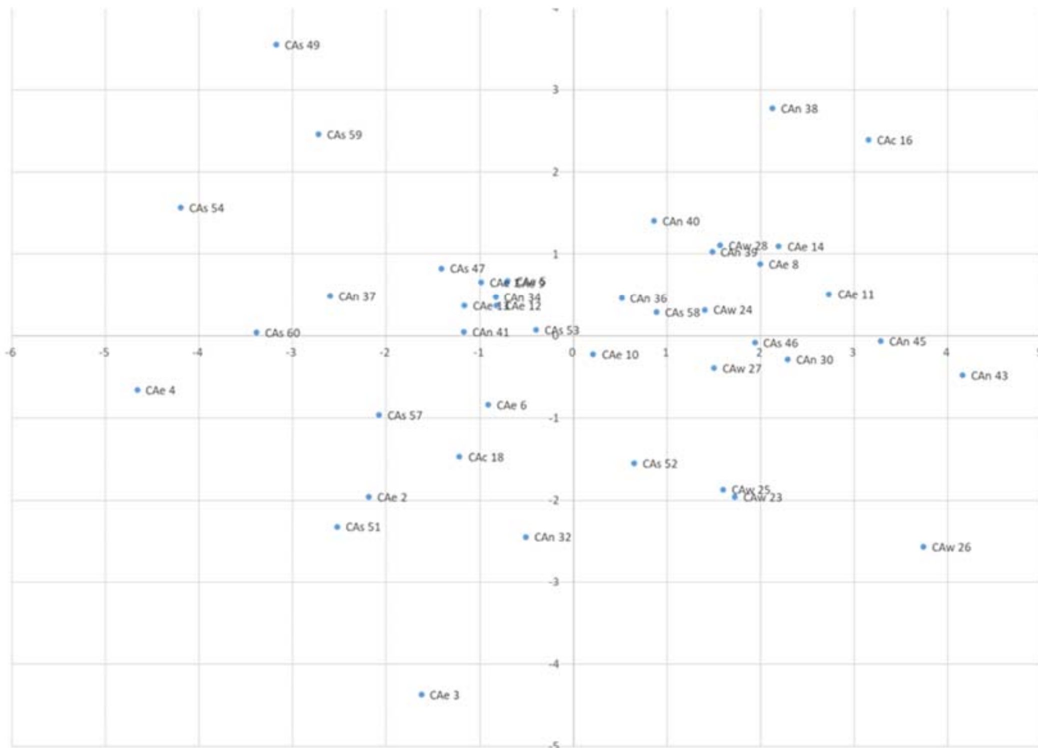


Fig. 2.4. Distribution on the first two principal coordinates of 43 fruits of cultivated melon from four regions in Cambodia. Principal component score of each accession was calculated in PCO analysis by using 16 morphological characters

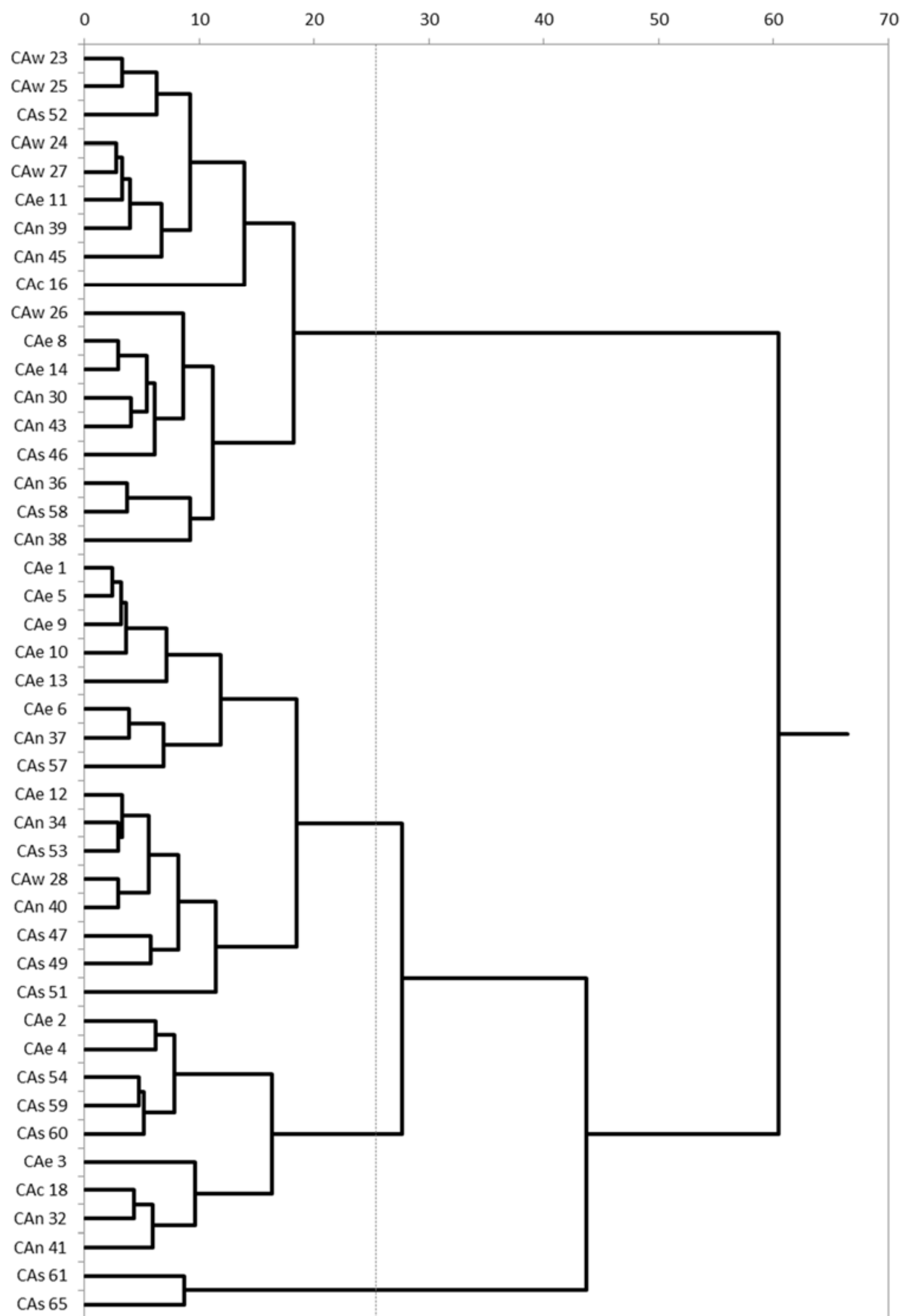


Fig. 2.5. Cluster analysis based on morphological data of Cambodian melon landraces

2.4 Discussion

Melon germplasm collection in Cambodia was conducted for four years (2014-2017), each year was implemented in each region (west region - 2014, east region - 2015, center and north region - 2016 and south region – 2017). 60 accessions of cultivated melon and two accessions of weedy melon were selected and analyzed in this study. In general, Cambodian melon landraces show monoecious type of sex expression and Brix value below 6.0, have oblong shape of fruits with smooth skin and seeds shorter than 9 mm in length (small-seed type). Melon landraces showing these traits were reported in Myanmar (Yi et al. 2009) and mountainous areas of Vietnam ("Dua thom" and "Montok") (Duong et al. 2021), showing the distribution of similar type of melon in these areas. Geographical variation of horticultural traits was also found in Cambodia. For example, fruit height and seed length were shorter in landraces from eastern region compared to those from western region, and the difference was statistically significant ($p<0.01$) (Table 2.4). Similarly, fruit weight was also different ($p<0.05$). These results might indicate the selection by local farmers. Although fruit morphology of Cambodian melon was similar with that of varieties *conomon* and *makuwa*, but sex expression type was different. According to Pitrat (2016) sex expression is the key character to distinguish Conomon, Makuwa, and Chinesis Groups with Mormodica and Acidulus Groups in eastern Asia. Conomon, Makuwa, and Chinesis Groups are showing andromonoecious while Mormodica and Acidulus Groups are monoecious. Different type of sex expression also reported between Chinese thin-skinned melon and vegetable melon types (Luan, Delannay, and Staub 2008). The dendrogram using genetic similarities (Jaccard's coefficient) revealed by RAPD markers indicated that both Chinese thin-skinned melons had genetic affinities with Indian accession, while they were most distant from Chinese net or non-netted thick skin (Luan, Delannay, and Staub (2008).

Cambodian melon landraces had similar fruit traits and sugar concentration from other countries. Tanaka et al. (2016) suggested that melon landraces collected from eastern Cambodia were grouped as either elongated with non-sweet flesh or globular with sweet flesh which is similar to the pattern of the East Asian *C. melo* var. *conomon* and var. *makuwa,* respectively (Kitamura, 1950). Similar type of fruit traits also found in our study. According to Tadaka (1979, 1983), *C. melo* var. *conomon* and var. *makuwa* both posses' genes that confer resistance to Fusarium wilt, gummy stem blight, and cucumber mosaic virus. By observing, the fruit traits of Cambodian melon indicated that Cambodian melon represents an important source of disease resistance genes and can be utilized as breeding materials.

The study of Yi et al. (2009) showed that Myanmar melons mainly consisted of three groups (Momordica – large seed type and Conomon and Agrestis – small seed type) and the genetic diversity of Indian melon was conserved in Myanmar. Meanwhile, all Vietnamese melons (Nhi et al. 2010 and Duong et al. 2021), were clustered into small seed type and had close genetic relationship with Conomon group except for one group collected from northern west of Vietnam called "Dua thom" which shared the same gene pool with small seed type from Myanmar, Bangladesh, and east India. The field survey (Tanaka et al., 2016 and 2017) showed they shared fruit traits from Momordica group and Conomon group. Cambodian melons featured by elongated fruit shape and wide variation in seed length. Whereas Yunnan and Laos melons had oblong fruits and Vietnamese melons varied from oblong to elongated shape. In consequence, Cambodian melons might be contained genetic component different from the neighbor countries. In this study, the cluster analysis showed that all Cambodian melons classified into four clusters among them cultivated melon belongs to cluster I, II, III and weedy melons in cluster IV. Therefore, comparative analysis of genetic variability of Cambodian melon and melon germplasm of diverse origins is needed to clarify genetic diversity of Cambodian melon and their genetic relationship from neighboring countries.

More specifically, genetic analysis of Cambodian landraces is indispensable to understand the distribution of groups Conomon and Makuwa and genetic relationship with Vietnamese landraces.

Table 2.2 List of Cambodian melon landraces analyzed in this study

Accession No.	Collection No.	Province	Region	Year of collection	Seed size
CAe 1	15CJV-C83-1	Ratanakiri	East	2015	Small
CAe 2	15CJV-C100	Ratanakiri	East	2015	Small
CAe 3	15CJV-C94-1	Ratanakiri	East	2015	Small
CAe 4	15CJV-C94-2	Ratanakiri	East	2015	Small
CAe 5	15CJV-C76	Mondolkiri	East	2015	Small
CAe 6	15CJV-C40-1	Mondolkiri	East	2015	Large
CAe 7	15CJV-C40-2	Mondolkiri	East	2015	Small
CAe 8	15CJV-C19	Mondolkiri	East	2015	Small
CAe 9	15CJV-C132-1	Stung Treng	East	2015	Small
CAe 10	15CJV-C132-2	Stung Treng	East	2015	Small
CAe 11	15CJV-C138	Stung Treng	East	2015	Small
CAe 12	15CJV-C139	Stung Treng	East	2015	Small
CAe 13	16CJVC-83	Stung Treng	East	2016	Small
CAe 14	16CJVC-107	Kratie	East	2016	Small
CAe 15	16CJVC-107	Kratie	East	2016	Small
CAc 16	16CJVC-19	Kampong Thom	Center	2016	Small
CAc 17	16CJVC-19	Kampong Thom	Center	2016	Small
CAc 18	16CJVC-11-1	Kampong Thom	Center	2016	Small
CAc 19	16CJVC-11-2	Kampong Thom	Center	2016	Small
CAc 20	15CJV-C9	Tboung Khmum	Center	2015	Small
CAw 23	58	Battambang	West	2014	Small
CAw 24	65	Battambang	West	2014	Small
CAw 25	68	Battambang	West	2014	Large
CAw 26	68	Battambang	West	2014	Small
CAw 27	78	Banteay Meanchey	West	2014	Small
CAw 28	16CJVC-52	Banteay Meanchey	West	2016	Small
CAw 29	16CJVC-52	Banteay Meanchey	West	2016	Small
CAn 30	16CJVC-72	Preah Vihear	North	2016	Small
CAn 31	16CJVC-72	Preah Vihear	North	2016	Small
CAn 32	16CJVC-74	Preah Vihear	North	2016	Large
CAn 33	16CJVC-74	Preah Vihear	North	2016	Small
CAn 34	16CJVC-80	Preah Vihear	North	2016	Small
CAn 35	16CJVC-80	Preah Vihear	North	2016	Small
CAn 36	16CJVC-100	Preah Vihear	North	2016	Large
CAn 37	16CJVC-100	Preah Vihear	North	2016	Small
CAn 38	16JCVC-16	Siem Reap	North	2016	Small
CAn 39	16JCVC-23	Siem Reap	North	2016	Small
CAn 40	16JCVC-37	Siem Reap	North	2016	Small
CAn 41	16JCVC-60	Siem Reap	North	2016	Small
CAn 42	16JCVC-60	Siem Reap	North	2016	Small
CAn 43	16CJVC-46	Oddar Meancheay	North	2016	Large
CAn 44	16JCVC-46	Oddar Meancheay	North	2016	Small
CAn 45	16CJVC-63	Oddar Meancheay	North	2016	Small
CAs 46	17CJVC-4	Svay Rieng	South	2017	Small

CAs 47	17CJVC-11	Svay Rieng	South	2017	Small
CAs 48	17CJVC-19-1	Prey Veng	South	2017	Small
CAs 49	17CJVC-19-2	Prey Veng	South	2017	Small
CAs 51	17CJVC-28	Takeo	South	2017	Small
CAs 52	17CJVC-48-1	Takeo	South	2017	Small
CAs 53	17CJVC-48-2	Takeo	South	2017	Small
CAs 54	17CJVC-87-1	Kompong Speu	South	2017	Large
CAs 55	17CJVC-87-2	Kompong Speu	South	2017	Small
CAs 56	17CJVC-87-3	Kompong Speu	South	2017	Small
CAs 57	17CJVC-92	Kompong Speu	South	2017	Small
CAs 58	17CJVC-52	Kampot	South	2017	Small
CAs 59	17CJVC-71	Koh Kong	South	2017	Small
CAs 60	17CJVC-64	Preah Sihanok	South	2017	Small
CAs 61	17CJVC-98	Kandal	South	2017	Small
CAs 62	17CJVC-101	Prey Veng	South	2017	Small
CAs 63	17CJVC-117	Kandal	South	2017	Small
CAs 64	17CJVC-121	Kandal	South	2017	Small
CAs 65	17CJVC-126	Kandal	South	2017	Small

Table 2.3 Morphological characteristics of 45 melon landraces collected from four regions of Cambodia

Accession No.	Cluster order traits	Internode length (cm)	Leaf blade length (cm)	Leaf blade width (cm)	Fruit shape	Ground color of the skin	Fruit groove	Fruit length (cm)	Fruit width (cm)	Fruit shape index	Fruit weight (Kg)	Brix (degree)	Flesh Thickness (cm)	Flesh texture	Flesh firmness	Seed length (mm)	Seed width (mm)
CAw 23	1	31.7	14.7	15.6	7.3	1.8	1.7	31.6	14.7	2.1	1.667	4.4	3.0	7.0	2.8	8.7	3.5
CAw 25	2	33.6	15.1	16.4	7.8	2.6	2.0	31.2	15.0	2.1	1.608	5.4	3.0	9.0	2.8	8.1	3.7
CAs 52	3	31.4	12.5	13.3	8.0	1.8	1.2	31.1	12.7	2.4	1.626	3.5	3.3	6.2	4.8	8.0	3.6
CAw 24	4	31.9	15.5	16.2	7.6	3.2	2.0	35.6	13.0	2.7	1.680	5.1	2.6	7.0	3.0	8.2	3.5
CAw 27	5	31.4	16.1	16.8	7.8	1.8	2.0	33.6	13.2	2.5	1.480	4.6	2.9	7.0	3.4	8.2	3.5
CAe 11	6	33.0	16.4	16.1	8.0	3.0	1.7	33.8	12.2	2.8	2.043	3.3	3.0	7.8	2.8	8.3	3.7
CAn 39	7	32.1	16.5	17.6	8.0	2.0	1.8	33.6	11.8	2.8	1.800	3.9	2.9	6.0	5.3	8.1	3.4
CAn 45	8	29.4	15.8	16.0	8.0	2.3	1.7	37.5	12.5	3.0	2.213	3.8	3.0	8.7	3.7	7.5	5.9
CAc 16	9	30.3	17.7	17.5	8.0	2.8	2.0	43.0	11.4	3.8	2.127	4.1	2.7	8.7	2.7	8.2	3.5
CAw 26	10	34.9	16.0	16.6	7.8	2.5	2.0	37.6	17.3	2.2	2.388	5.3	3.4	8.5	2.0	8.3	3.8
CAe 8	11	37.9	18.4	19.1	7.8	2.0	1.0	34.0	11.3	3.0	1.673	4.1	3.0	8.0	3.0	8.2	3.5
CAe 14	12	38.8	16.9	18.3	8.0	2.0	2.0	35.2	11.8	3.0	1.950	4.2	2.7	7.0	4.0	7.9	3.5
CAn 30	13	36.7	16.1	16.5	8.0	2.8	1.5	35.9	13.5	2.7	2.368	4.8	3.1	6.0	4.3	8.0	3.5
CAn 43	14	37.0	16.7	17.4	8.0	2.0	2.0	36.5	13.8	2.7	2.613	3.5	3.0	9.0	2.3	8.4	3.8
CAs 46	15	38.0	18.0	19.1	8.0	2.0	1.3	32.8	14.7	2.2	1.740	4.1	3.0	8.3	6.3	7.8	3.8
CAn 36	16	35.3	16.6	17.6	8.0	1.0	1.0	33.2	11.6	2.9	1.740	4.0	2.9	3.0	4.0	7.6	3.2
CAs 58	17	34.2	15.5	17.4	8.0	2.5	1.0	35.5	11.4	3.1	2.300	5.5	3.1	3.0	3.0	7.9	3.1
CAn 38	18	41.2	18.4	19.6	8.0	3.0	2.0	31.8	10.4	3.1	1.574	3.5	2.6	3.8	3.8	8.2	3.4
CAe 1	19	34.3	15.1	16.9	7.6	2.8	1.4	25.6	10.6	2.4	0.989	4.8	2.3	7.0	3.6	7.9	3.3
CAe 5	20	33.7	15.5	17.0	8.0	2.0	1.2	24.5	10.0	2.4	0.975	3.7	2.3	8.2	2.8	7.9	3.4
CAe 9	21	32.1	15.9	17.4	7.8	3.0	1.8	24.3	10.4	2.3	0.994	4.9	2.4	9.0	3.0	7.6	3.5
CAe 10	22	34.0	15.0	16.3	7.8	3.6	1.8	26.6	11.0	2.4	1.312	5.1	2.9	9.0	2.2	7.6	3.5
CAe 13	23	31.5	17.9	19.0	7.5	1.5	1.0	24.4	11.1	2.2	1.250	4.7	2.8	5.0	4.5	7.4	3.2
CAe 6	24	33.6	15.6	20.1	2.3	2.5	1.0	24.8	10.5	2.4	1.575	5.8	2.6	8.5	4.3	8.5	3.6
CAn 37	25	35.0	13.7	19.0	3.5	2.3	1.0	24.4	9.1	2.7	1.100	5.0	2.2	7.0	4.8	7.6	3.2
CAs 57	26	30.8	13.6	19.5	2.0	2.0	1.0	27.3	10.3	2.6	1.433	5.7	2.7	4.3	3.3	7.6	3.5
CAe 12	27	30.1	14.9	15.5	8.0	2.2	1.0	29.6	11.1	2.7	1.392	4.5	2.5	4.6	3.4	7.6	3.4
CAn 34	28	31.2	15.3	15.8	8.0	2.0	1.0	28.1	10.8	2.6	1.328	4.2	2.8	6.0	5.3	7.5	3.5
CAs 53	29	29.0	14.8	15.5	7.7	1.7	0.7	29.0	11.2	2.6	1.354	4.1	2.4	7.0	5.0	8.5	3.8
CAw 28	30	29.6	16.1	16.7	8.0	3.0	2.0	30.1	11.7	2.6	1.545	3.4	2.7	8.5	4.5	8.4	3.6
CAn 40	31	29.8	17.0	17.7	8.0	3.0	2.0	28.3	11.1	2.5	1.494	4.1	2.5	7.4	3.2	8.1	3.5
CAs 47	32	25.8	16.1	15.7	8.0	2.0	1.0	29.3	11.0	2.7	1.223	4.7	2.5	4.5	4.0	7.5	3.3
CAs 49	33	27.3	14.0	13.9	8.0	2.7	1.7	27.5	7.3	3.8	0.574	4.8	1.6	5.7	5.0	7.1	3.2
CAs 51	34	25.6	13.8	14.0	7.0	1.8	0.4	22.9	13.6	1.7	1.164	5.4	2.6	3.4	3.8	7.8	3.5
CAe 2	35	30.1	13.7	15.5	6.2	2.2	1.2	20.9	11.9	1.8	1.190	5.4	2.8	8.0	3.5	7.3	3.4
CAe 4	36	26.5	14.5	15.9	7.0	2.0	1.0	17.0	9.1	1.9	0.705	6.0	2.4	8.3	3.3	6.5	2.8
CAs 54	37	31.2	15.3	17.0	7.8	3.3	0.8	16.6	7.6	2.2	0.575	5.5	1.8	7.0	4.0	7.2	3.1
CAs 59	38	29.6	17.1	17.9	7.7	3.0	1.0	20.1	7.7	2.6	0.567	4.7	1.9	5.7	3.0	7.5	3.4
CAs 60	39	32.0	17.3	18.1	7.0	2.0	0.0	20.0	10.3	2.0	0.950	6.0	2.2	7.0	5.0	7.4	3.3
CAe 3	40	37.6	13.9	15.6	5.6	2.0	1.0	14.2	13.6	1.0	1.058	5.4	3.3	8.6	2.6	7.5	3.4
CAc 18	41	36.0	14.3	16.0	7.0	2.0	1.2	20.8	11.0	1.9	1.010	4.9	2.6	7.0	2.8	8.1	3.4
CAn 32	42	36.8	16.2	17.3	6.0	2.0	1.0	19.7	14.0	1.4	1.355	4.7	3.1	6.0	3.5	7.8	3.5
CAn 41	43	36.5	17.5	18.2	7.0	2.0	2.0	20.6	10.9	1.9	1.070	5.8	2.6	3.0	4.5	7.8	3.4
CAs 61	44	35.0	10.3	11.0	4.8	2.0	0.0	3.9	3.5	1.1	0.029	6.5	0.7	7.0	6.0	4.6	2.1
CAs 65	45	39.6	12.7	17.1	4.0	2.8	1.0	4.9	4.0	1.2	0.043	8.8	0.6	7.0	4.6	4.7	2.2

Chapter 3

Analysis of genetic diversity and population structure in Cambodian melon landraces using molecular markers

3.1 Introduction

Crop landrace possesses various traits which enabled adaptation to local conditions and farming practices and have been established through long period of natural and artificial selection. Some of these traits were inherited from their ancestral wild species, while others were acquired after domestication for adaptation to new environments encountered (Cortinovis et al. 2020). In case of wheat vernalization genes, for adaptation to warm conditions, cultivated wheat acquired spring alleles of *Vrn-A1*, *Vrn-B1*, and *Vrn-D4* after domestication and spread of wheat cultivation (Yan et al. 2004; Kippes et al. 2015). By combining these alleles, new cultivars with various types of adaptability can be developed. Therefore, local landraces are important genetic resources for crop breeding. Genetic diversity in landrace population also provides important evidence to uncover crop evolution history as already reported in many crop species (Pourkheirandish et al. 2015).

Reflecting long history of cultivation, various types of usage and adaptation to various parts of the world, melon (*Cucumis melo*) is known as most diversified among Cucurbitaceae crops, and great diversity is reported in morphological traits such as fruit weight and seed length which ranged from 50 g to 15 kg and from 4.5 mm to 15.0 mm, respectively (Nuñez-Palenius et al. 2008; Akashi et al. 2002). *C. melo* is divided into 19 horticultural groups based primarily on geographical origin, morphology, and horticultural traits: Agrestis, Kachri, Chito, Tibish, Acidulus, Momordica, Conomon, Makuwa, Chinensis, Flexuosus, Chate, Dudaim, Chandalak, Indicus, Ameri, Cassaba, Ibericus, Inodorus, and Cantalupensis (Pitrat 2016). Fujishita and

Nakagawa (1973) focused on seed size variation and indicated that melon is classified into large-seed type (≥9.0 mm) and small-seed type (<9.0 mm). Melon accessions of groups Cantalupensis and Inodorous are mostly classified as large-seed type and groups Agrestis, Conomon, and Makuwa as small-seed type according to Fujishita (1983) and Akashi *et al.* (2002). In Asia, groups Dudaim, Flexuosus, Chandalak, Ameri and Inodorus of large-seed type are mainly distributed in west and central Asia and India, whereas groups Chinensis, Conomon, Makuwa and Acidulus of small-seed type in southeast and far-east Asia and India (Stepansky *et al*. 1999; Akashi *et al*. 2002; Yashiro *et al*. 2005; Tanaka *et al*. 2007; Pitrat *et al*. 2008; Tanaka *et al*. 2013).

Among Asian melon, small- and large-seed types proved to be diversified in independent maternal lineages, Ia and Ib, respectively, by the analysis of chloroplast genome (Tanaka *et al*. 2013). India is considered as the secondary center of diversity (McCreight, 2004; Dhillon et al. 2007; Fergany et al. 2011), and both of small- and large-seed types and both of Ia and Ib types are frequently distributed. However, landraces of Conomon and Makuwa are not reported in India. Conomon and Makuwa are mostly distributed in areas from China to Japan and considered to be diversified in small-seed melon with Ia type cytoplasm in areas somewhere lying between India and China (Akashi *et al*. 2002; Tanaka *et al*. 2007; Serres-Giardi and Dogimont, 2012). In contrast to India and far-east Asia, little is known about melon landraces in southeast Asia. Myanmar melon landraces were first investigated by Yi et al. (2009) and proved to possess large genetic variation as like Indian melon. Myanmar melon consists both of small- and large-seed types, and they classified small-seed type accessions as Conomon, Momordica, or Agrestis. However, most of Myanmar Conomon accessions were clustered separately from Conomon and Makuwa of far-east Asia. Although one accession was regarded as Makuwa, they could not rule out the possibility of its recent introduction from other countries such as China and Japan. More

recently, Nhi et al. (2010) and Duong et al. (2021) analyzed Vietnamese melon landraces and showed that all except one accession were small-seed type. Vietnamese melon consisted of seven cultivar groups among which “Dua le” and “Dua vang” were regarded as Makuwa and “Dua bo” and “Dua gang-andromonoecious” as Conomon. In contrast, “Dua thom” and “Montok” showed genetic similarity with Indian and Maynmar landraces. Based on these results, the presence of groups Conomon and Makuwa was first confirmed in Vietnam. Therefore, to uncover the origin of groups Conomon and Makuwa, melon landraces of Cambodia, Laos, and Thailand should be investigated.

Recently, we have conducted germplasm collection expedition in Cambodia from 2014, and successfully introduced germplasms of melon landraces (Matsunaga et al. 2015; Tanaka et al. 2016; Tanaka et al. 2017; Tanaka et al. 2020). Genetic resources of Cambodian melon are not available in major Genebanks other than NARO Genebank, Japan, and are expected to contribute to breeding for disease resistance and to the analysis of diversification of melon in Asia. More specifically, genetic analysis of Cambodian landraces is indispensable to understand the distribution of groups Conomon and Makuwa and genetic relationship with Vietnamese landraces. Therefore, in this study, we aimed to uncover genetic diversity and genetic structure of Cambodian melon using RAPD and SSR markers and discussed genetic diversification in Asian melon.

3.2 Materials and methods

Plant Materials

Among Cambodian melon landraces collected during 2014-2017, 62 accessions were selected to cover most of the part of Cambodia and divided into five geographical groups, i.e., east, center, west, north, and south. The detail of materials is summarized in Table 3.1 and Fig. 3.1. These accessions have been introduced to NARO Genebank, Japan, with SMTA (Standard Material Transfer Agreement). A total of 229 accessions were used as reference, which included Conomon (9), Makuwa (11), Agrestis (6), Cantalupensis (10), Inodorus (8), and accessions from different areas (Vietnam: 58, Myanmar: 36, Thailand: 5, Yunnan (China): 5, Xinjiang (China): 24, Central Asia: 14, Pakistan: 11, Afghanistan: 9, Iran: 10, Spain: 8, the USA: 5). Two accessions of the wild cucumber *C. sativus* var. *hardwickii* were also used as the outgroup. Seed length and width were measured for 10 seeds of each accession, and accessions were classified as large (≥9.0 mm length) or small-seed (<9.0 mm length) types, according to Akashi *et al.* (2002).

3.2.2 Methods

DNA extraction

Seeds of each accession were germinated on wet filter paper in a Petri dish and later transferred into the pots. They were grown in an incubator maintained at 30ºC with a 16 h light-8 h dark cycle at a light intensity of 46.5 $\mu Ms^{-1}m^{-2}$. After two weeks, cotyledons from one seedling of each accession were ground individually in the liquid nitrogen. Total DNA was extracted by using the cetyl-trimethyl-ammonium bromide (CTAB) method (Murray and Thompson, 1980) with minor modifications. The quality and quantity of each DNA sample were determined with a spectrophotometer.

RAPD analysis

Ten random primers which were selected for their ability to detect polymorphism and for their stability in PCR amplification were used for RAPD analysis according to Nhi *et al*. (2010) (Table 3.2). PCR amplification was carried out in a 10 µl mixture containing 50 ng genomic DNA, 1 µl PCR buffer (Sigma®, St. Louis, MO, USA: 10 mM Tris-HCl; pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, 0.1 mM dNTP, 0.5 µM primer, and 0.25 U *Taq* polymerase (Sigma) by using iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR cycle was started with an initial denaturing step at 95°C for 3 min, 40 cycles at 93°C for 1 min, 40°C for 2 min, and 72°C for 2 min. Duration of annealing temperature at 40ºC and the duration of extension at 72ºC were modified to 45 seconds and 1.5 min, respectively, for three primers A31, A57 and B86. The final extension step was at 72°C for 5 min for all primers. After the amplification, samples were electrophoresed on a 1.5% agarose gel (GenePure LE, BM Bio, Tokyo, Japan) at a constant voltage of 100 V using a horizontal gel electrophoresis system (Mupid-2, Cosmo Bio, Tokyo, Japan). Then the PCR products were visualized with ethidium bromide staining and their polymorphism were evaluated.

SSR analysis

Seven SSR markers showing high polymorphism in Vietnamese landraces selected by Nhi *et al*. (2010) were used for the analysis of Cambodian melon landraces (Table 3.2). These SSR markers were developed by Ritschel et al. (2004) and Fukino et al. (2007). The PCR mixture was the same as RAPD analysis, but the primer concentration was changed to 0.25 µM each. The PCR cycle was started with an initial denaturing step at 95°C for 3 min, 35 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min. The final extension step was at 72°C for 5min. PCR

products were electrophoresed on 10% nondenatured polyacrylamide gel at a constant voltage of 260 V. Gels were stained in the same way as for RAPD analysis.

Genotyping of CmACS7

CmACS7 genotype was determined for sex expression analysis using the Cleaved Amplified Polymorphic Sequences (CAPS) marker developed by Boualem et al. (2008). The detail of PCR condition was given in Duong et al. (2021).

Data analysis

DNA fragments were scored as present (1) or absent (0) for RAPD markers. For SSR, DNA fragments were scored based on their size from the smallest (1) to the largest. From these data, the polymorphic information content (PIC) was calculated after Botstein et al. (1980). Genetic similarity (GS) was calculated after Apostol *et al.* (1993), using the formula GS = (N11 + N00)/T, where N11 and N00 was the number of positive and null bands, respectively, shared between two accessions and T was the total number of bands scored. The genetic distance (GD) between them was calculated by using the formula GD = 1 - GS. Gene diversity (D) within each group and genetic distance (GD) between each group were calculated according to Weir (1996) and Nei (1972), respectively. A dendrogram was constructed by PHYLIP version 3.5.c (Felsenstein, 1993), based on the GD matrix, with the unweighted pair group with arithmetic mean (UPGMA) method. Principal coordinate analysis (PCO; Gower 1966) based on the GS matrix was done to show multiple dimensions of each population in a scatter-plot.

The population structure and the degree of admixture were estimated using Bayesian clustering procedure of STRUCTURE 2.3.4 (Pritchard *et al*. 2000). First the program was run to assume the number of distinct populations defined as K using the admixture model with correlated allele frequencies. The parameter sets were configured as follow: the length of burn-in

period and the number of Markov chain Monte Carlo (MCMC) repetitions after burn-in were 10000, 50000, respectively, for each K ranging from 1 to 10 and running 20 iterations. The most plausible number of clusters was determined in the Structure Harvester program (Earl and vonHoldt, 2012) by calculating the distribution of the ΔK statistics as described by Evanno *et al*. (2005) and the performance of cluster matching across different *K* values was implemented through Cluster Markov Packager Across *K* (CLUMPAK) (Kopelman *et al*. 2015).

3.3 Results

Characterization of Cambodian melon landraces

Most of Cambodian melon (56/62) were small-seed type as like those of Yunnan (China), Myanmar, Vietnam, Conomon, Makuwa and Agrestis (Table 3.3). In contrast, large-seed type was common in areas from Xinjiang (China) to USA and also in Cantalupensis and Inodorus, while both of large- and small-seed types were frequent in areas from Central Asia to Iran.

Sex expression type determined by CAPS analysis of *CmACS7* also differed among melon populations (Table 3.3). All of the Cambodian melon accessions (62/62) proved to be monoecious. The monoecious type was frequent also in neighboring countries such as Yunnan (China), Myanmar, and Dua thom (Vietnam). In contrast, andromonoecious type was frequent in Conomon, Makuwa, Vietnam except Dua thom, in areas from from Xinjiang (China) to USA, and also in Cantalupensis and Inodorus. Both of monoecious and andromonoecious types were frequent in areas from Central Asia to Afghanistan.

RAPD and SSR analysis

Ten RAPD markers produced 12 polymorphic bands whose size ranged from 700 bp to 1520 bp, and the average number of marker bands per primer was 1.2 (Table 3.2). The average of PIC index was 0.297, being ranged from 0.047 (B32_700) to 0.452 (B99_1400). SSR analysis with

seven primer pairs generated 70 bands polymorphic in 291 accessions of melon and the average number of marker bands per primer was 10.0. The PIC index ranged from 0.664 (CMN04-07) to 0.891 (CMBR2) and was almost double compared with that of RAPD markers (Table 3.2).

Gene diversity (D) in Cambodian melon ranged from 0.105 (Center) to 0.246 (South) and was 0.228 in 62 accessions (Table 3.3). This value was smaller compared with those in neighboring countries such as Vietnam (0.333 in 61 accessions), Myanmar (0.428) and Thailand (0.328), and nearly equivalent to those of Conomon and Makuwa (0.227 and 0.168).

Genetic relationship between Cambodian melon and reference accessions

Genetic distance (GD) among 291 accessions ranged from 0.000 (CAn33 vs Can44 etc.) to 0.947 (P142 vs X8), while the maximum GD was 0.474 (CAs46 vs CAe9 etc.) in Cambodian accessions (data not shown). A dendrogram was constructed based on these GD values, and 291 accessions were grouped into three major clusters which were further separated into 25 sub clusters (Fig. 3.2). Cluster I comprised of 115 accessions which included landraces from Cambodia (59/62) and the neighboring countries such as Vietnam (Dua thom), Yunnan (China), Thailand, Myanmar (Table 3.4). Accessions of Conomon, Makuwa, and Agrestis formed cluster II (68 accessions), together with landraces from Vietnam except Dua thom. Cluster III (108 accessions) consisted of Cantalupensis, Inodorus, and accessions from Xinjiang (China) to USA. Landraces of Myanmar were classified into three clusters, in good accordance with the highest D value (0.428).

Structure analysis indicated the presence of three main populations in 291 accessions studied (Fig. 3.2), based on the ΔK value, and 61, 101, and 93 accessions were assigned to populations P1, P2, and P3, respectively, using a Q-value threshold of 70% (Table 3.4). The remaining 36 accessions were regarded as admixture type. The classifications of three clusters and three populations were highly associated, as 86.1%, 88.2%, and 85.2% of accessions in clusters I, II,

and III were assigned to P1, P2, and P3, respectively. Accessions of admixture type were mostly found in marginal subclusters such as If, IIg, IIIa. Accordingly, Cambodian accessions belonged to P2 together with those from Vietnam (Dua thom), Yunnan (China), Thailand, and Myanmar, and genetic relationship detected by cluster analysis was reproduced by structure analysis. Genetic relationship was also visualized on scatter plot of PCO1 (21.3%) and PCO2 (16.5%) (Fig. 3.3). Accessions of three populations were distinctly separated and admixture accessions located among three groups.

Three clusters were also characterized by seed length and sex expression type (Tables 3.3 and 3.4). Most of accessions of cluster I (111/115) and II (65/68) commonly had seeds shorter than 9 mm in length and were classified as small-seed type. In contrast, sex expression type differed between clusters I and II, and monoecious type and andromonoecious type were frequent in clusters I (108/115) and II (56/68), respectively. Cambodian melon proved to be small-seed type and monoecious type (56/62), as like those of Vietnam (Dua thom), Yunnan (China), and Myanmar, showing close similarity among these populations. Accessions of cluster III were mostly large-seed type (80/108) and andromonoecious type (87/108).

Genetic relationship between 24 melon populations

Genetic distance (GD) between 24 populations calculated from the RAPD and SSR data ranged from 0.018 (Cam-N vs Cam-S) to 0.984 (Makuwa vs China-X) (Table 5). GD among five populations of Cambodian melon ranged from 0.018 to 0.097 and was 0.062 on average. These values were nearly equivalent to those among three Vietnamese populations of Dua bo, Dua gang and Dua le (0.035 to 0.082), among Conomon and Makuwa (0.047) and among five populations from Xinjiang (China) to Iran (0.044 to 0.112), indicating that Cambodian melon was not genetically differentiated among geographical populations.

Genetic relationship between 24 populations was visualized by cluster analysis (Fig. 3.4) and by PCO analysis (Fig. 3.5). In accordance with the results of accession-based analysis, 24 populations were separated into three groups and Cambodian populations related closely to those in neighboring countries.

3.4 Discussion

Previous studies suggested that Conomon and Makuwa were originated from small-seed melon with Ia type cytoplasm in areas somewhere lying between India and China (Akashi *et al*. 2002; Tanaka *et al*. 2007; Serres-Giardi and Dogimont, 2012). Thereafter, we analyzed genetic structure of melon landraces from Myanmar (Yi et al. 2009) and Vietnam (Nhi et al. 2010; Duong et al. 2021). These studies indicated genetic differentiation in melon landraces in Southeast Asian countries and highlighted the importance of genetic studies using landraces from Cambodia and Laos. Recently, a melon germplasm collection was conducted in Cambodia during the period of 2014-2017, in western region (2014), eastern region (2015), central and northern regions (2016) and southern region (2017), by the joint expedition team of NARO (Japan) and CARDI (Cambodia). The geographical distribution, horticultural characteristics, and usage of melon landraces in Cambodia was first fully described by their reports (Matsunaga et al. 2015; Tanaka et al. 2016; Tanaka et al. 2017; Tanaka et al. 2020). Using part of this collection, in this study, we evaluated genetic diversity of Cambodian melon, using RAPD and SSR makers, and compared with those in neighboring countries to discuss the origin of Conomon and Makuwa.

India is rich in genetic diversity of melon and both large- and small-seed types are frequent (Akashi *et al*. 2002; Tanaka *et al*. 2007). In contrast, small-seed type is frequent in Myanmar

(36/41, Yi et al. 2009), Cambodia (56/62, Table 3.3), and Vietnam (61/61, Table 3.3), indicating the trend of increase of small-seed type from India to Vietnam. Genetic diversity also showed similar geographical cline. D was larger in India compared to Myanmar (Yi et al. 2009), while it was highest in Myanmar (0.428) followed by Vietnam (0.333) and Cambodia (0.228). However, among Vietnamese melon groups, Dua thom grown in the mountain areas is geographically and genetically differentiated from melon groups in plain areas, Dua bo, Dua gang and Dua le (Nhi et al. 2010; Duong et al. 2021), and D was 0.154 in Dua thom and 0.184 in population of plain areas. These results indicated the selection against large-seed type melon and decrease of genetic diversity from India towards east.

The similarity in fruit morphology (Matsunaga et al. 2015; Tanaka et al. 2016; Tanaka et al. 2017; Tanaka et al. 2020) and the results mentioned above might support the hypothesis that Conomon and Makuwa were originated from small-seed type melon somewhere in these areas. However, the results summarized in Table 3.4, Figs. 3.4 and 3.5 clearly demonstrated the presence of two groups of melon in southeast Asia. Conomon and Makuwa formed one group together with accessions from plain areas of Vietnam, and those from Myanmar to mountain areas of Vietnam formed another group. In addition, the sex expression type estimated by CAPS analysis of *CmACS7* was also different between two groups. The former group was characterized by monoecy, while the latter by andromonoecy. Therefore, it was concluded that Cambodian melon is not directly related with the establishment of Conomon and Makuwa. To further confirm this conclusion, landraces from Thailand, Laos, and Yunnan (China) should be studied in detail.

Table 3.1. List of Cambodian melon landraces analyzed in this study

Accession No.	Collection No.	Province	Region	Year of collection	Seed size	Seed length (mm)	Cluster	Population
CAe 1	15CJV-C83-1	Ratanakiri	East	2015	Small	8.1	I a	P2
CAe 2	15CJV-C100	Ratanakiri	East	2015	Small	7.7	I d	P2
CAe 3	15CJV-C94-1	Ratanakiri	East	2015	Small	8.2	I d	P2
CAe 4	15CJV-C94-2	Ratanakiri	East	2015	Small	7.0	I a	P2
CAe 5	15CJV-C76	Mondolkiri	East	2015	Small	8.3	I a	P2
CAe 6	15CJV-C40-1	Mondolkiri	East	2015	Large	9.4	I a	P2
CAe 7	15CJV-C40-2	Mondolkiri	East	2015	Small	7.5	I a	P2
CAe 8	15CJV-C19	Mondolkiri	East	2015	Small	7.3	I d	P2
CAe 9	15CJV-C132-1	Stung Treng	East	2015	Small	7.9	I d	P2
CAe 10	15CJV-C132-2	Stung Treng	East	2015	Small	6.3	I d	P2
CAe 11	15CJV-C138	Stung Treng	East	2015	Small	8.6	I d	P2
CAe 12	15CJV-C139	Stung Treng	East	2015	Small	8.2	I d	P2
CAe 13	16CJVC-83	Stung Treng	East	2016	Small	7.6	I d	P2
CAe 14	16CJVC-107	Kratie	East	2016	Small	7.7	I c	P2
CAe 15	16CJVC-107	Kratie	East	2016	Small	6.7	III a	P2
CAc 16	16CJVC-19	Kampong Thom	Center	2016	Small	8.6	I d	P2
CAc 17	16CJVC-19	Kampong Thom	Center	2016	Small	7.3	I d	P2
CAc 18	16CJVC-11-1	Kampong Thom	Center	2016	Small	8.6	I d	P2
CAc 19	16CJVC-11-2	Kampong Thom	Center	2016	Small	7.4	I d	P2
CAc 20	15CJV-C9	Tboung Khmum	Center	2015	Small	8.6	I a	P2
CAw 23	58	Battambang	West	2014	Small	8.2	I a	P2
CAw 24	65	Battambang	West	2014	Small	7.9	I a	P2
CAw 25	68	Battambang	West	2014	Large	9.0	I e	P2
CAw 26	68	Battambang	West	2014	Small	7.1	I d	P2
CAw 27	78	Banteay Meanchey	West	2014	Small	7.6	I c	P2
CAw 28	16CJVC-52	Banteay Meanchey	West	2016	Small	8.5	I c	P2
CAw 29	16CJVC-52	Banteay Meanchey	West	2016	Small	6.6	I c	P2
CAn 30	16CJVC-72	Preah Vihear	North	2016	Small	8.5	I d	P2
CAn 31	16CJVC-72	Preah Vihear	North	2016	Small	7.2	I a	P2
CAn 32	16CJVC-74	Preah Vihear	North	2016	Large	9.3	I a	P2
CAn 33	16CJVC-74	Preah Vihear	North	2016	Small	7.6	I a	P2
CAn 34	16CJVC-80	Preah Vihear	North	2016	Small	8.4	I d	P2
CAn 35	16CJVC-80	Preah Vihear	North	2016	Small	7.1	I d	P2
CAn 36	16CJVC-100	Preah Vihear	North	2016	Large	9.1	I a	P2
CAn 37	16CJVC-100	Preah Vihear	North	2016	Small	8.2	I a	P2
CAn 38	16JCVC-16	Siem Reap	North	2016	Small	8.1	I c	P2
CAn 39	16JCVC-23	Siem Reap	North	2016	Small	7.8	I e	P2

CAn 40	16JCVC-37	Siem Reap	North	2016	Small	7.6	I c	P2
CAn 41	16JCVC-60	Siem Reap	North	2016	Small	7.7	I c	P2
CAn 42	16JCVC-60	Siem Reap	North	2016	Small	6.9	I d	P2
CAn 43	16CJVC-46	Oddar Meancheay	North	2016	Large	9.3	I d	P2
CAn 44	16JCVC-46	Oddar Meancheay	North	2016	Small	7.7	I a	P2
CAn 45	16CJVC-63	Oddar Meancheay	North	2016	Small	8.0	I a	P2
CAs 46	17CJVC-4	Svay Rieng	South	2017	Small	6.9	III a	Mix
CAs 47	17CJVC-11	Svay Rieng	South	2017	Small	8.5	I a	P2
CAs 48	17CJVC-19-1	Prey Veng	South	2017	Small	8.5	I a	P2
CAs 49	17CJVC-19-2	Prey Veng	South	2017	Small	7.4	I d	P2
CAs 51	17CJVC-28	Takeo	South	2017	Small	7.7	I d	P2
CAs 52	17CJVC-48-1	Takeo	South	2017	Small	7.5	I a	P2
CAs 53	17CJVC-48-2	Takeo	South	2017	Small	6.6	II a	P2
CAs 54	17CJVC-87-1	Kompong Speu	South	2017	Large	9.5	I a	P2
CAs 55	17CJVC-87-2	Kompong Speu	South	2017	Small	8.3	I a	P2
CAs 56	17CJVC-87-3	Kompong Speu	South	2017	Small	7.3	I a	P2
CAs 57	17CJVC-92	Kompong Speu	South	2017	Small	7.9	I d	P2
CAs 58	17CJVC-52	Kampot	South	2017	Small	8.0	I a	P2
CAs 59	17CJVC-71	Koh Kong	South	2017	Small	7.7	I a	P2
CAs 60	17CJVC-64	Preah Sihanok	South	2017	Small	8.7	I a	P2
CAs 61	17CJVC-98	Kandal	South	2017	Small	4.9	I e	P2
CAs 62	17CJVC-101	Prey Veng	South	2017	Small	5.5	I e	P2
CAs 63	17CJVC-117	Kandal	South	2017	Small	5.1	I e	P2
CAs 64	17CJVC-121	Kandal	South	2017	Small	4.7	I e	P2
CAs 65	17CJVC-126	Kandal	South	2017	Small	4.7	I e	P2

Table 3.2. Primer sequence of 10 RAPD and seven SSR markers used in this study and the size of polymorphic fragments

Marker	Primer sequence	Size of RAPD amplicon (bp)	No. of alleles per locus	PIC
RAPD				
A20	TTGCCGGGACCA	1100	2	0.353
		800	2	0.364
A22	TCCAAGCTACCA	1520	2	0.216
A31	GGTGGTGGTATC	800	2	0.338
A41	TGGTACGGTATA	930	2	0.162
A57	ATCATTGGCGAA	800	2	0.364
B32	ATCATCGTACGT	900	2	0.447
		700	2	0.047
B68	CACACTCGTCAT	1078	2	0.330
B71	GGACCTCCATCG	1220	2	0.426
B86	ATCGAGCGAACG	1350	2	0.066
B99	TTCTGCTCGAAA	1400	2	0.452
SSR				
CMBR2	F: TGCAAATATTGTGAAGGCGA R: ATCCCCACTTGTTGGTTTG	-	12	0.891
CMBR83	F: CGGACAAATCCCTCTCTGAA R: GAACAAGCAGCCAAAGACG	-	9	0.825
CMBR120	F: CTGGCCCCCTCCTAAACTAA R: CAAAAAGCATCAAAATGGTTG	-	6	0.699
CMN04-03	F: ATCACAGAGACCGCCAAAAC R: GGTTGAAGATTGCGCTTGAT	-	9	0.829
CMN04-07	F: GAAAGCATTAAATATGGCATTGG R: AAGCTTAACAGCTTCCAGGG	-	8	0.664
CMN04-40	F: CACCTGACGATAGGGGTGTT R: AGTATTCGGGTTGGCAAAAA	-	9	0.795
CMN61-44	F: TGTTGGAGTTTAATGAGGAAGGA R: AGAGAAGATGAATGGGGCAC	-	17	0.854

Table 3.3. Number of accessions examined in each population and their characterization based on seed length, sex expression and gene diversity

Population	No. of accessions			Gene diversity
	Total	Small-seed type	Monoecious type	
Cambodia				
East	15	14	15	0.201
North	16	13	16	0.171
Center	5	5	5	0.105
South	19	18	19	0.246
West	7	6	7	0.183
Yunnan (China)	5	5	4	0.084
Thailand	5	2	1	0.328
Myanmar	36	32	34	0.428
Viet Nam				
Dua thom	20	20	20	0.154
Dua dai	1	1	1	-
Dua bo	14	14	0	0.142
Dua gang	13	13	1	0.150
Dua le	8	8	0	0.176
Dua vang	2	2	0	-
Conomon	9	8	1	0.227
Makuwa	11	11	2	0.168
Agrestis	6	6	3	0.105
Xinjiang (China)	24	0	0	0.246
Central Asia	14	7	4	0.351
Pakistan	11	7	4	0.322
Afghanistan	9	3	4	0.252
Iran	10	2	1	0.353
Spain	8	0	0	0.156
USA	5	0	0	0.219
Cantalupensis	10	0	0	0.278
Inodorus	8	0	0	0.240
Total	291	197	142	0.220

Table 3.4. Number of melon accessions classified into 25 subclusters and three populations in Fig. 2.2

Population	No. of accessions	Cluster No.																									Population structure			
		Ia	Ib	Ic	Id	Ie	If	Ig	Ih	IIa	IIb	IIc	IId	IIe	IIf	IIg	IIIa	IIIb	IIIc	IIId	IIIe	IIIf	IIIg	IIIh	IIIi	IIIj	IV	P1	P2	P3	Mix
Cambodia																															
East	15	5	-	1	8	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	15	-	-
North	16	7	-	3	5	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16	-	-
Center	5	1	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-
South	19	9	-	-	3	5	-	-	-	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	18	-	1
West	7	2	-	3	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7	-	-
Yunnan (China)	5	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-
Thailand	5	-	2		-	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	1	-	-	3	2	-
Myanmar	36	-	2	1	-	6	7	5	5	-	-	-	-	-	-	2	1	4	1	-	-	-	-	-	-	2	-	-	13	2	21
Viet Nam																															
Dua thom	20	1	16	1	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	19	-	1
Dua dai	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Dua bo	14	-	-	-	-	-	-	-	-	-	8	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14	-	-	-
Dua gang	13	-	-	-	-	-	1	-	-	1	8	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11	-	-	2
Dua le	8	-	-	-	-	-	-	-	-	-	-	5	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8	-	-	-
Dua vang	2	-	-	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-
Conomon	9	-	-	-	-	-	-	-	-	-	3	-	-	3	1	2	-	-	-	-	-	-	-	-	-	-	-	9	-	-	-
Makuwa	11	-	-	-	-	-	-	-	-	-	1	-	-	6	4	-	-	-	-	-	-	-	-	-	-	-	-	11	-	-	-
Agrestis	6	-	-	-	-	-	-	-	-	-	5	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	-	-	-
Xinjiang (China)	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	7	10	2	2	-	-	-	-	-	-	24	-
Central Asia	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7	2	-	1	3	-	1	-	-	-	12	2
Pakistan	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	2	-	-	-	1	2	2	-	-	-	9	2
Afghanistan	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	4	2	-	-	1	-	-	-	-	-	9	-
Iran	10	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	1	3	-	-	-	4	-	-	-	-	-	7	3
Spain	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7	-	-	-	-	1	-	-	-	-	-	8	-
USA	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	1	-	-	-	-	5	-
Cantalupensis	10	-	-	-	-	-		1		-	-	-	-	-	-	-	8	-	1	-	-	-	-	-	-	-	-	-	-	7	3
Inodorus	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	-	5	1	-	-	-	-	8	-
Hardwickii	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	2	-
Total	293	25	20	9	21	20	9	6	5	3	25	13	7	9	5	6	11	5	20	23	15	2	3	19	4	6	2	61	101	95	36
Association with population structure																															
P1	61	-	-	-	-	-	1	-	-	0	24	13	7	9	5	2	-	-	-	-	-	-	-	-	-	-	-				
P2	101	25	20	9	21	20	1	1	2	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-				
P3	95	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	5	2	20	23	15	2	3	15	4	3	2				
Mix	36	-	-	-	-	-	7	4	3	2	1	-	-	-	-	4	5	3	-	-	-	-	-	4	-	3	-				

Table 3.5. Genetic distance between 24 populations based on the frequency of 10 RAPD and seven SSR markers

Population	Cambodia-East	Cambodia-North	Cambodia-Center	Cambodia-South	Cambodia-West	Yunnan (China)	Thailand	Myanmar	Vietnam-Dua thom	Vietnam-Dua bo	Vietnam-Dua gang	Vietnam-Dua le	Conomon	Makuwa	Agrestis	Xinjiang (China)	Central Asia	Pakistan	Afgha-nistan	Iran	Spain	USA	Cantalu-pensis
Cambodia-North	0.045																						
Cambodia-Center	0.055	0.097																					
Cambodia-South	0.045	0.018	0.089																				
Cambodia-West	0.071	0.064	0.078	0.057																			
Yunnan (China)	0.243	0.203	0.311	0.181	0.250																		
Thailand	0.165	0.186	0.246	0.166	0.206	0.268																	
Myanmar	0.137	0.123	0.226	0.110	0.136	0.200	0.114																
Vietnam-Dua thom	0.138	0.103	0.203	0.123	0.146	0.141	0.194	0.121															
Vietnam-Dua bo	0.431	0.425	0.570	0.385	0.478	0.511	0.445	0.353	0.518														
Vietnam-Dua gang	0.373	0.397	0.493	0.359	0.429	0.517	0.455	0.337	0.482	0.035													
Vietnam-Dua le	0.541	0.565	0.676	0.524	0.611	0.675	0.541	0.472	0.662	0.077	0.082												
Conomon	0.448	0.505	0.553	0.427	0.528	0.524	0.454	0.386	0.575	0.086	0.060	0.089											
Makuwa	0.568	0.618	0.695	0.553	0.651	0.676	0.554	0.502	0.721	0.115	0.101	0.059	0.047										
Agrestis	0.608	0.567	0.818	0.499	0.623	0.615	0.627	0.479	0.660	0.100	0.093	0.118	0.119	0.117									
Xinjiang (China)	0.529	0.560	0.669	0.523	0.577	0.553	0.322	0.308	0.563	0.841	0.825	0.942	0.868	0.984	0.936								
Central Asia	0.407	0.460	0.510	0.415	0.455	0.536	0.271	0.250	0.501	0.662	0.620	0.697	0.654	0.731	0.753	0.060							
Pakistan	0.485	0.533	0.588	0.488	0.496	0.676	0.267	0.302	0.624	0.677	0.631	0.699	0.665	0.715	0.760	0.109	0.099						
Afghanistan	0.472	0.514	0.595	0.478	0.523	0.636	0.291	0.313	0.602	0.723	0.720	0.751	0.775	0.844	0.867	0.070	0.044	0.089					
Iran	0.420	0.445	0.549	0.408	0.452	0.618	0.254	0.249	0.549	0.557	0.545	0.522	0.585	0.586	0.627	0.112	0.048	0.089	0.058				
Spain	0.495	0.533	0.652	0.494	0.546	0.613	0.302	0.312	0.579	0.717	0.690	0.804	0.716	0.812	0.855	0.137	0.144	0.177	0.152	0.159			
USA	0.504	0.492	0.651	0.440	0.523	0.621	0.296	0.325	0.619	0.665	0.647	0.670	0.703	0.791	0.824	0.215	0.169	0.230	0.184	0.138	0.200		
Cantalupensis	0.384	0.453	0.479	0.417	0.457	0.629	0.276	0.262	0.547	0.525	0.455	0.540	0.467	0.522	0.563	0.268	0.150	0.209	0.206	0.164	0.292	0.303	
Inodorus	0.362	0.373	0.462	0.324	0.388	0.533	0.222	0.265	0.477	0.577	0.581	0.651	0.625	0.710	0.762	0.185	0.108	0.164	0.141	0.115	0.209	0.130	0.161

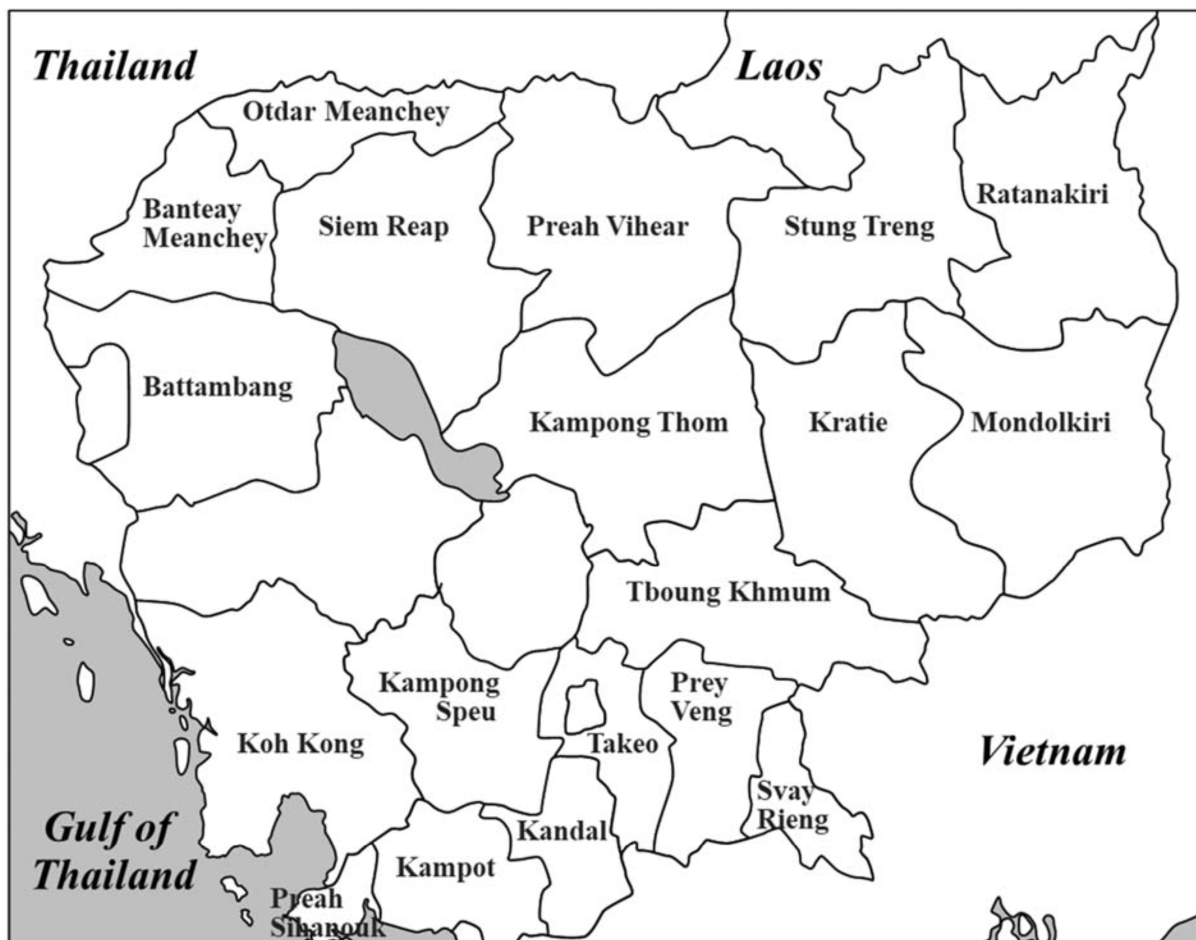


Fig. 2.1. Map of Cambodia. Nineteen provinces, where melon landraces were collected, were indicated.

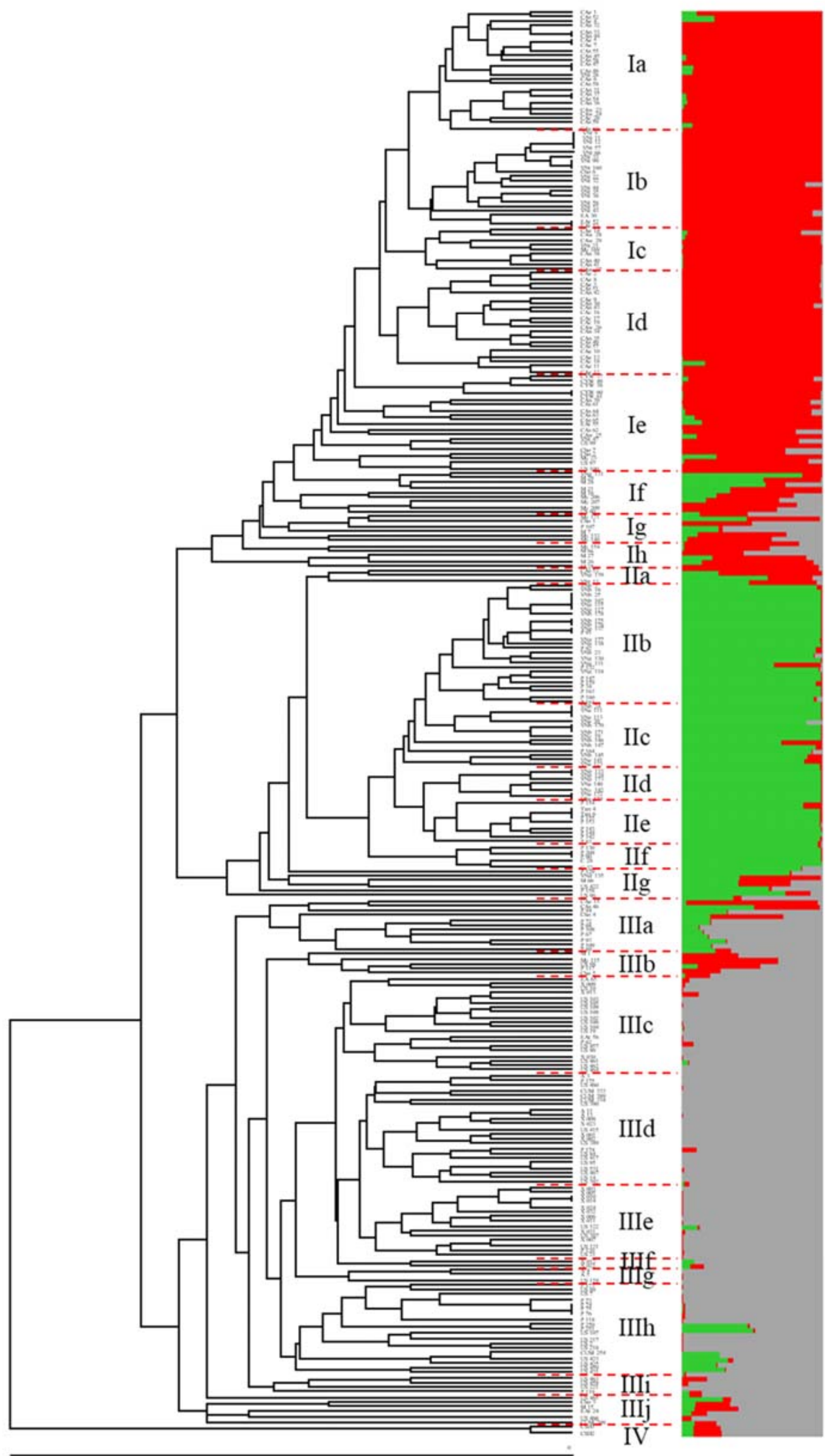


Fig. 2.2. Genetic relationship between 293 accessions revealed by UPGMA cluster analysis based on genetic distance, and population structure inferred by STRUCTURE using the admixture model.

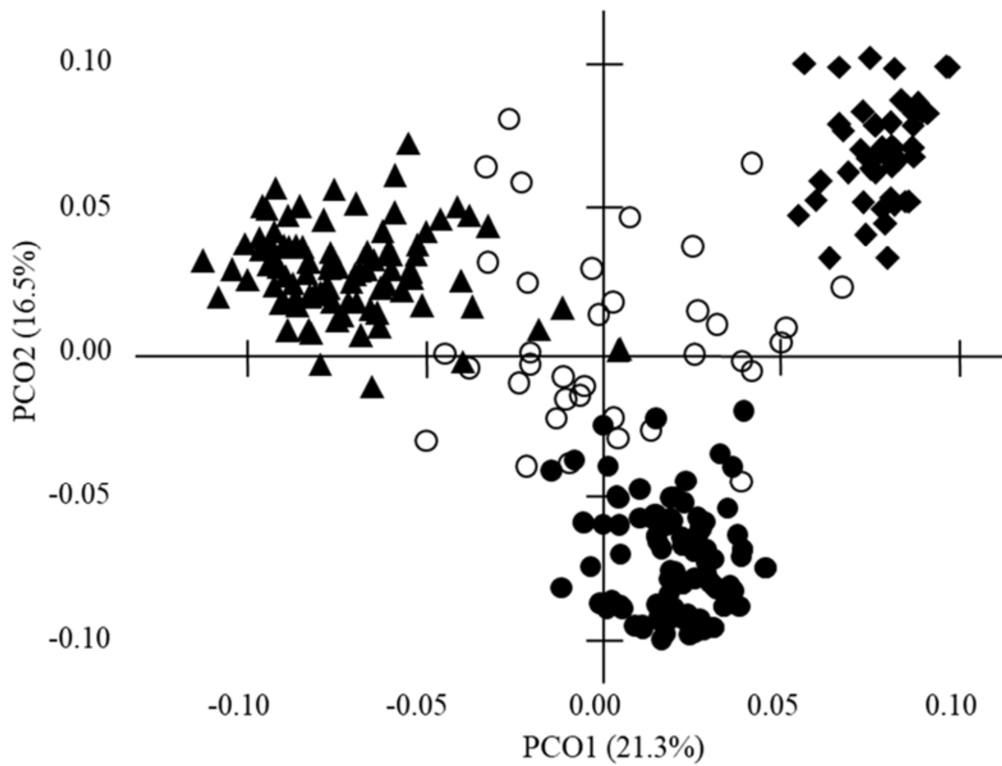


Fig. 2.3. Distribution of 293 accessions on the first two principal coordinates. Accessions are indicated with symbols unique to each population; P1 (◆), P2 (●), P3 (▲), and Admixture (○).

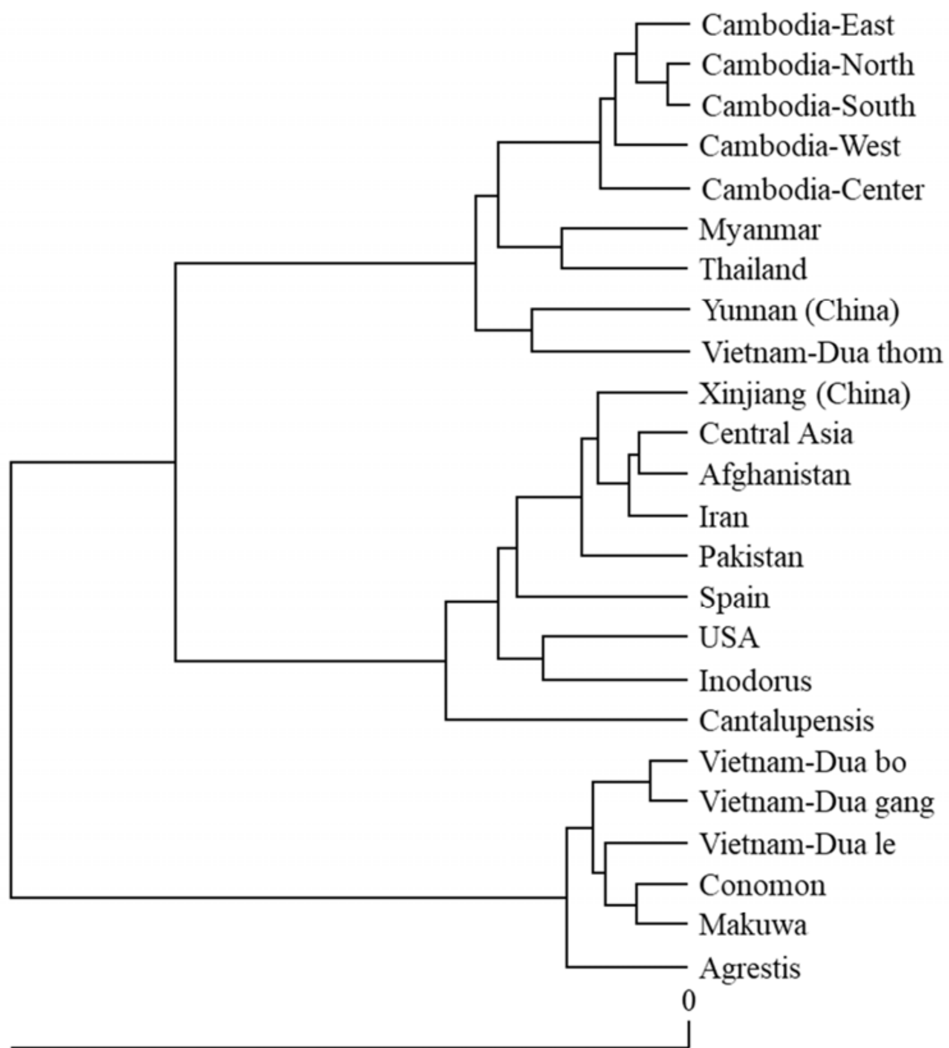


Fig. 2.4. Genetic relationship between 24 populations of melon, revealed by UPGMA cluster analysis based on genetic distance.

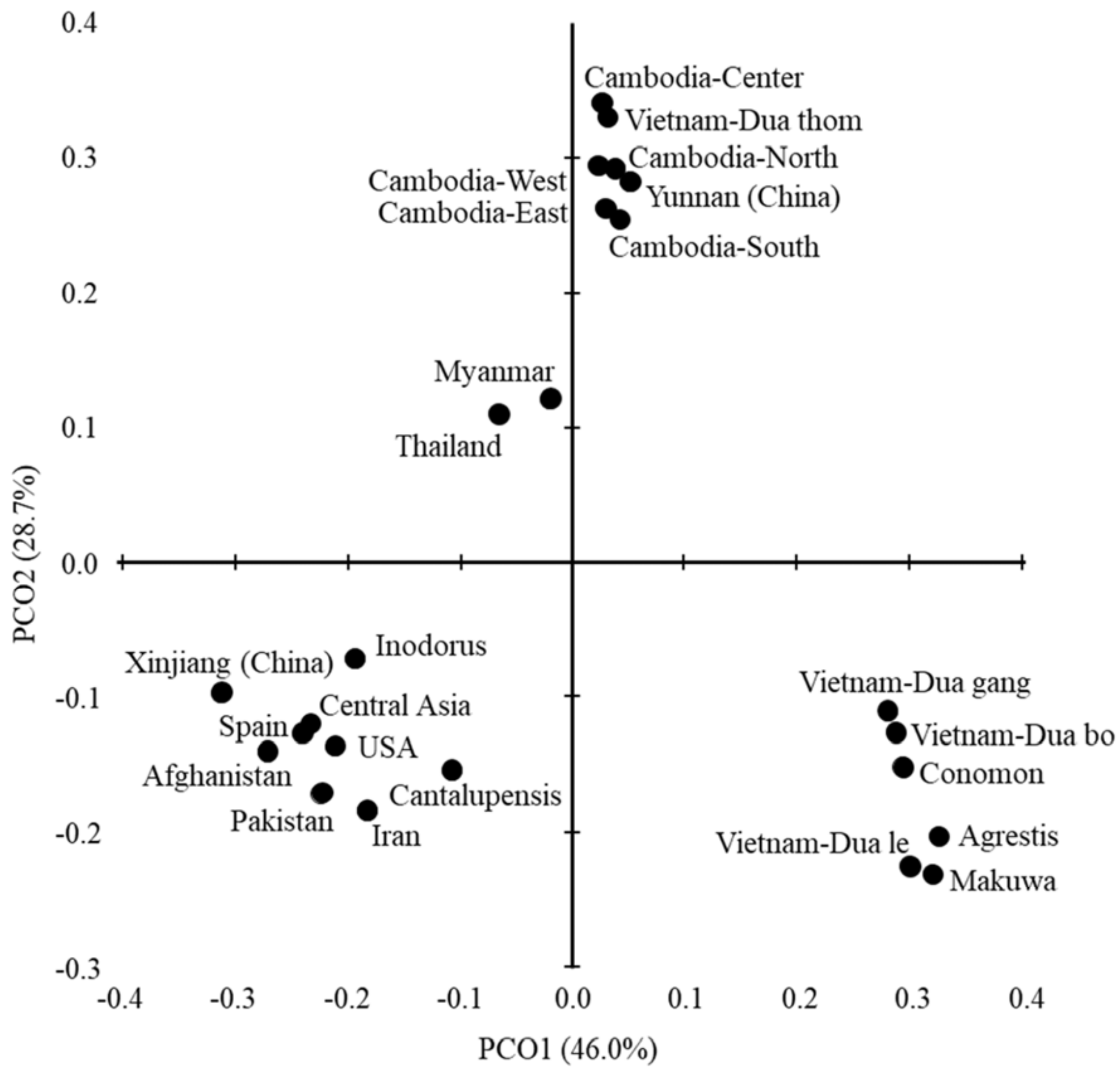


Fig. 2.5. Distribution of 24 populations of melon on the first two principal coordinates.

Chapter 4

Production of interspecific hybrids between melon and wild species of *Cucumis*

4.1 Introduction

Melon (*Cucumis melo* L.) and cucumber (*Cucumis sativus* L.) are important horticultural crops, and are grown worldwide to be consumed as dessert, vegetable, or ornamental fruit. For sustainable and stable production of melon and cucumber, resistance against diseases and pests has been improved by introducing resistant genes from genetic resources such as improved cultivars and local landraces of these crops. However, the resistance is often overcome by the occurrence of new races of pathogens or biotypes of pests, and new resistant cultivars should be bred. Currently, genetic resources resistant to *Fusarium oxysporum* f. sp. *melonis* race 1.2y, a new race causes Fusarium wilt, are required for melon breeding in Japan.

Among the major cucurbit vegetables, melon (*Cucumis melo* L.) has one of the greatest polymorphic fruit types and botanical varieties. Some melon fruits have excellent aroma, variety of flesh colors, deeper flavor, and more juice compared to other cucurbits. Despite numerous available melon cultivars, some of them are exceedingly susceptible to several diseases (Zitter *et al*. 1996). The genetic background carrying the genes for tolerance and/or resistance for those diseases is found in wild melon landraces (Guillaume & Boissot 2001; Dias *et al*. 2004). Interspecific hybridization is used to improve crops by transferring specific traits, such as pest and stress resistance, to crops from their wild relatives (Chen *et al*. 2000). When applicable, this approach is a very effective method of gene transfer.

Interspecific crossing is potentially important for cultivated melon and cucumber improvement since they are two most economic crops in genus *Cucumis* which are susceptible to about 45 different pathogens (Olczak *et al*., 2011). Within genus *Cucumis,* there are many wild species which considered containing resistant genes such as Fusarium wilt, downy mildew, powdery mildew, aphids, and viruses. An exchange of genes between the cultivated and feral or semi-feral species of *Cucumis* would open a vast potential resource of variability for exploitation by breeders attempting to improve cultivated melons and cucumbers. In other words, efforts to create interspecific hybrids become more critical and meaningful. However, the success of interspecific hybridization is still limited. One of the reasons is postzygotic failure of hybrid embryos which is often due, not to incompatibility between parental chromosomes but incompatibility problems in the endosperm (Deakin *et al*., 1971). In such cases, embryos from interspecific hybridization must be rescued; otherwise, they will fail due to embryo abortion and/or endosperm degeneration. Embryo rescue by tissue culture technique offers a means to recover starving embryo prior to abortion and subsequent regeneration of hybrid plant.

In interspecific crosses, embryo abortion can occur at any developmental stage. Besides, following embryo isolation and culture, variable response of embryo *in vitro* is common, including formation of leafy structure, swollen roots, non-responsive embryos; callus formation, deformed shoot/cotyledon growth and the rate of abnormal plants are always high. Therefore, determination the correlation between the optimal embryo stage of excision and optimal culture medium to obtain normal hybrid plant is extremely essential.

The development of embryo can be divided into two phases comprising heterotrophic (from proembryo stage to early heart stage) and auto trophic (from late heart stage to late mature)

(Raghavan, 1966). So that, embryo excised at the optimal stage could result in better embryo germination rates and vigorous growth *in vitro*. Embryo excised earlier than optimal stages are difficult to excise and have a lesser chance of survival *in vitro* but also require a more complex medium for growth and subsequent germination. Embryo excised too late during fruit development increases the risk of abortion (Shen *et al*., 2011). Custer *et al*., (1990) carried out embryo culture of three species in *Cucumis* (*C. sativus, C. zeyheri and C. metuliferus*) at different embryo ages on different culture media. The size of embryo varies across species, stages. As a result, strong effect of age was found.

In general, wild species harbors resistance against diseases and pests, and often crossed with crops to introduce resistant gene(s). However, interspecific hybridization between crops and wild species is often disturbed by various kinds of reproductive barriers such as cross incompatibility, hybrid weakness, hybrid sterility and so on. Attempts to overcome reproductive barriers have been made in many crop species. In cucumber, (Chen *et al*. 1997) succeeded in producing interspecific hybrid with *C. hystrix*, and the introgression of wild genes which show resistance to gummy stem blight and downy mildew. In melon, on the contrary, it is still impossible to produce interspecific hybrids. Although (Norton & Granberry 1980) reported successful production of fertile hybrids between *C. metuliferus* and *C. melo*, this result was not reproducible. In *Cucumis melo*, despite many attempts, melon is not cross-compatible with any other *Cucumis* species (Chen *et al.* 2000). Incompatibility is characterized by delayed growth of pollen, or arrested pollen tube growth in the stigma or inability of pollen tube to reach the ovules (pre-fertilization) (Kishi & Fujishita 1969), as well as the lack of cell division of zygote and abortion of endosperm (post-fertilization) (Kishi & Fujishita 1970). Several approaches in interspecific hybridization have been used to overcome the hybridization barriers but no one has efficient in

Cucumis distant hybridization. Until now, interspecific hybridization in *C. melo* is still attracting to scientists and in some wild relatives, *C. metuliferus* E. Meyer ex Naudin (nematode resistance) and *C. figarei* Naudin (virus resistance) have been more interested. However, the result of this hybridization is still limited because the lack of techniques and knowledge of species relationship. Therefore, the system for stable production of interspecific hybrids should be established to enable the use of wild genetic resources in melon breeding. The objective of this study was to develop interspecific hybrids by crossing *Cucumis melo* with its related wild species that can be used for genetic improvement of melon.

4.2 Materials and methods

4.2.1 Plant materials

C. melo cv. 'Harukei 3 gou' and its related wild species *C. sagittatus* were used as experimental plant material. 'Harukei 3 gou' is a pure line cultivar of "Earl's melon" which is known by its excellent fruit quality. *C. sagittatus* grows wild in southern part of Africa (Ghebretinsae *et al*. 2007) (Table 4.1). It was indicated by our previous studies that the chloroplast type of *C. sagittatus* is the most-close to that of cultivated melon among wild species of *Cucumis*. Seeds of these plant materials were provided by the Okayama University, Japan.

4.2.2 Crossing and Embryo culture techniques

These materials were grown in greenhouse heated during winter 2016, 2017, and 2018. Cross pollination was carried out using *C. melo* cv. 'Harukei 3 gou' (H3) as male parent and two accessions of *C. sagittatus* (US 161 and US 162), hybrid A (US 161 X H3) and hybrid B (US 162 X H3) as female parent. Female flowers should be pollinated in the morning (7:00–10:00 AM), since the pollen grains show the greatest survival rate in this period (Robinson & Decker-

Walters 1997). The fruits were harvested three weeks after pollination, and immature seeds containing hybrid embryo were cultured on agar solidified MS (Murashige & Skoog 1962) medium with or without plant hormone (Table 4.2). At first the surface of the fruit was sterilized with NaClO and Tween20 (10 minutes). After that washed twice with distilled water and immature seeds were excised. They were cut into half and half seeds containing embryo were cultured on MS hormone free solid medium containing 20g/L sucrose, 8g/L agar at pH 5.8, and MS medium supplemented with different kinds of plant hormone (Table 4.2) at pH 5.8. They were incubated in a growth chamber at 25^0C under fluorescent lighting with a 12h light: 12h dark photoperiod. Subcultures was carried out every month. Healthy well-developed plantlets were removed from in vitro culture vessels and acclimated.

4.2.3 Confirmation of hybrids

To confirm if the regenerated plants are interspecific hybrids, DNA was extracted from the true leaves by using the CTAB (cetyl trimethyl ammonium bromide) method (Murray & Thompson 1980) with minor modifications. Optical density value which represented for quality and quantity of extracted DNA was estimated by measuring the absorbance at 260nm and 280nm measured by spectrophotometer (DU 530, Beckman) at two kinds of wavelength (260 nm and 280 nm).

DNA was analyzed by using three DNA markers, among which CMCCT144 is an SSR in nuclear genome and 1F1R-1600-2in and STS-UBC114 are mtIREP (mitochondrial Inter Repetitive marker) in mitochondrial genome (Table 4.3). MtIREP marker was used because of its paternal inheritance (Havey *et al.* 1998). Amplification reactions were performed using iCycler (Bio-Rad, Hercules, CA, USA). The PCR cycle for MtIREP marker started with an

initial denaturing step at 95^0 C for 3 min, 35 cycles at 95^0C for 1 min, 60^0C for 1 min, and 72^0C for 2 min. The final extension step was at 72^0C for 5 min (Table 4.4).

Further, to detect the hybrid the PCR cycle for SSR marker started with an initial denaturing step at 94^0 C for 3 min, 35 cycles at 94^0C for 30 second, 55^0C for 1 min, and 72^0C for 1 min. The final extension step was at 72^0C for 5 min (Table 4.4). PCR mixture was done in a 10 µl mixture containing 100ng genomic DNA, 10 x PCR buffer (Sigma®), 0.1mM dNTP, 2.0mM MgCl2, Taq DNA polymerase (Sigma®), Forward Primer and Reverse Primer of 20pM, sterile Milli-Q water (Table 4.5). After amplification, samples underwent electrophoresis on 1% agarose gel (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.

Table 4.1. Plant Materials used for interspecific hybridization

Name of the species	Line number *	Country of origin	Cultivar name
C. melo	Harukei 3 gou	Japan	Earl's melon
C. sagittatus	US161	South Africa	
	US162	South Africa	

*Number registered at faculty of Agriculture, Okayama University, Japan.

Table 4.2. Medium used for embryo culture

Culture medium	Basic medium	Additive components
Hormone free	MS	20 g/L sucrose, 8 g/L agar
IAA	MS	3.0 mg/L IAA, 20 g/L sucrose, 8 g/L agar
BAP	MS	1.0 mg/L BAP, 20 g/L sucrose, 8 g/L agar

MS = Murashige and Skoog (1962)

IAA = 3-indoleacetic acid (heteroauxin)

BAP = 6-benzylaminopurine

Table 4.3. Primers used in this study

Primer name		Sequence (5′→ 3′)
1F1R-1600-2in	F1	ATATGAACCCGTCACCTGGA
	R1	CCTACGGACCAGAAAGTCAAA
STS-UBC114	F	GAGACCTAGTTTGCCAGCAG
	R	ACCGAGACGGATACATCGAC
CMCCT144	F	CAAAAGGTTTCGATTGGTGGG
	R	AAATGGTGGGGGTTGAATAGG

Table 4.4. PCR reaction setup

Primer	Step	Temperature (^0C)	Time	Cycle
1F1R-1600-2in	Pre-denaturation	95	3m	
STS-UBC114	Denaturation	95	1m	
	Annealing	60	1m	35
	Extension reaction	72	2m	
	Final extension	72	5m	
CMCCT144	Pre-denaturation	94	3m	
	Denaturation	94	30s	
	Annealing	55	1m	35
	Extension reaction	72	1m	
	Final extension	72	5m	

Table 4.5. Composition of PCR reaction

Component	Volume (µl)
10 x PCR buffer (Sigma®)	1.00
0.1mM dNTP	0.80
2.0mM MgCl2	0.60
Taq DNA polymerase (Sigma®)	0.10
Forward Primer	0.50
Reverse Primer	0.50
Sterile Milli Q	4.50
100ng/µl template DNA	2.00
Total	10.00

4.3 Results

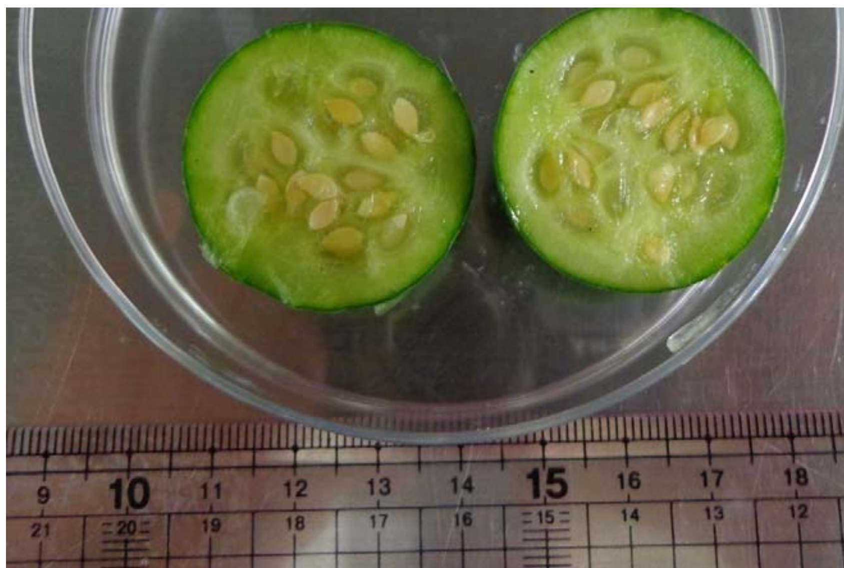
4.3.1 Crossing and embryo culture

Interspecific hybridization is a very effective method in gene transfer, which contributes to improve crops by transferring useful traits from their wild relatives. However, there is a cross incompatibility between cultivated melon and wild species of *Cucumis*. It is a big barrier to promote cross breeding of melon. *C. melo* cv. 'Harukei 3 gou' and its related wild species *C. sagittatus* were used as experimental plant material in this study. Based on the previous study (Matsumoto *et al*. 2012) cross pollination was carried out using *C. melo* cv. 'Harukei 3 gou' as male parent and two accessions of *C. sagittatus* (US 161 and US 162) as female parent. Cross pollination was done in 2016, 2017, and 2018. Cross pollination was mostly successful. Fruits developed normally and obtained 190, 375, and 220, fruits in 2016,2017, and 2018 respectively (Fig. 4.1). However, as seed development stops at young stage, no germinable seeds can be obtained (Fig. 4.2). Therefore, from these fruits, a total of 16903, 28798, and 17850 immature seeds were excised and cultured on MS medium with or without plant hormone (Table 4.1) in 2016,2017, and 2018 respectively. Callus induction became visible from one week to eight weeks after inoculation on all the culture medium but with very low frequency which is 0.88%-2.25% (Table 4.6, Fig. 4.3a) in 2016. We observed 246 calli induced from the cultured embryos (Table 4.6). Shoot developed from twenty-nine embryos among which sixteen also developed root (Fig. 4.3b and 4.4). Healthy well-developed plantlets were removed from in vitro culture vessels and acclimated. After the satisfactory development in root system, the small plantlets were transplanted in small plastic pot in growth chamber for proper hardening of the plantlets and finally these were planted in earthen pots. As a result, four plants (0.02%) were successfully germinated (Fig. 4.5) in 2016.

Table 4.6 Hybrid embryos and hybrid plants derived from culture from crosses of *C. sagittatus* and *C. melo* in 2016

Cross combination	Medium	Number of fruits	Number of embryos	Number of callus induction (ratio)	Number of shoots regenerated (ratio)		Number of regenerated plants (ratio)
					Without root	With root	
US 161 x H3	Hormone free	57	6219	78 (1.25%)	6 (0.10%)	1 (0.02%)	-
	BAP	28	1466	19 (1.30%)	5 (0.34%)	1 (0.07%)	1 (0.07%)
	IAA	23	1284	19 (1.48%)	3 (0.23%)	-	-
US 162 x H3	Hormone free	53	6002	99 (1.65%)	8 (0.13%)	11 (0.18%)	2 (0.3%)
	BAP	12	911	8 (0.88%)	2 (0.22%)	-	-
	IAA	17	1021	23 (2.25%)	5 (0.49%)	3 (0.29%)	1 (0.10%)
Total		190	16903	246 (1.46%)	29 (0.17%)	16 (0.09%)	4 (0.02%)

4.1 Hybrid fruit bearing *C. sagittatus*



4.2 Immature seeds of hybrid fruit

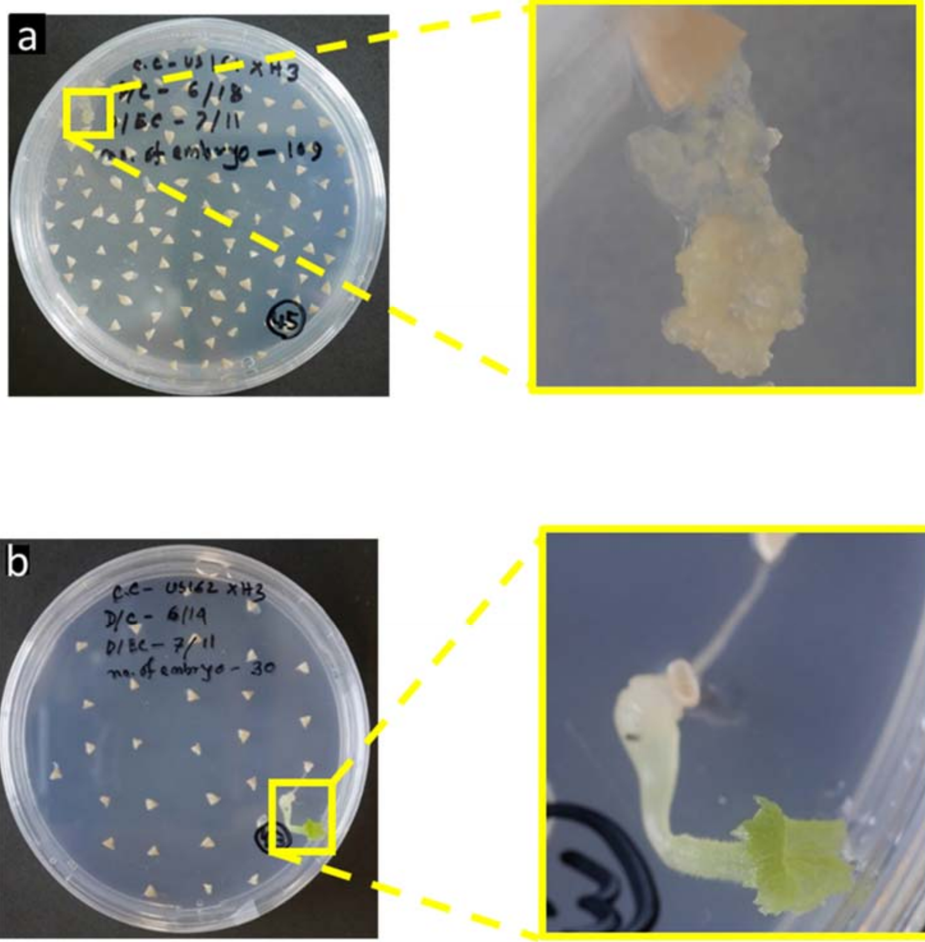


Fig. 4.3 Shoot differentiation from immature embryos. a. callus initiation and b. shoot development and proliferation.

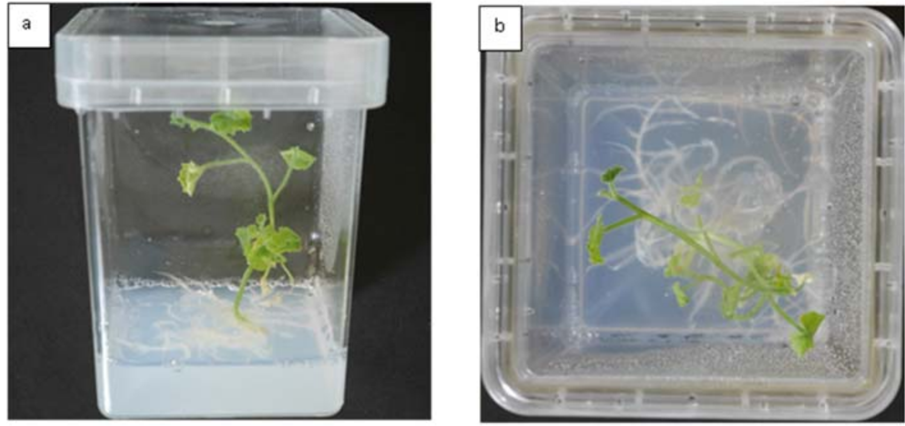


Fig. 4.4 Initiation of roots from regenerated shoot of melon. a. side view and b. top view of rooted melon plantlet in 2016

In 2017, from the above crosses 375 fruits were obtained. Therefore, from these fruits, a total of 28798 immature seeds were excised and cultured on MS medium (Table 4.7). We observed 423 calli induced from the cultured seeds (Table 4.7). Shoot also developed from 61 embryos among which eight also developed root (Fig 4.7 B, and C). In comparison with the result in 2016 (Table 4.6), the number of obtained fruits and the number of cultured embryos in 2017 increased by two times (128 fruits and 11673 embryos in 2016) but the ratio of callus induction or shoot regeneration were almost the same. Even the percentage of germinated normal plantlets had the decreasing trend (0.03% in 2016 and 0.013% in 2017). Here we observed three types of regeneration in 2017: 1. Callus formation (no organogenesis was observed from seeds; only callus without stems, roots, or leaves); 2. Abnormal plant formation (indirect organogenesis from seeds was observed, such as the formation of stems, roots or leaves on callus) and 3 normal plant formation (directly germinated from seeds) (Fig 4.7 A, B and C). In 2018, 219 fruits were obtained. 459 calli induced from the cultured seeds and 17 germinated seeds also observed from the cultured seeds (Table 4.8) but it's not developed as organized plant or plantlets.

Fig 4.5. Germinated Plants established on soil in 2016

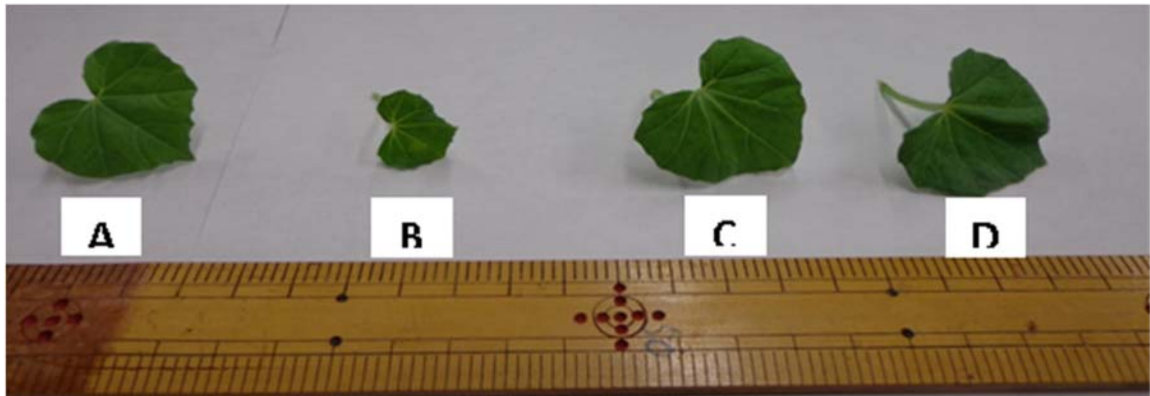


Fig 4.6. Leaf of four germinated plants in 2016

Table 4.7 Hybrid embryos and hybrid plants derived from culture from crosses of *C. sagittatus* and *C. melo* in 2017

Cross Combination	Medium	Number of fruits	Number of embryos	Numbers of seeds response	Number of calli induction (ratio %)	Number of shoots regenerated (ratio %)	Number of normal plants (ratio %)
US 161 x H3	MS	34	2589	85 (3.28)	69 (2.66)	12 (0.46)	-
US 162 x H3	MS	19	1059	17 (1.60)	17 (1.60)	-	-
Hybrid A x H3	MS	19	1249	20 (1.60)	16 (1.28)	-	3 (0.24)
Hybrid B x H3	MS	303	23901	428 (1.79)	321 (1.34)	45 (0.15)	1 (0.003)
Total		375	28798	550 (1.91)	423 (1.47)	57 (0.19)	4 (0.013)

Table 4.8 Hybrid embryos, germinated plantlets and callus derived from culture from crosses of *C. sagittatus* and *C. melo* in 2018

Cross combination	Number of fruits	Number of seeds cultured	Number of seeds response	Number of seeds germinated	Number of calli induction
Hybrid A X H3	41	3278	96 (2.92)	0	96
Hybrid B X H3	52	4,278	108 (2.52)	3	105
Hybrid D X H3	57	4,828	113 (2.34)	6	107
US162 X H3	70	5,466	159 (2.90)	8	151
Total	220	17,850	476 (2.67%)	17 (0.09%)	459 (2.57 %)

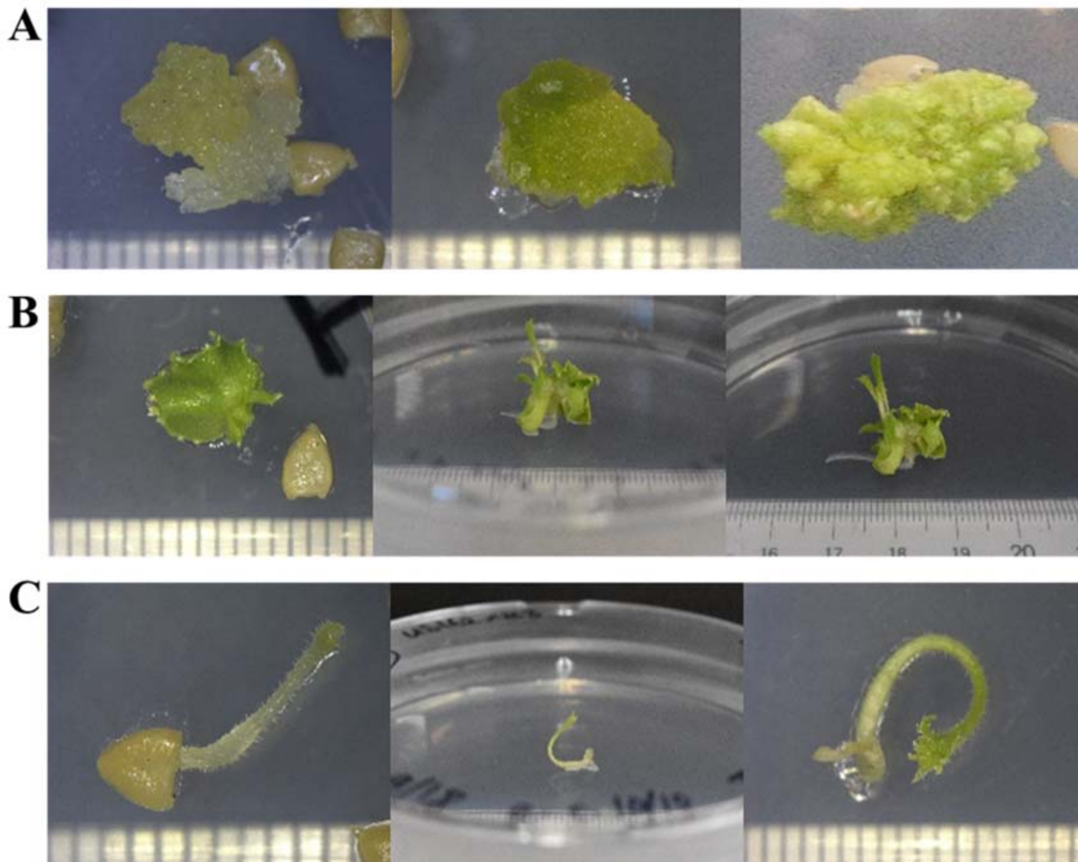


Fig. 4.7 Various types of regeneration from isolated half seeds of melon in 2017. A. callus formation, B, C abnormal plant formation.

4.3.2 Confirmation of hybrids

For confirming the germinated plants are interspecific hybrids, DNA was extracted from the true leaves from different vines of the germinated plants and DNA was analyzed by using three DNA markers, among which CMCCT144 is an SSR in nuclear genome and 1F1R-1600-2in and STS-UBC114 are mtIREP in mitochondrial genome. MtIREP marker was used because of its paternal inheritance (Havey *et al*. 1998). By the analysis of CMCCT144, an SSR marker of nuclear genome, a different size of specific band was amplified from parental lines in 2016. An SSR marker of nuclear genome, a 290bp sequence specific to *C. sagittatus* was commonly

amplified from the four germinated plants, while a 220bp sequence from ‘Haru 3’ was not detected (Fig. 4.9). In case of mtIREP marker STS-UBC114, which inherit paternally, a marker band of pollen parent ‘Haru 3’ was not amplified in the four plants in 2016. These results indicated that these plants obtained were not hybrid. On the contrary, in case of mtIREP marker of the mitochondrial genome, a marker band specific to male parent (Harukei 3 gou) was amplified only in one germinated plant (1F1R-1600-2in). Other germinated plants did not show any band which is similar to male parent. So, the analysis of another mtIREP marker 1F1R-1600-2in revealed the presence of ‘Haru 3’ sequence of mitochondrial genome in several vines of one plant, suggesting the fertilization with ‘Haru 3’ (Fig 4.9). Furthermore, among the four-germinated plants one plant is different from the other three plants (Fig 4.5 B). The leaf of the plant is smaller than the other three plants leave (Fig 4.6). In 2017 and 2018 we didn’t get any regenerated plant. So, further study is required to confirm if they are interspecific hybrid between *C. sagittatus* and ‘Haru 3’

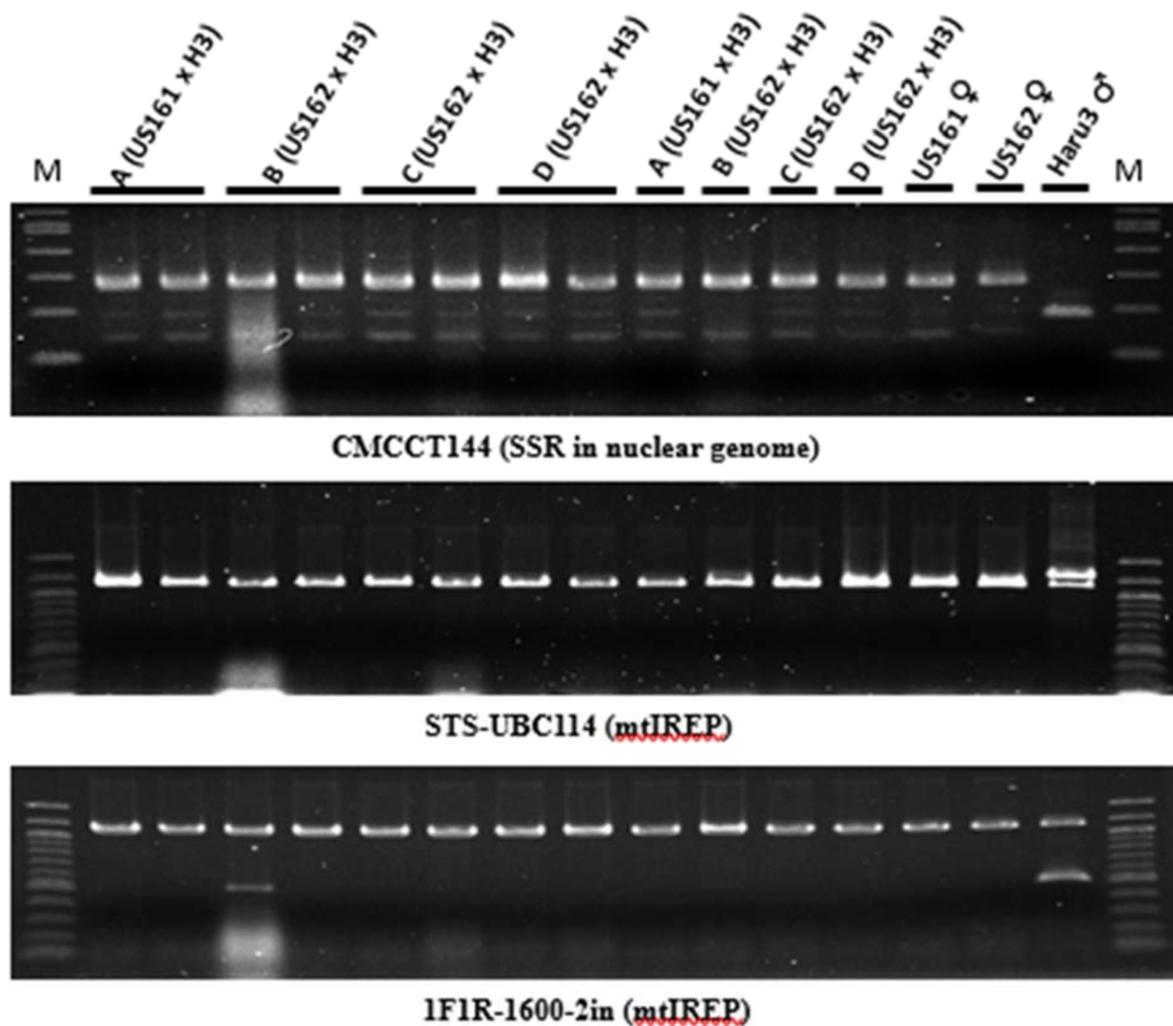


Fig. 4.9 Hybridity test by marker analysis in mitochondria (mt) and nuclear genome (ng) in 2016

4.4 Discussion

Besides the traditional methods in plant breeding, tissue culture is extensively used for induction of genetic variability to produce novel plant genotypes which are high yielding, resistant to insect pests and diseases and which display increased photosynthetic efficiency.

Variability can be generated through tissue culture by using different explant tissues ranging from hypocotyls, cotyledons, vegetative meristem to reproductive tissues that can be induced to differentiate into new plantlet. According to (Kraus *et al*. 1981) early induction and higher callus growth is expected to produce more variability by producing polypoids and aneuploids. Using a nascent embryo rescue technique, Deakin *et al*., (1971) have successfully obtained an interspecific hybrid between the distantly related species *C. sagittatus* and *C. melo* L. that could not be obtained by conventional techniques.

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). However, the archaeological record, suggests that African melon domestication started at least 5000–6000 years ago, perhaps earlier than in Asia. According to Endl *et al*. (2018) melon was domesticated at least twice: in Africa and Asia. modern melon cultivars go back to two lineages, which diverged ca. 2 million years ago. One is restricted to Asia (*Cucumis melo* subsp. *melo*), and the second one (*C. melo* subsp. *Meloides*), is restricted to Africa (Endl *et al*., 2018). The Asian lineage has given rise to the widely commercialized cultivar groups and their market types, while the African lineage gave rise to cultivars still grown in the Sudanian region.

C. sagittatus is genetically close to *C. melo* L. 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 F1 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*. The hybrid between *C. sagittatus* and *C. melo* L. may also serve as a bridge plant for transferring genes from other species into *C. melo* and overcome crossability barriers in *Cucumis* species, because wild species of *Cucumis* will become the source of useful germplasm for the genetic improvement of *C. melo* L. In this study, we succeeded to obtained only germinated plant. At first it seems to be hybrid plant but unfortunately, it's not clear is it hybrid or not? Further study is required to confirm if they are interspecific hybrid between *C. sagittatus* and 'Haru 3'. From the practical aspect, the hybrid plants are male sterile, and so far, no seeds were obtained. Therefore, chromosome doubling, or backcrossing should be done to obtain genetically stable hybrid which can be utilized in melon breeding. In addition, pollination method and culture condition should be reconsidered to increase the regeneration efficiency.

Chapter 5

General Conclusions

Cambodia has a border with Vietnam and a long history of cultural interchange including crop seeds. However, genetic resources of Cambodian melon landraces were not available at all outside Cambodia. The Japanese expedition team including Okayama University started field research in Cambodia from 2014, collaborating with the Cambodian Agricultural Research and Development Institute (CARDI), and successfully introduced melon seeds into Japan with the Standard Material Transfer Agreement (SMTA). Field survey was conducted in four regions to cover whole area of Cambodia (West in 2014, East in 2015, Center and North in 2016, and South in 2017). From the samples collected, 60 accessions of cultivated melon and two accessions of weedy melon were selected and analyzed in this study. For each accession, five plants were grown and a total of 37 horticultural traits were evaluated. In general, Cambodian melon landraces show monoecious type of sex expression and Brix value below 6.0, have oblong shape of fruits with smooth skin and seeds shorter than 9 mm in length (small-seed type). Melon landraces showing these traits were reported in Myanmar and mountainous areas of Vietnam ("Dua thom" and "Montok"), showing the distribution of similar type of melon in these areas (Nhi et al. 2010 and Thuy et al. 2021). Geographical variation of horticultural traits was also found in Cambodia. For example, fruit height and seed length were shorter in landraces from eastern region compared to those from western region, and the difference was statistically significant ($p<0.01$). Similarly, fruit weight was also different ($p<0.05$). These results might indicate the selection by local farmers. Although fruit morphology of Cambodian melon was similar with that of varieties *conomon* and *makuwa*, sex expression type was different.

Gene diversity of Cambodian melon was 0.228 which was equivalent to those of groups Conomon and Makuwa and smaller than those of Vietnamese and Central Asian landraces. Phylogenetic tree constructed from genetic distance matrix classified 293 accessions into three major clusters. Small-seed type accessions from east and southeast Asia formed clusters I and II, which were distantly related with cluster III consisted of large-seed type melon from other areas. All Cambodian melon belonged to cluster I except three accessions, along with those from Thailand, Myanmar, Yunnan (China) and "Dua thom" from northwestern part of Vietnam, indicating genetic similarity in these areas. In addition, Cambodian melon was not differentiated among geographical populations. Conomon and Makuwa were classified into cluster II, together with melon groups from plain areas of Vietnam. The presence of two groups of melon in southeast Asia was also indicated by population structure and PCO analysis. According to Nhi et al. (2010) and Duong et al. (2021) Vietnamese melon consisted of seven cultivar groups among which "Dua le" and "Dua vang" were regarded as Makuwa and "Dua bo" and "Dua gang-andromonoecious" as Conomon. In contrast, "Dua thom" and "Montok" showed genetic similarity with Indian and Maynmar landraces. Based on these results, the presence of groups Conomon and Makuwa was first confirmed in Vietnam. The similarity in fruit morphology (Matsunaga et al. 2015; Tanaka et al. 2016; Tanaka et al. 2017; Tanaka et al. 2020) and the results mentioned above might support the hypothesis that Conomon and Makuwa were originated from small-seed type melon somewhere in these areas. In overall, two distinct groups of melon in Southeast Asia were described. Conomon and Makuwa formed one group together with accessions from plain areas of Vietnam, and those from Myanmar to mountain areas of Vietnam formed another group. In addition, the sex expression type estimated by CAPS analysis of *CmACS7* was also different between two groups. The former group was characterized by

monoecy, while the latter by andromonoecy. These results indicated close genetic relationship between Cambodia and the neighboring countries and suggested that Cambodian melon is not directly related with the establishment of Conomon and Makuwa. To further confirm this conclusion, landraces from Thailand, Laos, and Yunnan (China) should be studied in detail.

In our third study, we succeeded to obtained only germinated plant. At first it seems to be hybrid plant but unfortunately, it's not clear is it hybrid or not? Further study is required to confirm if they are interspecific hybrid between *C. sagittatus* and 'Haru 3'. From the practical aspect, the hybrid plants are male sterile, and so far, no seeds were obtained. Therefore, chromosome doubling, or backcrossing should be done to obtain genetically stable hybrid which can be utilized in melon breeding. In addition, pollination method and culture condition should be reconsidered to increase the regeneration efficiency.

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References

Akashi, Y., Fukuda, N., Wako, T., Masuda, M., Kato, K. (2002). Genetic variation and phylogenetic relationships in East and South Asian melons, *Cucumis melo* L., based on the analysis of five isozymes. Euphytica, 125, 358 - 396.

Apostol, B.L., Black, W.C.I.V., Miller, B.R., Reiter, P. and Beaty, P. J. (1993). Estimation of the number of full sibling families at an oviposition site using RAPD-PCR markers: applications to the mosquito *Aedes aegypti*. Theor. Appl. Genet. 86, 991 - 1000.

Botstein, D., White, R. L., Skolnick, M. and Davis, R.W. (1980). Construction of genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32, 314 - 331.

Boualem A, Fergany M, Fernandez R, Troadec C, Martin A, Morin H, Sari MA, Collin F, Flowers JM, Pitrat M, Purugganan MD, Dogimont C, Bendahmane A (2008). A conserved mutation in an ethylene biosynthesis enzyme leads to andromonoecy in melons. Science. 321, 836-838.

Chen, J.F. et al., 1997. Successful interspecific hybridization between Cucumis sativus L. and C. hystrix Chakr. Euphytica, 96(3), pp.413–419.

Chen, J.F., Adelberg, J. & others, 2000. Interspecific hybridization in Cucumis-Progress, problems, and perspectives. Hort. Science, 35(1), pp.11–15.

Cortinovis G, Vittori VD, Bellucci E, Bitocchi E, Papa R (2020). Adaptation to novel environments during crop diversification. Current Opinion in Plant Biology 56, 203-217.

Costa, R., G. Pereira, I. Garrido, M.M. Tavares-de-Sousa, F. Espinosa (2016) Comparison of RAPD, ISSR, and AFLP Molecular Markers to Reveal and Classify Orchardgrass (Dactylis glomerata L.) Germplasm Variations. PLoS One 11(4)

Custers, J.B.M. and Bergervoet, J.H.W., 1990. In vitro culture of embryos of *Cucumis* spp: heart-stage embryos have a higher ability of direct plant formation than advanced-stage embryos. Sex plant report. 3: 152-159.

Deakin, J.R., Bohn, G.W. and Whitaker, T. W., 1971. Interspecific hybridization in Cucumis. J. of Economic Botany. 25(2), pp. 195-211.

Dhillon NPS, Monforte AJ, Pitrat M, Pandey S, Singh PK, Reitsma KR, Gracia-Mas J, Sharma A, McCreight JD (2012). Melon landraces of India: Contributions and Importance. *In*: Plant Breeding Review volume 35. Janick J (ed). Wiley-Backwell, New Jersey, pp.85-150.

Dhillon NPS, Ranjana R, Singh PP (2007). Diversity among landraces of Indian snap melon (*Cucumis melo* var. *momordica*). Genet Resour Crop Evol 54:1267-1283.

Dias, R. de C.S. et al., 2004. Resistance to melon vine decline derived from Cucumis melo ssp. agrestis: Genetic analysis of root structure and root response. Plant Breeding, 123(1), pp.66–72. Available at: http://doi.wiley.com/10.1046/j.1439-0523.2003.00944.x [Accessed January 23, 2017].

Du, Y. and Scheres, B., 2018. Lateral root formation and the multiple roles of auxin. J. of Expt. Botany. 69:2 pp 155-167.

Duong, T. T., Dung, T. T., Tanaka, K., Nhi, P. T. P., Shigita, G., Imoh, O. N., Hidetaka Nishida, H and Kato, K. (2021). Distribution of two groups of melon landraces and inter-group hybridization enhanced genetic diversity in Vietnam. Breed. Sci. 7: 564–574.

Earl, D.A. and Vonholdt, B.M. (2012). STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Resour. 4, 359 - 361.

Endl, J., Achigan-Dako, E. G., Pandey, A. K., Monforte, A. J., Pico, B., & Schaefer, H. (2018). Repeated domestication of melon (*Cucumis melo* L.) in Africa and Asia and a new close relative from India. American Journal of Botany, 105(10), 1662 - 1671.

Evanno, G., Regnaut, S., Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol, 14, 2611 - 2620.

Felsenstein, J. 1993. PHYLIP: phylogeny inference package (version 3.5c). University of Washington, Seattle.

Fergany, M., Kaur, B., Monforte, A. J., Pitrat, M., Rys, C., Lecoq, H., Dhillon, N. P. S., Dhaliwal, S.S. (2011). Variation in melon (*Cucumis melo*) landraces adapted to the humid tropics of southern India. Genet Resour Crop Evol, 58, 225 - 243.

Fujishita, N. (1983). Genetic diversity and phylogenetic differentiation in melon. Curr. Top. Plant Breed. 24: 3-21.

Fujishita, N. and K. Nakagawa (1973). About seed (embryo) and fruit development of Cucumis species (1). Effects of growth regulator affected apomixis and parthenocarpy of C. melo. J. Japan. Soc. Hort. Sci. 42 (Suppl.2). 186-187 (in Japanese).

Fukaki, H., Okushima, Y. and Tasaka, M., 2005. Regulation of lateral root formation by auxin signaling in Arabidopsis. Plnt biotech. 22 (5), pp. 393-399.

Fukino, N., Sakata, M., Kunihisa, M. and Matsumoto, S. (2007). Characterization of novel simple sequence repeat (SSR) markers for melon (*Cucumis melo* L.) and their use for genotype identification. J. of Hort. Sci. and Biotech, 82(2) 330-334.

Ghebretinsae, A.G., Thulin, M. & Barber, J.C., 2007. Relationships of cucumbers and melons unraveled: molecular phylogenetics of Cucumis and related genera (Benincaseae, Cucurbitaceae). American Journal of Botany, 94(7), pp.1256–1266. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/21636491 [Accessed January 23, 2017].

Goldberg, Robert; Paiva, Genaro; Yadegari, Ramin (October 28, 1994). "Plant Embryogenesis: Zygote to Seed". Science. 266 (5185): 605–614.

Gonzalo, M. J., A. Díaz, N.P.S. Dhillon, U.K. Reddy, B. Picó and A.J. Monforte (2019) Re-evaluation of the role of Indian germplasm as center of melon diversification based on genotyping- by-sequencing analysis. BMC Genomics: 1–13.

Gower, J.C. (1966). Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika, 53:325-338.

Guillaume, R. &Boissot, N., 2001. Resistance to Diaphaniahyalinata (Lepidoptera: Crambidae) in *Cucumis* species. Journal of Economic Entomology, 94(3), pp.719–723. Available at: http://jee.oxfordjournals.org/cgi/doi/10.1603/0022-0493-94.3.719 [Accessed January 23, 2017].

Havey, M.J. et al., 1998. Differential transmission of the Cucumis organellar genomes. Theoretical and Applied Genetics, 97(1–2), pp.122–128.

Jeffrey, C. (1980) A review of the Cucurbitaceae. *Bot. J. Linn. Soc.* 81: 233–247.

Jung, J., G. Park, J. Oh, J. K Jung, E. J. Shim, S.M. Chung, G.P. Lee, and Y. Park (2020) Assessment of the current infraspecific classification scheme in melon (Cucumis melo L .) based on genome wide single nucleotide polymorphisms. Horticulture, Environment, and Biotechnology 61: 537–547

Jurgens, and Gerd (May 19, 1995). "Axis formation in plant embryogenesis: cues and clues". Cell. 81 (4): 467–470.

Kippes N, Debernardi JM, Vasquez-Gross H, Akpinar BA, Hikmet B, Kato K, Chao S, Akhunov E, Dubcovsky J (2015). Identification of the *VERNALIZATION 4* gene reveals the origin of spring growth habit in ancient wheats from South Asia. PNAS 112, E5401-E5410.

Kishi, Y. & Fujishita, N., 1969. Studies on the interspecific hybridization in the genus Cucumis I Pollen germination and pollen tube growth in selfings and incompatible crossings. Journal of the Japanese Society for Horticultural Science, 38(4), pp.329–334.

Kishi, Y. &Fujishita, N., 1970. Studies on the interspecific hybridization in the genus Cucumis. II. Pollen tube growth, fertilization, and embryogenesis of post-fertilization stage in incompatible crossing. Jap Soc Hort Sci J.

Kitamura, S. (1950). Notes on *Cucumis* of Far East. Acta Phytotax et Geobot, 14, 41 - 44.

Kopelman, K., Naama, M., Mayzel, J., Jakobsson, M., Rosenberg, N.A., Mayrose, I. (2015) CLUMPAK: a program for identifying clustering modes and packaging population structure inferences across *K*. Mol. Ecol. Resour., 1179 – 1191

Kraus, J.E., Handro, W. & Dietrich, S.M.C., 1981. Growth, peroxidase activity, and protein content in stem pith and callus tissues from haploid and diploid Nicotiana tabacum plants. Physiologia Plantarum, 51(2), pp.157–162.

Lopez-Sese AI, Staub JE, Gomez-Gullamon ML (2003). Genetic analysis of Spanish melon (*Cucumis melo* L.) germplasm using a standardized molecular marker array and reference accessions. Theor Appl Genet 108: 41-52.

Luan, F., Delannay I. and Staub J.E. (2008) Chinese melon (*Cucumis melo* L.) diversity analyses provide strategies for germplasm curation, genetic improvement, and evidentiary support of domestication patterns. *Euphytica* 164: 445–461.

Malik, A. A., Vashisht, V.K., Singh, K., Sharma, A., Singh, D. K., Singh, H., Monforte, A. J.,

McCreight, J. D., Dhillon, N. P. S. (2014). Diversity among melon (*Cucumis melo* L.) landraces from the Indo-Gangetic plains of India and their genetic relationship with USA melon cultivars. Genet Resour Crop Evol, 61, 1189 - 1208.

Mallick, M.F. R. and Masui, M. (1986). Origin, distribution and taxonomy of melons. Scientia Horticulturae, 28 (3), 251 - 261.

Matsumoto, Y. et al., 2012. Temperature-dependent enhancement of pollen tube growth observed in interspecific crosses between wild Cucumis spp. and melon (C. melo L.). Scientia Horticulturae, 138, pp.144–150.

Matsunaga H, Matsushima K, Tanaka K, Theavy S, Heng SL, Channa T, Takahashi Y, Tomooka N (2015). Collaborative exploration of the Solanaceae and Cucurbitaceae vegetable genetic resources in Cambodia, 2014. AREIPGR, 31, 169 〜 187.

McCreight, J. D. (2004). Isozyme variation in Indian and Chinese melon (*Cucumis melo* L.) germplasm collections. J. AMER. SOC. HORT. SCI, 129 (6), 811 - 818.

Mliki A, Staub JE, Sun Z, Ghorbel A (2001). Genetic diversity in melon (*Cucumis melo*L.): an evaluation of African germplasm. Genet Resour Crop Evol 48: 587-597.

Monforte AJ, Garcia-Mas J, Arus P (2003). Genetic variability in melonbased on microsatellite variation. Plant Breed 122: 153-157.

Monforte, A.J., Aurora Diaz, Ana Caño-Delgado, and Esther Van Der Knaap (2014) The Genetic Basis of Fruit Morphology in Horticultural Crops: Lessons from Tomato and Melon. Journal of Experimental Botany 65(16): 4625–3

Murashige, T. & Skoog, F., 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum, 15(3), pp.473–497.

Murray, G.C. and Thompson W. F. (1980) Rapid isolation of high molecular weight DNA.

Nucleic Acids Res. 8, 4321 - 4325.

Murray, M.G. & Thompson, W.F., 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research, 8(19), pp.4321–4326.

Nakata E, Staub JE, Lopez-Sese Al, Katzir N (2005). Genetic diversity of Japanese melon cultivars (*Cucumis melo* L.) assessed by random amplified polymorphic DNA and simple sequence repeat markers. Genet Resour Crop Evol 52: 405-419.

Nei, M. (1972) Genetic distance between populations. Am. Nat. 106, 283 - 292.

Nhi, P. T. P., Akashi, Y., Hang, T. T. M., Tanaka, K., Aierken, Y., Yamamoto, T., Nishida, H., Long, C., Kato, K. (2010). Genetic diversity in Vietnamese melon landraces revealed by the analyses of morphological traits and nuclear and cytoplasmic molecular markers. Breeding Science, 60, 255 - 266.

Norton, J.D. &Granberry, D.M., 1980. Characteristics of progeny from an interspecific cross of Cucumis melo with C. metuliferus. Journal of the American Society for Horticultural Science, 105(2), pp.174–180.

Nuñez-Palenius HG, Gomez-Lim M, Ochoa-Alejo N, Grumet R, Lester G, Cantliffe DJ (2008). Melon fruits: Genetic diversity, physiology, and biotechnology features. Crit Rev Biotechnol. 28, 13-55.

Olezak, H, Marcinkowska and Niemirowicz, K. 2011. The genetic basis of resistance to downy mildew in Cucumis spp. latest developments and prospects. J.Appl gen. 52, pp. 249-255.

Pitrat, M. (2008). Melon. In Prohens, J. and Nuez, F.[eds], Handbook of plant breeding – Vegetables I: *Asteraceae*, *Brassicaceae*, *Chenopodiaceae*, and *Cucurbitaceae*, 238 - 317.

Pitrat M (2016). Melon genetic resources: Phenotypic diversity and horticultural taxonomy. In:

Grumet, R., N. Katzir and J. Garcia-Mas (eds.) Genetics and genomics of Cucurbitaceae, Springer, Cham, pp. 25-60.

Pourkheirandish M, Hensel G, Kilian B, Senthil N, Chen G, Sameri M, Azhaguvel P, Sakuma S, Dhanagond S, Sharma R, Mascher M, Himmelbach A, Gottwald S, Nair S, Tagiri A, Yukuhiro F, Nagamura Y, Kanamori H, Matsumoto T, Willcox G, Middleton CP, Wicker T, Walther A, Waugh R, Fincher GB, Stein N, Kumlehn J, Sato K, Komatsuda T (2015). Evolution of the grain dispersal system in barley. Cell 162, 527-539.

Pritchard, J.K., Stephens, M., Donnelly, P. (2000). Inference of population structure using multi-locus genotype data. Genetics, 155, 945 - 959.

Raghami, M., Lopez-Sese Al, Hasandokht, Z.Z., Moghadam, M.R.F. and Kashi, A. (2014). Genetic diversity among melon accessions from Iran and their relationships with melon germplasm of diverse origins using microsatellite markers. Plant Syst Evol 300: 139-151.

Raghavan, 1966. Nutrition, growth, and morphogenesis of plant embryos. Biol. Rev, 41, pp. 1-58.

Ritschel PS, Lins TCL, Tristan RL, Buso GSC, Buso JA, Ferreira ME (2004). Development of microsatellite markers from an enriched genomic library for genetic analysis of melon (*Cucumis melo* L.). BMC Plant Biol. 4: 9.

Robinson, R.W. & Decker-Walters, D.S., 1997. Cucurbits., Cab international.

Sebastian, P., Schaefer, H., Telford, I. R. H., & Renner, S. S. (2010). Cucumber (*Cucumis sativus*) and melon (C. *melo*) have numerous wild relatives in Asia and Australia, and the sister species of melon is from Australia. Proceedings of the National Academy of Sciences, 107(32), 14269 - 14273.

Serres-Giardi, L. and Dogmont, C. (2012). How microsatellite diversity helps to understand the domestication history of melon. In N. Sari, I. Solmaz, and V. Aras [eds.], Cucurbitaceae

2012. In Proceedings of the Xth EUCARPIA meeting on genetics and breeding of Cucurbitaceae, 254 - 263, University of Cukurova, Antalya, Turkey.

Shen, X., Gmitter, F.G and Grosser, J.W, 2011. Immature embryo rescue and culture. J. of Plant Embryo culture. pp 75-92.

Staub JE, Lopez-Sese Al, Fanourakis N (2004). Diversity among melon landraces (*Cucumis melo* L.) from Greece and their genetic relationships with other melon germplasm of diverse origins. Euphytica 136: 151-166.

Stepansky, A., Kovalski, I., and Perl-Treves, R. (1999). Intraspecific classification of melons (*Cucumis melo* L.) in view of their phenotypic and molecular variation. Plant Systematics and Evolution, 217, 313 - 333.

Takada K (1979). Studies on the breeding of melon resistant to cucumber mosaic virus. III. Inheritance of resistance of melon to cucumber mosaic and other characteristics. Bulletin of the Vegetable and Ornamental Crops Research Station. Series A 5: 71-79 (in Japanese with English summary).

Takada K (1983). Breeding and characteristics of disease-resistant melon varieties (Lines 'Anȯ No. 1, No. 2 and No. 3'). Bulletin of the Vegetable and Ornamental Crops Research Station. Series A 11: 1-22 (in Japanese with English summary).

Tanaka K, Nishitani A, Akashi Y, Sakata Y, Nishida H, Yoshino H, Kato K (2007). Molecular characterization of South and East Asian melon, *Cucumis melo* L., and the origin of Group Conomon var. *makuwa* and var. *conomon* revealed by RAPD analysis. Euphytica, 153, 233 - 247.

Tanaka, K., Akashi, Y., Fukunaga, K., Yamamoto, T., Aierken, Y., Nishida, H., Long, C., Yoshino, H., Sato, Y., Kato, K. (2013). Diversification and genetic differentiation of

cultivated melon inferred from sequence polymorphism in the chloroplast genome. Breeding Science, 63, 183 - 196.

Tanaka, K., Duong, T. T., Yamashita, H., Lay Heng, S., Sophany, S., Kato, K. (2016). Collection of Cucurbit crops (*Cucurbitaceae*) from Eastern Cambodia, 2015. AREIPGR, 32, 109 - 137.

Tanaka, K., Shigita, G., Sophea, Y., Thun, V., Sophany, S., Kato, K. (2017). Collection of melon and other Cucurbitaceous crops in Cambodia in 2016. AREIPGR, 33, 175 - 205.

Tanaka K, Shigita G, Dung TP, Sophea Y, Thun V, Sophany S, Kato K (2020). Collection of melon and other Cucurbitaceous crops in Cambodia in 2017. AREIPGR, 35, 121-146.

Weir, B.S. (1996). Genetic data analysis II. Sinauer Associate Inc. Publisher, Massachusetts.

Whittaker, T. W., and G.N. Davis (1962). Cucurbits: botany, cultivation, and utilization. Inter-science, NY, USA.

Yan L, Helguera M, Kato K, Fukuyama S, Sherman J, Dubcovsky J (2004). Allelic variation at the *VRN-1* promoter region in polyploid wheat. Theor. Appl. Genet. 109, 1677-1686.

Yashiro, K., Iwata, H., Akashi, Y., Tomita, K., Kuzuya, M., Tsumura, Y., Kato, K. (2005). Genetic relationship among East and South Asian melon (*Cucumis melo* L.) revealed by AFLP analysis. Breeding Science, 55, 197 - 206.

Yi, S. S., Akashi, Y., Tanaka, K., Cho, T. T., Khaing, M. T., Yoshino, H., Nishida, H., Yamamoto, T., Win, K., Kato, K. (2009). Molecular analysis of genetic diversity in melon landraces (*Cucumis melo* L.) from Myanmar and their relationship with melon germplasm from East and South Asia. Genet Resour Crop Evol, 56, 1149 - 1161.

Zitter, T.A., Hopkins, D.L. & Thomas, C.E., 1996. Compendium of cucurbit diseases. St. Paul, MN, itd: American Phytopathological Society Press.