Studies on Diversity and Evolutionary Relationship among South Asian Germplasms of *Cucumis sativus* and *C. melo*

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General Introduction

Cucumber, *Cucumis sativus* ($2n=2x=14$) and melon, *Cucumis melo*($2n=2x=24$) are two common vegetable crops worldwide, which are both within the 20 most important vegetables (FAO, 2008). Cucumber is also an ideal system for studying several important biological processes such as sex determination (Tanurdzic *et al.*, 2004; Wang *et al*., 2010), gene transfer among nuclear and organellar genomes (Havey, 1997; Alverson *et al.*, 2011), and phloem physiology (Lough and Lucas, 2006; Zhang *et al.*, 2010a). Within the genus *Cucumis*, two groups (African group and Asian Group) are generally accepted, they are defined by geographic origin and chromosome number. Geographically, Chromosome number of the African group is $2n=2x=24$. Africa that contains the most numerous wild species was assumed to the origination of *Cucumis, melo* (Kirkbride *et al*, 1993). *Cucumis sativus* is indigenous to South Asia and estimated to be domesticated about 300 years ago, soon was cultivated in the South and East of the Himalayas. Diversity in *Cucumis sativus*, the Asian group, is not such abundant as in *Cucumis melo*, the African group. Cucumber, *Cucumis sativus* is thought to be the representative of Asiatic group with the chromosome number $2n=14$ (Jeffrey, 1980; Whitaker and Davis, 1962). Recent study proposed some different opinions about the origination of *Cucumis*. It was supposed that melon and cucumber are both of Asian origin and have numerous previous overlooked species-level relatives in Australia and around the Indian Ocean (Patrizia *et al.*, 2010).

There are thirty species within *Cucumis, melo*, but only two species within *Cucumis, sativus* (*C. sativus* and *C. hardwickii*). Interestingly, *Cucumis hystrix* which is the only $2n=2x=24$ species native to Asia has the same chromosome number with *Cucumis melo*, but morphological and biochemical characteristics similar to *Cucumis sativus*. It is thought to be the only species of *Cucumis* with the chromosome number of $2n=2x=24$ in Asia and a possible bridge between cucumber and melon (Kirkbride *et al.*, 1993; Zhang L. B. *et al*., 2006). Also, *Cucumis hystrix* is cross-incompatible with *Cucumis sativus*, which narrows the genetic background of cucumber breeding. So, investigation on the diversity and genetic relationship of some new *Cucumis* germplasms could be a help for the benefit of breeding program and evolutionary explanation.

The diversity of *Cucumis sativus* is relatively low comparing with other cross-fertilized species of *Cucumis,* such as *Cucumis melo.* Genetic erosion has resulted in a depletion of both the number of crop species and the genetic diversity expressed by the amount of genetic variation within a species (Frankel, 1972; Harlan, 1975). In the early part of this century, the U.S. government undertook a plant collection expedition to acquire vegetable germplasm including *Cucumis* from Asia. Since 1953, cucumber (*Cucumis sativus* and *Cucumis hardwichii*) germplasm donated by international public and private sources formatted an U.S National Plant Germplasm System (NPGS). In 1996, genetic variation within the U.S. cucumber collection was assessed by examining the variation of 21 polymorphic isozyme loci (Melic *et al.*, 1996). Then genetic variation in cucumber (*Cucumis sativus*) accessions from India was assessed by examining at the same 21 polymorphic isozyme loci. The results were compared with what examined

previously and different accessions were grouped with their relationships (Staub *et al*., 1997). Furthermore, 67 cucumber (*Cucumis sativus*) accessions from China were assessed with the similar method. Comparing all the accessions, it was concluded that Chinese and Indian accessions represented the most diverse genetic variation in the NPGS cucumber collection, and further collection in Indian and China would be strategically important for increasing genetic diversity among the cultivars (Staub *et al.*, 1997).

In the case of melon, the genetic diversity among 67 Japanese melon cultivars was evaluated by RAPD and SSR markers in 2005. These cultivars were clustered to three horticulture variety groups: Group Cantalupensis, Group inodorus, and Group conomon (Nakata *et al.,* 2005). In China, the important secondary center of diversity, the genetic diversity was firstly assessed in 2008. Sixty-eight Chinese *Cucumis melo* cultivars were evaluated by RAPD markers. The results supported the previously described theoretical melon domestication patterns constructed from historical and archeological (Luan *et al.,* 2008).

Karyotype analysis is a basic method for cytogenetic study, which describes the overall nature of the chromosome morphology. FISH is a powerful cytological tool for integration of genetic and physical pattern (Jiang and Gill, 2006). In the early stage, because of the small sizes of the mitotic chromosomes, similar chromosome lengths or/and arm ratios and poor dyeing, cytological studies of cucumbers appeared much discrepancy among the experiments (Dane *et al*., 1991; Ramachandran and Seshadri, 1986; Trivedi and Roy, 1970). With the proceeding of *Cucumis* study, some important

work have been done, such as achievement of chromosome identification of cucumber and melon (Chen *et al.,* 1998; Hoshi *et al*., 1998) and cucumber genome sequence program (Huang S. *et al*., 2009). The tandem satellite DNA sequences locationg at specific chromosome positions such as centromeric, subtelomeric or intercalary, are adopted as probes of FISH. These tandem repeat sequences not only made karyotype analysis easily, comparison of the diverse distribution of the repetitive sequences also revealed their phylogenetic and genetic relationships (Liu *et al*., 2010; Zhao *et al*., 2010). Here, 4 types of tandem repetitive DNAs sequence (Type I, II, III and IV) were adopted for study these South Asian germplasm and some Japanese cucumber cultivars.

Geographically, all the accessions studied in this work, are from south Asia which is thought to be the first center of diversity in *Cucumis*. Accessions were evaluated in this study are just from south Asia, one of places representing the most diverse genetic variation. The results could be a reference for breeding improvement.

Besides providing karyotype analysis of Indian cucumber cultivar and Japanese cucumber cultivar, comparison of the distribution of the repetitive DNA sequences also help to establish the basis of the evolutionary relationships within *Cucumis* varieties.

Chapter 1

MORPHOLOGICAL AND MOLECULAR DIVERSITY AMONG SOUTH ASIAN GERMPLASMS OF *CUCUMIS SATIVUS* AND *CUCUMIS MELO*

1.1 Introduction

Cucumber, *Cucumis sativus* (2*n*=2*x*=14), and melon, *Cucumis melo*(2*n*=2*x*=24), are two common vegetable crops worldwide, which are both within the 20 most important vegetables (FAO, 2008). Cucumber is also an ideal system for studying several important biological processes such as sex determination (Tanurdzic *et al.,* 2004; Wang *et al*., 2010), gene transfer among nuclear and organellar genomes (Havey, 1997; Alverson *et al.*, 2011), and phloem physiology (Lough and Lucas, 2006; Zhang *et al.*, 2010a). Within the genus *Cucumis*, two groups (African group and Asian Group) are accepted (Dilson, 2002). They are defined by geographic origin and chromosome number. Geographically, Chromosome number of the African group is 2*n*=2*x*=24. Africa, which contains the most numerous wild species, was assumed to be the place of origin of *C. melo* (Kirkbride *et al.*, 1993). *C. sativus* is indigenous to South Asia and estimated to be domesticated about 300 years ago, and was soon cultivated in the South and East of the Himalayas. Diversity in *Cucumis sativus*, the Asian group, is not as abundant as in that *C. melo*, the African group. Cucumber, *Cucumis sativus,* is thought to be the representative of Asian group with the chromosome number 2*n*=14 (Jeffrey, 1980; Whitaker and Davis, 1962). On the other hand, a recent study proposed a different opinion about the origin of *C. melo* and *C. sativus*. It was supposed that melon and cucumber are both of Asian origin and have numerous previous overlooked relatives in Australia and around the Indian Ocean which were overlooked previously (Patrizia *et al.*, 2010).

It was known that there are thirty subspecies in *C. melo*, but only two in *Cucumis sativus* (*C. sativus* var *sativus* and *C. sativus* var *hardwickii*). *C. sativus* var *hardiwickii* (2*n*=14) is a wild, sympatric botanical variety of *C. sativus* var. *sativus,* which grows in the foothills of Himalayas mountains. This botanical variety has the multiple fruiting and branching habits which are not present in *C. sativus* var *sativus*. *C. sativus* var *hardwickii* is also cross-compatible with *C. sativus*. var *sativus.* Therefore, *C. sativus.* var *hardiwickii* represent the extreme in variation in *C. sativus* and has potential for increasing the genetic diversity available for breeding commercial cucumber (Staub *et al*., 1997).

Cucumis melo is thought to contain the most diverse varieties in the genus *Cucumis* (Robinson and Decker-Walters, 1999). Significant morphological variations exist in fruit traits such as color, size, shape, texture, and taste. In 2005, 67 Japanese melon cultivars were evaluated by RAPD and SSR markers. These cultivars were clustered to three horticulture variety groups: Group Cantalupensis, Group inodorus, and Group conomon (Nakata *et al.,* 2005). In China, the important secondary center of diversity, the genetic diversity of *C. melo* was firstly assessed in 2008. Sixty-eight Chinese *C. melo* cultivars were evaluated by RAPD markers (Luan *et al.,* 2008). The results indicated that Chinese accessions are a rich resource of genetic diversity for plant improvement. Further, to show the genetic diversity of Chinese Hami Melon, 120 melon accessions, including 24 accessions of Hami melon, were analyzed by RAPD markers and Hami melon were classified as the large-seed type (Yasheng Aierken *et al.,* 2011). In *Cucumis sativus*, 16 selected cucumber cultivars were divided into four groups by evaluating the traits related

with fruit flavor such as taste, aroma and dry matter and the correlation of these traits was found in 2008 (Behera *et al.,*2008). Recently, a high density (97.5% of chromosome were covered) genetic map of cucumber was constructed, which was composed of 1,369, including 1,152 SSR, 192 SRAP, 21 SCAR and one STS loci (Zhang W. *et al.*, 2010). Genetic diversity assessments and linkage map construction can increase the effectiveness of breeding programs (Fan *et al*., 2006).

Elucidating the phylogenetic relationships in the genus *Cucumis* is of great importance, because the closest relatives and natural composition can provide valuable information to improve melon and cucumber breeding (Chung *et al*., 2006). By analyzing combined chloroplast sequences for 123 of the 130 genera of Cucurbitaceae, it was concluded that *Cucumis sativus* evolved within an Austrilian/Asian clade and is phylegenetically far from *C. melo* than the current morphological classification (Susanne *et al.,* 2007). Recently, by using DNA sequences from plastid and nuclear markers for 100 accessions from Africa, Australia, and Asia, it was shown that melon and cucumber are both of Asian origin and have some species-level relatives in Australia which were overlooked previously and further investigation of wild species should concentrate on Asia and Australia (Patrizia *et al*., 2010). Here, 18 accessions (*C. sativus* and *C. melo*) were collected from South Asian for wild species investigation. It is expected to supply more evidence for elucidating the phylogenetic relationships in the genus Cucurbitaceae. Meanwhile, morphological descriptions of these accessions enriched the genetic diversity of *Cucumis sativus* and *C. melo*.

1.2 Materials and Methods

1.2.1 Plant materials

The materials used in this study comprised of eighteen genotypes of cucumber and melon collected from South Asian countries, most of which are from India (Table 1.1). The field work was conducted at College Orchard, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, India. All of the eighteen germplasms were planted with two replications inside the shade net house and each replication had eight plants. All the recommended crop production and protection measures were adopted for raising a healthy crop.

1.2.2 Evaluation of quantitative and qualitative characters

Morphological characters were recorded using five plants in each replication according to combined standards as described by National Bureau of Plant Genetic Resources (NBPGR). In total, 32 traits including nine quantitative and 23 qualitative characters were recorded, which are shown in Table 1.2 with their abbreviations.

1.2.2.1. Quantitative characters

a) Primary branches per plant: The number of branches borne on the main stem was counted and expressed as primary branches per plant.

b) Internodal length: The length between two subsequent nodes was measured from 10th to 15th node on the main vine and the mean expressed in centimeters.

c) Leaf petiole length: The length was measured in five fully developed leaf petioles from the base of attachment with the stem to the point where the leaf intersects petiole and the mean expressed in centimeters.

d) Leaf length: The length was measured in five fully developed leaves from the tip of the leaf to the point where the leaf intersects petiole and the mean expressed in centimeters.

e) Leaf width: Those leaves used for recording the length were used for measuring the width at the broadest place of the leaf lamina and the mean expressed in centimeters.

f) Peduncle length: The length was measured in five randomly selected fruits at fully ripe stage from the base of attachment with the stem to the point where the fruit intersects peduncle and the mean expressed in centimeters.

g) Fruit length: The length was measured in five randomly selected fruits at fully ripe stage from stalk end to the tip of the fruit and the mean expressed in centimeters.

h) Fruit girth: Those fruits used for recording the length were used for measuring the girth at the middle portion and the mean expressed in centimeters.

i) Fruit weight: The weight of five randomly selected fruits at fully ripe stage was recorded and the mean expressed in grammes.

1.2.2.2. Qualitative characters

a) Plant growth: (3=determinate, 5=semi-determinate and 7=indeterminate)

b) Stem shape: (3=round, 5=angular and 99=others)

c) Stem color: (1=yellow, 3=light green, 5=green, 7=dark green and 99=others)

d) Stem pubescence density: (3=sparse, 5=medium and 7=dense)

e) Stem pubescence type: (3=soft, 5=intermediate and 7=hard)

f) Leaf shape: (1=orbicular, 2=ovate, 3=elliptical, 4=reniform, 5=cordate, 6=triangular, 7=entire, 8=trilobite, 9=pentalobate and 99 = others)

g) Leaf margin: (1=crenate, 2=denticulate, 3=entire, 4=lobate, 5=serrate, 6=serrulate, 7=spiny, 8=undulate and 99=others)

h) Leaf color: (3=light green, 5=green, 7=dark green and 99=others)

i) Leaf glossiness: (3=dull, 5=intermediate and 7=glossy)

j) Leaf pubescence density: (3=sparse, 5=medium and 7=dense)

k) Leaf pubescence type: (3=soft, 5=intermediate and 7=hard)

l) Flower color: (1=white, 3=cream, 5=yellow, 7=orange and 99=others)

m) Fruit shape: (1=globular, 2=flattened, 3=oblate, 4=elliptical, 5=pyriform, 6=ovate, 7=acorn, 8=elongated and 99=others)

n) Stem end fruit shape: (1=depressed, 3=flattened, 5=rounded and 7=pointed)

o) Blossom end fruit shape: (1=depressed, 3=flattened, 5=rounded and 7=pointed)

p) Fruit skin lustre:(3=matt, 5=intermediate, 7=glossy and 99=others)

q) Fruit skin texture: (3=smooth, 5=rough, 7=spiny and 99=others)

r) Fruit skin hardness:(3=soft, 5=intermediate, 7=hard and 99=others)

s) Predominant fruit skin color: (1=white, 2=yellow, 3=cream, 4=pale green, 5=green, 6=dark green, 7=orange, 8=brown, 9=grey and 99 = others)

t) Secondary fruit skin color: (0=absent, 1=white, 2=yellow, 3=cream, 4=pale green, 5=green, 6=dark green, 7=orange, 8=brown, 9=grey and 99=others)

u) Secondary fruit skin color pattern (0=absent, 1=spotted, 3=striped, 5=netted, 7=short streaked, 9=long streaked and 99=others)

v) Fruit mesocarp (flesh) color (1=white, 3=cream, 5=green, 7=orange and 99=others)

w) Seed color (1=white, 3=cream white, 5=yellow, 7=light-brown and 99=others)

1.2.3 Extraction of DNA

Genomic DNA was extracted from fresh leaves. Seeds were sown on filter paper in petri dish and grown in room temperature. A-week-old seedlings were ground by using mortar and pestle in liquid nitrogen to fine powder and genomic DNA was extracted by using the CTAB method (Doyle and Doyle, 1987). DNA samples were diluted to 5 ng/ µl for polymerase chain reaction (PCR) amplification.

1.2.4 *rbcL* and *matK* gene Sequencing

PCR amplification was performed in a 10 μL mixture containing: 5 ng genomic DNA, 0.01 μmol primer, 0.4 mmol dNTP, 0.05 U ExTaq (TaKaRa, Japan), 1x PCR Buffer. PCR reaction was performed in Takara PCR thermal cycler (Takara, Japan) and the cycle can be described as an initial denaturing step at 95℃ for 5 min, then 30 cycles of at 95℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 25 sec. The final extension step was at 72℃ for 10 min. The PCR products were subjected to sequencing by using ABI 3130 Genetic Analyzer. Data were collected using an ABI PRISM 7000 sequence detection system (Applied Biosystems, http://www.appliedbiosystems.jp) and analyzed according to the instruction manual. Primers used in PCR and shotgun sequencing are shown in Table 1.3. Consensus sequences were assembled and edited by using the program GENETYX-MAC version 14 (Genetyx, Japan) followed by manual adjustments.

1.2.5 Data analysis

Principle Component Analysis (PCA) and K-means cluster analysis was performed by using R version 2.15.0 for statistical computing. Phylogenetic analysis was performed under Neighbor-joining (NJ) and maximum likelihood (ML) methods. Sequences of *rbcL* and *matK* were aligned with “clustalw2” on the website: http://www.ebi.ac.uk/Tools/msa/clustalw2/. The resulting aligned data matrix was prepared in MEGA5.05. Bootstrap method is taken for the test of phylogeny. The number of bootstrap replications was 1000. The model of NJ tree was Maximum Composite Likelihood, and the model of ML tree was Tamura-Nei model.

1.3 Results and Discussion

1.3.1 Quantitative and qualitative characterization

Fruit appearances of eighteen accessions are shown in Fig. 1.1, and their morphological performance are shown in Table 1.4 and 1.5. For the nine quantitative characters, there was a large divergence among the values of fruit weight. *C. melo* 'N78' and *C. melo* 'Andaman' had the biggest fruit weights 1,390 g and 1,350 g, respectively. These two values exceeded all the other accessions ranging from 210 g of *C. sativus* 'Long Green' to 850 g *C. melo* 'Theni'. *C. melo* cultivars had bigger fruit weights than those of *C. sativus* cultivars. *C. melo* accessions were almost above 500 g and the mean was 825 g. On the other hand, *C. sativus* accessions were 420 g on average, and 400 g less than *C. melo*. The exceptions were *C. melo* 'Mysore' and *C. sativus* 'AAUC-2', being the lightest *C. melo* and the heaviest *C. sativus*, with the weight of 290 g and 790 g, respectively (Table 1.4). Lopez-Sese *et al.* (2003) reported that the mean fruit weight for a collection of 125 Spanish accessions was 1.5 kg, while the mean fruit weight of Villaconejos landraces also from Spain was 2.08 kg. It was also reported the variability in fruit weight among Spanish varieties was 0.8 to 4.95 kg (Escribano and Lâzaro, 2008). The range of fruit weight in the present study was 0.29 to 1.39 kg, not as heavy as the Spanish group. The difference might be due to the different collection region, as well as the different breeding process in the long history of cultivation between the Mediterranean region and South Asian region.

The other eight quantitative traits didn't show significant difference as fruit weight.

We divided these traits into two groups, namely, fruit-related traits and stem and leaf-related traits. The first group included fruit length, fruit girth and peduncle length. According to the results of fruit girth and length, it was shown that the fruit size of *C. melo* was bigger than *C. sativus* except one cucumber accession 'Shantung Thorny', which was bigger than *C. melo* 'Mysore'. 'CM-1' and 'Andaman' were the biggest ones in melon accessions. The second group were the traits including leaf width, leaf length, No. of primary branches, intermodal length and leaf petiole length, which showed smaller variance than fruit-related characters.

By Principle Component Analysis (PCA) and K-means cluster analysis, eight quantitative values were covered by three principle components (PC1, PC2 and PC3). Contribution of variables in each principle components was calculated (Table 1.6). Fruit length and fruit girth dominated in PC1 and PC2, respectively. Five characters, No. of primary branches, internodal length, leaf petiole length, leaf length and leaf width, had the similar contribution to PC3. Based on the result of principle component analysis, eighteen accessions were classified into three groups (Fig. 1.3). Group1 contained nine accessions (seven *sativus* accessions: AAUC-2, Perundurai, Nepal Local, IC-4079, Cus125/71, Long Green, and Nanhai2 Aouri and two *melo* accessions: Olakkur and Mysore) and Group2 contained six accessions (two *sativus* accessions: Shantung Thorny, JL-3 Dhillon, and four *melo* accessions: Andaman, N-78, Pudukkottai and Mayilam). Group3 contained three *C. melo* accessions (CM-1, Theni, and Muskmelon Orange). The relative dispersion among accessions within Group2 was greater than the other two. Group3 contained the least number of accessions, i. e., *C. melo* cultivars only. Group1

was mainly constituted of *C. sativus* accessions, while Group2 and Group3 contained most of *C. melo* accessions. The dispersion among *C. melo* accessions was greater than that among *C. sativus* accessions.

Characteristics on twenty-three qualitative traits of eighteen accessions are shown in Table 1.5. Traits observed were varied from stem characters, leaf characters, fruit characters and seed characters. Same growth patterns were shown in all the plants observed and all of them were indeterminate. For stem characters, all of the accessions had angular stems with different degrees of green color. 'Long Green' was the only one with light green stem, which was lighter than the others. Besides, 'AAUC-2' displayed sparse stem pubescence and 'JL-3 Dhillon', 'Nepal Local', 'Perundurai' and 'IC-4079' showed dense stem pubescence. Other accessions were between them. For leaf characters, five types of leaf shape were recorded, namely, entire, cordate, triangular, reniform and pentalobate. Among them, the entire shape was common, including ten accessions showing entire leaves with spiny margin. All accessions had green leaves with different density, and 'Perundurai' was the only one without leaf glossiness. Pubescence was also observed on leaves as on stems in all accessions, and the leaf pubescence was softer than those on stem. The pubescence trait is closely related to plant defense system. It is under investigation for map-based cloning by SSR markers (Cai *et al*., personal communication). The biggest diversity was appeared on the fruit-related characteristics, as shown with the results of quantitative characteristics. Except 'AAUC-2', the fruit shapes of all the *C. sativus* accessions were elongated. For *C. melo* accessions, fruit shapes of 'Mayilam' and 'CM-1' were pyriform. 'Mysore' was the only elliptical and

'Muskmelon Orange' and 'Theni' were globular. Fruit characters were further recorded on fruit skin luster, texture and hardness, which are related to the appearance and taste. 'Nanhai 2 Aouri', 'AAUC-2', 'IC-4079', and 'Mysore' did not have fruit skin luster. Fruit skin textures of all the *C. melo* accessions were smooth and those of *C. sativus* acceesions were rough or spiny. Three *C. melo* accessions, 'Olakkur', 'Theni', and 'Andaman' had soft fruit skins. 'Nepal local', 'N-78', and 'Mysore' had harder fruit skin than 'Olakkur', 'Theni', and 'Andaman', but softer than the others. Besides, all of accessions had yellow flowers and white or cream seeds, expect that the seeds of 'Long Green' were green.

1.3.2 Phylogenetic analysis

The lengths of *matK* and *rbcL* genes ranged 750-1067 bp, sequence were submitted to DNA Data Base of Japan (DDBJ). The aligned data matrix combined with *matK* and *rbcL* gene was 1,728 bp long. Based on the aligned matrix, phylogenetic trees were made by Neighbor-joining and Maximum Likelihood method (Figs. 1.4 and 1.5).

On the topology of *C. sativus*, 'JL-3 Dhillon' showed divergence from other accessions. Fruit morphologies of 'JL-3 Dhillon', the longest fruit length and yellow fruit skin, differed from other *C. sativus* (Tables 1.4 and 1.5). In a previous phylogenetic study of *Cucumis*, some special morphological traits were circumscribed within some certain clades (Sebastian *et al*., 2010). Species of the Asian/Australian clade had more of less ellipsoid fruits that stay green or turn yellow-orange at maturity. *C. ritchiei/C. maderaspatanus* clade was characterized by smooth and round fruits that turn red at maturity (Sebastian *et al*., 2010). Also here in the present study, this correlation between

morphological traits and clade divergence at molecular level was shown in the case of 'JL-3 Dhillon'.

CGN23965 'I.C 4079' has the scientific name *C. sativus* group *Gherkin*, while CGN24494 'Nepal local' has the scientific name *C. sativus* var. *sikkimensis* (TANU and CGN). In the phylogenetic tree, 'I.C 4079' and 'Nepal local' did not show big divergence with other accessions, both of them were nested in the same clade. Two Chinese accessions 'Cus 125/71' and 'Nanhai 2 Aouri' were nested in the same clade as well as the accessions from different areas, India and Nepal. One of reasons for narrow molecular divergence could be due to the accessions derived from narrow areas of India and Himalaya.

According to the previous works and present study, comparing with *C. sativus,* genetic diversity in *C. melo* seems to be much bigger. African melon group has 30 species that is divided into six subgroups (Kirkbride, 1993), and ancient melon varieties were distributed throughout South Asia, which could be the first genetic diversity center (Robinson and Decker, 1997). *C. melo* accessions studied in the present study were mainly introduced from three institutions, namely, India Institute of Vegetable Research (IIVR), Tamil Nadu Agriculture University (TNAU) and National Bureau of Plant Genetic Resources (NBPGR), USA. They distributed all around India, but in the phylogenetic tree, the accessions from different places nested in the same clade with each other. A big divergent nodes was found with *C. melo* 'CM1', branching with the other accessions. Special morphological traits were also found in accession 'CM1', such as the longest intermodal length and unique fruit color (Fig. 1.2). 'Muskmelon Orange' branch

was close to 'CM1', and they were grouped into Cluster 2 in the cluster analysis (Fig. 1.3).

The fruit shapes of 'Muskmelon Orange' and 'Theni' were globular, being distinguished from the other *C. melo* accessions. This discrepancy was not supported by the phylogenetic results. Besides, 'Mayilam' branch was the biggest divergent node. It shared the same group with 'N-78" in cluster analysis in spite of that they were far from each other in phylogenetic trees. So in *C. melo*, correlations between morphological traits and phylogenetic tree were not as supportive as those in *Cucumis sativus*. It could be concluded that *C. melo* holds bigger diversity in morphology than *C. sativus*, which has caused more complex factors for analyzing phylogenetic relationships.

In conclusion, eighteen *Cucumis* accessions derived from South Asia, where represents the most diverse genetic resources, were evaluated morphologically and at the molecular level. It was shown that, although large morphological diversity was found among *C. melo* and *C. sativus* accessions, genetic diversity based on the molecular analyses showed larger diversity in *C. melo* than in *C. sativus*. Thus, it would be necessary to investigate phylogenetic relationships at the molecular level among *Cucumis* species using accessions collected more widely in Asia, Africa and Australia continents.

1.4 Tables and Figures

Table 1.1. Cucumber and melon accessions in this study

Acc. No.	Germplasm	Sp.	Source	Country
1	AAUC 2	*C. sativus*	IIVR	India
2	Andaman	*C. melo*	TNAU	India
3	CM 1	*C. melo*	TNAU	India
4	Cus 125/71	*C. sativus*	CGN	China
5	IC 4079	*C. sativus*	CGN	India
6	JL 3 Dhillon	*C. sativus*	CGN	India
7	Long Green	*C. sativus*	CGN	India
8	Mayilam	*C. melo*	TNAU	India
9	Muskmelon Orange	*C. melo*	TNAU	India
10	Mysore	*C. melo*	TNAU	India
11	N 78	*C. melo*	NBPGR	India
12	Nanhai 2 Aouri	*C. sativus*	CGN	China
13	Nepal Local	*C. sativus*	CGN	Nepal
14	Olakkur	*C. melo*	TNAU	India
15	Perundurai	*C. sativus*	TNAU	India
16	Pudukkottai	*C. melo*	TNAU	India
17	Shantung Thorny	*C. sativus*	CGN	China
18	Theni	*C. melo*	TNAU	India

IIVR: India Institute of Vegetable Research

TNAU: Tamil Nadu Agriculture University

CGN: The Centre for Genetic Resources, the Netherlands

NBPGR**:** National Bureau of Plant Genetic Resources

Table 1.2. Characters were observed and their abbreviations

Characters	Abb.	Standards
Fruit Weight (g)	FW	The weight is fruits at fully ripe stage
Peduncle Length (cm)	PL	The length from the base to fruit intersects peduncle
Fruit Length (cm)	FL	The length from stalk end to the tip of the fruit
Fruit Girth (cm)	FG	The girth at the middle portion
Leaf Length (cm)	LL	The length from the tip to the leaf intersects petiole
No. of Primary Branches	NPB	The number of branches borne on the main stem
Leaf width	LW	The width at the broadest place of the leaf
Internodal Length (cm)	IL	The length between two subsequent nodes
Leaf Petiole Length (cm)	LPL	The length from the base of to the intersects petiole
Plant growth	PG	Determinate, semi-determinate and indeterminate
Stem Shape	SS	Round, angular and others
Stem Color	SC	Yellow, light green, green, dark green and others
Stem Pubescence Density	SPD	Sparse, medium and dense
Stem Pubescence Type	SPT	Soft, intermediate and hard
Leaf Shape	LS	Orbicular, ovate, elliptical, reniform, cordate, triangular, entire, trilobite, pentalobate and others
Leaf Margin	LM	Crenate, denticulate, entire, lobate, serrate, serrulate, spiny, undulate and others
Leaf Color	LC	Light green, green, dark green and others
Leaf Glossiness	LG	Dull, intermediate and glossy
Leaf Pubescence Density	LPD	Sparse, medium and dense
Leaf Pubescence Type	LPT	Soft, intermediate and hard
Flower Color	FC	White, cream, yellow, orange and others
Fruit Shape	FS	Globular, flattened, oblate, elliptical, pyriform, ovate, acorn, elongated and others
Stem end Fruit Shape	SFS	Depressed, flattened, rounded and pointed
Blossom End Fruit Shape	BEFS	Depressed, flattened, rounded and pointed
Fruit Skin Luster	FSL	Matt, intermediate, glossy and others
Fruit Skin Texture	FST	Smooth, rough, spiny and others
Fruit Skin Hardness	FSH	Soft, intermediate, hard and others
Predominant Fruit Skin Color	PFSC	White, yellow, cream, pale green, green, dark green, orange, brown, grey and others
Secondary Fruit Skin Color	SFSC	Absent, white, yellow, cream, pale green, green, dark green, orange, brown, grey and others
Secondary Skin Color Pattern	SSCP	Absent, spotted, striped, netted, short streaked, long Streaked and others
Fruit Mesocarp Color	FMC	White, cream, green, orange and others
Seed Color	SC	White, cream white, yellow, light-brown and others

Table 1.3. Primer sequences for PCR and shotgun sequencing of *matK* gene and *rbcL* gene

Primer	Sequence
*mat*K (Forward)	5'- CTATATCCACTTATCTTTCAGGAGT -3'
*mat*K (Reverse)	5'- AAAGTTCTAGCACAAGAAAGTCGA -3'
rbcL (Forward)	5'- ATGTCACCACAAACAGAAACTAAAGC -3'
rbcL (Reverse)	5'- CTACGTCTGGAAGACCTGCGAATCC -3'

Table 1.4.The value of 9 quantitative characteristics of 18 accessions

Accessions	FW (g)	PL (cm)	FL (cm)	FG (cm)	LL (cm)	LW (cm)	NPB	IL (cm)	LPL (cm)
Cus 125/71	330	2.9	21.5	14.9	12.7	16.9	10.5	7.5	11.9
Long Green	210	6.1	17.3	16.9	12.5	17.1	6.5	4.9	17.3
JL-3 Dhillon	500	1.6	36.5	15.5	14.8	16.1	8.5	8.7	10.4
Nanhai 2	230	3.6	16.9	15.3	13.9	15.9	6.5	9.3	9.7
Aouri									
Nepal Local	590	2.9	22.5	20.4	15.5	16.1	9.5	8.2	9.9
Shantung	360	1.3	35.9	14.8	15.5	18.3	9.5	12.1	10.3
Thorny									
AAUC-2	790	3.3	24.3	18.7	15.7	20.1	8.5	14.5	15.3
Perundurai	400	2.5	23.9	18.3	12.7	17.5	12.5	8.5	12.5
IC-4079	390	2.5	26.9	21.1	14.3	16.5	5.5	9.9	9.9
N-78	1390	2.9	32.9	21.3	14.9	13.3	10.5	9.3	12.7
Mysore	290	3.3	21.3	24.2	8.2	12.7	5.5	5.6	7.5
CM-1	820	7.1	33.7	39.3	14.3	16.3	8.5	12.2	8.6
Olakkur	640	3.9	22.7	26.3	13.5	13.3	5.5	8.5	12.3
Mayilam	770	5.6	30.3	27.9	13.7	15.5	6.5	7.7	11.9
Muskmelon	520	3.1	14	35.3	11.9	14.5	9.5	9.1	13.6
Orange									
Pudukkottai	795	5.3	46.3	18.9	12.1	14.4	6.5	7.9	11.7
Theni	850	2.6	18.9	39.3	14.9	17.7	8.5	6.5	14.3
Andaman	1350	3.2	40.7	29.9	14.6	17.9	12.5	10.6	14.7

Table 1.5. The value of 23 qualitative characteristics of 18 accessions

Accessions	PL	SS	SC	SPD	SPT	LS	LM	LC
Cus 125/71	indeterminate	angular	dark green	medium	hard	entire	spiny	dark green
Long Green	indeterminate	angular	light green	medium	hard	entire	spiny	green
JL-3 Dhillon	indeterminate	angular	dark green	dense	intermediate	entire	spiny	dark green
Nanhai 2 Aouri	indeterminate	angular	dark green	medium	hard	entire	spiny	dark green
Nepal Local	indeterminate	angular	dark green	dense	intermediate	cordate	others	dark green
Shantung Thorny	indeterminate	angular	dark green	medium	hard	entire	spiny	dark green
AAUC-2	indeterminate	angular	green	sparse	intermediate	entire	spiny	dark green
Perundurai	indeterminate	angular	dark green	dense	intermediate	entire	spiny	green
IC-4079	indeterminate	angular	dark green	dense	intermediate	entire	spiny	dark green
N-78	indeterminate	angular	green	medium	hard	entire	spiny	light green
Mysore	indeterminate	angular	green	medium	hard	triangular	spiny	light green
CM-1	indeterminate	angular	dark green	medium	hard	entire	spiny	green
Olakkur	indeterminate	angular	green	medium	hard	reniform	undulate	light green
Mayilam	indeterminate	angular	green	medium	hard	pentalobate	lobate	light green
Muskmelon Orange	indeterminate	angular	green	medium	hard	pentalobate	lobate	light green
Pudukkottai	indeterminate	angular	dark green	medium	hard	reniform	entire	light green
Theni	indeterminate	angular	green	medium	hard	reniform	entire	light green
Andaman	indeterminate	angular	green	medium	intermediate	cordate	spiny	light green

Accessions	LG	LPD	LPT	FC	FS	SFS	BEFS
Cus 125/71	intermediate	dense	soft	yellow	elongated	rounded	pointed
Long Green	intermediate	sparse	soft	yellow	elongated	rounded	rounded
JL-3 Dhillon	intermediate	dense	soft	yellow	elongated	pointed	pointed
Nanhai 2 Aouri	glossy	dense	soft	yellow	elongated	rounded	rounded
Nepal Local	glossy	medium	hard	yellow	elongated	rounded	rounded
Shantung Thorny	glossy	dense	soft	yellow	elongated	pointed	pointed
AAUC-2	glossy	dense	soft	yellow	others	flattened	flattened
Perundurai	dull	dense	soft	yellow	elongated	flattened	rounded
IC-4079	intermediate	dense	soft	yellow	elongated	rounded	pointed
N-78	intermediate	medium	hard	yellow	elongated	pointed	rounded
Mysore	intermediate	dense	soft	yellow	elliptical	pointed	rounded
CM-1	intermediate	dense	soft	yellow	pyriform	rounded	rounded
Olakkur	intermediate	medium	intermediate	yellow	others	flattened	flattened
Mayilam	intermediate	dense	soft	yellow	pyriform	pointed	depressed
Muskmelon Orange	dull	dense	soft	yellow	globular	flattened	flattened
Pudukkottai	intermediate	dense	soft	yellow	elongated	rounded	rounded
Theni	intermediate	sparse	intermediate	yellow	globular	flattened	depressed
Andaman	intermediate	medium	hard	yellow	elongated	pointed	rounded

Accessions	FSL	FST	FSH	PFSC	SFSC	SSCP	FMC	SC
Cus 125/71	glossy	spiny	hard	dark green	pale green	cream	spotted	white
Long Green	intermediate	rough	hard	brown	white	green	striped	green
JL-3 Dhillon	intermediate	spiny	hard	pale green	yellow	cream	striped	white
Nanhai 2 Aouri	matt	spiny	hard	pale green	yellow	cream	striped	white
Nepal Local	glossy	spiny	intermediate	brown	absent	absent	striped	white
Shantung Thorny	intermediate	spiny	hard	yellow	absent	absent	spotted	white
AAUC-2	matt	spiny	hard	brown	orange	cream	netted	white
Perundurai	intermediate	spiny	hard	pale green	green	white	netted	cream
IC-4079	matt	rough	hard	brown	yellow	others	spotted	white
N-78	glossy	smooth	intermediate	green	yellow	cream	spotted	white
Mysore	matt	smooth	intermediate	yellow	absent	absent	spotted	white
CM-1	glossy	smooth	hard	dark green	pale green	cream	spotted	white
Olakkur	intermediate	smooth	soft	pale green	yellow	cream	spotted	cream
Mayilam	intermediate	smooth	hard	yellow	dark green	white	netted	cream
Muskmelon Orange	glossy	others	hard	yellow	dark green	cream	short streaked	white
Pudukkottai	glossy	smooth	hard	yellow	pale green	orange	netted	white
Theni	intermediate	smooth	soft	yellow	dark green	cream	short streaked	white
Andaman	intermediate	smooth	soft	yellow	pale green	orange	spotted	white

Table 1.6. Eight quantitative characters contribution to three principle components

Quantitative characters	PC1	PC2	PC3
No. of Primary Branches	0.041166083	0.027369843	0.457822813
Internodal Length	0.08581916	0.045566541	0.422407785
Leaf Petiole Length	-0.047422225	-0.00040903	0.452534753
Leaf Length	0.052677711	0.015715088	0.384663641
Leaf width	0.016545044	-0.028699686	0.48660623
Peduncle Length	-0.008258262	0.068007104	-0.134810083
Fruit Length	0.935473105	0.328750878	-0.06483991
Fruit Girth	-0.332362377	0.939894501	0.007247136

Fig. 1.1. Photographs of fruits of 9 *Cucumis. Sativus* accessions. scale:5cm.

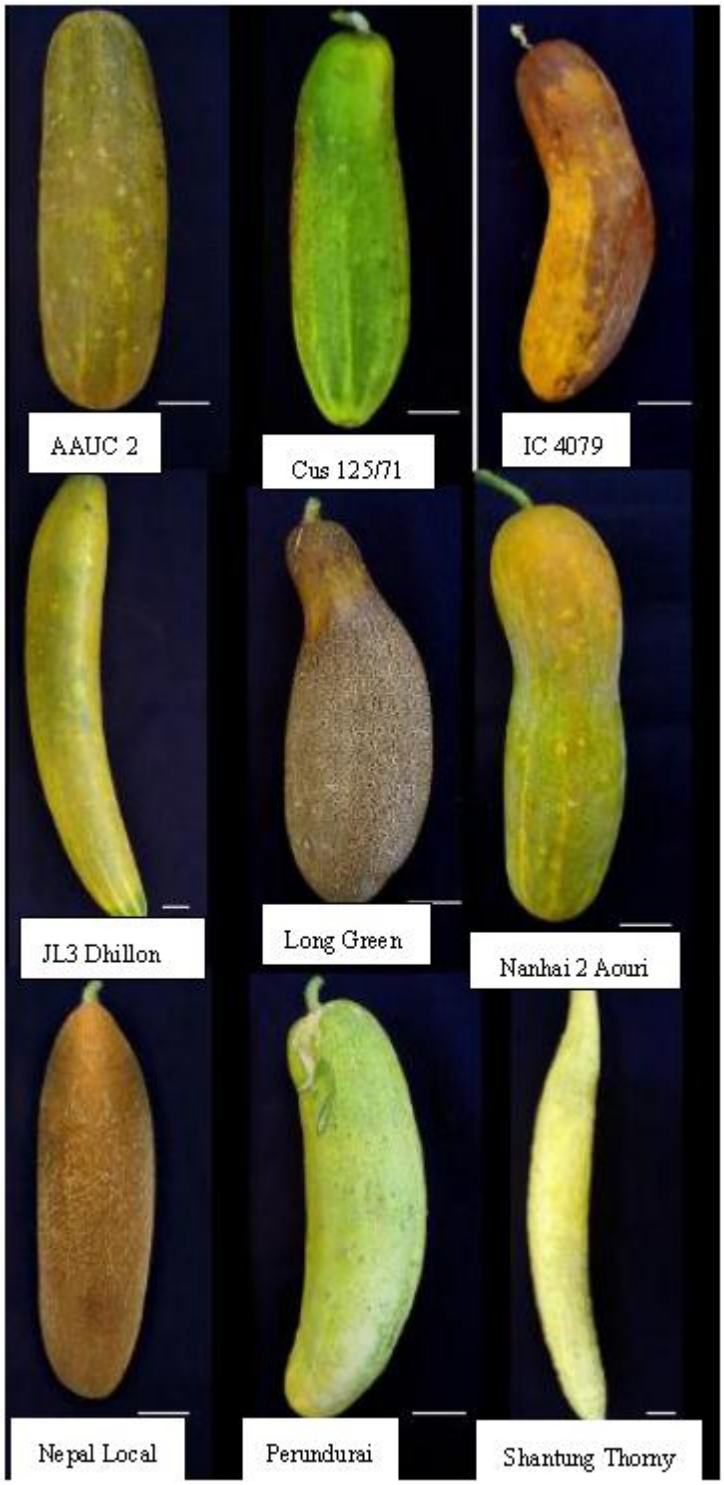


Fig. 1.2. Photographs of fruits of 9 *Cucumis. melo* accessions . scale:5cm.

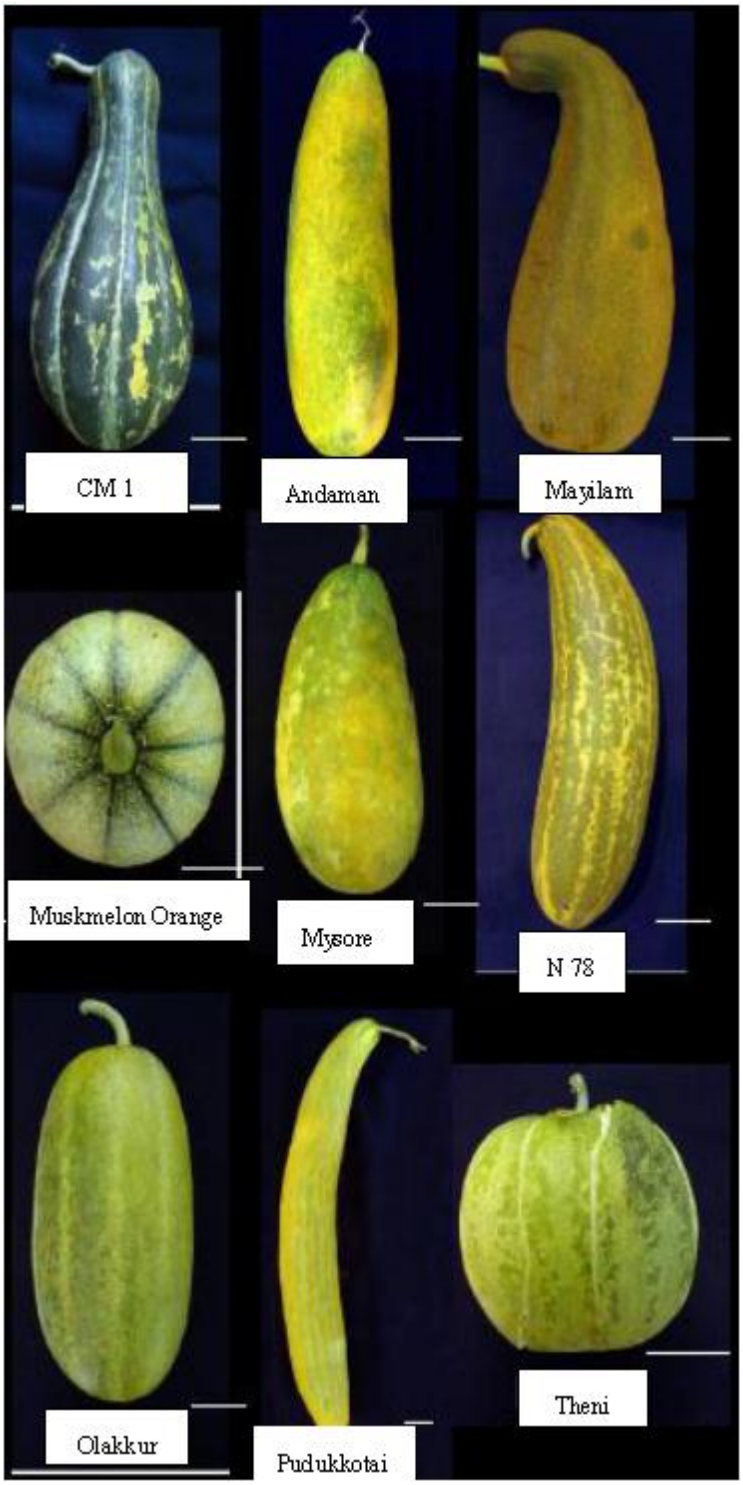
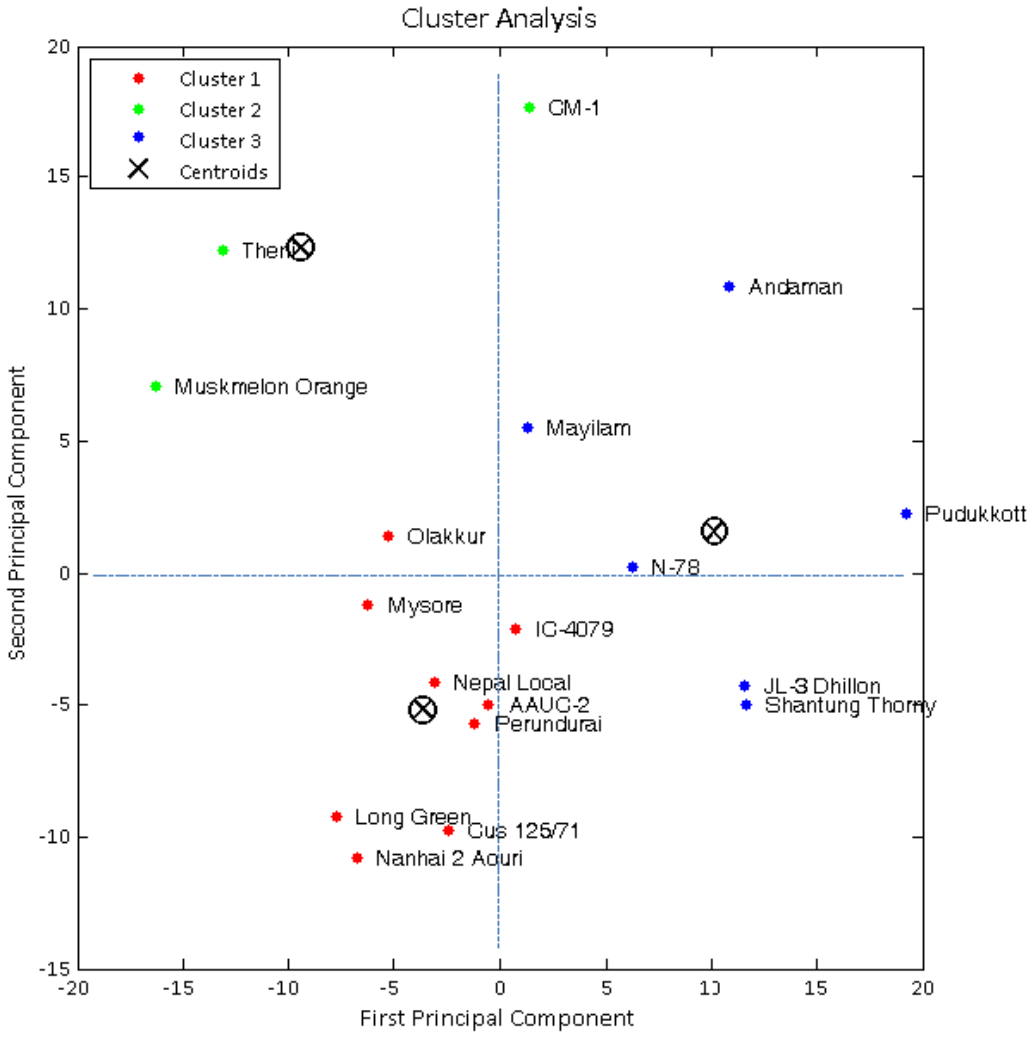
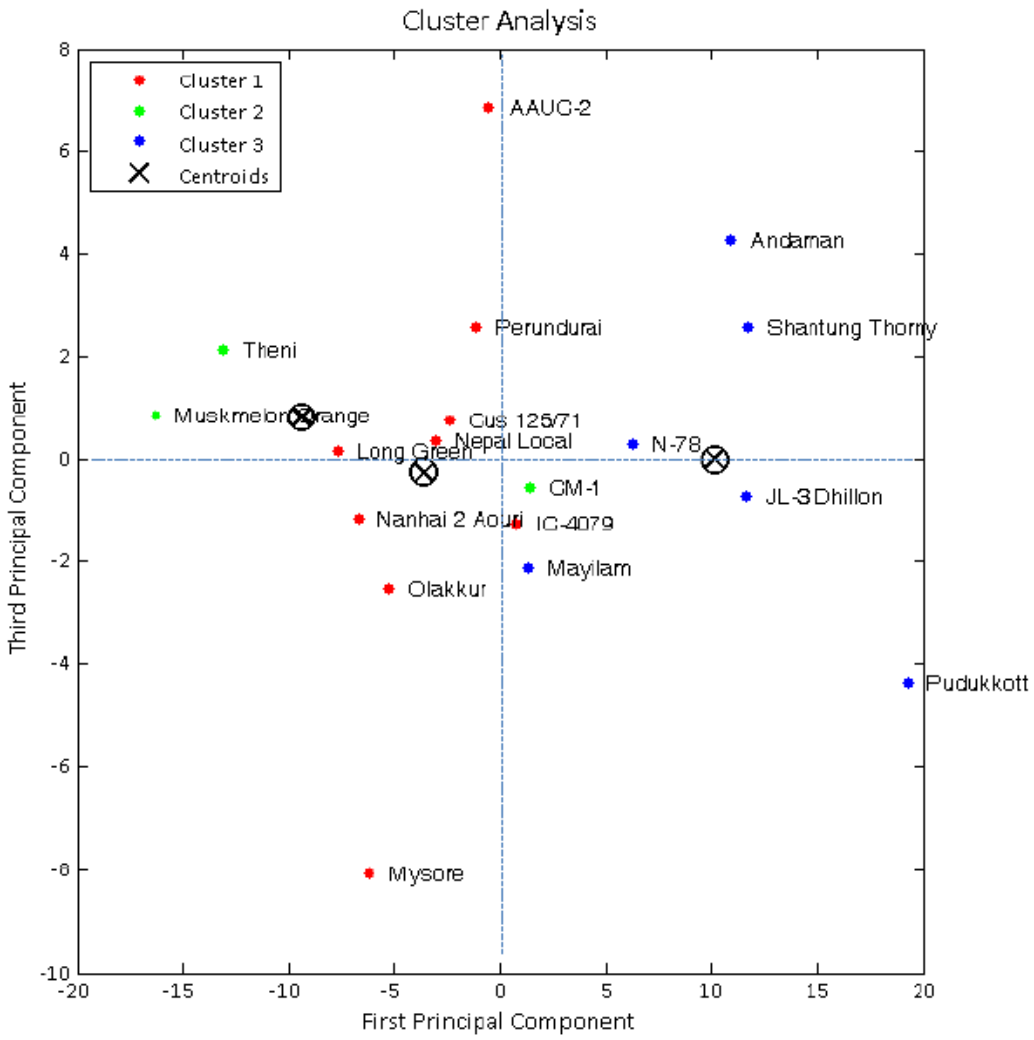


Fig. 1.3. K-means cluster after principle analysis of eighteen accessions collected in this study. A: Distribution on the first and second principle component of eighteen accessions; B: distribution on the first and third principle component of eighteen accessions; C: grouping details in the table.



A


Cluster Analysis
Cluster 1
Cluster 2
Cluster 3
Centroids
AAUC-2
Andaman
Perundurai
Shantung Thorny
Theni
Muskmelon Orange
Cus 125/71
Nepal Local
Long Green
N-78
CM-1
JL-3 Dhillon
Nanhai 2 Aouri
IC-4079
Mayilam
Olakkur
Pudukkott
Mysore
Third Principal Component
First Principal Component
8
6
4
2
0
-2
-4
-6
-8
-10
-20
-15
-10
-5
0
5
10
15
20

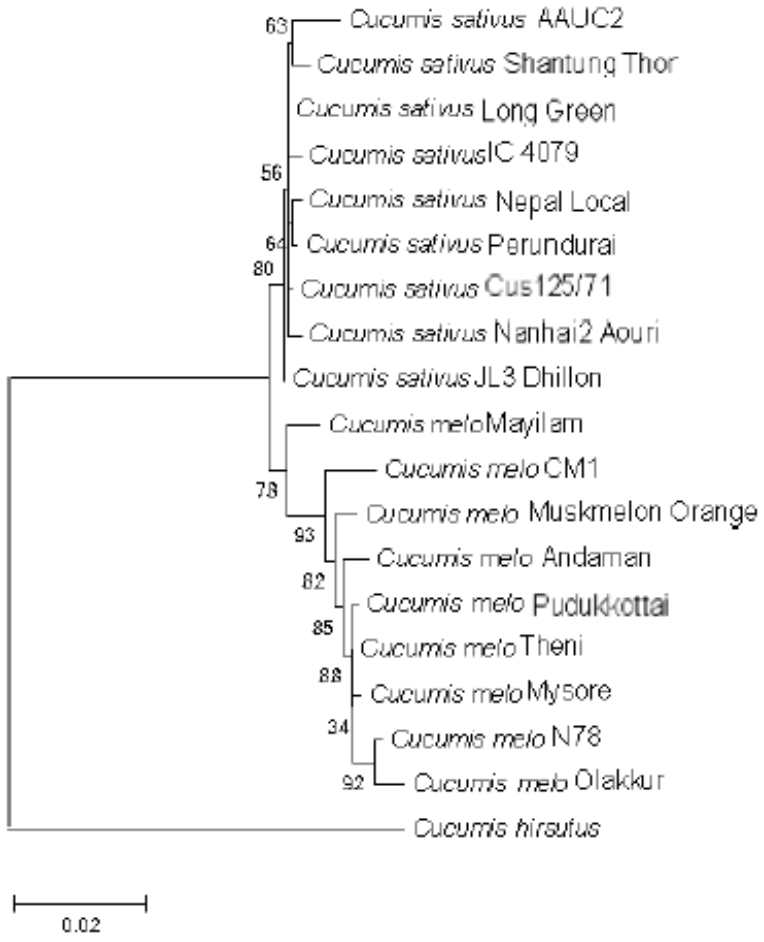
B

Name of accessions	cluster
Cus 125/71	R
Long Green	R
JL-3 Dhillon	B
Nanhai 2 Aouri	R
Nepal Local	R
Shantung Thorny	B
AAUC-2	R
Perundurai	R
IC-4079	R
N-78	B
Mysore	R
CM-1	G
Olakkur	R
Mayilam	B
Muskmelon Orange	G
Pudukkottai	B
Theni	G
Andaman	B

C

Fig. 1.4. Neighbor-Joining tree for 18 germplasms in this study based on combined sequence of *rbcL* and *matK* genes. The tress is rooted on *Cucumis hirsutus*. Bootstrap values based on 1000 replicates above branches.

Fig. 1.5. Maximum likelihood tree for 18 germplasms in this study based on combined sequence of *rbcL* and *matK* genes. The tress is rooted on *Cucumis hirsutus*. Bootstrap values based on 1000 replicates above branches.



Chapter 2

KARYOTYPE ANALYSIS AND COMPARISON OF SOUTH ASIAN AND JAPANESE CUCUMBER VARIETIES

2.1 Introduction

Cucumber (*Cucumis sativus*, $2n=2x=14$) is extensively cultured all over the world, and is ranked within the 20 most important vegetables (FAO, 2008). Different ecotypes are usually partitioned according to the local culture environment and consumption custom. Asia, especially South Asia, where is considered to be the place of origin of *C. sativus,* holds the most varieties (Jeffrey, 1980). This point was supported by the isoenzyme analysis of germplasms in the collection of the National Plant Germplasm System founded by USDA (Staub *et al.*, 1997). It was shown that Chinese and Indian accessions represented the most diverse genetic variation in the world.

Karyotype analysis is a basic method of cytogenetic study, which describes overall nature of chromosome morphology. Because of small sizes of the mitotic chromosomes, similar chromosome lengths or/and arm ratios and poor dyeing characteristics, cytogenetic studies of cucumbers appeared much discrepancy among the researchers (Dane, 1991; Trivedi and Roy 1970). Chromosome identification of cucumbers was firstly realized by C-band staining, however, the distribution patterns of the C bands obtained at that time were different among the reports (Chen *et al*. 1998; Hoshi *et al*. 1998; Ramachandran and Seshadri, 1986). In the later studies, it was proposed that the tandem satellite DNA sequences were usually located at specific chromosome positions (Sharma and Raina 2005). These specific positions were broadly classified and described as centromeric, subtelomeric or intercalary which were constituted of tandem repetitive sequences. The probes, such as 5S, 45S ribosomal DNAs (rDNAs) and other tandem

repeated sequences have been commonly used for fluorescent *in situ* hybridization (FISH)-based karyotyping in plant species. Two cucumber repeats, CsRP1 and CsRP2, were isolated from RAPD markers (Koo *et al*. 2005). They were 1,003 and 439 bp long, respectively. CsRP1 was detected on the intercalary region of chromosome 1, and the pericentromeric heterochromatic region of the other chromosomes. The signals of CsRP2 were detected on chromosome 1, 2, 3, 4 and 7. Based on the distribution of these two RAPD-based repeats and 5S rDNA, an integrated pachytene chromosome was established (Koo *et al*. 2005).

Tandem repetitive DNA is the most dominant component accounting for about 20-30% of the total nuclear DNA in cucumber (Ganal *et al*. 1986). Most of them, ~90%, are mainly specified to four types, Type I, II, III and IV, with the length ranging 177 bp to 360 bp (Ganal and Hemleben 1988). Coupling with the release of the complete genome of cucumber (Huang *et al*. 2009), it was shown that these four types of tandem repetitive DNA were the main components of the cucumber genome. An integrated metaphase chromosome karyotype in cucumber was built by analyzing the distribution of these four types of tandem repetitive sequences (Han *et al*. 2008). Also, probes of tandem repetitive sequences were also applied in the studies on the phylogenetic relationships within *C. sativus*. Repetitive sequences in the genome play an important role during the evolution (Sharma and Raina 2005), so the closer relatives often display similar distribution patterns of tandem repetitive sequences. Based on different distribution patterns of tandem repetitive sequences, a previous study concluded that the relationship between a Chinese cucumber variety and *C. sativus* var. *xishuangbannesis* was closer than that of *C.*

sativus var. *xishuangbannesis* and *C. sativus* var. *hardwickii* (Zhao *et al.* 2010).

In the present study, four types of tandem repetitive DNAs, Type I, II, III and IV were firstly used as FISH probes in Japanese and Indian cucumber varieties. Karyotypes of somatic metaphase chromosome of an Indian variety 'Long Green' (*C. sativus,* $2n=2x=14$) and a Japanese variety 'Hokutosei' (*C. sativus*, $2n=2x=14$) are presented and efficiencies of using these repetitive DNA are discussed.

2.2 Materials and Methods

2.2.1 Plant materials and DNA probes

Seeds of an Indian cucumber variety (*C. sativus*) 'Long Green' and a Japanese cucumber variety (*C. sativus*) 'Hokutosei' were kindly provided by Tamil Nadu Agriculture University (TNAU), India and 'Tohuku' Seeds Co., Japan, respectively. Seeds were sown on the wet filter paper in Petri dishes under room temperature. Root samples were collected at 1-2 cm long and stored in 3:1 (v/v) ethanol/acetic acid fixative solution. Probes of Type I/II, III, IV are kindly provided by Dr. Koo DH, Department of Horticulture, University of Wisconsin, USA. Their features are as follows: Type I/II satellite DNA: subtelomere specific 180 bp repeat; Type III satellite DNA: centromere specific 170 bp repeat; Type IV satellite DNA: subtelomere or/and pericentromere specific 360 bp repeat.

2.2.2 Chromosome preparation and FISH

Chromosome preparation for FISH was followed as previously described (Zhao *et al*. 2010). Briefly, the roots harvested from germinated seeds were directly put into 3:1 (v/v) ethanol/acetic acid fixative and stored in 4℃. Root tips were digested with 10μl enzymatic mixture (3% Cellulose Onozuka RS, Yakult; 1.5% Pectolyase Y-23, Seishin, in 0.01M sodium citrate, pH 4.6) at 37℃ for 35-40 minutes for maceration. Squashing was made in 45% acetic acid and slides were stored at -80℃ over 12 h or on dry ice for 2 h. FISH was carried out according to the protocol by Ohmido and Fukui (1997). Plasmids harboring tandem repetitive sequence were labeled with biotin-16-dUTP (Roche) and

digoxingenin-11-dUTP (Roche) by nick translation, respectively. Biotin-16-dUTP and digoxingenin-11-dUTP were detected with fluorescein isothiocyanate (FITC)-streptavidin conjugates (Molecular Probes) and anti-digoxingenin-rhodamine (Roche), respectively. Chromosome DNAs were counterstained with 4', 6-diamidino-2-phenylindole (DAPI).

2.2.3 Chromosome measurements and analysis

All images were captured digitally using a CCD camera attached to Leica DMRXA2 fluorescence microscope with three different filter sets. Image analysis was performed by Adobe Photoshop v5.0 (Adobe System Inc.). Six cells were used for measuring length of chromosome by 'Image J' and for construction of ideograms. Chromosomes were numbered as 1-7 according to their sizes.

2.3 Results and Discussion

2.3.1 Distribution of tandem repeat sequences and karyotyping in an Indian cucumber variety 'Long Green'

Metaphase chromosomes of Indian cucumber variety 'Long Green' were prepared for FISH analysis (Fig. 2.1 A). FISH on mitotic metaphase chromosomes showed that Type III repeats hybridized strictly to the centromeric regions on six pairs of chromosomes except Chromosome 7 (Fig. 2.1 B and B'). In a previously study, Type III repeat signals were detected on each of seven metaphase chromosomes of three cucumber accessions (Zhao *et al*. 2010), which was different with the results in the present work. The intensities and sizes of the FISH signals varied significantly among the six pairs of chromosomes, suggesting that different chromosome have variable copies of Type III repeats. Four pairs of chromosomes (Chromosome 1, 2, 3 and 5) had strong and broad signals, two pairs of chromosomes (Chromosome 4 and 6) had relatively weak signals which were not strong but clear (Fig. 2.1 B').

Type I/II and Type IV FISH signals were both located in telomeric regions. In 'Long Green', Type IV signals were detected on Chromosome 1, 2 and 7. They were on the short arms of Chromosome 1 and 7, and long arm of Chromosome 2 (Fig 2.1 B'). However, Type IV FISH signals were not detected on Chromosome 3, 4, 5 and 6, though a small Type IV signal coming from other chromosome was detected on Chromosome 6 (Fig 2.1 B'). Type I/II signals were detected on all the seven pairs of chromosomes. Chromosome 4 and Chromosome 7 had Type I/II signals only on short arms and the other five pairs of chromosomes had the signals on both arms, though signals on Chromosome

3 and 5 were relatively weak (Fig 2.1 C'). The results supported the previous conclusion that the signals of Type I/II and Type IV were located adjacently (Han *et al*. 2008). Variances in signal sizes and intensities were also found in Type I/II and Type IV repeats, indicating that differences in copy number exist among seven chromosomes.

Distribution of tandem repeat sequences is used for precise identification of chromosomes. Characteristics of individual chromosome, relative lengths, arm ratios and types are shown in Table 2.1. The relative chromosome lengths varied 9.48 to 17.41. All the well-condensed chromosomes were prepared from the cells at metaphase, and the arm ratios were 1.07 to 1.68. Two pairs of chromosomes were obviously longer than the others, and they also had stronger signals of the tandem repeats. The longest Chromosome 1 and the shortest Chromosome 7 were 'sm' type, and the other five pairs were all 'm' type.

It was estimated that the highly repeated satellite DNA of *C. sativus* accounted for 20-30% of the total nuclear DNA (Ganal *et al*. 1986). Type I, II, III and IV of tandem repeat sequences accounted for over 90% of the satellite DNA in the genome (Huang *et al*. 2009). The distribution patterns of the tandem repeats on the metaphase chromosome of *C. sativus* 'Long Green' were detected with a large quantity, although their distribution pattern in 'Long Green' was different with other cultivars in the previous study. The reason should be that tandem repeat sequence was not only major component of telomere and centromere of chromosome region but also represents a fast-evolving portion of the genome. Some examples of how the satellites have undergone rapid evolution within a

genus had been reported (Ohmido *et al*. 2001; Sharma and Raina 2005).

2.3.2 Distribution of tandem repeat sequences and karyotyping in a Japanese cucumber variety 'Hokutosei'

DAPI staining clearly showed the morphologies of the chromosomes in Japanese variety 'Hokutosei' (Fig. 2.2 A). Each chromosome was named from number 1 to 7 in the order of their length. The range of relative length among seven chromosomes was from 8.67 to 21.15. There were two sm type chromosomes and five 'm' type chromosomes (Table 2.1). Another study about karyotype of a Japanese cucumber variety showed different results. The range of relative length of *C. sativus* cv. 'Jibai' was 10 to 19, and contained three 'm' type chromosomes, three 'sm' type chromosomes and one 'st' type chromosome (Sato *et al*., 2007). Comparing with the Indian variety, variance between individual chromosomes was also found. This kind of inconsistency was also displayed among different melon varieties (Liu *et al*., 2010). In cucumber, this inconsistency may be caused by different materials or operation. Thus, more reliable cytogenetic markers like tandem repeat sequences are necessary.

FISH by two types of tandem repeat sequence on mitotic metaphase chromosomes showed that Type III repeat was located on centromeric regions of Chromosomes 2, 3, 4 and 6, both arms of Chromosome 1, and long arm of Chromosome 7 of this Japanese cucumber variety. The Type III signal was weak on Chromosome 4 and no signal was detected on Chromosome 5 (Fig. 2.2 B and B'). The result was different with the previous study that all the chromosomes had centromeric Type III signals which located

on centromeric regions (Zhao *et al*. 2010). Type I/II tandem repeat was detected on the telomeric regions of all seven chromosomes. They were located on both ends of five pairs of chromosome, except on the short arms of Chromosome 3 and 7 (Fig. 2.2 B'). Variances in signal sizes and intensities reflect that differences in copy number still exist among individual chromosomes. One of the homologous chromosomes of Chromosome 5 looked shorter than the other, which was caused by the fold (Fig. 2.2 B and B'). Ideograms of Indian cucumber variety 'Long Green' and Japanese cucumber variety 'Hokutosei' metaphase chromosomes are shown in Fig. 2.3, which displays the positions of the Type I/II and Type III. Compared with the Chinese cucumber variety in previous study (Zhao *et al*. 2010), there are six pairs of chromosomes in Indian cucumber varieties, but four pairs of chromosomes in Japanese cucumber variety, which have the same distribution patterns of Type I/II and Type III with Chinese cucumber variety. For Type I/II probe, all the detected signals were located in telomeric region. There are two chromosomes with one-arm Type I/II signals in Indian and Japanese varieties, but only one in Chinese variety. For Type IV probe, signals were detected on the arms of two chromosomes in Japanese variety. But they were all centromeric in Indian and Chinese cucumber varieties. According to comparison of Type I/II and Type IV, relationship between Indian varieties and Chinese varieties seems closer than Japanese varieties. But more probes distribution is necessary for further comparison.

Repetitive DNA sequences including satellites and transposable elements are the main components of genomic DNA (San *et al*. 1996; Kumar and Bennetzen 1999). Meanwhile they play an important role during the evolution (Sharma and Raina 2005). According to

different distribution patterns of the DNA repetitive sequences, it was clarified that the relationship between cucumber varieties and *C. sativus* var. *xishuangbanna* was closer than that between *C. sativus* var. *xishuangbanna* and *C. sativus* var. *hardwickii* (Zhao *et al*. 2010). Comparison of the distribution of more repetitive DNA sequences, such as transposable elements and satellite DNAs, between Indian cucumber variety 'Long Green' and Japanese cucumber variety 'Hokutosei' may help to explain the phylogenetic relationships in *C. sativus*.

In conclusion, karyotypes of Japanese and Indian cucumber varieties were constructed using tandem repetitive sequence probes. Four types of tandem repetitive sequence as three types of FISH probes, type I/II, III and IV, were successfully applied to Indian cucumber varieties. Type I/II and Type III were successfully applied to Japanese cucumber varieties. That would be useful information for analyzing karyotypes and integrating physical and genetic maps, as well as phylogentic analysis in the genus *Cucumis*.

2.3.3 Chromosome morphology of an Indian melon variety 'Andaman'

Chromosome morphology of Indian melon variety 'Andaman' is shown in Fig. 2.4 A. Chromosome in metaphase cell was stained with DAPI. Twenty-four chromosomes were well-spread and distinctive. They displayed different morphology and staining intensity. Karyotype of 'Andaman' is shown in Fig. 2.4 B. Individual chromosome was named from chromosome 1 to 12 in the order of their lengths. Characteristics of individual

chromosome, relative lengths, arm ratios and types, are shown in Table 2.2. The range of relative length among 12 chromosomes was from 6.76 to 11.13. Four chromosomes, chromosome1, 2, 3 and 4 were longer than the others and had clearer centromeric regions. Chromosome 5 was the only SM type with the arm ration of 1.69. There was no ST type was found in this Indian melon variety. Chromosomes of *Cucurbitaceae* plants are usually small and difficult for cytogenetic analysis, especially in *Cucumis melo* (Trivedi and Roy, 1970; Dane and Tsuchiya, 1976). Karyotypes of *Cucumis melo* are still with inconsistent results according to chromosome morphology. It was reported that a karyotype in muskmelon, $2n=2x=24$=16m+4sm+4stm in 1986 (Ramachandram and Seshadri). Karyotype of 'Hetian' (*Cucumis melo spp. melo* Pang) was described as $2n=2x=24$=20m+2sm+2sm (Ma *et al.,* 1995). In 2005, the karyotype of 'Jiashi' melon (*Cucumis, melo* L. thick skin type) and 'Huangjin' melon (*Cucumis, melo* L. thin skin type) were descripted as $2n=2x=24$=14m+10st (Zhang *et al.,* 2005). The inconsistency of karyotype may due to the small chromosome size, similar chromosome length or/and different materials. The Indian *Cucumis, melo* variety 'Andaman' could be a good material for melon karyotyping analysis, as its origination is the center of diversity, but also the chromosome morphology is more distinctive.

Chromosome identification is critical for cytogenetic studies. The somatic metaphase chromosomes of melon are small and with similar size, making it difficult to distinguish individual chromosomes accurately (Chen *et al.,* 1999). Recently, a set of cucumber fosmids clones were used as probes in melon FISH and some of them could be assigned to distinctive melon chromosomes (Liu *et al.,* 2010). This kind of cross-species molecular

cytogenetic markers facilitated chromosome identification in melon karyotype analysis. In the future, development of more reliable molecular cytogenetic makers and selection of suitable materials is necessary for getting a consistent melon karyotype.

2.4 Tables and figures

Table 2.1 Characteristics of individual chromosomes of Indian cucumber variety 'Long Green' and Japanese cucumber variety 'Hokutosei'

	Chromosome	Relative length	Arm ratio	Type
'Long Green'	1	17.41	1.66	sm
	2	16.73	1.16	m
	3	14.49	1.07	m
	4	14.15	1.33	m
	5	13.80	1.59	m
	6	13.58	1.23	m
	7	9.84	1.68	sm
'Hokutosei'				
	1	21.55	1.83	sm
	2	17.50	1.22	m
	3	14.90	1.56	m
	4	14.28	1.43	m
	5	12.22	1.47	m
	6	11.11	1.15	m
	7	8.67	1.84	sm

Relative length: 100x(chromosome length/total complement length).
Arm ratio: length of the long arm/length of the short arm.
m: median type chromosome; sm: sub-median type chromosome.

Table 2.2 Characteristics of individual chromosomes of Indian melon 'Andaman'

Chromosome	Relative length	Arm ratio	Type
1	11.13	1.45	m
2	10.43	1.17	m
3	10.09	1.15	m
4	9.70	1.09	m
5	8.77	1.69	sm
6	7.98	1.18	m
7	7.45	1.28	m
8	7.17	1.29	m
9	7.04	1.17	m
10	6.77	1.14	m
11	6.77	1.31	m
12	6.76	1.36	m

Relative length: 100x(chromosome length/total complement length).
Arm ratio: length of the long arm/length of the short arm.
m: median type chromosome; sm: sub-median type chromosome.

Fig. 2.1. FISH analyses of Indian cucumber variety 'Long Green'. A: Metaphase chromosomes stained with DAPI. B and B': Type III (green) and Type IV (red) signals. Red arrows indicate the chromosomes not having Type III signals. C and C': Type I/II signals (green). Bars: 5µm.

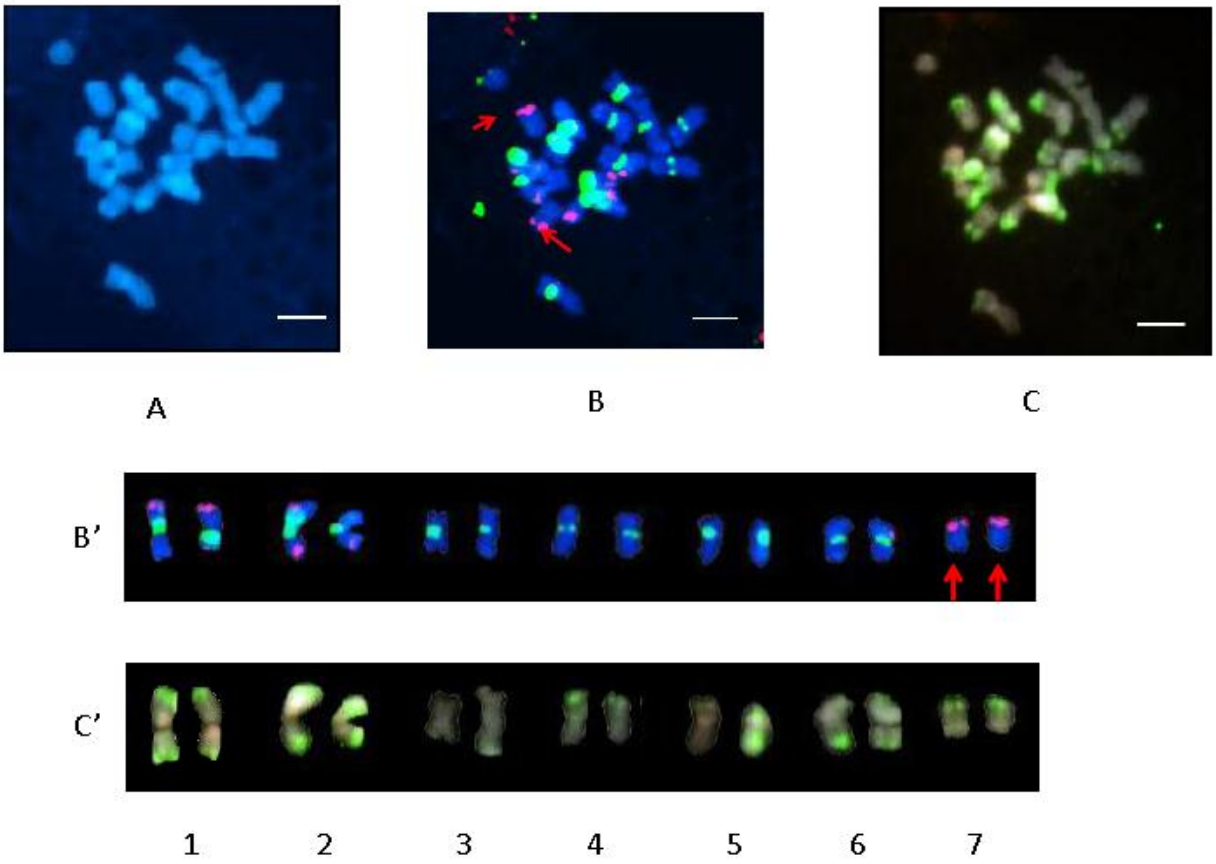


Fig. 2.2. FISH analyses of Japanese cucumber variety 'Hokutosei'. A: Metaphase chromosomes stained with DAPI. B and B': Type III (red) and Type I/II (green) signals. Red arrows indicate Type III signals on the arms of Chromosome 1 and 7. Yellow arrows indicate the fold chromosome. Bar: 5µm.

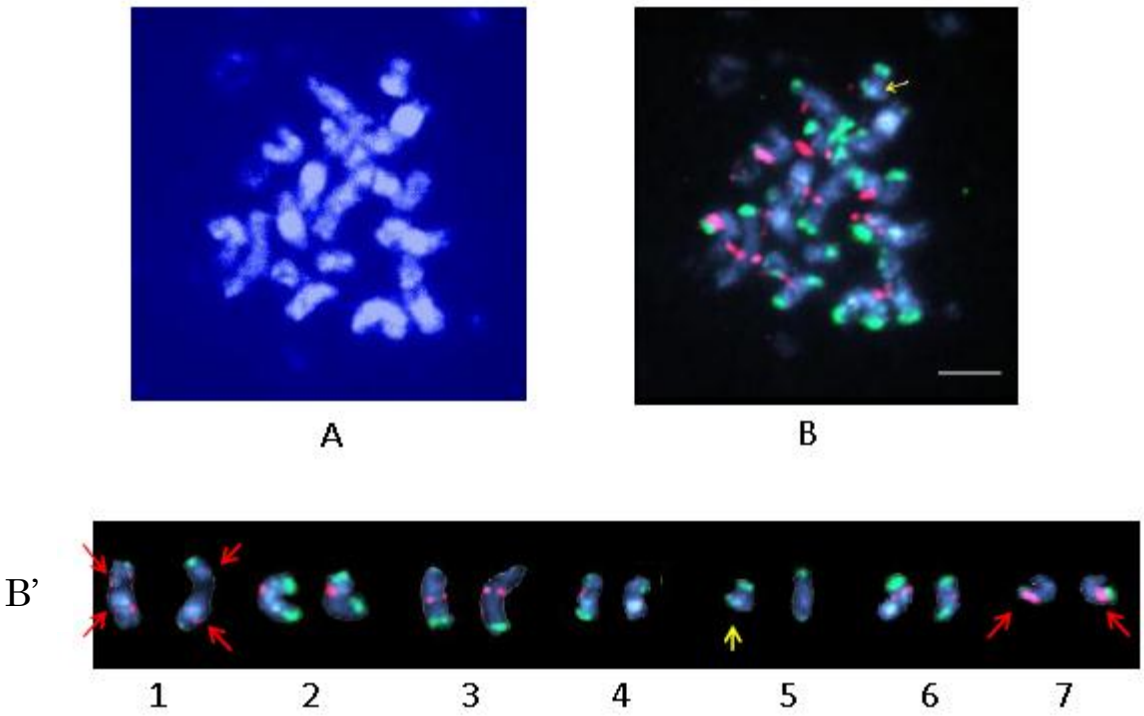
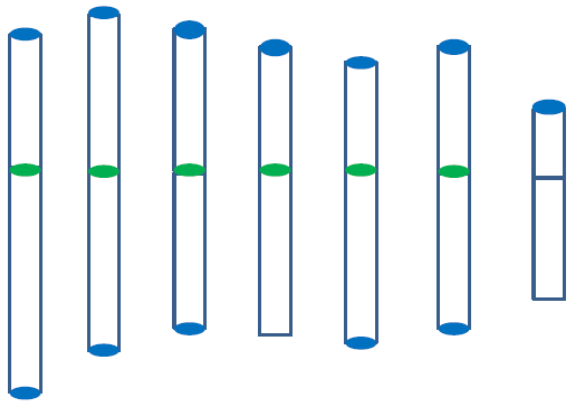
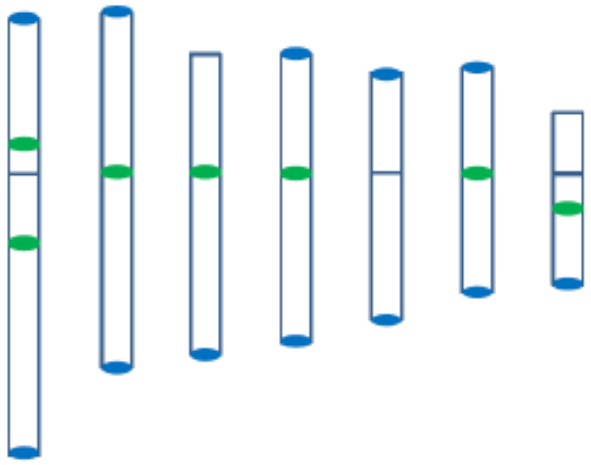


Fig. 2.3. Ideograms of Indian cucumber variety 'Long Green' (A) and Japanese cucumber variety 'Hokutosei' (B) (Blue: Type I/II, Green: Type III).

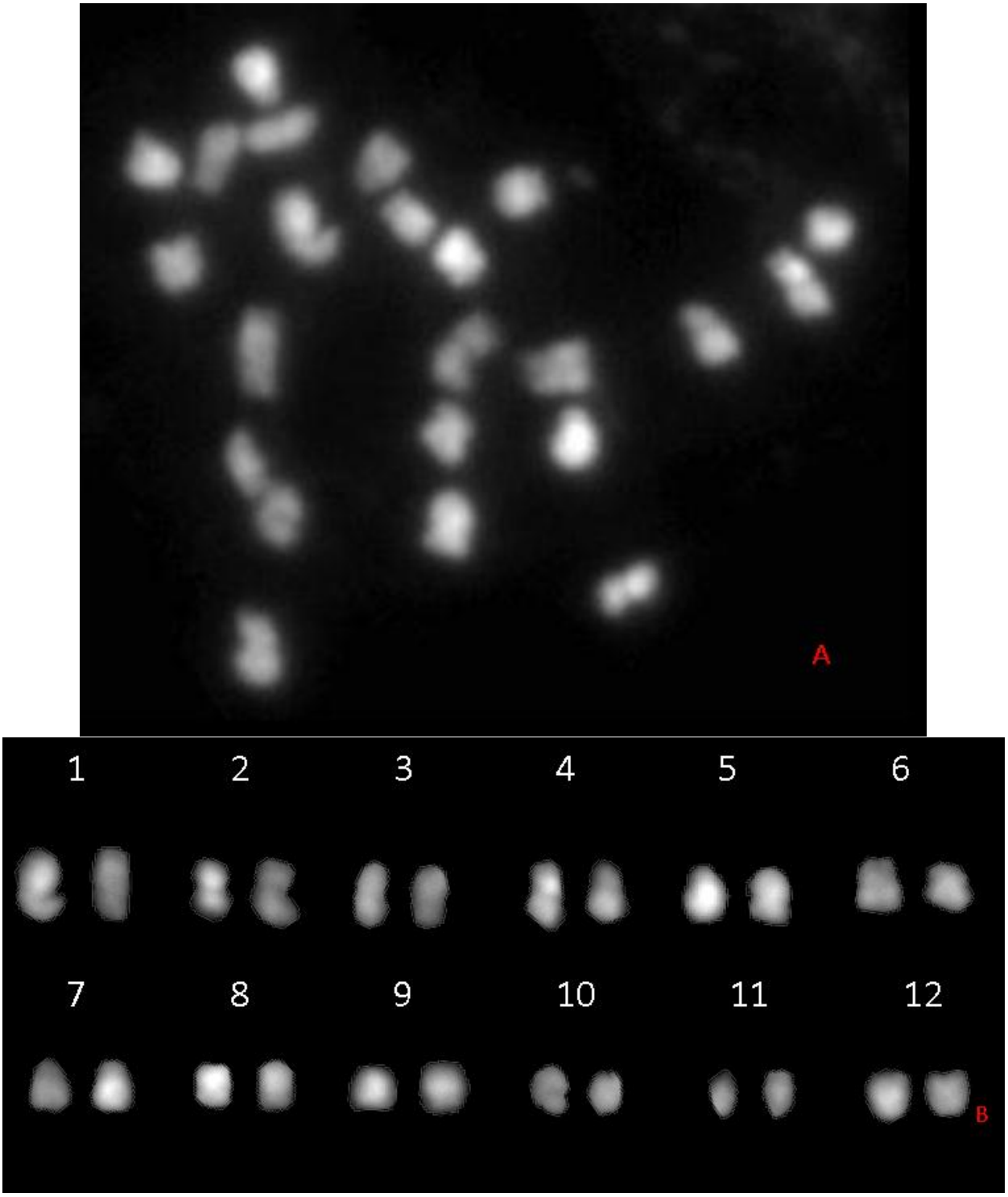


A



B

Fig. 2.4. (A): Chromosome morphology of *Cucumis melo* Andaman in metaphase cell (B): karyotype of metaphase chromosomes.



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List of Publications

1. Chi Zhang, Arun S. Pratap, S,Natarajan, L. Pugalendhi, Shinji Kikuchi, Hidenori Sassa, Natesan Senthil and Takato Koba (2012) Diversity and genetic relationship of Cucumis sativus and C. melo among South Asian Germplasm (ISRN Agronomy, in press)

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3. Wei-Wei Zhang, Jun-Song Pan, Huan-Le He, Chi Zhang, Zheng Li, Jun-Long Zhao, Xiao-Jun Yuan, Li-Huang Zhu, San-Wen Huang, Run Cai, (2012) Construction of a high density integrated genetic map for cucumber (*Cucumis sativus* L.) Theor Appl Genet 124:249–259

4. X. J. Yuan, J. S. Pan, R. Cai, Y. Guan, L. Z. Liu, W. W. Zhang, Z. Li, H. L. He, C. Zhang, L. T. Si, L. H. Zhu. (2008) Genetic mapping and QTL analysis of fruit and flower related traits in cucumber (*Cucumis sativus* L.) using recombinant inbred lines. Euphytica 164:473–491