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MASTER THESIS

T.C.
NİĞDE ÖMER HALİSDEMİRUNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL GENETIC ENGINEERING

CHARACTERIZATION OF SOMATIC METAPHASE CHROMOSOMES IN BOTTLE GOURD (*Lagenaria siceraria*)

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Supervisor
Associate Professor Dr. AHMET LATIF TEK

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Kamran KHAN tarafından **Doç. Dr. Ahmet Latif TEK** danışmanlığında hazırlanan **"Sukabağı (*Lagenaria siceraria*) somatic metafaz kromozomlarının karakterizasyonu"** adlı bu çalışma jürimiz tarafından Niğde Ömer Halisdemir Üniversitesi Fen Bilimleri Enstitüsü Tarımsal Genetik Mühendisliği Dalı'nda Yüksek Lisans tezi olarak kabul edilmiştir.

(The study titled **"Characterization of somatic metaphase chromosomes in bottle gourd (*Lagenaria siceraria*)"** and presented by **Kamran KHAN** under the supervision of **Assoc. Prof. Dr. Ahmet Latif TEK ,** has been recognised as Master thesis by the jury at the Department of Agricultural Genetic Engineering of Niğde Ömer Halisdemir University Graduate School of Natural and Applied Sciences.)

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/ /2020

Prof. Dr. Murat BARUT
DIRECTOR

THESIS CERTIFICATION

I certify that this thesis has been duly written by me, to the best of my knowledge. All the necessary information provided in the thesis is scientific and in accordance with the academic rules. All the help received in conducting the research and all other sources used have been duly acknowledged.

Kamran Khan

ÖZET

SUKABAĞI (*Lagenaria siceraria*) SOMATİK METAFAZ KROMOZOMLARININ KARAKTERİZASYONU

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Danışman : Doç. Dr. Ahmet Latif TEK

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Kromozom yapısı temelinde, sitogenetik çalışmalar türlerin genomik ve genetik özellikleri hakkında önemli bilgiler sağlar. Türlerin tanımlanmasında ve ıslah programlarının ilerelemesinde kritik bir rol oynamaktadır. Bu çalışmada, AT ve GC nükleotitleri açısından zengin bölgeleri, farklı hücre döngüsü aşamalarında nükleolar düzenleyici bölgeleri (NOR) tespit etmek için CMA_3/DAPI ve gümüş nitrat boyaması (bantlama yöntemleri) kullanılmıştır. Kromozom çalışmaları için ribozomal genler (rDNA'lar) önemli sitogenetik belirteçlerdir. Bu araştırmanın temel amacı, su kabağının metafaz kromozomları üzerinde ribozomal genlerin (rDNA'lar) 45S ve 5S lokus sayılarını ve yerlerini FISH tekniği yardımıyla tespit etmektir. Elde ettiğimiz sonuçlar, interfaz aşamasında 45S sinyalinin, profazda, subtelomerik fazda veya terminal bölgedeki metafaz kromozomlarında sırasıyla 4, 4 ve 2 adet, sinyalin bulunduğunu göstermektedir. Metafaz kromozomlarının sub-telomerik veya terminal bölgesinde 5S'in varlığı kayıd edilmiştir. Bu sonuçlar, Kabakgiller türleri arasındaki kromozom yapısı, genom yapısı ve filogenetik ilişkiler hakkında önemli bilgiler sağlamaktadır.

Anahtar Sözcükler: sitogenetik markörler, rDNA, FISH, sub-telomerik, genom yapısı

SUMMARY

CHARACTERIZATION OF SOMATIC METAPHASE CHROMOSOMES IN BOTTLE GOURD (*Lagenaria siceraria*)

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On the basis of chromosome structure, cytogenetic studies provide an important understanding of the genomic and genetic features of the species. Cytogenetic studies play a critical role in the recognition of species and development of breeding programs. CMA_3/DAPI banding and silver nitrate staining methods were used to detect the AT-rich and GC-rich regions as well as nucleolar organizer regions (NORs) on the different mitotic stages. For the chromosome study, ribosomal genes (rDNAs) are important cytogenetic markers. The main aim of this research was to find numbers and locations of ribosomal genes (rDNAs) 45S and 5S on metaphase chromosomes of bottle gourd by using FISH. The results showed that there were 4 signals of 45S were present in interphase, 4 signals of 45S in prophase, 4 signals of 45S on metaphase chromosomes at the sub-telomeric or terminal region. However, there were 2 signals of 5S present at the sub-telomeric or a terminal region of metaphase chromosomes. These results will provide important knowledge about chromosome, genome structure and phylogenetic relationships in Cucurbitaceae species.

Keywords: Cytogenetic markers, rDNA, FISH, Sub-telomeric, Genome structure

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SYMBOLS AND ABBREVIATIONS

Symbols	Description
Gr	Gram
mM	Millimolar
%	Percentage
µl	Microliter
ml	Milliliter
mg	Milligram
M	Molar
µm	Micrometer
mm	Millimeter
ºC	Degrees celsius
pg	Picogram
Mbp	Mega base pair
kb	Kilobase
cm	Centimeter

Abbreviations	Description
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
FISH	Fluorescence *In Situ* Hybridization
NOR	Nucleolar Organizer Region
CMA_3/DAPI	Chromomycin A_3/4′-6-Diamidino-2-Phenylindole

CHAPTER I

INTRODUCTION

The family Cucurbitaceae includes cultivated species like melons, squashes, cucumbers, gourds, and pumpkins that are normally known as cucurbits (Robinson and Decker-Walters, 1999). This family contains many genera that are utilized for nourishment, pharmaceutical, medicinal and residential purposes; due to these advantages, a few species from this family become financially important globally (Ng, 1993). The members of the Cucurbitaceae family broadly dispersed all over tropical and sub-tropical regions. Many species of this family like cucumber, gourd, melon, watermelon, and pumpkins are produced worldwide to fulfill the human food and financial requirements. Cucurbits are also produced for use as ornaments, containers and several other purposes. The total production of vegetables in Turkey is approximately 24 million tons with roughly a cultivation area of 1 million ha. From total vegetable production, 35% of production comes from the species which belong to family Cucurbitaceae.

Bottle gourd (*Lagenaria siceraria*) belongs to the *Lagenaria* genus and the member of the Cucurbitaceae family also called opo squash or calabash containing 2n=2x=22 number of chromosomes and it is a diploid plant. A close genetic relationship has been revealed of bottle gourd with other economically important cucurbit species such as melon, watermelon and cucumber phylogenetically (Xu et al., 2011). The genome size of this plant is approximately 334 Mb (Achigan-Dako et al., 2008). Bottle gourd is a consumable, medicinal and useful plant species in the Cucurbitaceae family. In the world, the mature fruit of bottle gourd is used as containers, bowl, music instruments, and decorative purposes and sometimes as the fishing float. Seeds, shoots, tendrils, and leaves are used for cooking purposes. Seeds of bottle gourd are also used in oil production. Seeds of bottle gourd, young leaves and tendrils are also used for medical purposes. Bottle gourd fruit has been traditionally used for cardioprotective, cardiotonic, diuretic, aphrodisiac and remedy for certain poisons and scorpion sting. There is a great number of morphological variations are present in the accessions present in Turkey although it is not the native origin of bottle gourd (Yetisir et al., 2008).

According to the current perspective, cytogenetic is the science of chromosomes in the extensive sense which plays a vital role in discovering the purpose and chromosomes behavior by using light, electron microscope and other advanced molecular tools (Sybenga, J., 2012). Complete and new information on genome structure, gene function, and meiotic recombination can be provided by cytogenetic studies (Heslop-Harrison, 1991).

The chromosome is defined as the structure which is packaged by DNA and histone proteins. In the nuclei of all eukaryotic cells, chromosomes are the major carrier of genetic material. Chromosomes are formed from a molecule of linear DNA and the related proteins that bound together (Heslop-Harrison and Schwarzacher, 2011). Each chromosome structurally has a chromosome arm(s), centromere, telomere, euchromatin, and heterochromatin (Richards, E.J et al., 1998). A primary constriction in every chromosome is known as the centromere. A chromosome is divided by centromere into 2 parts or arms. The p arm is used to label the short arm of the chromosome. The q arm is used to label the long arm of the chromosome. The characteristic shape of the chromosome is given by the position of the centromere on each chromosome. The position of the centromere on each chromosome is also very helpful to detect the position of specific genes.

Cytogenetic studies require the best quality metaphase preparation that contains a high amount of proper metaphase spread that is necessary for chromosome banding methods and *in situ* hybridization. For the mitotic chromosome preparation, the meristematic plant tissues that contain actively dividing cells root tip meristems are one of the most commonly used. Other plant tissues can also be used such as leaf meristems (Anamthawat-Jonsson, 2004), calli or protoplast. Plants rigid cell wall and cellular waste create difficulties in the preparation of chromosomes that create problems in modern cytogenetic techniques like genomic and fluorescence *in situ* hybridization. In-plant cytogenetics, a typical tissue is root tip meristematic cells to get metaphase chromosomes. The condition of mitosis in root tips can be effectively affected by the pretreatment of root tips with different chemicals (Darlington and La cour, 1969). Many pretreatment chemicals like 8-hydroxyquinoline, ice-cold water, colchicine, and alpha-bromonaphthalene contribute a vital job in the collection of cells at metaphase and condense chromosomes during mitosis (Singh, 1993).

For identifying nucleolar organizer regions (NORs) on chromosomes, the most frequently used method is silver nitrate staining because of its effectiveness and ease of performance (Rufas et al., 1982). A quick method to reveal the ribosomal gene behavior for the calculation of protein synthesis rate is the silver staining of NORs. Tandem repeat genes are present in nucleolar organizer regions (NORs) which are called ribosomal genes (rDNA). Ribosomal genes (rDNA) are responsible for all cellular protein synthesis when transcribed into the ribosomal RNAs (Sumner, 2003). For the chromosomes in various plants, Chromomycin A_3 (CMA_3) GC-rich specific fluorochrome and DAPI the AT-rich specific fluorochrome banding techniques are entirely dependable and effective (Hizume, 1991). The GC-rich guanine-cytosinespecific fluorochrome Chromomycin A_3 (CMA_3) and the AT-rich adenine-thymine specific fluorochrome DAPI play an important role in chromosome analysis as well as useful for determining the phylogenic relationships of Citrus and other plant species (Yamamoto et al., 2007).

In the plant genomes, there are many regulatory elements, classes of repetitive DNA sequences and many different or a low copy number of coding sequences are present (Schmidt and Heslop-Harrison, 1996). Repetitive DNAs that have coding functions are called genes for ribosomal RNA (rRNA). The huge portion of the plant genome of about 90-95% of the nuclear DNA contains repetitive DNA sequences. In the plant genome, there are two different families are present in ribosomal RNA (rDNA) genes called 45S and 5S. Many copies of the intergenic spacers and coding sequences as tandem repeats are also present in the plant genomes (Katsiotis et al., 2000). Non-transcribed spacers and tandem repeat units of the 18S-5.8S-26S ribosomal genes are present in the 45S rRNA. NOR (nucleolar organizer regions) is developed by each unit that is normally 10kb long in plants (Heslop-Harrison, 2000). In 5S rRNA, there is anon-transcribed spacer and a conserved coding region of 120 bp is present. The location of both 45S and 5S rRNA can be present at one or more positions for every set of chromosomes (Heslop-Harrison and Schwarzacher, 2011). In cytogenetic studies, 45S or 5S rRNAs can be used as important cytogenetic markers (Vasconcelos et al., 2010), in the cucurbits relative studies have to some extent been incomplete (Li et al., 2007).

A molecular cytogenetics technique FISH plays an important role to find the relationship between intra- and inter-specific species. FISH with ribosomal genes (rRNA) as probes is a frequently used tool especially in chromosomes with small size (Leitch and Heslop-Harrison, 1992). The FISH technique is used to detect the interphase nucleus, DNA fibers, and target sequence position by the method of binding labeled nucleic acid on to the chromosomes (Li et al., 2015).

Main aims of the study are given below:

1- Identify and characterize the chromosomes in bottle gourd by using the squash root tip method.
2- Identification of nucleolar organizer regions (NORs) on metaphase chromosomes by using silver nitrate staining.
3- Identification of AT-rich regions and GC-rich regions on metaphase chromosomes by using CMA_3/DAPI staining method.
4- Physical mapping of ribosomal genes (rDNA) 45S and 5S on somatic metaphase chromosomes in *L. siceraria* by using the FISH technique.

CHAPTER II

LITERATURE REVIEW

2.1 General Information of Cucurbitaceae Family

Recently scientists give significant importance to the members of this family in breeding programs that help to advance the cucurbit quality because these crops produce a large amount of income (Weng and Sun, 2012). Botanically this family is extremely focused in which most of the plants are climbing plants and medium in size. There are 100 genera and 800 species are present in this family which is mostly allocated to tropical or subtropical and some of the species are present into temperate climate. Out of 100 genera, 20 genera are used for cooking purposes which generally involved in the utilization of the mature fruit, whole young fruit, and seeds. From total 100 genera, three genera (1) *Cucumis* (cucumbers, melons) (2) *Cucurbita* (Pumpkins, Squash) (3) *Citrullus* (watermelons) are among in the top 10 rankings in economic importance between the vegetable crops of the world and many others have local importance. They are called major cucurbits. For smallholder farmers, the members of some genera are vital especially in the South, East and Southeast Asian countries like bottle gourd (*Lagenaria siceraria*), sponge and ridge gourd (*Luffa ssp*.), bitter gourd (*Momordica charantia*),snake gourd (*Trichosanthes ssp*.) and wax gourd (*Benincasa hispida*). These crops are considered minor cucurbits from a universal point of view because these crops are grown by smallholder farmers, especially in Asia.

2.2 Status of Cucurbitaceae Family in Turkey

The most commonly produced cucurbits in Turkey are watermelon, melon, cucumber, summer and snake squash, and pumpkin. Some other species from Cucurbitaceae family are also produced in Turkey like *Lagenaria siceraria* (bottle gourd), *Luffa cylindrica* (sponge gourd), *Momordica charantia* (bitter melon) and *Cucumis melo* (snake melon), but these are considered minor cucurbits because they are produced by smallholder farmers in Turkey. Turkey is considered as a microcenter for many landraces as well as for Cucurbitaceae species like muskmelon (*C. melo)* [subtropical and tropical Africa], watermelon (*C. lanatus)* [subtropical and tropical Africa], cucumber (*C. sativus)*

[Central Asia and Himalayas], butternut squash (*C. moschata)* [South America] and pumpkin (*C. pepo*) (South America). All these Cucurbitaceae landraces are still produced in almost all regions of Turkey that mostly change in shape and taste. Cucurbitaceae species are used as food, pickling, containers and medicinal purposes in Turkey.

2.3 General Description of Bottle Gourd *(Lagenaria siceraria*)

Bottle gourd *(Lagenariasiceraria*) is an important underutilized crop (van Wyk, 2011). This crop is grown by smallholder farmers. The *Lagenaria* genus consists of five other wild species, namely: *L. abyssinica* (Hook f.), Jeffrey, *L. rufa* (Gilg.), *L. breviflora* (Benth.) Roberty, Jeffrey, *L. sphaerica* (Sonder) Naudin and *L. guineensis* (G. Don) Jeffrey. In Africa, all six species are found, which is supposed to be the native place of genetic diversity for *L. siceraria*. *Lagenaria siceraria* is the only main developed species. *L. siceraria ssp.siceraria and L. siceraria ssp.asiatica.* are two morphologically different sub-species of bottle gourd that have been known within the species of *L. siceraria.* Significantly, the initial partial sequence of bottle gourd genome displayed a 90% sequence similarity to the cucumber genome (Xu et al., 2011).

2.4 Botanical Description of Bottle Gourd (*Lagenaria siceraria)*

Bottle gourd is a fast-growing annual vine climbing by means of tendrils (Figure 2.1 a). Bottle gourd is a monoecious species with male and female flowers found separately on the leaf axils of the same plant (Achigan-Dako et al., 2008; Singh, 2008). Bottle gourd is a greatly cross- pollinated crop despite the fact that it is a monoecious crop. There are also some wild, non-cultivated species that are present which have dioecious (male and female flowers found on different plants) and andromonoecious sex forms (sex forms bearing male and perfect flowers). There is a great diversity is present in bottle gourd accessions with respect to fruit size and shape in Turkey (Singh, 2008; Yetişir et al., 2008; Xu et al., 2014).Some common fruit shapesof different accessions of Turkey (Figure 2.1 b) (Yetişir et al., 2008).

a b

Figure 2.1. Bottle gourd plant showing white flower and climbing nature (a) and some common fruits of different accessions of Turkey to show the size and shapes of different fruit of bottle gourd plant (b (Yetişir et al., 2008)

2.5 Nutritive Value of Bottle Gourd

There is a large number of essential minerals (iron, phosphorus, potassium, zinc and magnesium), dietary fiber, amino acids and vitamins (vitamins B and E) are present in the young edible fruits (Milind and Satbir, 2011). Blood pressure can be controlled by the juice of its fruit because it has high potassium content (Milind and Satbir, 2011). It also plays a vital role in weight loss because it has high dietary fiber and low fat and cholesterol levels. Diseases like liver diseases, hypertension, liver diseases, flatulence, ulcer, and diabetes mellitus can also be treated by using fresh bottle gourd juice and also used as a diuretic (Ghule et al., 2007).

2.6 Bottle Gourd History, Origin, and Distribution

In tropics, bottle gourd is one of the earliest crops grown by mankind (Erickson et al., 2005; Clarke et al., 2006). Bottle gourd was consumed by humans in the new world almost 15000 years ago and 12000 years ago in the old world according to archaeological data. In the Americas (New World) bottle gourd production and consumption started from around 9000 - 10000 B.C. (before present), 6000 - 10000 B.C. in East Asia and 4000 - 5000 B.C. in Africa according to same archaeological data. Bottle gourd was native to tropical Africa and spread to the Americas (New World) by the oceanic movement or human transportation. The conclusion of the Whitaker study

that Africa is the native place of bottle gourd was supported by Heiser (1980). There is still very little significant verification data is available to decide the original native place of this species between Africa and America (Heiser, 1980). The Ancient dispersal and distribution of the bottle gourd in the Americas, Asia, and Oceania is shown in Figure 2.2.

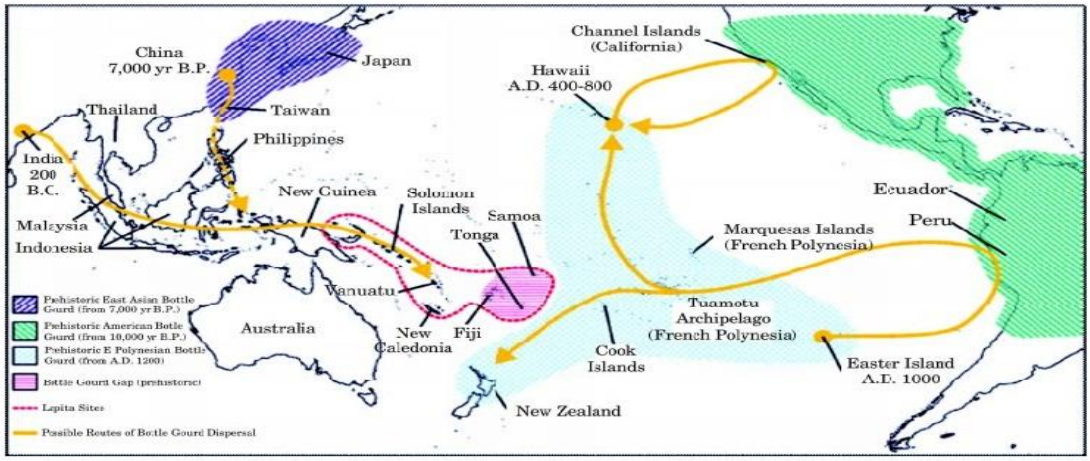


Figure 2.2. Ancient dispersal and distribution of the bottle gourd (*Lagenaria siceraria*)Americas, Asia, and Oceania. Bottle gourd is present in East Asian countries from 7000 Yr B.C. shown by dark blue lines. Bottle gourd is present in the Americas from 10000 Yr B.C. shown by the green area. Distribution of bottle gourd to other countries shown by the yellow line. (Clarke et al., 2006)

2.7 Economic Importance of Bottle Gourd (*Lagenaria siceraria)*

Bottle gourd is a consumable, remedial and useful plant species in the Cucurbitaceae family (Decker-Walters et al., 2001). In the world, the mature fruit of bottle gourd is used as a container, bowl, music instruments, and decorative purposes and sometimes as the fishing float. Seeds, shoots, tendrils, and leaves are used for cooking purposes. Seeds of bottle gourd also used as oil extraction. Due to the presence of large amounts of the protein, minerals, lipids and fatty acids in the seed of bottle gourd (Essien et al., 2013) make it perfect for human food or for inclusion into animals feed (Ogunbusola et al., 2010). Seeds of the bottle gourd, tendrils, and young leaves are also used for medicinal functions. Bottle gourd fruit has been usually used for cardiotonic, diuretic, cardioprotective, aphrodisiac and remedy for certain poisons and scorpion sting.

Mostly this crop is cultivated for its fruit when it is used as a vegetable harvested young and harvested mature when used as a utensil, bottle, or pipe harvested mature. In most regions of Asia and Africa fresh fruit of bottle gourd which generally has a light green smooth skin and a white soft tissue used as a stir-fry or vegetable soup. Sweet, pickles and other tasty dishes can also be prepared by using its tender edible fruits. It is recommended for the curing of minor illness due to the cooling effect on the stomach and easy digestibility (Singh, 2008).

2.8 Agricultural Importance of Bottle Gourd

Bottle gourd is used as a rootstock for watermelon against soil-borne diseases such as low soil temperature, Fusarium wilt, high pH, salinity and excessive water in soil (Yetisir et al. 2006). Recently, seedless watermelon produced by pollinating with bottle gourd pollen (Sugiyama et al., 2014). That's why plant breeders are strongly attracted to studying the germplasm of bottle gourd to use in watermelon breeding. Bottle gourd (*L. siceraria)* has been commonly used like rootstock mainly for watermelon for several years in eastern Asia (China, Japan, and Korea). In recent times, in Europe and U.S.A as a rootstock bottle gourd has received major attention as an alternative approach to handle soil-borne diseases such as fusarium wilt.

2.9 Current Information on Bottle Gourd Genome

Very little molecular genetics/genomic resources for bottle gourd are present openly up till now. The bottle gourd genome size is approximately 334 Mb (Achigan-Dako et al., 2008). Though bottle gourd genome size is relatively small but still very few DNA sequences of this plant present in the public DNA database, due to this reason it is difficult to detect the genes of this plant and to study their functions. Up until now DNA markers that are locus-specific such as sequence-tagged site (STS), microsatellite (SSR) and single nucleotide polymorphism (SNP) markers are not present (Xu et al., 2011). Unluckily, for this plant a very less molecular data is accessible and also molecular data about the genetic relationship with other family species such as cucumber (*Cucumis sativus)* that is considered as a model plant for Cucurbitaceae family members is not clear (Srivastava et al., 2014).The evolution of gene families and the phylogenetic tree of 12 species is shown in Figure 2.3 (Xie et al., 2019).

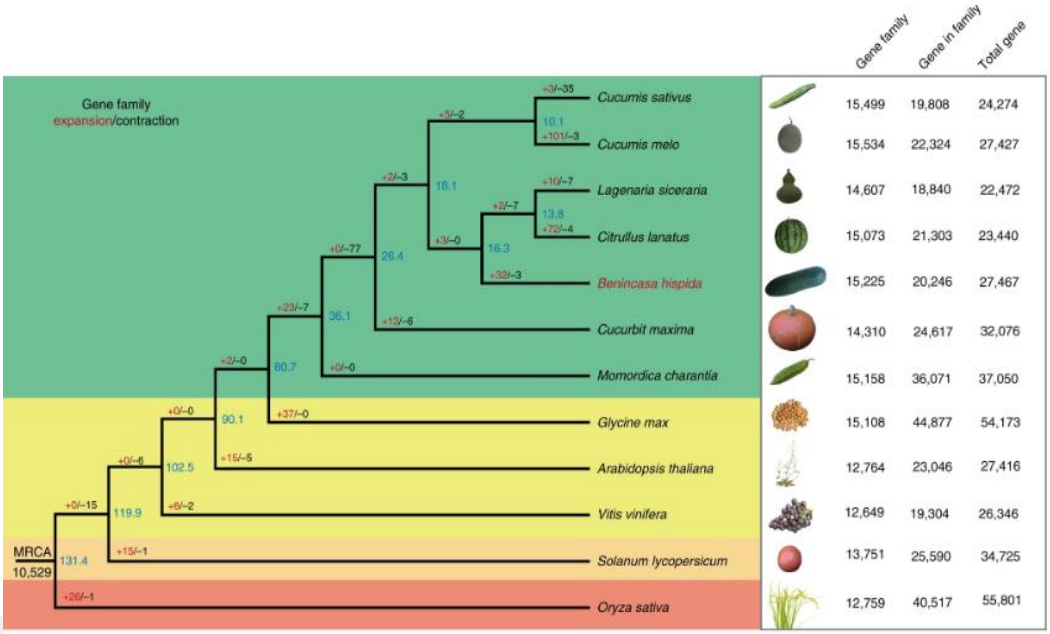


Figure 2.3. Evolution of gene families and phylogenetic tree of 12 species (Cucumber, muskmelon, bottle gourd, watermelon, wax gourd, buttercup squash, bitter squash, soybean, *Arabidopsis thaliana*, common grapevine, tomato, rice (Xie et al., 2019)

For genetic investigation in bottle gourd several molecular markers have been developed named as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSRs) chloroplast markers, sequence-related amplified polymorphism (SRAP), amplified fragment length polymorphism (AFLP), single nucleotide polymorphism (SNPs), allozyme markers, start codon targeted and inter-simple sequence repeats (ISSR) (Koffi et al., 2008). New technique such as restriction site-associated DNA sequencing (RAD-Seq) was used for genome-wide analysis. Their results showed that 45,066 perfect microsatellite repeat-motifs are present in the genome and also found scaffolds/contigs sequences data. They also studied nucleotide repeats in the genome and found that 34.3% tetra nucleotide repeats, 30.73% tri nucleotide repeats were present while the percentage of di-, penta- and hexa nucleotide repeats were 21.03%, 9.6% and 4.3%, respectively (Xu et al., 2014).

In the genome of bottle gourd, 84 disease-resistant genes are present (Wu et al., 2017). A large number of disease-resistant genes are available in the genome of this plant than cucumber, melon, and watermelon but lesser than Rosacea species, rice and Arabidopsis. On all 11 chromosomes, these genes were present but a high number of

genes were present at chromosome no 8 and 11 that was 21 and 19 genes, respectively. Gourd Base genome browser is used to detect the disease-resistant genes as well as peptides, mRNAs and other genes of interest that give a graphical explanation on the genome of this plant (Wang et al., 2018).

To introduce better quality varieties the old breeding techniques of bottle gourd should be shifted to new breeding and genomic techniques because the genome of this plant has not been completely sequenced and very less genomic resources openly available (Xu et al., 2011, 2014). Developed simple sequence repeat (SSR) markers for this plant used in many analyses and should be engaged to display population of segregation and to detect breeding lines that hold desirable traits for this plant in marker-assisted breeding. It will make possible in the fast releasing of improved bottle gourd varieties (Abdin et al., 2015). The SSR markers transferability is considerable because they can be used in the study of the genetics of related Cucurbitaceae species like the bottle gourd. They are also very helpful for the interest of breeders to develop linkage maps for this plant (Yildiz et al., 2015).

2.10 Status of Bottle Gourd in Turkey

Although Turkey is not the native place of genetic variation for bottle gourd (*Lagenaria siceraria*), the accessions of bottle gourd exhibit great variation mainly in the shape and size of fruit (Yetisir et al., 2008). A great number of different accessions of bottle gourd are usually grown for many purposes like food, decorative, containers and musical instrument in villages and most of the small cities of Turkey depending on the features of the fruit. 25 years ago, many accessions of bottle gourd in numerous cities of Turkey were grown but now accessions have slowly vanished because now a day's utensils are preparing from plastic rather than bottle gourds. Now in Turkey bottle gourd is cultivated in some small areas for human utilization and decorative uses. But still, in Southern and Western Turkey, there are significant variations present among bottle gourd (*Lagenaria siceraria*) accessions. The growing areas of bottle gourd in Turkey are shown in Figure 2.4.

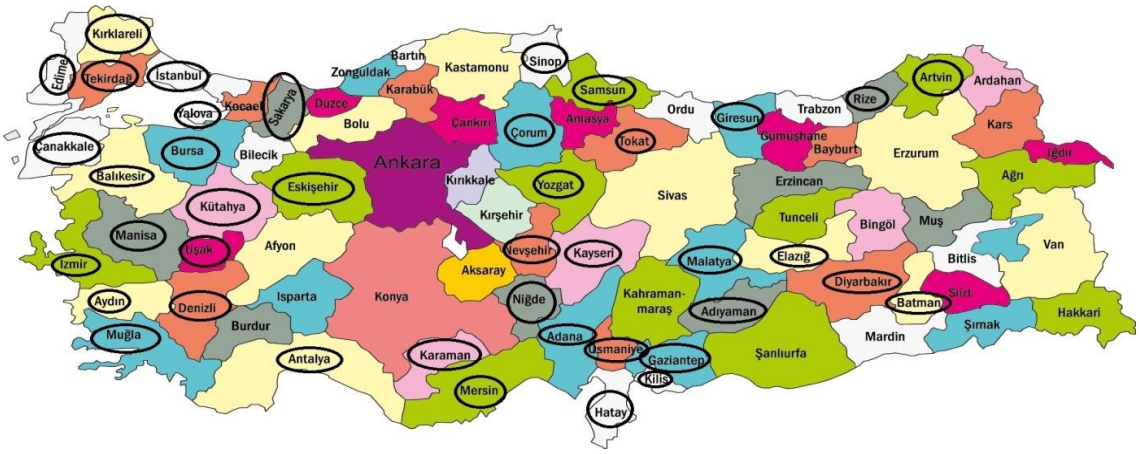


Figure 2.4. Growing areas of bottle gourd (*Lagenaria siceraria*) in Turkey (Yetişir et al., 2008)

2.11 Brief Description of Molecular Cytogenetics

The advancement in the cytogenetic tools played a vital role in the programs of breeding to influence both genetic structure and the crop-to-wild gene transfer system in wheat (Sybenga, 2012), maize and watermelon. Molecular cytogenetic studies explain phylogenetic relationships (Heslop-Harrison, 1991) and give valuable knowledge for plant breeding (Guo et al., 2013) as well as polyploidy, physically mapping specific DNA sequences, and genome rearrangements (Sybenga, 2012).

2.12 Brief Introduction of Chromosomes and Squash Root Tip Method

Chromosomes performed a vital role in the evolution of organisms as a unit of inheritance. The very first step in genome analysis is the characterization and identification of chromosomes. Many kinds of cytogenetic analysis can be done like chromosomal rearrangements, chromosomal assignment of genes, chromosome identification and others due to the construction of cytogenetic maps (Zhao et al. 2015). The structure of a chromosome is shown in Figure 2.5. Good hybridization results are required for the best quality of the chromosomes preparations. The preparation of chromosomes should be flat, well spread and have many chromosomes with good structure. There should not be any cytoplasmic and other cellular residue remains in the chromosomes. Therefore, many scientists developed expensive protocols to remove the

debris from chromosomes based on enzymatic digestion of the cell wall (Bowler et al., 2004), protoplast isolation and drop method (Andras et al., 1999).

The analysis of mitotic cell division could be very helpful for chromosome characterization because the chromosomal structural makeup is balanced in this cell division and can be seen clearly. Complete components of chromosome inside the cell are known as Karyotype. To determine the identity of an organism like study the biological changes, the study of the evolution process, genetic disability and taxonomical unit of organisms, an important tool to study these functions in organisms is karyotype (Jones and Luchsinger 1979). The fundamental studies for the development of economically essential plant species, karyotyping and FISH as cytogenetic study tools are essential for this purpose (Vasconcelos et al., 2010). Chromosome characterization becomes easier and more dependable after the development of molecular cytogenetic techniques (Fukui, 2005).

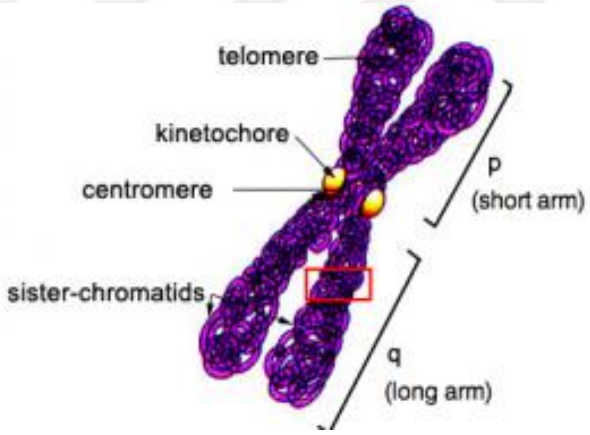


Figure 2.5. Chromosome structure (Estandarte, 2012)

2.13 Brief Description of Ribosomal Genes (rDNA)

Plant genome contains thousands or tens of thousands of families which differ in motif length, copy number and arrangement (Heslop-Harrison, 2000). Still, it is not completely reported the reasons for having large amounts of repetitive DNA and a huge range in the genome. It is assumed that these repetitive DNA performed a major role in the balance and protection of chromosome structure or they are involved in the

identification and proper separation of chromosomes in mitosis and meiosis (Kubis et al., 1998).

Important information for chromosome study can be provided by the characteristics of 45S and 5S rDNAs with chromosomes (Heslop-Harrison, 2000). By using FISH with ribosomal genes mapping on chromosomes we can get knowledge about the identification of chromosomes, structure of chromosomes, association and differentiation of rDNAs in strongly associated species, chromosomes development and species pattern in important economic and medicinal plants (Zhao et al. 2011). Arrangement of ribosomal genes coding for 45S and 5S rRNA is shown in Figure 2.6.

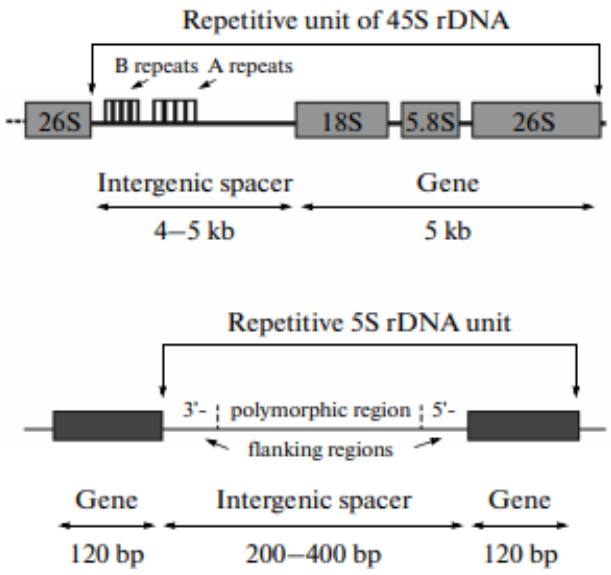


Figure 2.6. Arrangement of ribosomal genes coding for 45S and 5S rRNA (Shcherban, A.B., 2015)

2.14 Brief Description of FISH

The molecular tool in cytogenetics for the analysis of phylogenetic relationships at the chromosomal level is FISH. For studying genome structure, constructing a physical map of the genome, chromosome identification, studying the spatial location and dynamic changes of chromatin in cell cycles, analyzing DNA molecular composition of specific chromosome region and chromosome evolution FISH has been widely used for this purpose (Li et al., 2015). The steps in the FISH technique are shown in Figure 2.7.

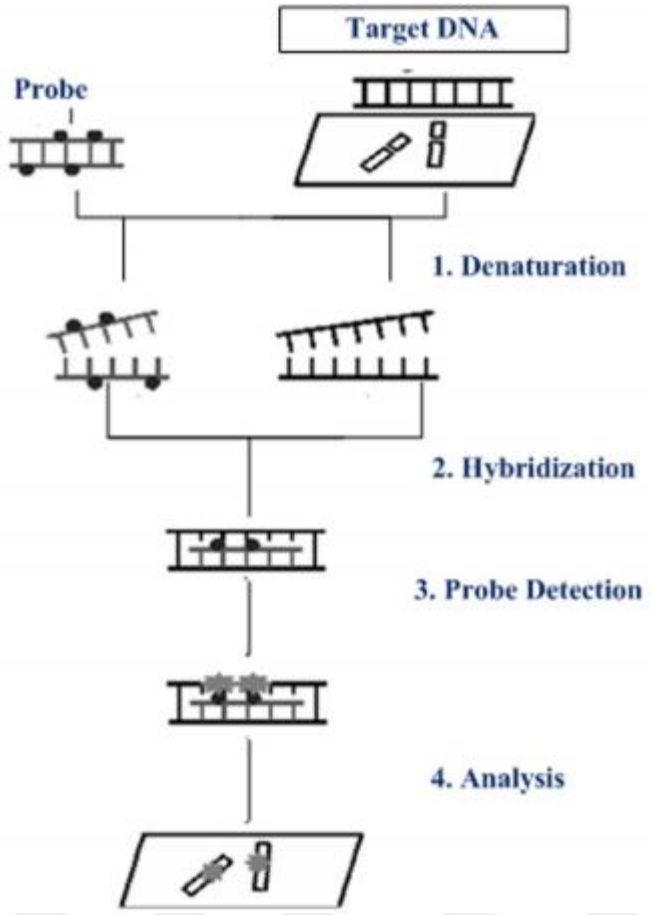


Figure 2.7. Steps of the FISH technique (Ratan et al., 2017)

FISH is a molecular technique that performs an outstanding role to map cytogenetic markers on chromosomes (El-Twab and Kondo, 2006). It also provides information aboutthe genome structure and detection of chromosomes. We can get knowledge about chromosomal relocation during evolution and phylogenetic relationships by the utilization of rDNAs and identified cytogenetic markers (Miao et al., 2016). Small chromosomes are present in many cucurbit species that are complicated to identify but FISH performs an excellent job to detect the structure of chromosomes and give knowledge about small chromosomes that can be utilized in programs of breeding (Waminal et al., 2011; Waminal and Kim, 2012).

The identification of chromosomes properly in many plants by using species-specific repetitive sequences has been done by FISH. The physical positions of 5S and 45S rRNAs reported in barley, tomato and wheat (Leitch I J and Heslop Harrison J S, 1992), garlic and *Aegilopesum belluata* (Castilho A and Heslop Harrison J S, 1995). The physical mapping of ribosomal genes, microsatellite, and transposable DNA sequences

on sugar beet chromosomes has been done by using FISH (Schmidt T and Heslop-Harrison J S, 1996).

2.15 Previous Work Done on Ribosomal Mapping in Bottle Gourd

Previously, ribosomal mapping in bottle gourd is done by Waminal et al., 2012 and no other report is present for ribosomal mapping in bottle gourd. Their results showed that on interphase two signals of 5S and four signals of 45S as well as on metaphase two signals of 5S and four signals of 45S rDNA signals were observed. They also mentioned that on metaphase chromosomes two 5S rDNA signals were closed to the two signals of 45S rDNA as well as two 5S rDNA signals were closed to the two signals of 45S rDNA in the interphase nuclei. On the short arm of chromosome two and three two signals of 45S rDNA were present. On the short arm of chromosome 9 and 10, two signals of 5S rDNA along with two signals of 45S rDNA were present together. There is no report is present for the silver staining and CMA_3/DAPI staining of metaphase chromosome of bottle gourd.

Table 2.1. Previous results of ribosomal mapping in bottle gourd (by Waminal et al., 2012

Common name	No of 45S ribosomal genes signals	No of 5S ribosomal gene signals	No of NORs by silver nitrate staining	Chromomycin A_3/DAPI Staining signals	Reference
Bottle gourd	4	2	-	-	Waminal et al., 2012

2.16 Previous Work Done on Ribosomal Mapping on Cucurbitaceae Family Members in Worldwide

A study was done on the positions of heterochromatic bands, 45S and 5S rDNA regions which were present on the chromosomes in three cultivars of cucumber (*Cucumis sativus* L.) (Tagashira et al., 2009). Their study results explained that most of the chromosomes in three cultivars showed the heterochromatic region on both terminal and pericentromeric regions by using the DAPI bands. Although between European and Eastern Asian cultivars the patterns of these bands were different. Their FISH results

revealed that on the chromosomes 1, 2 and 5 there were the major 45S rDNA sites present around the centromeric region and on the chromosomes 3 and 7 the minor 45S rDNA sites were present around the centromeric region. They did not observe the positional difference of this region between three cultivars but in chromosome 5 they noticed size variation. Furthermore, they noticed that in all three cultivars 5S rDNA site was present in the interstitial region of chromosome 6. They concluded from research that in the heterochromatic region the difference of chromosome structure among cucumber cultivars was present. By careful study and evaluation of the position and size of the heterochromatic region, they noticed the genetic difference among cucumber cultivars.

Four wild Cucurbitaceae Species were investigated that named *Citrullus lanatus* (Thunb.) Mansf. var. citroides L. H. Bailey (2n = 22), *Melothria japonica* Maxim. (2n = 22), *Sicyos angulatus* L. (2n = 24), and *Trichosanthes kirilowii* Maxim. (2n = 66, 88, 110 cytotypes) (Waminal and Kim., 2015). All species were diploids, except for *T. kirilowii*, which included hexa-, octa- and decaploid cytotypes (2n = 6x = 66, 8x = 88, and 10x = 110). They reported that cytogenetic studies based on chromosome composition give basic knowledge about the genetic and genomic properties of a species that can be useful in a breeding program. In this research, they used the FISH technique with 5S and 45S ribosomal DNA (rDNA) probes to identify the ploidy level, chromosome structure, and genomic distribution of 5S and 45S ribosomal DNA (rDNA) in four wild Cucurbitaceae species. Their research results mentioned that all species had small metaphase chromosomes in the range of 2–5 μm. The 45S rDNA signals were confined to a small area distally compared to the 5S rDNA. There was one signal of 45S and two signals of 5S rDNA present in *C. lanatus* var. citroides and *M. japonica* but in *M. japonica* one signal of 45S was adjacent to 5S on one chromosome. Two signals of 5S and 45S rDNA loci were adjacent to each other in *S. angulatus.* Five signals each for 45S and 5S rDNA in the hexaploid *T. kirilowii* cytotype but three signals of 45S were adjacent to 5S.

A study was conducted on three Japanese cultivars of *Momordica charantia* by using the fluorescent staining method (Kido et al., 2016). A large amount of well-spread metaphase chromosomes was produced by using enzymatic digestion. There was the same somatic chromosome number 2n=22 present in studied cultivars. The majority of

chromosomes of all cultivars had early condensing segments at the proximal regions in condensation behavior from prophase to metaphase stage. Many chromosomes showed decondensed segments at the distal regions in both arms at prophase or earlier stage of metaphase. On the basis of centromere positions, there were twenty-two metacentric chromosomes present in three cultivars. Cultivars showed different sizes and numbers of Chromomycin A_3 (CMA_3) positive and DAPI negative (CMA_3/DAPI-) satellites with fluorescent staining. In *M. charantia* 'Abashi-goya' and *M. charantia* 'Naga-goya' four CMA_3/DAPI- satellites were present on the other hand in *M. charantia* 'Shiro-goya' two satellites were present. At one end of sat-chromosomes, all satellites were present. In all cultivars weakly CMA_3/DAPI sites were revealed at the proximal regions or primary constrictions of many chromosomes.

To find out cytogenetic interaction between closely related species, physical mapping of 5S and 45S rDNA by using the FISH method is an excellent tool. A study was conducted on the number and position of 5S and 45S rDNA loci in all *Citrullus* species and subspecies (Li et al., 2016). The study results showed that there were two 45S rDNA loci and one 5S rDNA locus which was present adjacent to one of the 45S rDNA loci on the same chromosome in the cultivated watermelon (*C. lanatus* subsp. *vulgaris*), *C. lanatus* subsp. *mucosospermus*, *C. colocynthis*, and *C. naudinianus*. There were one 45S rDNA locus and two 5S rDNA loci present on a different chromosome in *C. ecirrhosus* and *C. lanatus* subsp. *Lanatus*. One 5S and one 45S rDNA locus were present on different chromosomes in *C. rehmii.* A great diversity was present between these species in the distribution scheme of rDNAs. The researchers of this study concluded that evolutionary closeness was present between cultivated watermelon (*C. lanatus* subsp. *vulgaris*), *C. lanatus* subsp. *mucosospermus*, *C. colocynthis* however, *A. naudinianus* was more closely connected to *Cucumis* than to *Citrullus*, and act as a link bridge between the *Citrullus* and the *Cucumis*.

By cytogenetic studies based on chromosome composition,we can get important and basic information about the genetic and genomic characteristics of a species that can be helpful for species recognition and breeding programs. For karyotyping tandem repeats (TRs) like the 45S rDNA, 5S rDNA and telomeric repeats are present everywhere in nuclear genomes and are good cytogenetic markers. The karyotypes of three exotic cucurbit species named as *Cucumis melo* var. *flexuosus* (L.) Naudin (2n = 24),

Melothria pendula L. (2n = 24) and *Trichosanthes anguina* L. (2n = 22) were studied by using triple-color FISH with 45S, 5S probes to find out the cytogenetic distribution of the 45S, 5S ribosomal genes (rDNA) on chromosomes (Pellerin et al., 2018). The study results showed that *T. anguina* had larger chromosomes (3.2-5.4 μm) as compared to *C. melo* var. *flexuosus* and *M. pendula* (1.5-2.2 μm and 1.8-2.5 μm). In *C. melo* var. *flexuosus* one pair of 5S and two pairs of 45S rDNA signals were present. Four pairs of 45S rDNA and two pairs of 5S rDNA signals were present in *M. pendula*. Three pairs of 45S rDNA and two pairs of 5S rDNA signals were present in *T. anguina*. In *M. pendula* and *T. anguina*, co-localized signals of 5S and 45S were noticed but not in *C. melo* var. *flexuosus*.

2.17 Summary of Literature Review

Very less amount of studies has been found for examination or detection of somatic metaphase chromosomes and mitotic phases under light microscope in meristems cells of root tips in bottle gourd (*Lagenaria siceraria*) with different stains like acetocarmine and DAPI as well as physical mapping of ribosomal genes (rDNA) on somatic metaphase chromosomes by FISH technique. The aims of the study are to identify and characterize the chromosomes in bottle gourd by using the squash root tip method. Silver nitrate staining of metaphase chromosomes of bottle gourd to identify NORs. CMA_3/DAPI staining of metaphase chromosomes of bottle gourd to identify AT-rich and GC-rich regions. Physical mapping of ribosomal genes (rDNA) 45S and 5S on metaphase chromosomes in *L. siceraria* by using FISH technique. This research work will provide useful information about chromosome structure, genome structure and phylogenetic relationship of bottle gourd. This study will also provide background for the next cytogenetic studies in Cucurbitaceae family species.

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental Site Description

The research about "Characterization of somatic metaphase chromosomes in bottle gourd (*Lagenaria siceraria*)" was conducted at Plant Chromosome Laboratory of Department of Agricultural Genetic Engineering, Ayhan Şahenk Faculty of Agricultural Science and Technology, Niğde Ömer Halisdemir University, Turkey.

3.2 Plant Material

Bottle gourd (*Lagenaria siceraria*) seeds were used for research. The name of the population of seeds was Sandıklı which was used in this research (Figure 3.1. a). The plant material was the root of the plants coming to this population. The seeds were collected by Dr. Ahmet Latif Tek.

3.3 Germination of Seeds

The seeds were washed with distilled water to remove the contamination from the outer surface of seeds. Filled a plastic container with tap water and put the seeds in it to soak for 24 hours (Figure 3.1. b). The seeds were placed on cotton in the Petri dish to increase the rate of germination of seeds (Figure 3.1. c). The seeds were germinated after 3-4 days after sowing (Figure 3.1.).

a b c d

Figure 3.1. Bottle gourd seeds population Sandıklı used in the study (a), germination method (b), seeds on cotton in Petri plates (c) and germination of seeds after 3-4 days after sowing (d)

3.4 Optimization of Pretreatment Method

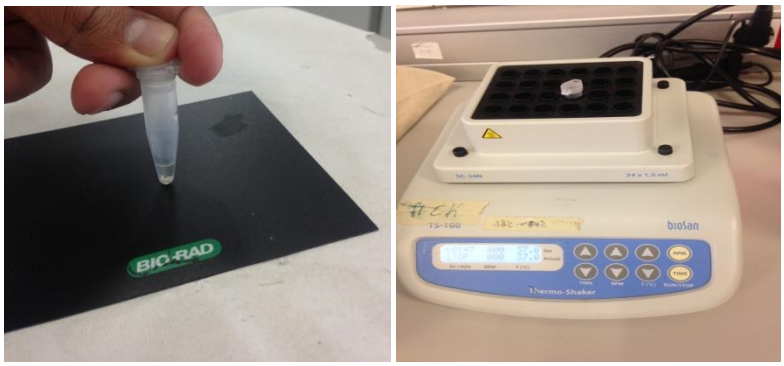
Root tips were cut 1-2 cm long and collected for somatic metaphase chromosome preparation and also for examination of different stages of mitosis. For the pretreatment of roots, 2 mM 8-hydroxyquinoline, cold treatment (+4ºC) and ice-cold water methods were used with different time periods. After pretreatment of roots, roots were washed with distilled water (dH_2O) 3 times for 5 minutes each. After washing, roots were put on tissue paper to dry. After drying, the roots were put in Carnoy's solution (3 parts of 95-100% ethanol and 1 part of glacial acetic acid) for 24 hours in the refrigerator (+4ºC). After 24 hours, the roots were washed with distilled water 3 times for 5 minutes each. Roots were placed on tissue paper to dry. After drying, the roots were placed in 1N HCl for 20 minutes. After hydrolyzation in 1N HCl, the roots were placed into 1% acetocarmine to stain the mitotic chromosomes for 24 hours in the refrigerator (+4ºC). Slides were prepared by cutting the root tips 1-2 mm long with a safety razor anddarkly staining with 1% acetocarmine. 1-2 mm long root tips were put on the slides. Few drops of 1% acetocarmine were dropped on the slides where 1-2 mm long root tips were present. Coverslips were placed on the slides. Root tips were squashed with a toothpick. The excess amount of acetocarmine was removed with tissue paper by gently press the coverslip with the thumb without moving it. Slides were checked under a light microscope by using a 100X lens with emulsion oil. The mitotic index % and mitotic phase % of three different pretreatment methods were calculated by the formulas

$$\text{Mitotic index \%} = \frac{\text{Number of dividing cells}}{\text{Number of dividing cells+Number of non dividing cells}} \times 100 \quad (3.1)$$

$$\text{Mitotic phase \%} = \frac{\text{Number of mitotic phase cells}}{\text{Number of dividing cells}} \times 100 \quad (3.2)$$

3.4.1 Chromosome oreparation with 4′-6-diamidino-2-phenylindole (DAPI) staining

Enzyme digestion with the flame-dry method was done as firstly roots were washed with distilled water 3 times for 5 minutes on the shaker. After washing with distilled water, roots were washed with 30 mM potassium chloride (KCl) 3 times for 5 minutes on the shaker. After that root tips were cut 1-2 mm long. Enzyme mixture was prepared that contained 50 µl 30 mM potassium chloride (KCl) + 30 µl 5x (Cellulose+Pectinase) enzyme + 20 µl distilled water (dH2O). Root tips were placed in the enzyme mixture (Figure 3.5.1 a). The enzyme mixture was placed in the incubator at 37ºC for incubation for 1:35 hours (Figure 3.5.1 b). After incubation, the enzyme mixture was replaced with distilled water and washed the roots to remove the enzyme for 30 minutes on ice. Slides were prepared as firstly prepared 3:1 solution which contained 3 parts 100% methanol and 1 part glacial acetic acid in a separate Eppendorf tube and placed it into the refrigerator (-20 ºC). Slides were prepared on the freezing board. Root tips were squashed completely with forceps on the slides. After squashed, pipetted a few drops of 3:1 solution on the slides. Slides were placed on the burning flame for a few seconds. Slides were air-dried for 1 hour. Pipetted 10 µl DAPI on slides for observation. Slides were checked with A.2 Olympus AX10 microscope using an oil lens (100X magnification). Photographs were captured with Zeiss Axiocam 702 mono chromatic camera attached to an Olympus AX10 imager view ZEN 2.3 lite software.



a b

Figure 3.2. Roots in enzyme (a) and roots for incubation (37ºC) in an incubator (b)

3.5 Silver Nitrate Staining

Silver nitrate stain was prepared as 1 ml of sodium citrate buffer (pH=3.4) was taken in the Eppendrof tube. 1 g of silver nitrate ($AgNO_3$) was added in the sodium citrate buffer (For 100 ml sodium citrate buffer, 2.40 g Sodium Citrate dehydrate + 0.34 g citric acid + 97.26 ml dH2O). The short centrifuge was done to dissolve the silver nitrate ($AgNO_3$) in sodium citrate buffer.

Slides were prepared for silver staining as firstly prepared the moisture chamber and adjust the temperature of the incubator at 45ºC. Slides were put in the moisture chamber and pipetted 250 µl of silver nitrate stain per slide. After putting the silver nitrate stain on the slides, slides were covered with nylon cloth. Slides were placed in the incubator at 45ºC for 40 minutes in the moisture chamber. After incubation, slides were washed with distilled water 3 times for 5 minutes and removed the nylon cloth from slides. After washing, slides were air-dried. A drop of glycerol was pipetted on the slides and covered the slides with coverslips.

3.6 Chromomycin A_3/DAPI Staining

For the preparation of McIlvaine's buffer firstly prepared stock solution as 5.670 g sodium phosphate was dissolved in 200 ml of distilled water and 2.10 g of citric acid was dissolved in 100 ml of distilled water in a separate glass jar. After the preparation of the stock solution, a working solution was prepared as pH 7.0 took 164.70 ml from the stock solution of sodium phosphate and 35.3 ml from the stock solution of citric

acid and dissolved in a glass jar. Pipetted 50 ml from the solution and diluted it with 50 ml distilled water in a separate glass jar. After dilution of Mcllvaine's buffer, 0.102 g of magnesium chloride ($MgCl_2$.6H2O) was added in Mcllvaine's buffer for the final concentration of 5 mM.

For the Preparation of Chromomycin A_3 (CMA_3) stain, pipetted 2 ml of Mcllvaine's buffer which has a final concentration of 5 mM in Eppendorf tube. Pipetted 0.5 mg Chromomycin A3 in few drops of 100% methanol and dissolved it completely. After that Chromomycin A_3 solution was added in 2 ml of Mcllvaine's buffer which had a final concentration of 5 mM and dissolved it completely.

Slides were prepared for ChromomycinA_3/DAPI (CMA_3/DAPI) staining as 20 µl of Chromomycin A_3 solution was pipetted on slides and covered the slides with a plastic paper. Slides were put in dark for 20 minutes on the moisture chamber. Slides were washed with distilled water (dH_2O) and removed the plastic paper from slides. Air-dried the slides for 1 hour in dark. For observation, pipetted 10 µl of DAPI solution on the slides and covered the slides with coverslips. Slides were placed in an incubator at 37 ºC for 2 days.

3.7 Fluorescence *in Situ* Hybridization (FISH)

3.7.1 Chromosome preparation

Chromosome preparation was done by enzyme digestion with the flame-dry method already mentioned in section 3.4.1.

3.7.2 Bacterial transformation

For the preparation of 45S and 5SrDNA probes, pTa71 and pTa794plasmid DNA's were used, respectively (Gerlach and Bedbrook, 1979; Gerlach, 1980). To isolate the clones, conserved regions of rRNA genes wereused for 45S and coding sequences of rRNA which were present in the wheat plant used for 5S (Gerlach and Bedbrook, 1979; Gerlach, 1980). LB media (For 100 ml LB,1 g tryptone + 0.5 g NaCl + 0.5 g yeast extract + 1 g agar) was prepared. LB media was pipetted in separate plates under the

laminar flow conditions. After that, 100 µl 50% glycerol + *E.coli* (XLN-1 Blue) bacteria were taken from stock solution pipetted on the LB agar plates and spread it evenly under laminar flow conditions. The plates were placed in an incubator at 37ºC for the growth of bacteria.

3.7.3 Miniculture and plasmid isolation

Miniculture was done to isolate single colony bacteria. Miniculture solution contained (3 µl LB media + 3 µl ampicillin). After 8 hours of a single colony, bacteria were produced. 100 ml media culture which contained *E. coli* (XLN-1 Blue) bacteria transferred in the incubator at 37ºC overnight. The silica matrix plasmid miniprep DNA purification method was used for plasmid isolation and purification of DNA. From the cells of 100 ml *E. coli*, the pellet was produced by centrifugation at 16000 RPM for 30 seconds. Cell pellet was resuspended in 500 µl resuspension solution (50 µl 1M Tris-HCl + 20 µl 0.5M EDTA + 20 µl RNase 5 mg/ml + 930 µl dH2O) by short vortexing. 500 µl alkaline lysis solution (10 µl 0.2M NaOH + 100 µl 1% SDS + 890 µl dH2O) was added and the tube was inverted many times. The Eppendorf tube was placed at room temperature for 2 minutes. 500 µl neutralization solution (1.32M KOAc, pH=4.8) was added and the tube was inverted many times. The Eppendorf tube was centrifuged at 16000 RPM for 5 minutes, supernatant transferred to a fresh tube that contained 2500 µl 6M NAI solution and mixed well by inverting the tube. 50 µl silica matrix was added in the Eppendorf tube, mixed well and placed the tube at room temperature for 5 minutes. By the centrifugation at 16000 RPM for 10 seconds the matrix pellet was produced. The supernatant was removed from the matrix pellet Eppendorf tube and gently tap the inverted tube to remove the liquid. The matrix was washed by resuspending in 500 µl washing solution (25 ml 50% ethanol + 500 µl 10 mM Tris-HCl pH= 7.5 + 1000 µl 100 mM NaCl + 100 µl 1mM EDTA + 23.5 ml dH_2O) and vortexed vigorously. After that, by centrifugation at 16000 RPM for 10 seconds a matrix pellet was produced. The supernatant was removed from the matrix pellet Eppendorf tube and gently inverted the tube to remove excess liquid. This procedure was repeated twice. Centrifugation was done again for 10 seconds and the residual liquid was carefully pipette off from the tube. 100 µl sterile water was added to resuspend the pellet by short vortex and the tube was placed at 70ºC for 2 minutes. Centrifugation was done at 16000 RPM for 2 minutes

and 36-38 µl supernatant that contained the eluted plasmid DNA was transferred in another Eppendorf tube. The DNA was stored in -20ºC.

3.7.4 Probe preparation and labeling

A volume of 16 µl DNA of 45S and 4 µl of digoxigenin was pipetted into an Eppendorf tube. The same volume was taken for 5S. Both tubes were placed in a thermocycler at 15ºC overnight. After that, 1 µl of 5M EDTA was added in both tubes and again placed them in a thermocycler at 65ºC for 10 minutes. Digoxigenin nick translation kit was used for probe labeling according to the manufacturer instructions (Roche, Germany). 45S and 5S probes were labeled by the nick translation method. Both probes were labeled with digoxigenin-11-dUTP (Roche, Accession No 11745816910).

3.7.5 Fluorescence *in Situ* hybridization (FISH) and signal detection

Hybridization solution was prepared which contain 10 µl hybridization mixture (1 g dextran sulfate + 4 ml formamide), 2.5 µl 20X SSC, 2.5 µl salmon sperm, 1.5 µl digoxigenin 45S probe, 1.5 µl digoxigenin 5S probe and 7 µl distilled water (dH2O) per slide in a separate Eppendorf tube. The hybridization solution was quick centrifuge and placed on ice for 5 minutes following an incubation period of 10 minutes at 95 ºC. A denaturation solution of 2X SSC and 70% formamide was prepared. Both solutions were placed on ice subsequently. Slides were washed 5 minutes each on a shaker with 70%, 90%, and 100% ethanol and air-dried. A volume of 100 µl denaturation solution was pipetted on slides. Slides were covered with coverslips and placed on a hot plate at 80 ºC for 2 minutes. Coverslips were gently removed from slides and transferred them in chilled 70% ethanol for 5 minutes on the shaker after which slides were transferred in 90% and 100% ethanol for 5 minutes washing. After air-drying slides, 25 µl hybridization mixture was pipetted onto slides and covered them with coverslips. After that slides were placed in a humid chamber and incubated overnight at 37 ºC.

After incubation, cover slips were removed from slides and transferred into 2X SSC for 5 minutes and 10 minutes washing following 5 minutes washing with 1X PBS on the shaker. A rhodamine-conjugated anti-digoxigenin antibody solution (0.5 µl rhodamine-conjugated anti-digoxigenin antibody, 25 µl 4X TNB and 75 µl distilled water (dH2O)

was prepared. A volume of 100 µl rhodamine-conjugated anti-digoxigenin antibody was pipetted onto the slides and covered with a parafilm. Slides were placed in a humid chamber and incubated at 37 ºC for 90 minutes. The slides were washed in 1X PBS for 5 minutes after incubation following 10 minutes staining with the DAPI solution. The slides were finally washed with 1X PBS again for 5 minutes and air-dried in a dark chamber. For observation, 15 µl antifade solution was pipetted on the slides and covered them with coverslips. Rhodamine-conjugated anti-digoxigenin antibody was used to detect the 45S and 5S signals. Slides were checked the same as previously mentioned in section 3.4.1

CHAPTER IV

RESULTS

4.1 Flow Chart of the Study

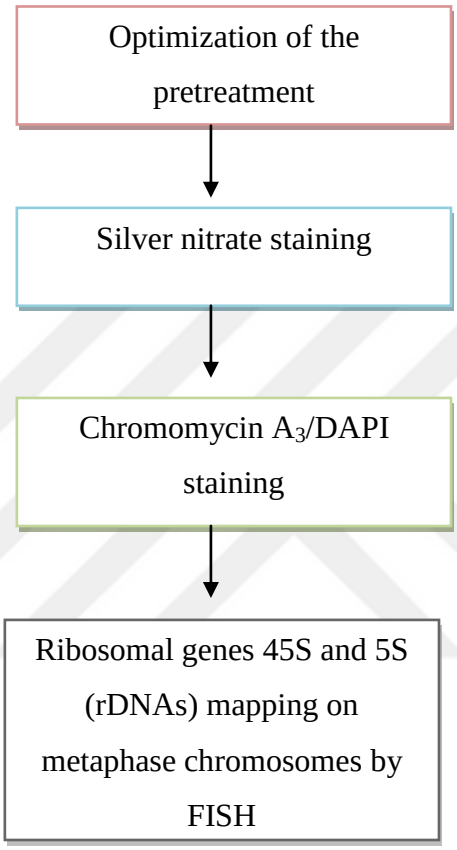


Figure 4.1. Flow chart of the study

4.2 Optimization of Pretreatment Method

For counting chromosomes, a better method had been developed in bottle gourd root tips by changing conventional techniques mentioned in other Cucurbitaceae species such as cucumber and watermelon. Root tips of bottle gourd were put in 8-hydroxyquinoline, cold treatment (+4 ºC) and ice-cold water with different time periods as shown in Table 4.1. Three different pretreatment methods were used to find the best pretreatment method that arrests a large number of somatic metaphase chromosome sets. There was a positive result of 8-hydroxyquinoline pretreatment with 24 hours in the preparation of chromosomes in the root tips that ends the development of spindles,

by arresting the chromosomes at the metaphase plate increased the number of metaphase cells, decrease the chromosome length with clear visible constrictions, and increased the cytoplasm viscosity. Fixation solution (3 parts of 100% ethanol and 1 part of glacial acetic acid) was used in this study. After fixation, the roots were hydrolyzed in 1N HCl for 20 minutes at room temperature because the root tips of bottle gourd were hard to squash on slides. This softens the root tips and enabled them to be easily squashed on slides for observation. After hydrolyzation, roots were put in 1% acetocarmine for 24 hours that allowed the best result for observation and counting of chromosomes. For the study of chromosomes in the different stages of mitosis in meristematic cells from bottle gourd root tips, the optimized procedure is very helpful for this purpose (Figure 4.2). Interphase is shown in (Figure 4.2 a). In the bottle gourd, the best stage for counting and studies the structure of chromosomes was metaphase (Figure 4.2 d). The chromosomes of bottle gourd were observed very small. Early and late prophase stages are given in (Figure 4.2 b,c). In bottle gourd, chromosome complement was observed symmetric and the majority were metacentric chromosomes as well as some were sub-metacentric. The mitotic index percentage of three different pretreatment methods with different time periods is shown in table 4.2. Graphical representation of the mitotic index percentage of different pretreatment methods with different time periods is shown in Figure 4.3.

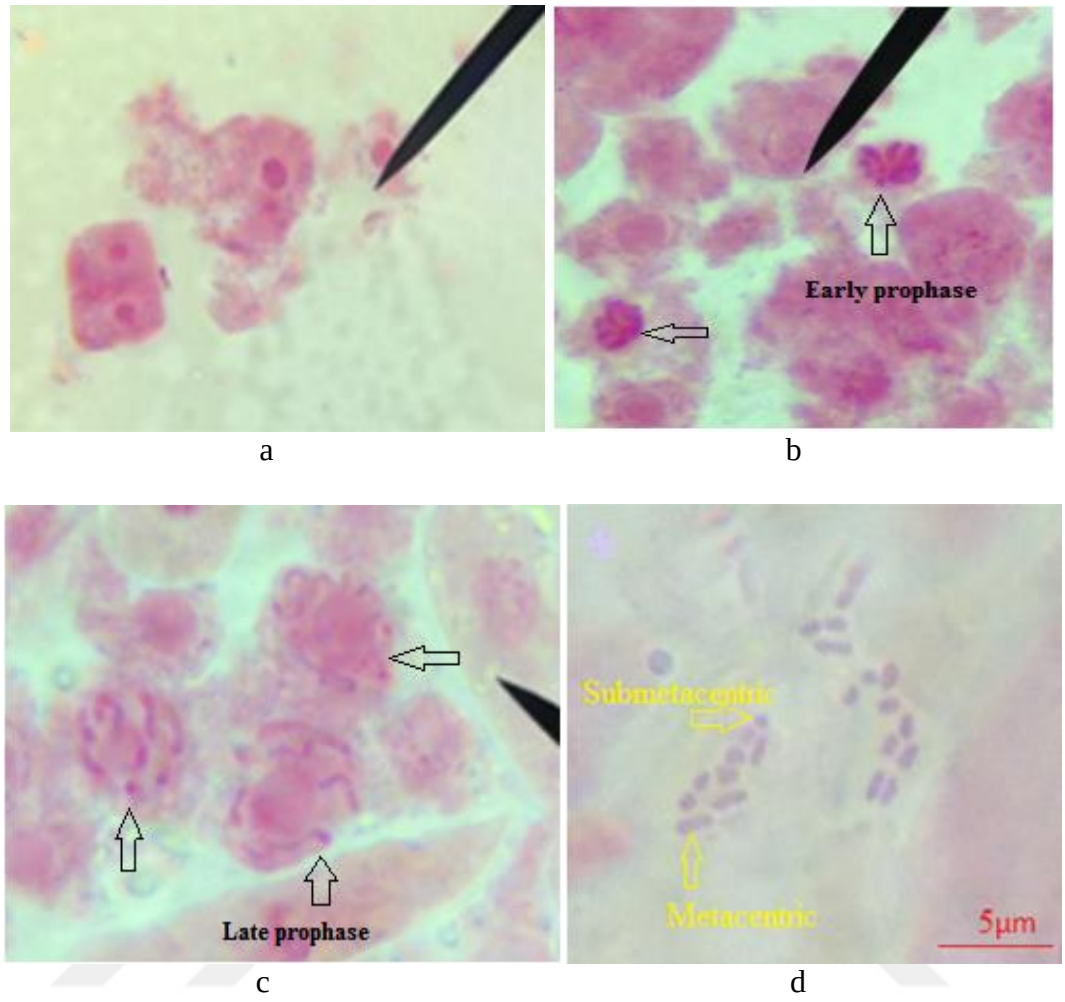


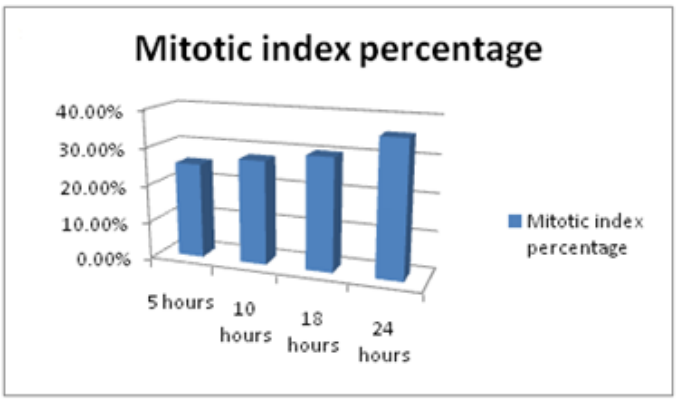
Figure 4.2. Mitotic stages stained with 1% acetocarmine interphase (a), early prophase (b), late prophase (c) and metaphase (d)

Table 4.1. Three different treatment methods with different time and 10 slides observation results per treatment method with different time under the light microscope

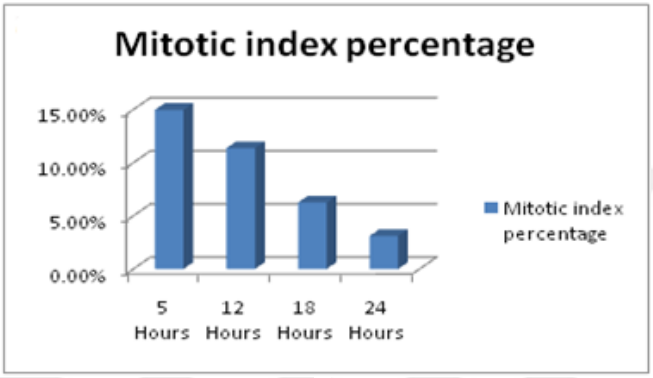
Pretreatment Method	**Concentration**	**No of roots in tube**	**No of slides**	**Results**
8-Hydroxyquinoline	2mM	10	10	Different time was used for the pretreatment of roots like 5, 10, 18 and 24 hours. The pretreatment of roots in 8-Hydroxyquinoline for 24 hours gave the best result because a higher amount of metaphase chromosome sets was observed. Interphase and prophase were also observed.
Cold treatment (+4 ºC)	_	10	10	Different time was used for the pretreatment of roots like 5, 12, 18 and 24 hours. There were fewer amount of metaphase chromosome sets observed as compared to 2 mM 8-Hydroxyquinoline. Interphase was also observed.
Ice-cold water	_	10	10	Different time was used for the pretreatment of roots like 2, 8, 12 and 15 hours. There was the lowest amount of metaphase chromosome sets were observed as compared to 2 mM 8-hydroxyquinoline and cold treatment (+4 ºC). Interphase was also observed.

Table 4.2. Mitotic index percentage and mitotic phases percentage as a result of different pretreatment methods and time. Data is collected on the basis of 10 slides for each treatment and time. Slides were observed under a light microscope with a 100X magnification lens. Data is collected on the basis of 10 root tips preparation for each pretreatment method and time. Mitotic index is calculated by the formula number of the dividing cells divided by the number of dividing cells + number of non-dividing cells multiplied by 100. The number of counted cells and the number of dividing cells were calculated by the observation of slides under the light microscope. Interphase, prophase and metaphase percentages were calculated by the formula number of mitotic phase cells divided by the number of dividing cells multiplied by 100.

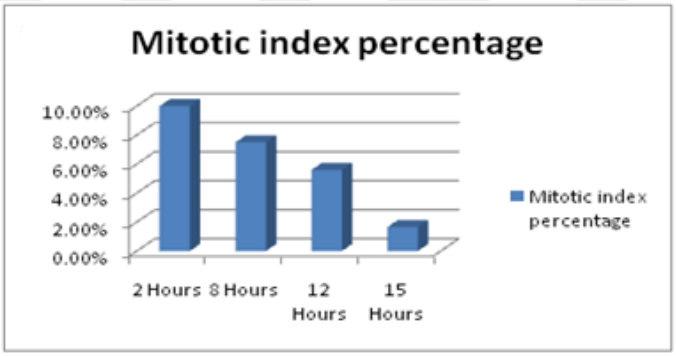
Pretreatment	**Time Period**	**No of roots in tube**	**No of slides**	**No of counted cells**	**No of dividing cells**	**Mitotic index %**	**Interphase %**	**Prophase%**	**Metaphase%**
8-Hydroxyquinoline	5h	10	10	1379	350	25.38	44.58	36.28	19.14
	10h	10	10	1500	415	27.66	41.50	38.30	21.20
	18h	10	10	1250	375	30.00	41.20	35.40	23.40
	24h	10	10	4500	1616	35.91	41.42	32.50	26.08
Cold treatment (+4)	5h	10	10	2560	383	14.96	49.54	36.30	14.16
	12h	10	10	1100	125	11.36	53.65	36.45	9.90
	18h	10	10	400	25	6.25	55.21	39.20	5.59
	24h	10	10	290	9	3.10	60.50	37.00	2.50
Ice-cold water	2h	10	10	1700	170	10.00	55.00	36.00	9.00
	8h	10	10	690	75	7.5	54.92	38.75	6.33
	12h	10	10	410	23	5.60	64.23	30.10	5.67
	15h	10	10	300	5	1.66	66.45	32.55	1.00



a



b



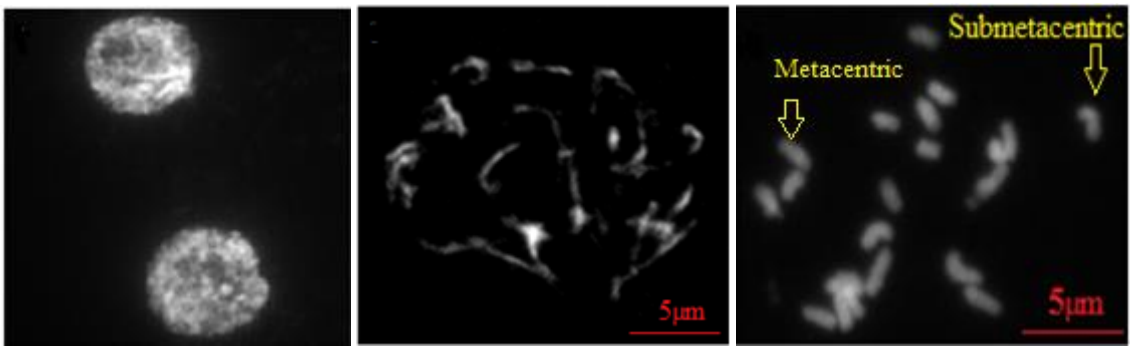
c

Figure 4.3. Mitotic index percentage of 2mM 8- hydroxyquinoline (a), mitotic index percentage of cold treatment (+4 ºC) (b) and mitotic index percentage of ice-cold water (c)

The mitotic index percentage of 2 mM 8- hydroxyquinoline was gradually increasing as the time of pretreatment was increasing. In the case of cold treatment (+4 ºC) and ice-cold water treatment methods, the mitotic index percentage was decreasing as the time of pretreatment was increasing. The 8-hydroxyquinoline pretreatment method gave the highest mitotic index percentage at 24 hours as compared to the other two pretreatment methods.

4.2.1 Chromosome preparation with 4′-6-diamidino-2-phenylindole (DAPI) staining

In *Lagenaria siceraria*, 2n=22 diploid number of chromosomes was present. Positive DAPI bands were present in interphase and metaphase chromosomes showed neither positive nor negative DAPI bands. In the interphase nucleus, positive DAPI bands were very clear as well as in prophase (Figure 4.4 a,b). In the case of the metaphase chromosome, most of the chromosomes were heterochromatin when stained with DAPI (Figure 4.4 c). In closely associated diploid species the presence or absence of the DAPI bands had been studied (Kondo et al. 1995).

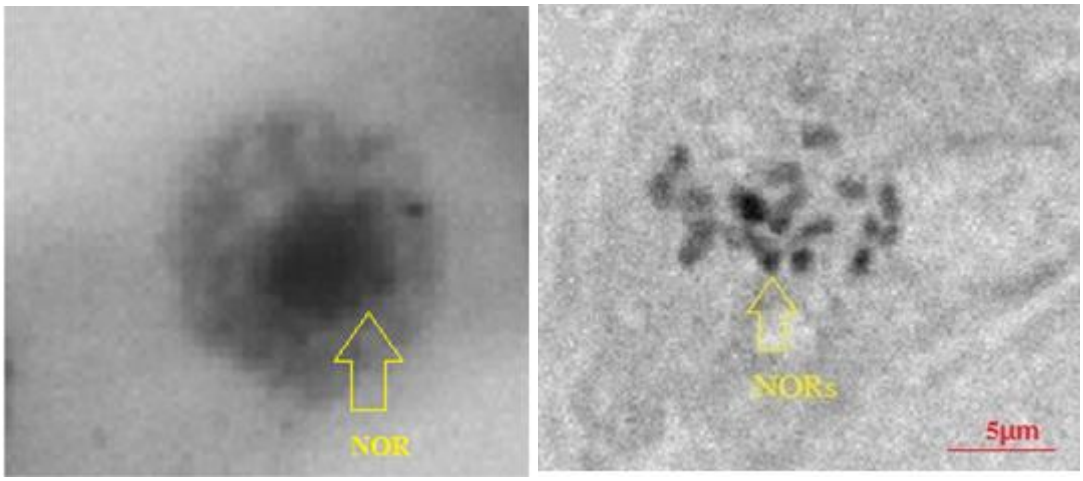


a Interphase b Prophase c Metaphase

Figure 4.4. 4′-6-diamidino-2-phenylindole (DAPI) staining of different mitotic stages

4.3 Silver Nitrate Staining

In bottle gourd, it is the first report on the use of silver nitrate staining for cytological investigation. Silver staining was done on the slides with enzymatic digestion method. Silver staining was done on slides to determine the activity of the rDNA sites, NORs regions, and numbers of nucleoli of mitotic cells. A well-stained nucleus was observed in interphase prepared by enzymatic digestion method. One silver-stained nucleus was observed in the interphase stage (Figure 4.5 a). Nucleolar organizer regions are present in the regions of the nucleolus during interphase called a fibrillar center (Sumner, 2003). On the metaphase chromosomes, four NOR regions were identified, suggesting that 45S rDNA sites were transcriptionally active (Figure 4.5 b). The number of NORs in interphase and metaphase stages is shown in Table 4.3.



a b

Figure 4.5. Silver-stained interphase (a) and silver-stained metaphase chromosomes (b) Scale bar = 5µm

Table 4.3. Number of NORs regions in interphase and metaphase as a result of silver staining

Parameter	Results
Population name	Sandıklı
Number of chromosomes	2n=2x=22
Number of NORs regions in interphase	1
Number of NORs regions on metaphase chromosomes	4
Number of 45S loci	4

4.4 Chromomycin A_3/DAPI (CMA_3/DAPI) Staining

In bottle gourd, it is the first report on the use of Chromomycin A_3/DAPI (CMA_3/DAPI) staining for cytological investigation. CMA3/DAPI staining was done on the slides with enzymatic digestion method. CMA_3/DAPI staining was done on slides to determine the GC-rich and AT-rich regions. Guanine-cytosine regions bind particularly with CMA_3 in helical DNA in the base-specific fluorescent staining methods or heterochromatin (Deumling and Greilhuber 1982) while DAPI binds exclusively to AT base pairs in the minor channel of DNA (Portugal and Waring 1988). GC-rich heterochromatin regions, which were always correlated with rDNA genes, were displayed by fluorochrome banding. In the interphase stage, 4 strong signals were observed (Figure 4.6 a,b,c). In the prophase stage, there were also 4 strong signals observed (Figure 4.6 d,e,f). 4 strong

signals were also detected on the metaphase chromosomes (Figure 4.6 g,h,i). On the metaphase chromosomes, all 4 positive CMA_3signals were present on a terminal or sub-telomeric region of two different chromosome pairs. These signals showed that these regions were GC-rich DNA regions. No positive DAPI band was observed on metaphase chromosomes in this population. The number of CMA_3 positive signals in interphase, prophase and metaphase stages is shown in Table 4.4.

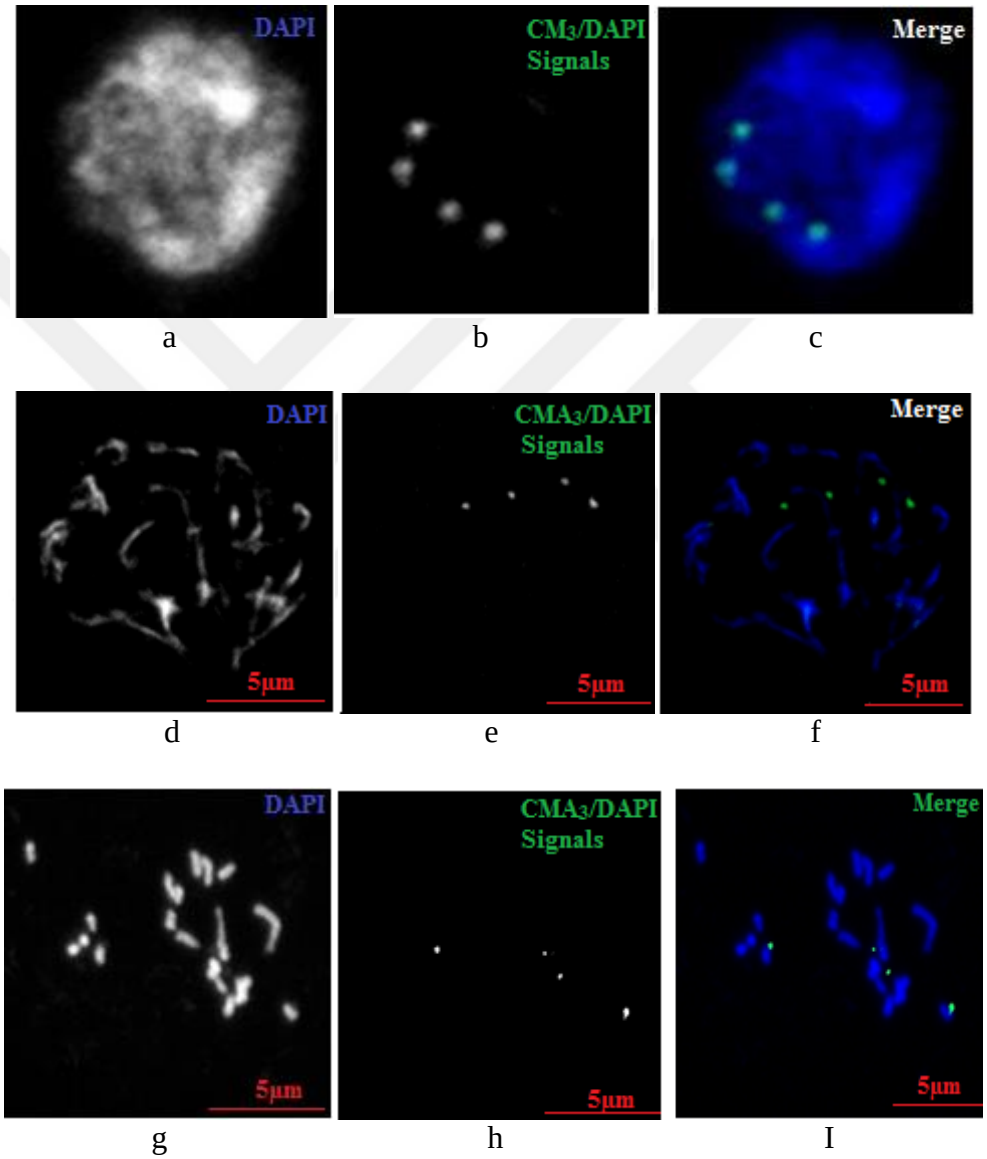


Figure 4.6. Chromomycin A_3/DAPI (CMA_3/DAPI) staining in interphase (a-c), prophase (d-f) and metaphase (g-i) Scale bar = 5µm

Table 4.4. Number of CMA_3 positive signals in interphase, prophase and metaphase stages

Parameters	Results
Name of species	*Lagenaria siceraria*
Number of chromosomes	2n=22
Number of CMA_3positive signals in interphase	4
Number of CMA_3positive signals in prophase	4
Number of CMA_3positive signals on metaphase	4

4.5 Fluorescence *in Situ* Hybridization (FISH)

In this study, one population of bottle gourd was used. To detect the position and number of 45S and 5S rDNA, FISH using digoxigenin-labeled probes was used.

4.5.1 Number of 45S rDNA signals on different mitotic stages

In the interphase stage, 4 strong signals of 45S were observed (Figure 4.7 a,b,c). 4 strong signals of 45S were also observed in the prophase stage (Figure 4.7 d,e,f). On the metaphase chromosomes, there were also 4 strong signals of 45S (Figure 4.7 g,h,i) that showed a similar pattern of the number of the 45S rDNA in different mitotic stages in this population. The number of FISH signals of the 45S probe in different mitotic stages is shown in Table 4.5.

4.5.2 Number of 5S rDNA signals on different mitotic stages

2 strong signals of 5S were observed in interphase (Figure 4.8 a,b,c) as well as 2 strong signals were observed in prophase (Figure 4.8 d,e,f) and 2 strong signals of 5S rDNA were observed on metaphase chromosomes (Figure 4.8 g,h,i). The number of FISH signals of the 5S probe in different mitotic stages is shown in Table 4.6.

4.5.3 Position of 45S rDNA signals

2 signals of 45S rDNA were present on the short arm of the two metaphase chromosomes and 2 signals of 45S rDNA were present on the short arm of two different

metaphase chromosomes. All 4 signals were present at sub-telomeric or terminal regions of metaphase chromosomes (Figure 4.7 g,h,i). All signals were clearly visible on metaphase chromosomes.

4.5.4 Position of 5S rDNA signals

2 signals of 5S were observed on the short arm of two metaphase chromosomes. Signals were present on the sub-telomeric or terminal region of the two metaphase chromosomes (Figure 4.8 g-l).

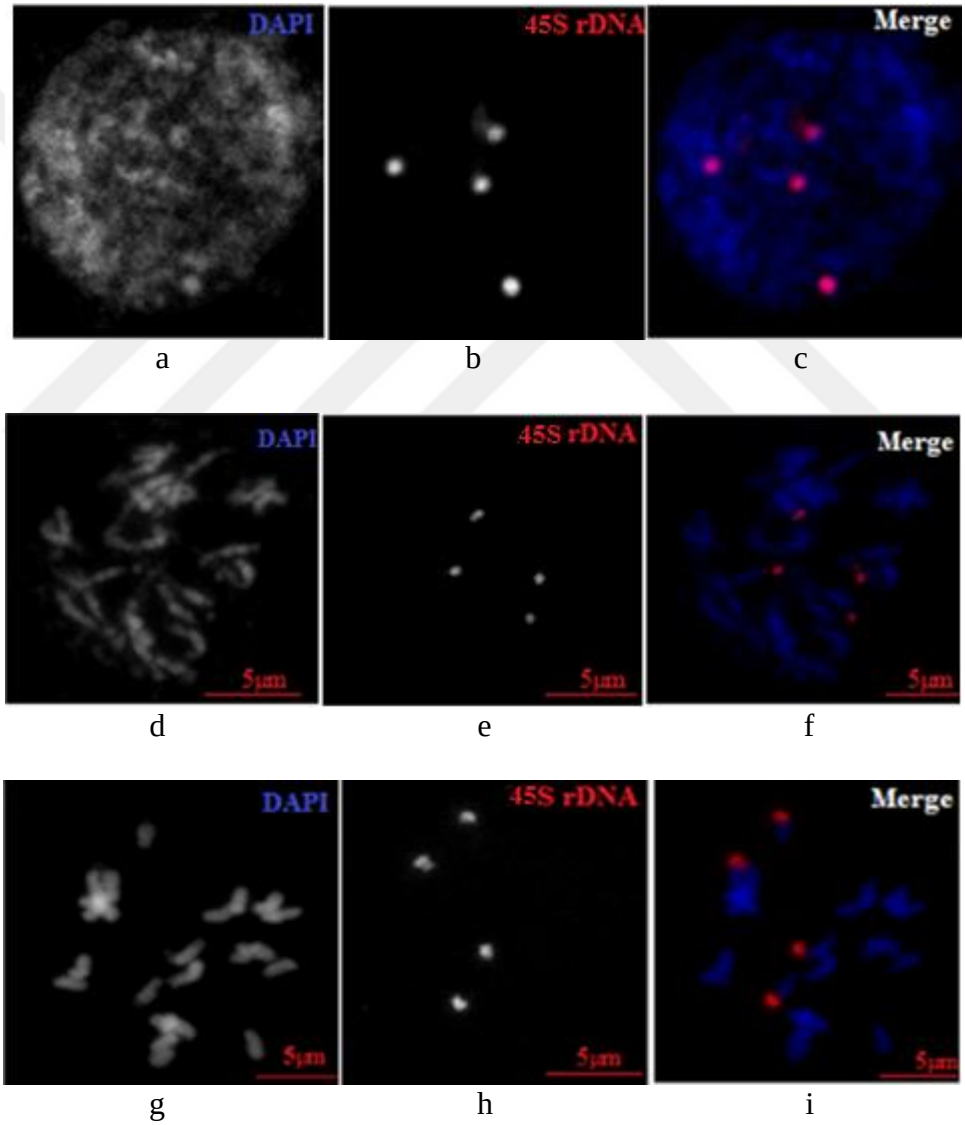


Figure 4.7. FISH with 45S probe interphase (a-c), prophase (d-f) and metaphase (g-i) Scale bar = 5µm

Table 4.5. Number of FISH signals of the 45S probe in different mitotic stages

Parameters	Results
Common name	Bottle gourd
Number of 45S signals in interphase	4
Number of 45S signals in prophase	4
Number of 45S signals on metaphase	4

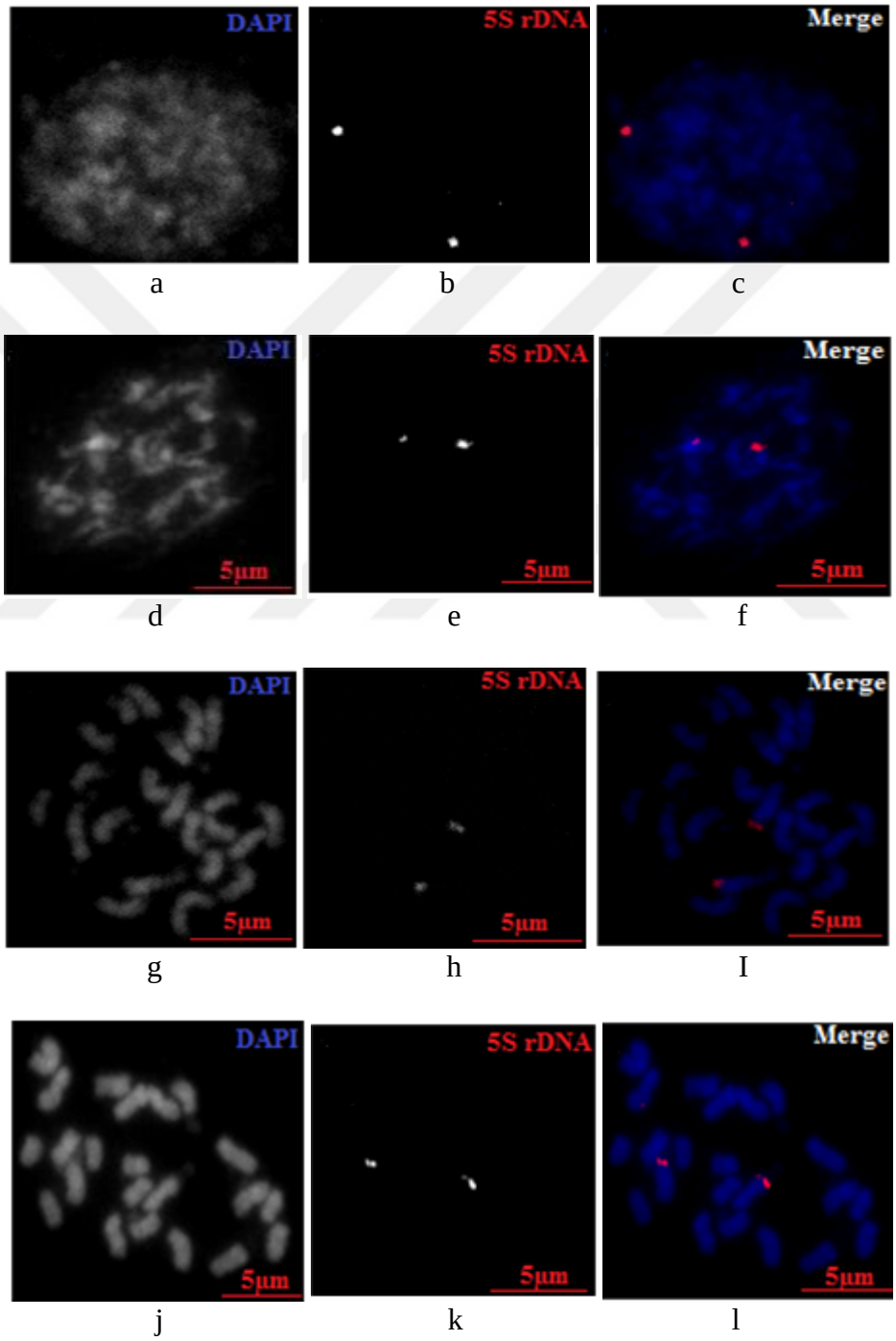


Figure 4.8. FISH with 5S probe interphase (a-c), prophase (d-f) and metaphase (g-l) Scale bar = 5µm

Table 4.6. Number of FISH signals of the 5S probe in different mitotic stages

Parameters	Results
Ploidy level	Diploid
Number of 5S signals in interphase	2
Number of 5S signals in prophase	2
Number of 5S signals on metaphase	2

Table 4.7. Summary of signals of NORs, CMA_3/DAPI, 45S rDNA and 5S rDNA in different mitotic stages

Parameters	Interphase	prophase	Metaphase
No of NORs	1	-	4
CMA_3/DAPI	4	4	4
45S rDNA	4	4	4
5S rDNA	2	2	2

CHAPTER V

DISCUSSION

The most common basic chromosome number of the Cucurbitaceae family is 11 or 12 (Waminal and Kim, 2015). In this study, one population of bottle gourd, which is a member of the Cucurbitaceae family, was investigated and exhibited a basic chromosome number of 11. The chromosome number in the Cucurbitaceae family varies from 14 to 48. The chromosome number in bottle gourd 2n=2x=22 is already reported (Waminal and Kim, 2012), and the results of this study present no divergence to the chromosome number. These results showed that subspecies of *Lagenaria siceraria* have the same ploidy level. To verify the AT-rich segments in the chromosomes of many plants, a fluorescent banding method such as 4′-6-diamidino-2-phenylindole (DAPI) is used that could give more information and significant chromosomal data for phylogenetic relationships of species (El-Twab et al., 2015). For that reason, DAPI has been used in some chromosome complements of closely associated taxa (El-Twab and Kondo, 2010). DAPI staining results of this study showed positive DAPI bands in the interphase and prophase stages but on metaphase chromosomes, there were neither positive nor negative bands present but chromosomes were mostly heterochromatic that verified the findings of Waminal and Kim, (2012).

For the detection of ribosomal gene activities and estimation of protein synthesis rate, silver staining of NORs can be used as a fast method. The amount of AgNOR protein is a good marker of cell explosion activity according to previous studies (Derenzini et al., 1994). That's why every silver-stained spot confirms the ribosomal gene activity and the amount of silver-stained proteins. There is no report is present on the silver staining of bottle gourd chromosomes up till now. It is the first report on the silver staining of the bottle gourd chromosomes. The results of this study showed that only one silver-stained nucleus was found in interphase nuclei. The nucleolus is the major and clearest position of NORs carrying ribosomal genes in interphase. For ribosomal transcription, all important elements are present inside the boundaries of interphase nucleolar organizer regions (Derenzini et al., 1990).

On the metaphase chromosomes, 4 NORs were observed at the sub-telomeric or a terminal region. These NORs showed that 45S rDNA was transcriptionally active at those regions which were later confirmed by chromomycin A_3/DAPI staining and FISH with the same number and location of 45S rDNA signals in metaphase. In the studied population of bottle gourd, the results obtained from AgNOR banding were significantly useful in the relationship of the detection of chromosomes and the detection of number and position of NORs. This method explains its importance for revealing intra- and inter-specific chromosome differences in the genus. Silver staining allowed us to detect active rDNA locations that were not detected by fluorochrome banding, even though they might have been known as CMA_3/DAPI regions. This method also allowed us to discriminate FISH signals. Hoshi et al., (1999) done silver staining on the cucumber (*Cucumis sativus*). Their results showed that one to two well silver-stained nucleoli were observed in interphase nuclei. They observed 2 dark nucleolar organizer regions (NORs) bands in mid-metaphase and the bands were present on the proximal region of mid-metaphase chromosomes. These study results do not match with Hoshi et al., (1999) results because the number of AgNORs in metaphase differs with the maximum number of nucleoli in interphase nuclei in some of the studied taxa. This is most likely due to the nucleolar relationship, the inclusion of the nucleolus during interphase (Lacadena et al. 1984), which also depends on the non-random position of NORs in the cell. The observed intraspecific variations with varying numbers of AgNORs on metaphase chromosomes resulted from the differences in rDNA locus activity, as detection with silver nitrate was not possible (Moscone et al. 1995). In the metaphase chromosomes, the noticed intraspecific variations with different numbers of AgNORs are due to the differences in rDNA locus activity. When loci were inactive it was difficult to detect with silver nitrate (Moscone et al. 1995).

To investigate the chromosomal polymorphism in crops and vegetables, the base-specific fluorescence dyes are used (Hayasaki et al. 2001). There is also no report that is present on the chromomycin A3/DAPI staining of bottle gourd chromosomes up till now. It is the first report on the chromomycin A_3/DAPI (CMA_3/DAPI) staining of the bottle gourd chromosomes. The results of the study showed that in the interphase stage 4 strong signals were observed (Figure 4.5 a,b,c). In the prophase stage, there were also 4 strong signals (Figure 4.5 d,e,f). 4 strong signals were also detected on the metaphase chromosomes (Figure 4.5 g,h,i). On the metaphase chromosomes, all 4 positive CMA_3

signals were present on a terminal or sub-telomeric region of two different chromosomes pairs. These signals showed that these regions were GC-rich DNA regions. In the FISH, there were also observed the same kind of results with the 45S rDNA probe. In the plant karyotype, it is common in the position of 45S rDNA and CMA_3 positive heterochromatic blocks (Marcon et al., 2005). No positive DAPI bands were observed on metaphase chromosomes in this population. Lombello and Pinto-Maglio, (2007) worked on the cytogenetic of bitter gourd (*Momordica charantia*) to describe the features of its heterochromatin and the distribution of rDNA sites by using CMA_3/DAPI and FISH techniques. Their results showed that there was no band present on the chromosomes with DAPI staining. When chromosomes stained with CMA_3 there were 4 positive bands present at the edges of the terminal region of chromosomes, which showed the presence of GC rich sites in this species. The results of CMA_3/DAPI of this study are matched with the Lombello and Pinto-Maglio, (2007) study results.

There are hundreds to thousands of repeat units present in the NOR-forming 45S rDNA and non-NOR-forming 5S rDNA (Singh et al., 2009). These two rDNA families are generally not positioned or associated with the same chromosomes even though some examples are present (Leitch and Heslop-Harrison, 1993). rDNA families have also been used in phylogenetic studies utilizing the changeable distribution scheme between species and populations (Drouin and Moniz de Sá, 1995). The characterization of chromosomes and genome structure of various species can be found by using FISH with probes targeting for the ribosomal rRNA genes (rDNAs) and other repeats (Leitch and Heslop-Harrison, 1992). In more than 1000 species ribosomal gene loci were studied by FISH recently giving important information on genome structure and inter-specific relationships that will result in improving the breeding designs. In this study, the results of the ribosomal mapping of bottle gourd chromosomes by using FISH showed that on the interphase stage 4 strong signals of 45S were observed (Figure 4.6 a,b,c). 4 strong signals of 45S were also observed in the prophase stage (Figure 4.6 d,e,f). On the metaphase chromosomes, there were also 4 strong signals of 45S (Figure 4.6 g,h,i) that showed the similar pattern of number of the 45S rDNA in different mitotic stages. 2 strong signals of 45S rDNA were observed on the short arm of two different metaphase chromosomes (Figure 4.6 g,h,i) as well as 2 other signals of 45S rDNA were present on the short arm of the two other different metaphase chromosomes. All 4 signals were present at sub-telomeric or terminal regions of metaphase chromosomes (Figure 4.6

g,h,i). In the case of 5S rRNA on interphase 2 strong signals were detected, 2 strong signals were detected on prophase and 2 signals of 5S were observed on the short arm of two metaphase chromosomes. Signals were present on the sub-telomeric or terminal region of the two metaphase chromosomes (Figure 4.7 d,e,f). The results of this study are similar to the results of Wiminal and Kim, 2012 study in which they found 4 signals of 45S rDNA on the terminal region of metaphase chromosomes and 2 signals of 5S rDNA which were also present on the terminal region of the metaphase chromosomes. In 80% of the studied species, 45S rDNA sites were especially present on the short arm and in the terminal regions usually forming a satellite (Lima-de-Faria, 1976). In this study, all the 45S rDNA were present on the short arm and in the terminal regions. May be due to homologous recombination limitation the distribution of 45S rDNA sites in the terminal regions occurred (Rao and Guerra 2012). 45S and 5S rDNA sites are located on different chromosomes in most species, whereas the two kinds of rDNAs 45S and 5S sites occur on the same chromosome at separate positions in some species.

In modern breeding programs, cytogenetic studies are very useful in phylogenetic analyses and provide important knowledge about the structure and evolution of genomes. The results of the study show that in *Lagenaria* genus rDNA sequences are excellent chromosome markers. The scheme of rDNA signals and bands could be very helpful in studies on the origin and evolution of various polyploid species.

The results of this study will provide useful knowledge for understanding the chromosomal and genome evolution of *Lagenaria* genus. This study will also provide important knowledge for the chromosome identification and phylogenetic relationships among the species of the Cucurbitaceae family.

CHAPTER VI

CONCLUSION

In this research, chromosomes structure, nucleolar organizer regions (NORs), A-T-rich and G-C-rich regions, physical mapping of ribosomal genes (rDNAs) 45S and 5S at different mitotic stages were studied in the bottle gourd by using conventional and new molecular cytogenetic tools. Squash root tip method was used for identification and characterization of chromosome number, morphology and structure of bottle gourd. The results showed that there were 2n=2x=22 chromosomes present in bottle gourd. Most of the chromosomes were metacentric and some chromosomes were sub-metacentric. The silver staining method was used to identify the NORs on the interphase and metaphase mitotic stages. One silver-stained nucleus was observed on interphase and 4 NORs were present on the metaphase chromosomes. The location of the NORs in metaphase showed the activity of 45S rDNA in those regions. AT-rich and GC-rich regions in different mitotic stages were determined by CMA_3/DAPI staining method. DAPI positive bands were present on interphase and prophase but there were no DAPI positive bands present on metaphase chromosomes. 4 strong CMA_3/DAPI signals were present on interphase, 4 strong CMA_3/DAPI signals were present on prophase and 4 strong CMA_3/DAPI signals were also present on metaphase chromosomes. These signals showed GC-rich regions. FISH was used to determine the positions of 45S and 5S rDNAs on different mitotic stages. 4 strong signals of 45S were found on interphase, 4 strong signals of 45S were present on prophase, 4 signals of 45S were present on metaphase chromosomes at terminal regions. In the case of 5S rRNA, 2 strong signals were detected on the interphase, 2 strong signals were detected on the prophase and 2 strong signals of 5S were found on metaphase chromosomes at the sub-telomeric or terminal region. The detected rDNAs signals will help in the mapping of the karyotype of *Lagnaria* genus chromosomes. These rDNAs signals will also play an important role in understanding the chromosomal evolution of genus *Lagnaria.* Very limited amount of literature is present for the cytogenetic studies in bottle gourd as well as in the other species of the Cucurbitaceae family. Due to this reason, this research has great importance. The results of this study showed that ribosomal gene (rDNAs) sequences, CMA_3/DAPI bands and nucleolar organizer regions (NORs) are very good chromosomes markers that contributed to detect the homologous chromosomes which

have rDNA signals from the studied population of bottle gourd. For the first time silver staining and CMA_3/DAPI staining was done on the bottle gourd chromosomes. So the data obtained from this study will be the guideline for future studies on the other species of the Cucurbitaceae family. The results of this research could contribute to providing the important knowledge on bottle gourd chromosomes and are considered to share in the progress of breeding and genome studies in bottle gourd. Still, more study required to understand the chromosome structure, phylogenetic relationships and genome structure of bottle gourd by using new cytogenetic tools like genomic *in situ* hybridization (GISH) and genome-wide analysis by next-generation sequencing techniques.

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