Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: Ahmet L. Tek, Hümeyra Yıldız, Kamran Khan, Bilge Ş. Yıldırım (2021) Chromomycin A3 banding and chromosomal mapping of 45S and 5S ribosomal RNA genes in bottle gourd. *Caryologia* 74(3): 3-8. doi: 10.36253/caryologia-1134

Received: November 12, 2020

Accepted: Semptember 24, 2021

Published: December 21, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

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Chromomycin A3 banding and chromosomal mapping of 45S and 5S ribosomal RNA genes in bottle gourd

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Abstract. Ribosomal DNAs and various banding patterns are landmarks in molecular cytogenetics providing useful information for karyotyping and addressing individual chromosomes. Bottle gourd is the only cultivated species of the *Lagenaria* genus with high genetic diversity. After CMA_3/DAPI fluorochrome banding we investigated the GC- and AT-rich regions in interphase nuclei of five different local accessions. Fluorescence *in situ* hybridization (FISH) was conducted to determine the number and location of 45S and 5S rDNAs in bottle gourd. Our results showed four strong CMA_3 regions in interphase and on mitotic metaphase chromosomes. FISH revealed four strong signals of 45S rDNA at the termini of two metaphase chromosome pairs and terminal 5S rDNA signals at another pair of chromosomes. The presence of four positive CMA3 bands colocalizes with four 45S rDNA signals in all bottle gourd accessions. Our results allow distinguishing two out of eleven chromosome pairs of bottle gourd.

Keywords: bottle gourd, chromomycin A3, fluorescence *in situ* hybridization, local accessions, ribosomal DNA, 5S, 45S.

INTRODUCTION

Bottle gourd, *Lagenaria siceraria*, is a member of the Cucurbitaceae family, also known as calabash or white-flowered gourd. It is a diploid crop species with 2n = 2× = 22 chromosomes (Beevy and Kuriachan 1996). The genome size of this plant is approximately 365 Mbp (Achigan-Dako et al., 2008). DNA markers revealed that Chinese bottle gourd and Turkish bottle gourd accessions have a close phylogenetic relationship within other cucurbit species (Xu et al., 2011; Yildiz et al., 2015). These findings have implications for the preservation of bottle gourd genetic diversity and advanced marker-assisted breeding studies (Xu et al., 2011; Yildiz et al., 2015). The Lagenaria genus consists of six species: *L. siceraria* (cultivated form), *L. sphaerica*, *L. rufa*, *L. breviflora*, *L. sphaerica*, and *L. guineensis*. All six species are naturally found in Africa, the supposed center of genetic diversity for *L. siceraria*.

Bottle gourd is the only cultivated species. In the tropics, it is one of the oldest crops (Erickson et al., 2005). Bottle gourd is cultivated for food, decoration, medicine, domestic utensils, musical instruments, containers, and fishing floats (Morimoto and Mvere, 2004, Xu et al., 2011). Some bottle gourd varieties are grown for their seeds which are rich in oil and essential amino acids (Achigan-Dako et al., 2008). Additionally, bottle gourd seedlings are used as a rootstock for watermelon against adverse effects in soil such as low temperature, high pH, salinity, excessive water as well as soil-borne diseases, such as Fusarium wilt (Yetisir et al., 2007). For seedless watermelon production, bottle gourd pollen has recently been utilized to pollinate watermelons (Sugiyama et al., 2014). Therefore, bottle gourd is a crop of great economic interest and a detailed characterization at the chromosome and genome level is desirable.

The plant genomes contain a significant amount of repetitive DNA sequences. Among them, ribosomal DNAs (rDNAs) encode the RNA components of ribosomes. Two structurally distinct gene families of rDNAs exist in plant genomes, specifically known as 45S and 5S rDNAs. Non-transcribed spacers and tandem repeat units of the 18S-5.8S-26S ribosomal genes are present in the 45S rDNA. The 5S rDNA genes consist of a non-transcribed spacer and a conserved coding region of 120 bp (Long and Dawid, 1980). 45S and 5S rDNA genes can be present at one or more positions within a set of chromosomes and be used as chromosomal markers (Long and Dawid, 1980; Lombello and Pinto-Maglio, 2007; Han et al., 2008; Heslop-Harrison and Schwarzacher, 2011; Li et al., 2016; Santos-Sanchês et al., 2019). CMA_3 (chromomycin A3), a GC-rich specific fluorochrome, and DAPI (4'-6-diamidino-2-phenylindole), an AT-rich specific fluorochrome, banding techniques can also be useful to differentiate between chromosomes (Kim et al. 2002). The bottle gourd and its relatives have small and morphologically similar chromosomes, and fluorescent chromosome staining techniques such as CMA_3 and DAPI might be helpful to distinguish them and proved to be useful for determining the phylogenetic relationships among plant species (Schweizer, 1976; Kim et al. 2002; Yamamoto et al., 2007; Volkov et al., 2017; Maragheh et al., 2019). Fluorescence *in situ* hybridization (FISH) has been widely used for constructing chromosomal maps, for chromosome identification, for studying the dynamic organization of chromatin in interphase nuclei as well as for studying chromosome homology and karyotype evolution (Lysak et al., 2006; Probst, 2018; Santos et al., 2020).

Previously, rDNA mapping in bottle gourd was investigated by Waminal and Kim (2012) and Li et al. (2016), although definite information is not available about origin of accessions used for comparison. These authors found four signals of 45S and two signals of 5S on metaphase chromosomes. There is no report for the CMA_3/DAPI staining of the metaphase chromosome of bottle gourd. We hypothesize that a combination of CMA_3/DAPI staining and FISH can be used for the determination of any possible chromosomal variability from different geographical origins. Given the high genetic diversity of bottle gourds, we tested a set of local varieties from Turkey for potential variability of rDNAs loci and differentiation of their metaphase chromosomes by CMA_3/DAPI staining.

MATERIALS AND METHODS

Plant Material

Bottle gourd (*Lagenaria siceraria*) seeds were obtained from a local population in Sandıklı, Afyonkarahisar, Turkey and used as a main accession for the research if not indicated otherwise. Additionally, four different accessions were used from provinces of Hatay, Niğde/Merkez, Niğde/Ulukışla, and Niğde/Bor (Turkey). These accessions with different seed morphology and high germination rates were chosen to cover different localities to determine variability. Moistened seeds were placed on sterile filter paper and germinated in petri dishes using double distilled water.

Mitotic chromosome preparation

The mitotic chromosome preparation was performed according to the published protocols (Tek et al. 2011) with the following modifications. Young root (~1 cm) tips were cut off with a razor blade, treated with 2 mM 8-hydroxyquinoline at room temperature, and subsequently, fixed in 3:1 methanol: glacial acetic acid for 24 h at -20 °C. After fixation, root tips were washed three times in distilled water and 30 mM potassium chloride, digested in an enzyme mixture, pH 4.5, containing 4% cellulase and 2% pectinase at 37 °C for 90 min. Digested root tips were washed in distilled water. Following a fixation step, slides were prepared by the flame-dry (Tek et al. 2011). Slides with suitable cells were selected using a phase-contrast microscope.

Chromomycin A3/DAPI staining

Chromosome staining with CMA_3 and DAPI was performed as described (Schweizer 1976). Briefly, slides

were stained for 20 min with CMA_3 (0.5 mg/ml) in Mcllvaine's buffer (pH = 7.0). Subsequently, slides were incubated at 37 °C for 2 days with DAPI (0.5 µg/ml) (Hasterok et al., 2006).

Probe preparation

To determine 45S and 5S rDNA sites, plasmid clones pTa71, and pTa794 were used, respectively (Gerlach and Bedbrook, 1979; Gerlach and Dyer 1980). Both rDNAs were labeled with digoxigenin-11-dUTP using a nick translation kit (Roche) according to the manufacturer's instructions.

Fluorescence in situ hybridization and signal detection

FISH was conducted according to Tek et al., (2011) with modifications. A hybridization mixture containing the denatured probe DNA in 50% formamide, 10% dextran sulfate, 2×SSC was applied. The slides with chromosomes were denatured in 70% formamide with 2×SSC at 80 °C for 2 min before hybridization at 37 °C overnight. The rhodamine-conjugated anti-digoxigenin antibody (Roche) was used to detect both 45S and 5S signals in independent experiments. Slides were checked using a fluorescence microscope at 63× magnification (Carl Zeiss Axio Imager.A2). Photographs were captured with monochromatic charge-coupled device (CCD) camera (Carl Zeiss Axiocam 702) operated with multichannel ZEN Pro Imaging software.

RESULTS AND DISCUSSION

Chromosomes of a local bottle gourd accession were examined for the presence of GC- and AT-rich heterochromatin regions by CMA_3/DAPI and by FISH for distribution of rDNAs. GC-rich heterochromatin regions, as displayed by fluorochrome banding, proved to be correlated with rDNA genes. In the interphase stage, four strong positive CMA_3 bands were observed (Fig. 1a-c). Four strong positive CMA_3 bands were also detected on the metaphase chromosomes (Fig. 1d-f). Among these positive CMA_3 bands were clear differences observed on the size and brightness on the interphase as well as the mitotic metaphase chromosomes (Fig. 1h, k, n, r). Two of the bands are small and less bright, while the other two are large and bright. These observations are consistent among all five accessions analysed indicating the similarity of the structural CMA_3 bands. On the metaphase chromosomes, all four bright and strong CMA_3 bands were present in termini regions of two pairs of chromosomes. Also FISH with the 45S rDNA probe yielded four signals. In plants, 45S rDNA loci and CMA_3 positive heterochromatic blocks often coincide spatially (Lombello and Pinto-Maglio, 2007; Maragheh et al., 2019; Santos-Sanchês et al., 2019). 5S rDNA sites were not detected by CMA_3/DAPI banding. The same number and position of ribosomal gene (45S rDNA) showed that CMA_3/DAPI positive bands may overlap with 45 rDNA sites. Similarly, our method, first with FISH experiment and subsequently with CMA_3/DAPI banding procedure on the same interphase nuclei, does allow conclusive evidence of overlap between 45S rDNA signals and CMA_3/DAPI positive bands (Fig. 2a-c, d-f). When we investigate CMA_3 bands in terms of size and intensity, a pair of chromosomes have big and another pair of chromosomes consistently have small signals in both metaphase and interphase stages whereas we did not detect clear differences on 45S rDNA signals using FISH (Fig. 2a-c, d-f). Nevertheless, these differences are more prominent as presented in Fig. 1 Lombello and Pinto-Maglio, (2007) worked on the bitter gourd (*Momordica charantia*), which is a member of the same family as bottle gourd. They found no band on the chromosomes with DAPI staining. Santos-Sanchês et al., (2019) conducted similar work on different melon accessions and reported a DAPI positive band on the metaphase chromosomes. Chromosomes stained with CMA_3 revealed four bands in terminal regions of chromosomes in this species. Our results of CMA_3/DAPI staining are in line with those obtained by Lombello and Pinto-Maglio, (2007) and Santos-Sanchês et al., (2019).

To detect the position and number of 45S and 5S rDNAs, FISH with digoxigenin-labeled probes was applied. In interphase nuclei, four strong red signals of 45S rDNA were observed (Fig. 2a-c). The size and intensity of all four red signals were similar, which is in contrast to our findings from the CMA_3 bands. Four strong 45S rDNA signals were also detected on the short arm ends of two mitotic metaphase chromosome pairs (Fig. 2g-i). Two strong signals of 5S rDNA were observed in interphase nuclei (Fig. 2j-l) as well as on metaphase chromosomes (Fig. 2m-o). There was no prominent difference in the size and intensity of 45S and 5S rDNA signals. The 5S rDNA signals appeared on the short arm of termini of a metaphase chromosome pair (Fig. 2o). The two rDNA families are usually not positioned on the same chromosomes, with some exceptions (Waminal and Kim, 2012; Li et al., 2016). In conclusion, rDNA loci and CMA_3 bands in accessions of *Lagenaria siceraria,* as in other species provide useful markers to distinguish at least two chromosome pairs individually. Also, our data

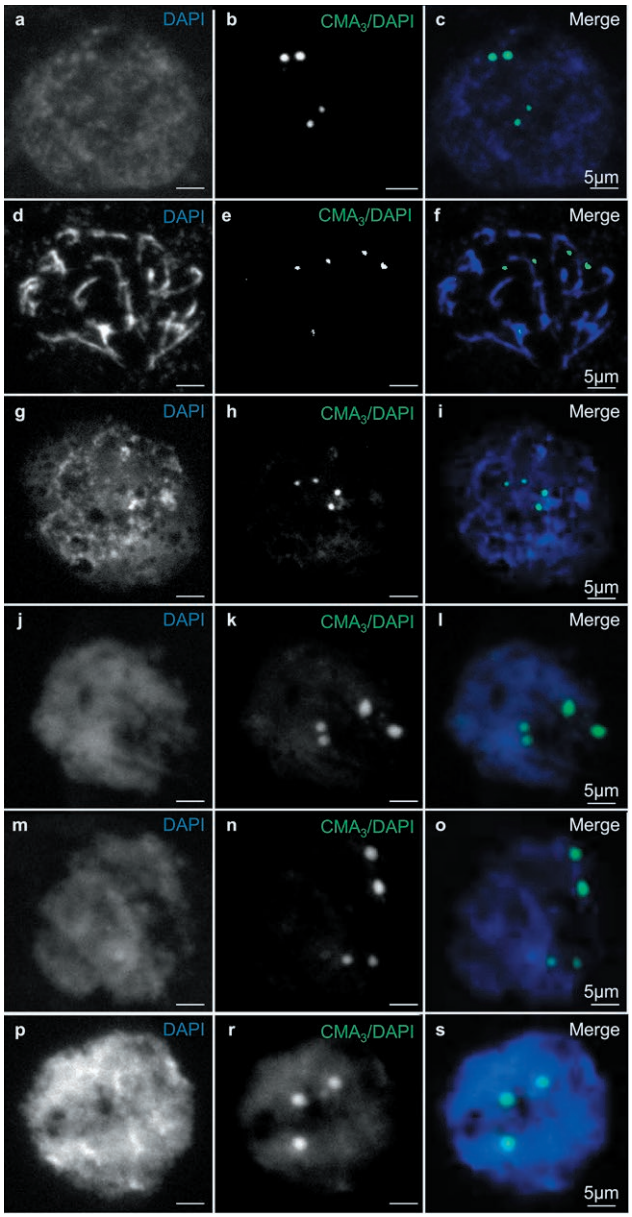

Figure 1. Chromomycin A_3/DAPI (CMA_3/DAPI) staining in *Lagenaria siceraria* nuclei and mitotic chromosomes (2n = 2× = 22) from five different local accessions. Interphase nuclei (a-c, g-i, j-l, m-o, p-s), prometaphase chromosomes (d-f), GC-rich loci stained with CMA_3 (b, e, h, k, n, r; green signals) and DAPI merge image (c, f, i, l, o, s) are shown on the chromosomes. Images are shown from the local accessions obtained from provinces of Sandıklı (**a-f**), Hatay (g-i), Niğde/Ulukışla (**j-l**), Niğde/Merkez (m-o), Niğde/Bor (p-s). Scale bar = 5 µm.

demonstrate that a relatively low level of intraspecific chromosomal diversity is present among morphologically different bottle gourd accessions.

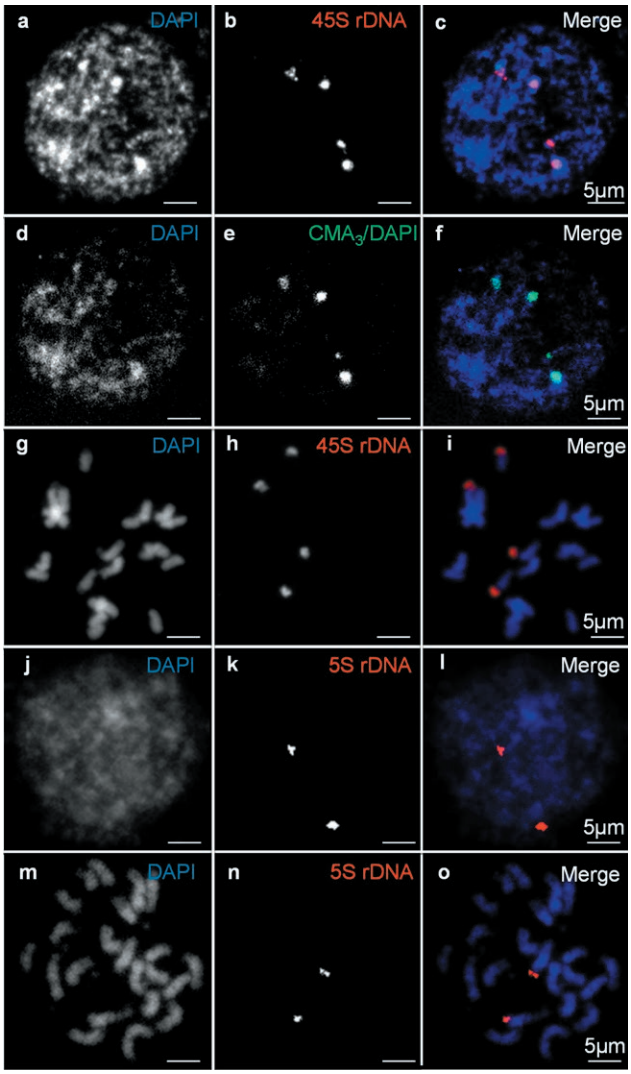

Figure 2. Localization of 45S and 5S rDNA in *Lagenaria siceraria* nuclei and mitotic chromosomes (2n = 2× = 22). DAPI stained interphase chromosomes (a-c), metaphase chromosomes (**g-i**), 45S rDNA loci labeled with digoxigenin (b, h; red signals of rhodamine), 45S rDNA loci merge image (**c, i**) is shown on the chromosomes. Interphase nuclei (d-f), GC-rich loci staining with CMA_3 (**e**; green signal). DAPI stained interphase chromosomes (**j-l**), metaphase chromosomes (m-o), 5S rDNA loci labeled with digoxigenin (k, n; red signals of rhodamine), 5S rDNA loci merge image (l, o) is shown on the chromosomes. Scale bar = 5 µm.

AUTHOR CONTRIBUTIONS

ALT conceived the study and designed the experiments. HY, KK and ALT performed the experiments. HY, KK, BŞY and ALT conducted data analysis. HY, KK, BŞY and ALT wrote the paper. All authors read and approved the final manuscript.

FUNDING

This work was partially supported by the INDEPTH-COST Action CA16212 and scholarship program from Ayhan Şahenk Foundation. HY and BŞY recognize the scholarship from YÖK 100/2000 Plant Genetics and Agricultural Biotechnology.

ACKNOWLEDGMENTS

We thank Prof. Ingo Schubert for critical reading and valuable suggestions on the manuscript and the lab members for technical assistance.

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