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Cytogenetics of two Indian varieties of *Momordica charantia* L. (bittergourd)☆

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ABSTRACT

Momordica charantia L.commonly known as bitter gourd is a widely cultivated cash crop in India with immense medicinal values. India has been one centre of diversification of two prominent taxonomic varieties *viz.* long-fruited variety, *M. charantia* var. *charantia* (MCC) and small-fruited variety, *M. charantia* var. *muricata* (MCM). *Momordica* species have long been known not to be amenable to conventional cytological techniques. After thorough technical standardization, karyotype variation between the two varieties as well as different populations of the same variety was compared and we primarily structured karyotypes of MCC and MCM populations that conformed to diploid count of 2n = 22 with slightly greater chromosome size in MCM (1.32μm–3.24 μm) than MCC (1.27μm–3.07 μm). Significant differences in genome size between MCC and MCM was also confirmed by flow cytometry for the first time in the two varieties of the species. Pronounced intraspecific dissimilarity in somatic chromosomes was established by EMA-Giemsa method revealing four nucleolar chromosomes in MCC populations and six nucleolar chromosomes in MCM populations. This difference in varieties was substantiated by fluorochrome banding which revealed four and six distal CMA^{+ve} signals in MCC and MCM respectively. Detailed inter- and intra- karyomorphometric evaluation ascertained higher tendency of karyotype asymmetry in MCC. EMA-DAPI technique was applied for meiotic analysis in MCC (n = 11) and MCM (n = 11) populations providing indications of regular meiotic behavior. These results will benefit the classification, the genetic basis of domestication and the genome studies of this widely cultivated cash crop.

1. Introduction

Momordica charantia L.is the largest cultivated species of *Momordica* out of the 60 existing species (Schaefer and Renner, 2011) world-wide. It is a monoecious annual climber belonging to the Momordiceae tribe of the Cucurbitaceae family (Schaefer and Renner, 2011) characterized by lobed leaves and jagged fruits with crocodile skin-like sculptured seeds (Behera et al., 2011). The species has been mostly valued for its anti-diabetic effect among several other medicinal applications (Grover and Yadav, 2004) since ages. Due to 'bitter' taste of edible parts of the plant (especially the fruits) justifying the name 'Bitter gourd', *M. charantia* was thought to have hypoglycaemic effect and consumed commonly in most Indian households as an effective alternative-drug for diabetic patients (Bharathi and John, 2013). The anti-diabetic feature of the plant has been successfully examined in clinical trials (Miura et al., 2001; Chen et al., 2003; Lo et al., 2017; Thent et al., 2017) as well.

It is well known that a huge genetic diversity occurs in *M. charantia* which has been reported within domesticated and wild populations of this species (Behera et al., 2008). India is one centre of diversity of *M. charantia* owing to huge domestication and agronomical practices. Two prominent varieties of *M. charantia* namely *M. charantia* var. *charantia* (long fruited variety) and *M. charantia* var. *muricata* (small fruited variety) were reported to occur in India in taxonomy (Chakravarthy, 1990; Nayar et al., 2006; Joseph and Antony, 2008; Bharathi and John, 2013). Molecular marker based assessment of the accessions (Dey et al., 2006; Behera et al., 2008) also pointed to the existence of considerable genetic diversity in *M. charantia* all together.

The *Momordica* species are not easily amenable to cytogenetic analysis due to small size of chromosomes, dense cytoplasmic background and inefficient stainability (Trivedi and Roy, 1972). These are probably the reasons for overall dearth in karyomorphological information on *M. charantia* till date. Though there are some earlier reports on chromosome counts and conventional karyotypes for *M. charantia* (Bhaduri and Bose, 1947; Trivedi and Roy, 1972; Lombello and Pinto-Maglio, 2007; Zaman and Alam, 2009; Bharathi et al., 2011;

☆ This paper is dedicated to the memory of our respected teacher & renowned Plant Cytogeneticist Professor Arun Kumar Sharma, who passed away on 6th July 2017.

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Waminal and Kim, 2012; Kausar et al., 2014; Kido et al., 2016), detailed investigation involving Indian populations of the intraspecific varieties are negligible (Beevy and Bai, 2013). In Cucurbitaceae species, enzymatic maceration and air drying method, EMA (Fukui, 1996) followed by Giemsa staining has been applied previously for improved karyomorphological analysis by resolving structural details of chromosomes and overcoming the technical limitations of conventional aceto-orcein schedules (Bhowmick et al., 2012; Bhowmick and Jha, 2015). EMA method based chromosomal preparations enable easy application of fluorochrome banding technique as a suitable upgradation in karyotyping in the era of molecular cytogenetics. The use of base specific fluorochromes like the guanine-cytosine (GC)-specific chromomycin A3 (CMA) and adenine-thymine (AT)-specific 4′,6-diamidino-2-phenylindole (DAPI) facilitates distinction of morphologically alike chromosomes which in a broader sense contribute to karyotype refinements and characterization of individual genotypes or varieties in particular. In the present study, we report EMA based karyotyping and meiotic analysis for the first time in two varieties of Indian bittergourd, MCC and MCM collected from different regions of India. We addressed detailed investigation of the two varieties of *M. charantia* involving the karyomorphometric parameters, fluorochrome banding patterns accompanied by flow cytometric nuclear genome size estimation.

2. Materials and methods

2.1. Plant materials

For the present study, seeds of 14 cultivated populations of *M. charantia* var. *charantia* (long-fruited variety of *M. charantia*, MCC) were collected from different parts of India *viz.* West Bengal, Odisha, Maharashtra, Karnataka and Kerala; seedling and fruits of a wild population of MCC were also collected from Kerala and seeds of 5 accessions of MCC were kindly provided by National Bureau of Plant Genetic Resources (NBPGR), Thrissur, Kerala. On the other hand, seeds of 6 cultivated populations of *M. charantia* var. *muricata* (small-fruited variety of *M. charantia*, MCM) were procured from localities in West Bengal and Odisha and 9 accessions of MCM were provided by NBPGR, Thrissur, Kerala.

A sufficient stress has been given to obtain a satisfactory seed germination frequency in the present study as a prerequisite of cytological investigation. Germination frequency of the seeds varied (0–40%) among the different populations collected and around 650 seeds from each variety were utilized for optimization of germination frequency. Seeds were washed, imbibed for 48 h, decoated and germinated in between moist filter papers in Petri plates in dark at 25–28 °C. The problem of recalcitrance could be overcome in 5/20 populations of MCC and 5/15 populations in MCM. Seedlings of each variety were also transferred to the experimental garden of the Department of Botany, University of Calcutta for growth, flowering and meiotic analysis. Herbaria were prepared and identified based on the distinguishable fruit sizes of the two taxonomic *M. charantia* varieties.

2.2. Standardisation of root-tip pretreatment for mitotic study

Chromosome pretreatment was conducted following previously reported works (Zaman and Alam, 2009; Bharathi et al., 2011; Waminal and Kim, 2012) with several modifications for improvement. Healthy primary roots (0.4- 0.5 cm in length) were excised from germinating seeds followed by pretreatment in several agents. Around 300 root tips were used for standardization of pretreatment schedule in the species using several pre-treating chemicals (0.01%–0.05% colchicine, saturated solution of p-Dichlorobenzene, 0.002 M hydroxyquinoline) at 9 °C − 20 °C for 3–8 hours. Root tips were also pretreated in distilled water at 4 °C for 12–24 h followed by fixation in 1:3 aceto-methanol and subsequently screened by aceto-orcein staining technique (Sharma and Sharma, 1980).

2.3. Chromosome preparation following EMA-Giemsa method

Pretreated and fixed root tips were subjected to enzymatic maceration and air drying method (Fukui, 1996) with effective modifications. Root tips were suspended in a cocktail of 1% cellulase (Onuzuka RS), 0.15% pectolyase (Y-23), 0.75% macerozyme (R-10) and 1 mM EDTA (pH 4.2) for 42 min. at 38 °C. The enzyme treated root tips were spread on frosted slides with freshly prepared fixative (1:3 aceto-methanol), air-dried and stained with 2% Giemsa solution (Merck,Germany) prepared in 1/15th phosphate buffer and kept for 20 min. Metaphase plates were observed under 100X objectives of Leitz GMBH microscope with attached ProgRes®CT5Jenoptik D07739 (Germany) camera for capturing photographs.

2.4. Fluorochrome staining of somatic metaphase chromosomes

Giemsa stained somatic metaphase slides were destained in 70% methanol and air-dried for application of fluorochrome staining with 4′-6-diamidino-2-phenylindole (DAPI, A-T specific) and chromomycin A3 (CMA, G-C specific) following the protocol of Schweizer (1976) with minor modifications (Jha and Yamamoto, 2012; Bhowmick et al., 2012; Nath et al., 2015). For DAPI staining, slides with metaphase chromosomes were stained with 0.1 mg/ml DAPI solution for different durations (ranging between 30mins- 60 min s) and observed under UV filter. Similarly, slides containing metaphase plates were incubated with 0.1 mg/ml CMA staining solution for varying durations of time (8 min s-45 min s) before observation in BV filter in order to standardize a suitable protocol to obtain chromosomal CMA signals. Because of small size of chromosomes, around 200 metaphase plates were studied to determine number and distribution of the DAPI and CMA signals. Images were captured by ProgRes®CT5 Jenoptik D07739 (Germany) camera attached to Zeiss Axioskop 2 fluorescent microscope. Image analysis and measurement of chromosomal fluorescent signals were done using the software ProgRes CapturePro2.8.8.

2.5. Preparation of meiotic chromosomes

Anthers from staminate flower buds of *M. charantia* var. *charantia* and *M. charantia* var. *muricata* plants were excised and fixed in Carnoy's fixative (acetic acid :ethanol: 1:3v/v) and stored at − 20 °C. For clarification of heavy cytoplasmic matter and chromosome visualization, anthers were stained with DAPI after enzymatic maceration following Fukui (1996) with modifications. Anthers were incubated in enzyme cocktail as reported for other Cucurbits (Bhowmick et al., 2012; Bhowmick and Jha, 2015) and macerated in fixative followed by staining with 0.1 mg/ml DAPI for 30 min in dark. Around 100 PMCs were screened for observation and understanding of meiotic configurations of chromosomes of *M. charantia* var. *charantia* and *M. charantia* var. *muricata*. Meiotic stages of the PMCs were observed under UV filter in Zeiss Axioskop 2 and photographs were captured in ProgRes®CT5 Jenoptik D07739 (Germany) camera.

2.6. Genome size estimation

Young leaves of 2 months old plants of three populations of *M. charantia* var. *charantia* (MCC 1, 4 and 5) and three populations of *M. charantia* var. *muricata* (MCM 1, 2 and 5) were chosen as source material for nuclei isolation and estimation of genome size following method reported for other Cucurbit species (Bhowmick and Jha, 2015). For nuclei isolation, trials with young leaves were conducted in Galbraith buffer (Galbraith et al., 1983) with two different pH values (pH 7.0 and 8.0) to see the efficacy of eliminating nuclei clumps. Leaves of *Pisum sativum* with reported 2C value of 9.09 pg (Doležel and Bartoš, 2005) was used as standard. Nuclei suspensions of sample and standard material were simultaneously analyzed using BD Accuri C6 PLUS Flow Cytometer and BD FACS Verse Flow Cytometer (BD Bioscience, U.S.A.)