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Research Article

Comparative Analysis in CMA Banding Patterns Between Wild and Cultivated *Citrus* Species of North-East India

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Abstract

Fluorochrome staining with chromomycin A3 (CMA) was used to characterize the CMA banding pattern of chromosomes of 5 wild and 5 cultivated species of *Citrus* collected from North-east India. All species used in this study had 2n = 18 chromosomes. These chromosomes were classified into five types based on the number and position of CMA-positive bands; B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without bands and Dst: type D with a satellite chromosome. Each species possessed three to five types of chromosomes and unique CMA banding pattern. It was exciting to note the presence of type D chromosomes of *C. maxima* and *C. ichangensis* with satellite body. The taxonomical position of the true basal species viz. *C. medica, C. reticulata* and *C. maxima* is further confirmed by their identical configurations in the CMA banding pattern. The CMA banding pattern for *C. ichangensis* (Papeda) shows close relations to the sub-genera *Citrus*. On the other hand the banding patterns in the wild species *C. indica* and *C. assamensis* has indicated their genomic hybridity in relation to the true basal species.

Keywords: Chromomycin A3; Karyotype; Citrus; Wild; North-East; Heterochromatin; Wild

Abbreviations

CMA: Chromomycin; DAPI: 4-6-Diamidino-2-Phenyl-Indole

Introduction

The wild and cultivated species of *Citrus* have been the focus of numerous taxonomic and evolutionary studies. The taxonomy of this genus is particularly confusing, varying the number of accepted species from 16 [1] to 162 [2]. Most wild species belong to the more primitive subgenus *Papeda* whereas the commercial varieties and related species are grouped/resolved into the subgenus *Citrus* [1]. India is rich in *Citrus* genetic resources, both in cultivated and wild and enjoys a remarkable position in the "*Citrus* belt of the world" due to her rich wealth of genetic resources [3]. Several earlier workers had attempted to study and classify Indian *Citrus* from botanical as well as horticultural perspective [4-10], but taxonomical ambiguities still remains. Apart from the most commonly cultivated taxa, such as citrons, lemons, limes, mandarins, sour oranges, sweet oranges, pummelos, and grapefruits, some species, viz. *C. indica* Tanaka (Memang narang), *C. macroptera* Montr. (Melanesian papeda), C. latipes (Swingle) Tanaka (Khasi papeda), C. ichangensis Swingle (Ichang papeda), *C. megaloxycarpa* Lush. (Sour pummelo) and *C. assamensis* Dutta et Bhattacharya (Ada jamir) were recorded to occur in wild/semi-wild/ semi-domesticated state in north-east India [3-6,8,11-15]. These wild species have great potential in improvement of Indian *Citrus* since these wild taxa are source for useful genes to combat biotic and abiotic stresses [15]. The plausible hybrid nature of several *Citrus* species, as well as the genetic similarity among different cultivars, including commercial ones, have been investigated by different methods, mainly by molecular markers [16]. Karyotype analysis revealed chromosome polymorphism between (and among) species of the genus *Citrus* [17-21].

The CMA banding patterns of a few cultivated Citrus and its relatives have been elucidated [17-20,22-29]. These studies demonstrated the existence of characteristic CMA banding patterns with a high level of diversity and heterozygosity in these chromosomes. Such cytogenetical information regarding the distributional pattern of euchromatin and heterochromatin has been demonstrated and correlated with the chromosomal evolution of Aurantioideae [30]. Hence these studies are undertaken to have a better understanding of the phylogenetic position and karyotype evolution of cultivated/wild Citrus taxa from north-east India using double staining with the fluorochromes chromomycin A3 (CMA) and 4-6-diamidino-2-phenyl-indole (DAPI).

Materials and Methods

The details of plant material used in the present investigation are detailed in table 1. For each species, wherever possible, a minimum of five individuals and more than one population were analyzed. For obtaining actively growing root tips, plants were raised in earthen pots and the root tips of about (0.5 - 1.0 cm) long were excised. All the root tips were pre-treated with 8-hydroxyquinoline (0.002M) for three hours at room temperature, fixed in ethanol-acetic acid (v/v, 3:1) and subsequently stored at 4°C until required. Enzymatic maceration and air drying were performed as described by Guerra [17] with minor modifications. For squash preparation

of chromosomes, the root tips were washed twice in distilled water, digested with a cellulose-pectinase cocktail solution (1 hr) and spread in 45 per cent acetic acid. After coverslip removal, the slides were aged for 3 days, followed by staining with CMA for 1h, counterstained with DAPI for 30 minutes and mounted in McIlvaine's (pH 7.0) buffer-glycerol v/v 1:1 [31,32]. Photomicrographs were taken on Leica DM 4000B fluorescent microscope.

SI. No	Species	Accession numbers	Status	Distribution in North-east India	Common names	CMA banding pattern
Sub genus *Citrus*						
1	*C. assamensis*	IC 285355	Wild	Jaintia Hills, Meghalaya	Ginger lime	2B+10D+6E
2	*C. indica*	IC 558179	Wild	Garo Hills, Meghalaya	Memang narang	1B+7D+10E
3	*C. jambhiri*	IC 278011	Cultivated	Khasi Hills, Meghalaya	Rough lemon	1B+11D+6E
4	*C. maxima*	IC 583271	Cultivated	Arunachal Pradesh	Pumello	1B+2C+6D+8E+1Dst
5	*C. medica*	IC 583259	Cultivated	Arunachal Pradesh	Citron	2B+1C+7D+8E
6	*C. reticulata*	IC 583264	Cultivated	Arunachal Pradesh	Mandarin	1B+9D+8E
7	*C. sinensis*	IC 558164	Cultivated	Meghalaya	Valencia	1B+2C+11D+4E
Sub genus *Papeda*						
8	*C. ichangensis*	IC 591460	Wild	Kohima, Nagaland	Ketsa chetfu	2B+2C+10D+3E+1Dst
9	*C. latipes*	IC 583263	Wild	Upper shillong, Meghalaya	Khasi papeda	1B+3C+10D+4E
10	*C. macroptera*	IC 558161	Wild	Meghalaya, Mizoram and Manipur	Malenesian papeda	2B+3C+13D

Table 1: Species used in the present investigation and their banding pattern.

Results

All species investigated showed 2n = 18 chromosomes. Chromosomes were classified into the following five types based on the number and position of CMA-positive bands [18,19,22,28]. The CMA+ banding pattern of each chromosome was classified into one of the following types to simplify karyotype description and comparison: B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without bands and Dst: type D with a satellite chromosome (Figure 1). The Citrus species analysed showed a large number of telomeric, and a few proximal, bands with enhanced CMA and quenched DAPI fluorescence (CMA+/DAPI-). The telomeric bands were dissimilar in size and were selectively located on the long arms. All chromosome complements showed a distinct banding pattern that was consistent between cells of each individual.

The similarity with a few exceptions in the banding pattern among the true species i.e. *C. medica* (2B+1C+7D+8E), *C. reticulata* (1B+9D+8E) and *C. maxima* (1B+2C+6D+8E+1Dst), exhibiting same number of type E chromosomes but differing in the number of type B and C chromosomes. The exceptions in the banding pattern of the true species are the presence of Dst type chromosomes in *C. maxima* and the total absence type C chromosomes in *C. reticulata.* The papedian taxa (*C. macroptera*, *C. latipes* and *C. ichangensis*) also showed relative similarities in their banding patterns where *C. latipes* had 1B+3C+10D+4E, *C. macroptera* had 2B+3C+13D and *C. ichangensis* had 2B+2C+10D+3E+1Dst exhibiting minor variation in the number of telomeric bands among the taxa however there was a total absence of E type chromosomes in *C. macroptera* and the presence of the telomeric band with satellite in *C. ichangensis.* The banding pattern of *C. sinensis* and *C. jambhiri* also revealed a pattern of 1B+11D+6E and 1B+2C+11D+4E respectively having same number of telomeric bands in the chromosome complements. The banding pattern of wild taxa of the sub-genus *Citrus* consisting of *C. indica* (1B+7D+10E) and *C. assamensis* (2B+10D+6E) which reveals the distinct similarity of the banding pattern with that of the true species.

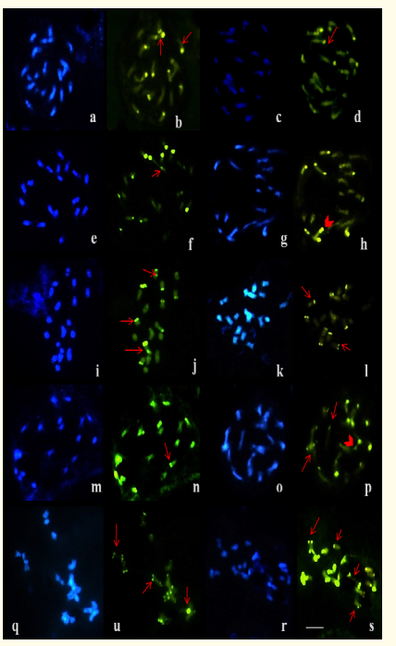

Figure 1: CMA staining of somatic chromosomes in 10 Citrus species of North-east India. a-b: Citrus assamensis, c-d: C. indica, e-f: C. jambhiri, g-h: C. maxima, i-j: C. medica, k-l: C. reticulata, m-n: C. sinensis, o-p: C. ichangensis, q-u: C. latipes, r-s: C. macroptera. Arrow indicates type B, C and D chromosomes. Arrow head indicates type Dst: See all figures Scale Bar represents 5 µm for all figures.

Discussion

Heterochromatin is an important factor in evolution [33] since it can interfere with phenomena such as DNA replication, chromosomal structure, gene expression and the cell cycle [34]. CMA+ or DAPI+ bands observed in many plant genera reveal the occurrence of AT- or GC-rich repetitive DNA sequences, respectively. Hence, CMA positive (CMA+) segment is high in GC-content and DAPI positive (DAPI+) segment is high AT-content [35]. Comparative studies of plant repetitive sequences are useful for exploring evolutionary relationships between plant species [36]. In this investigation of CMA banding pattern in 10 *Citrus* species from North-east India has revealed some concurrence with published data of *Citrus* CMA banding pattern. For example, heterochromatic blocks were found mostly at terminal regions of the chromosomes in all the species, wild and cultivated [17,22,37,38]. All these chromosomes are observed commonly in *Citrus*, and types D and E chromosomes are also predominant in *Citrus* and *Poncirus* [17-20,22,23,25,27,28]. In our observations too on *C. medica*, the chromosome banding pattern of the accession studied was 2B + 8D + 8E and coincides with earlier reports. This indicates that the CMA-positive bands are observed in both the units of homologous pair in C. medica, however it is not the case with rest of the species [17-19,22,23,27]. Such observations also support the basal nature but not the non-hybrid of *C. medica* [28]. Among *Citrus* cultivars, conservation of banding patterns has previously been demonstrated in several accessions of *C. sinensis* [17,22,38,39].

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On the other hand the banding pattern of *C. reticulata* is quite similar in configuration to *C. medica* in having same number of E type chromosomes and with only one B type chromosome (one telomeric and one proximal) and total absence of C type chromosomes (two telomeric bands). Cornelio., *et al.* [25] reported a very simple karyotype (having only D and E types), which was a characteristic feature of a true species as observed in *C. reticulata*. Since hybrids are more probably heteromorphic for chromosome types as compared to basal species, it is reasonable to assume that the homomorphic mandarin groups and *C. medica* may be regarded as true species that has contributed to the origin of several species and hybrids of the subgenus *Citrus*, as evidenced by molecular analysis of genomic DNA [16].

C. maxima configuration consisted of all the types of chromosomes (B,C,D and E) a characteristic feature of the species [17,22,23] with the exception of the presence of one Dst type having a satellite attached to it . The type Dst chromosome, found in *C. maxima* may have originated from a small chromosomal inversion, comprising part of the terminal heterochromatin of a large D chromosome, in this our observations are in agreement with that of Carvalho., *et al* [20].

The papedian taxa i.e. *C. latipes, C. macroptera* and *C. ichangensis* was consistent in heterochromatin banding pattern with all three taxa having C type chromosomes, a distinction from those of other species of sub genus *Citrus. C. ichangensis* possess a Dst type chromosome in its complement which stand out from other Papedas. *C. ichangensis*, a papedian taxa shows close resemblance with taxa belonging to sub genus *Citrus* based on cytogenetical data and inference of nuclear and chloroplast DNA sequence data [40-43] also our present observation on distributional pattern of heterochromatin supports the above view. However morphological features of *C. ichangensis* oppose such conclusions. The banding pattern for the wild species *C. assamensis* and *C. indica* has revealed their similarity with those of the true basal species *C. reticulata* and *C. medica* which could indicate the relationship between them. It has been reported that *C. indica* is closely related to *C. reticulata* and has also been suggested to be another true species or a progenitor species of cultivated *Citrus* [13,21]. Thus, the CMA banding pattern for both these wild species viz. *C. assamensis* and *C. indica* is reported for the first time in this study [44-52].

Conclusion

Understanding *Citrus* phylogeny, taxonomy and genetic variability is crucial for determining sampling strategy, controlling genetic erosion and establishing breeding programs which can be crucial for survival of the Indian *Citrus* industry. Wild relatives of cultivated *Citrus* species can be a major source of genetic variation for utilization in breeding programs aimed at crop improvement through transfer of disease resistance or other desirable agronomic traits. In *Citrus* the importance of wild germplasm has not yet been duly realized due to lack of substantial efforts in documentation and characterization of wild relatives of various locally cultivated *Citrus* species. This study demonstrated the variability in heterochromatin banding pattern in *Citrus* chromosomes. Moreover, the CMA staining method can also be used as a molecular cytogenetical marker for *Citrus* species, because each species exhibited a characteristic CMA banding pattern and the possibilities for the use of cytological data for evolutionary analysis and framing future breeding programmes.

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