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ORIGINAL ARTICLE

Heterochromatin characterization through differential fluorophore binding pattern in some species of *Vigna* Savi

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Abstract Heterochromatin regions are the most intensively studied and best known chromosome markers in plants. In *Vigna* species, blocks of constitutive heterochromatin were found either in the terminal or interstitial region of the chromosomes. The number and distribution of CMA^+ and $DAPI^+$ binding sites exhibit high chromosomal variability with characteristic unique banding patterns in all the eight taxa. A predominant feature was observed, i.e., most of the CMA^+ binding sites were in the terminal region of the short arm of some chromosomes while $DAPI^+$ binding sites were found mostly in the intercalary region of the chromosomes. The higher divergence in the heterochromatin blocks, as revealed by chromomycin A3 (CMA) binding pattern, in a few taxa, viz. *Vigna glabrescens*, *Vigna khandalensis*, and *Vigna mungo*, suggests that the processes of divergent evolution of repetitive sequences in genomic DNA involve a guanine-cytosine (GC)-rich region. On the contrary, *Vigna dalzelliana* had shown a prominent adenine-thymine (AT)-rich repetitive DNA sequence in terminal regions in the short arm of chromosomes while *Vigna umbellata* had shown in interstitial regions. The presence of prominent heterochromatic-rich regions, either GC- or AT-rich regions, does facilitate the rate of chromosomal rearrangements leading to restructuring of the karyotypes and thereby helping the species to attempt structural alterations as means of speciation.

Keywords Euchromatin · Fluorochrome · Heterochromatin · Karyotypes · *Vigna*

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Introduction

The genus *Vigna* (Fabaceae) comprises 104 described species (Lewis et al. 2005) and is grown in tropical and subtropical areas of the world. It is composed of both cultivated and wild species for which a rich diversity occurs in India. Twenty-four species of *Vigna* under the subgenus *Ceratotropis* are reported to occur in India (Sanjappa 1992), which partly represents the center of species diversity for all the three sections of subgenus *Ceratotropis*, known as Asiatic *Vigna*. Despite the presence of a rich diversity in India, these cultivated species and wild relative complexes have not been the subject of intense studies, which is essential for identification, classification, and management of the genetic resources.

Furthermore, distinct morphogenetic differences have been reported in few *Vigna* species, namely *Vigna aconitifolia*, *Vigna angularis*, *Vigna trilobata*, *Vigna umbellata*, *Vigna unguiculata*, and *Vigna vexillata* (Chandel 1984). However, morphological studies alone do not provide sufficient information to understand genetic diversity within the species and relatedness among the taxa, if any. Alternate approaches, including application of appropriate cytogenetical techniques, have now been increasingly adapted for estimating the genetic diversity in Asiatic *Vigna* species (Rao and Chandel 1991).

Cytogenetical data of different species of *Vigna* have been a matter of great importance due to a wide range of varieties with distinct and diffused geographical adaptability. The data on chromosome numbers and structure of the chromosome is of vital importance when studying the origin, evolution, and classification of taxa (Yang et al. 2005). There are significant variations in chromosome numbers among the different species reported for the genus *Vigna* ranging from $2n=18$, 20, 22, 23, 24, 44, etc. with the basic numbers of $x=9$, 10, and 12 (Adetulo 2006; Darlington and Wylie 1955; Frahm-Leliveld 1965; Froni-Martinus 1986; Kumar and Subramaniam 1987; Rao and Raina 2004; Sen and Bhowal 1960). The most frequently observed number was $2n=22$, which is a predominant chromosome number in many *Vigna* species. So far, polyploidy has been reported in only one species, i.e., *Vigna glabrescens*, based on the basic number $x=11$. To resolve the species relationships, modern molecular approaches at DNA (Goel et al. 2002) and chromosome (Galasso et al. 1995) levels have been adopted; however, ambiguity still remains at large.

In eukaryotes, contrary to prokaryotes, the DNA is packaged as a nucleoprotein complex called "chromatin." It is located in the nucleus and gets organized into several "chromosomes" during cell division. Based on how intensely the chromatin is stained, we can distinguish between euchromatin and heterochromatin. Since euchromatin is less intense, heterochromatin stains more densely, indicating thick DNA packing. The heterochromatin region is one of the chromosomal components that deserves most attention from cytogeneticists, and it has been extensively studied. It is the best worked out chromosome domain in plants and animals. The mechanism of differential staining of heterochromatin is still unclear, though it is certainly related to one of the most universal characteristics of heterochromatin, i.e., the presence of one or more families of tandem repetitive short DNA sequence(s). Heterochromatin is predominantly low in gene density and may still contain transcriptionally active genes (Nagaki et al. 2004). The heterochromatin region is commonly found in large blocks near centromeres, telomeres, and nucleolar organizers such as knobs. It is also observed in B chromosomes of certain taxa of grass (Ostergren 2010). Due to the lack of useful genes, the heterochromatin region is also referred to as a "genomic wasteland" or a repository of "junk" DNA (John 1998). Nowadays, this idea is obsolete, and in fact, in the past two decades, molecular genetic studies have implicated that the heterochromatin region also plays an important cellular function in the structural rearrangement of the chromosome (Dimitri et al. 2004, 2005; Corradini et al. 2007). The heterochromatin adjacent to the secondary constrictions, being composed of tandem repetitive 45S rRNA genes, forms large rDNA blocks identified as regions for C-banding and CMA staining (Fuchs et al. 1998).

Heterochromatic bands in plants have been analyzed mainly by C-banding or by base-specific fluorochrome staining (Guerra 2000) techniques. Fluorochrome banding techniques have played a major role in chromosome analysis since the early 1990s. Fluorochrome banding has the advantage of being a simpler, more reproducible, and less destructive method that provides more insights into molecular aspects as compared to C-banding (Guerra 1993). The fluorochromes such as chromomycin A3 (CMA) and 4-6-diamidino-2-phenylindole (DAPI) exhibit preferential staining for guanine-cytosine (GC)- and adenine-thymine (AT)-rich DNA sequences (Schweizer and Ambros 1994; Juliano et al. 2006) in such a way that each region of the chromosome may show a positive (+) or negative (−) result with the given fluorochrome, respectively, allowing the identification of different types of heterochromatin (Schweizer 1981).

In plants, the most commonly used fluorochromes are DAPI, which preferentially stains AT-rich regions, and CMA, which preferentially stains GC-rich regions. Using these two fluorochromes together, heterochromatin blocks can be characterized either as GC- or AT-rich regions and their distribution pattern at interphase and chromosomes, thereby distinguishing genomes (Guerra 2000). Such studies have been successfully carried out in a large number of plant taxa including *Azima tetracantha* (Guerra 1989), *Capsicum pubescens* (Moscone et al. 1993), *Cicer arietinum* (Galasso et al. 1996a), *Citrus hystrix* (Guerra 1985), *Haplopappus gracilis* (Ruffini Castiglione et al. 2008), *Phaseolus calcaratus* (Zheng et al. 1991), *Sesbania tetraptera* (Forni-Martins et al. 1994; Forni-Martins and Guerra 1999), *Vicia faba* (Greilhuber 1975), and *Vigna ambacensis* (Galasso et al. 1996b).

Most of the earlier reports on the cytogenetical aspects of both African and Asian *Vigna* are confined only to conventional approaches primarily based on chromosome counts and, in few cases, chromosome morphology, thereby indicating the intervention by modern techniques based on molecular aspects. Therefore, differential fluorochrome binding, a modern cytogenetical approach, was undertaken to determine the species differentiation and/or affinity towards GC/AT-rich regions in some of the *Vigna* species, viz. *V. aconitifolia*, *V. dalzelliana*, *V. glabrescens*, *V. hainiana*, *V. khandalensis*, *V. mungo*, *V. radiata*, and *V. umbellata.* In order to enhance our knowledge on the organization and distribution of euchromatin and heterochromatin blocks within the genome as well as chromosomes and to assess the diversity at interspecific level, the present work has been envisaged. Thus, the present investigations were taken up with the prime objective of analyzing the genome and chromosomal diversity of *Vigna* species.

Materials and methods

Eight species belonging to genus *Vigna* used in the present investigation was supplied by the National Bureau of Plant Genetic Resources (NBPGR) under a collaborative research

project funded by the World Bank through a consortium research program of the Indian Council of Agricultural Research (ICAR) through the National Agricultural Innovative Project (NAIP). Actively growing root tips of about 2 cm long were excised from germinating seeds on a moist filter paper in petri dishes at 25±2 °C pre-treated with 0.025 % colchicine (Sigma) for 3 h at room temperature (20±2 °C).

Prior to mitotic preparations, 15 root tip meristems of each species were digested in a 2 % cellulase and 20 % pectinase solution for 180 min at 37 °C. Individual root tip meristems were washed in distilled water, squashed in a drop of 45 % acetic acid, and frozen in liquid nitrogen. The slides were stained with DAPI (2 μg/ml)/glycerol (1:1, *v*/*v*) solution to allow selection of the best cells. Subsequently, they were destained in ethanol/glacial acetic acid (3:1, *v*/*v*) for 30 min and transferred to absolute ethanol for 1 h, both at room temperature. Slides were air-dried and aged for 3 days at room temperature. The aged slides were stained with CMA3 (0.5 mg/ml, 1 h) and DAPI (2 μg/ml, 30 min), mounted in McIlvaine's buffer (pH 7.0)/glycerol (1:1, *v*/*v*), and stored for 3 days (Schweizer and Ambros 1994). At least 15 clear mitotic metaphase preparations of the chromosome complements of every representative accession of each species were analyzed. Slides were analyzed under a Leica DM 4000 B microscope, and photographs were carried out with different filter combinations using a Leica CCD camera.

Result

The number and distribution of fluorophore binding sites in chromosomes stained with CMA and DAPI yielding in eight *Vigna* species have been summarized in Tables 1 and 2, and the data has been illustrated in Fig. 1.

Fluorophore binding pattern

The somatic chromosome number of all the taxa studied was $2n=22$ except for *V. glabrescens* which had shown $2n=44$. The CMA^+ and $DAPI^+$ binding sites were found either in the terminal or interstitial region of the respective chromosome, in all the eight taxa studied. The number and distribution of CMA^+ and $DAPI^+$ binding sites exhibit a high chromosomal variability with each taxon having characteristic and unique binding patterns in each taxon. The species-wise fluorophore binding patterns are described below.

V. aconitifolia

CMA^+ binding sites were found mostly in the terminal region of the chromosomes showing the positional mean (±SD) value of 2.4 (±0.7), and the number CMA^+ binding sites ranged from 2 to 7 (Table 1). $DAPI^+$ sites are also found mostly in the terminal region of the chromosomes, with a positional mean (±SD) value of 2.8 (±2.13). The number of $DAPI^+$ binding sites ranged from 2 to 8 (Table 2).

V. dalzelliana

In *V. dalzelliana*, CMA^+ binding sites were found consistently in the terminal and interstitial regions of the chromosomes showing the positional mean (±SD) value of 2.5 (±1.4) and 2.5 (±0.8), respectively (Table 1). The number of chromosomes showing different CMA^+ sites ranged from 2 to 6, while the $DAPI^+$ sites were found maximum in the terminal region showing a positional mean (±SD) value of 4.0 (±1) and ranged from 3 to 5 (Table 2).

V. hainiana

The correlation between the CMA^+ and $DAPI^+$ binding sites was found in *V. hainiana* (Tables 1 and 2); both the binding sites were found at the interstitial region showing the same positional mean (±SD) value of 2.6 (±0.5). The number of CMA^+ binding sites is 3 to 5 and $DAPI^+$ binding sites is 3 to 6.

Table 1 Distribution of CMA^+ sites in the chromosomes of *Vigna* species

Species	2*n*	Cells showing different nos. of CMA^+ sites									Mean (±SD) of cells showing CMA^+ sites with terminal or interstitial position	
		I	II	III	IV	V	VI	VII	VIII	IX	Terminal	Interstitial
V. aconitifolia	22	–	10	–	2	–	–	2	–	–	2.4 (±0.7)	2.0 (±1)
V. dalzelliana	22	–	6	2	2	2	2	–	–	–	2.5 (±1.4)	2.5 (±0.8)
V. hainiana	22	–	–	4	4	2	–	–	–	–	2.0 (±0.8)	2.6 (±0.5)
V. khandalensis	22	–	3	–	6	4	–	–	2	–	3.0 (±0.9)	2.3 (±1)
V. mungo	22	–	–	4	2	2	2	–	–	2	4.0 (±1.2)	2.0 (±0.8)
V. radiata	22	–	–	–	4	8	–	2	–	–	4.2 (±0.7)	2.5 (±0.5)
V. umbellata	22	–	–	4	4	2	2	2	–	–	2.6 (±0.7)	2.8 (±0.97)
V. glabrescens	44	–	2	–	2	5	–	–	1	5	3.1 (±0.9)	3.5 (±1.4)

Table 2 Distribution of DAPI$^+$ sites in the chromosomes of *Vigna* species

Species	2*n*	Cells showing different nos. of DAPI$^+$ sites									Mean (±SD) of cells showing DAPI$^+$ sites with terminal or interstitial position	
		I	II	III	IV	V	VI	VII	VIII	IX	Terminal	Interstitial
V. aconitifolia	22	–	2	2	6	–	–	–	2	–	2.8±2.13	1.8±0.6
V. dalzelliana	22	–	–	4	–	4	–	–	–	–	4.0±1	2.6±0.5
V. hainiana	22	–	–	2	2	2	4	–	–	–	2.2±0.7	2.6±0.5
V. khandalensis	22	–	4	–	6	–	–	–	4	–	2.6±1.3	2.8±1.2
V. mungo	22	–	2	2	6	–	–	2	–	–	1.6±0.5	2.7±1.4
V. radiata	22	–	–	6	6	–	–	2	–	–	2.1±0.3	2.2±1.3
V. umbellata	22	–	–	4	4	–	2	4	–	–	1.8±0.9	3.0±1.5
V. glabrescens	44	–	4	–	5	3	–	3	–	–	2.2±0.7	2.5±1

V. khandalensis

CMA$^+$ binding sites were found mostly in the terminal region of the chromosomes showing a positional mean (±SD) value of 3.0 (±0.9), and the number of CMA$^+$ binding sites ranged from 2 to 8, while the DAPI$^+$ sites were found mostly in the interstitial region of the chromosomes with a positional mean (±SD) value of 2.8 (±1.2). The number of different DAPI$^+$ binding sites ranged from 2 to 8 (Table 2).

V. mungo

In *V. mungo*, CMA$^+$ binding sites were found mostly in the terminal region of the chromosomes showing the positional mean (±SD) value of 4.0 (±1.2). The different CMA$^+$ binding sites ranged from 3 to 9, while the DAPI$^+$ binding sites were found commonly in the interstitial region showing a positional mean (±SD) value of 2.7 (±1.4), and the sites of DAPI$^+$ ranged from 2 to 7 (Table 2).

V. radiata

Majority of the CMA$^+$ binding sites were found at the terminal region of the short arm of the chromosomes having a positional mean (±SD) value of 4.2 (±0.7) (Table 1). The number of CMA$^+$ sites ranged from 4 to 7. DAPI$^+$ binding sites were found consistently in the terminal and interstitial regions of the chromosomes showing a positional mean (±SD) value of terminal 2.1 (±0.3) and interstitial 2.2 (±1.3), and the sites of DAPI$^+$ ranged from 3 to 7 (Table 2).

V. umbellata

Both the CMA$^+$ and DAPI$^+$ binding sites were found mostly in the interstitial region of the chromosomes showing the positional mean (±SD) value of 2.8 (±0.97) and 3.0 (±1.5), respectively. The number of CMA$^+$ and DAPI$^+$ binding sites also ranged from 3 to 7 (Tables 1 and 2).

V. glabrescens

In *V. glabrescens*, CMA$^+$ binding sites were found consistently in the interstitial region of the chromosomes showing a positional mean (±SD) value of 3.5 (±1.4). The number of CMA$^+$ binding sites ranged from 2 to 9 (Table 1), while the DAPI$^+$ sites were also found mostly in the interstitial region showing a positional mean value of 2.5 (±1), and the sites of DAPI$^+$ ranged from 2 to 7 (Table 2).

Discussion

The use of base-specific fluorochrome band detection techniques has been in vogue for the last few decades and is presently utilized for analysis of constitutive heterochromatin in eight *Vigna* species. Blocks of constitutive heterochromatin were found either in the terminal or interstitial region of the chromosomes. Data presented in Tables 1 and 2 has revealed some common characteristics in binding sites and the distribution pattern(s). However, DAPI$^+$ binding sites were found mostly in the interstitial region of the chromosomes whenever recorded. It is interesting to note that a higher divergence in CMA binding sites was observed in *V. glabrescens*, *V. khandalensis*, and *V. mungo*. The CMA binding sites in chromosomes, which are indicative of a GC-rich region, were recorded in the terminal region of the short arm of chromosome(s) in *V. aconitifolia*, *V. khandalensis*, *V. mungo*, and *V. radiata*. Preliminary observations by de A Bortoleti et al. (2012) have revealed that the CMA/DAPI binding pattern is a suitable approach for chromatin differentiation analysis in *V. radiata* and *V. unguiculata* and revealed that higher divergence could be observed with regard to the distribution pattern of heterochromatin of *V. radiata* when considering the distribution of these sequences with other phaseoloides previously described in the literature (Zheng et al. 1991, 1993; Fonseca et al. 2010). Similar conclusions can also be drawn from our

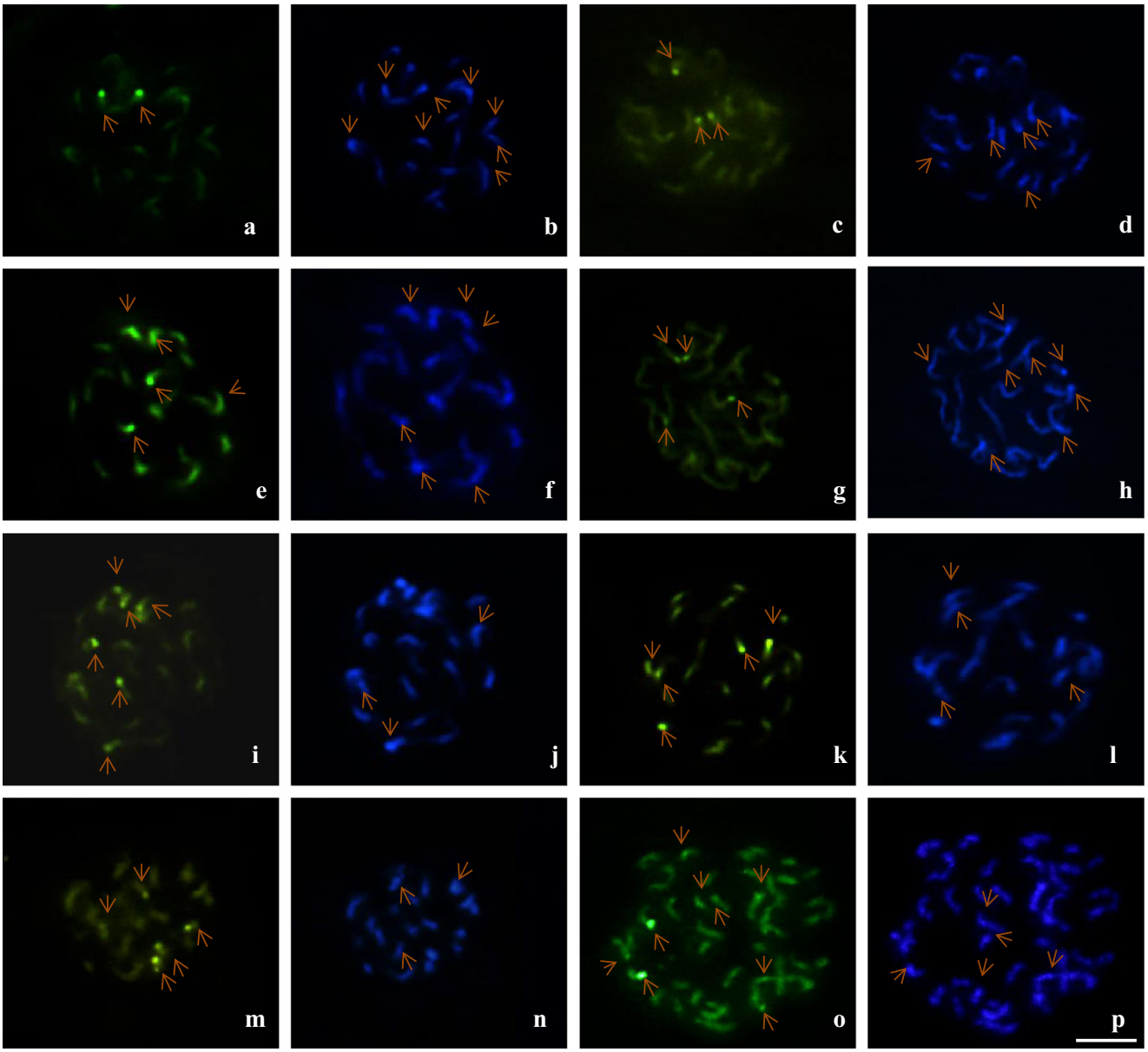

Fig. 1 Differentially stained mitotic chromosome complements in *Vigna* species: *V. aconitifolia* (**a**, **b**), *V. dalzelliana* (**c**, **d**), *V. hainiana* (**e**, **f**), *V. khandalensis* (**g**, **h**), *V. mungo* (**i**, **j**), *V. umbellata* (**k**, **l**), *V. radiata* (**m**, **n**), and *V. glabrescens* (**o**, **p**); *arrows* indicate CMA^+ and $DAPI^+$ sites. *Scale bar* −5 μm in all the figures

observation which has shown that the highest mean value of CMA^+ (4.2) at the terminal region of the short arm of the chromosome(s) in *V. radiata*. *V. glabrescens*, an important amphidiploid species, had shown the highest mean value of CMA^+ (3.5) at the interstitial region in both arms of a few chromosomes. This indicated a tendency for the accumulation of GC-rich repeats at the interstitial region of chromosomes. The higher divergence in heterochromatin blocks, as evidenced by CMA^+ staining especially consisting of GC base pairs, is possibly reflecting the processes of divergent evolution of repetitive sequences in the heterochromatin region of the genome.

Two species, *V. aconitifolia* and *V. khandalensis*, had shown a higher degree of divergence with DAPI staining. $DAPI^+$ binding pattern was distinct in *V. dalzelliana* and revealed the highest mean value of 4.0 at the terminal region of the short arm of the chromosomes, while *V. umbellata* had shown the highest mean value of $DAPI^+$ (3.0), suggesting that their heterochromatic region was rich in AT base composition at the interstitial region(s) of chromosomes. Similar conclusions were also drawn by Galasso et al. (1995), who found AT-rich repetitive DNA sequences from the *V. unguiculata* genome with 488 nucleotides in length, associated with the centromeric regions as detected by FISH. Polymorphism regarding the number and location of $DAPI^+$ binding sites could be recorded. This may be due to either minute deletion or high condensation of heterochromatic regions at the respective loci.

One of the important assumptions in the heterochromatin dispersion model proposed by Schweizer and Loidl (1987) is that interstitial binding sites in long arms are equidistant to the terminal binding site of short arms. In the present investigation, such interstitial binding sites corresponding to non-homologous terminal heterochromatin were occasionally observed, through several non-equidistant heterochromatin binding sites. For instance, chromosomes of all the taxa presently investigated have exhibited telomeric binding sites only in short arms and interstitial binding sites in long arms, although an exact equidistancing is not expected since different chromosome arms may have different condensation/decondensation patterns (Okada 1975; Fukui and Mukai 1988).

From the previous reports on karyomorphological studies in *Vigna* species (Rao and Chandel 1991; Rao and Raina 2004; Shamurailatpam et al. 2012), a predominance of metacentric and submetacentric chromosomes is being reported with a symmetrical nature of karyotype to a larger extent, but quite a diverse heterochromatic region within them. Similar conclusions were also drawn by John and King (1983) who found closely related species with very similar karyotypes but differed to a large extent in the heterochromatin content. Hence, we can conclude that chromosomal divergence occurred through structural alteration revealing in the heterochromatic regions of the genus *Vigna*, an adopted evolutionary process of the genus.

The presence of prominent heterochromatic-rich regions facilitates the rate of chromosomal rearrangements; this is based on the assumption that constitutive heterochromatin acts as a hot spot for the occurrence of chromosome rearrangements (Yunis and Yasmineh 1971; Peacock et al. 1982; John and King 1983; Chaves et al. 2004). Wichman et al. (1991) opined that rapidly evolving families or variants of highly repetitive satellite DNA can promote chromosome structural rearrangements that reshape karyotypes and thereby help the species to attempt in structural alterations as means of speciation.

In general, increment in the volume of heterochromatin probably developed along with the evolution of the genus (Ikeda 1988). This phenomenon could also be observed in *Vigna*, an advanced genera that had showed a heterochromatin-rich chromosome configuration that might have been involved in the diversification of this genus (Miranda et al. 1997; Guerra 2000). With the help of CMA and DAPI differential staining, it is now possible to develop marker chromosomes for authentic identification in *V. radiata* varieties, which allow accurate determination of individual chromosome pairs (Mahbub et al. 2007). This has opened up new possibilities and allowed the visualization of chromosomal markers associated with heterochromatin in *Vigna* species. Since these basic features provide important information for further construction of cytological maps, their reassessment is relevant for future marker associations. This characterization may be valuable in the future for inferences regarding chromosomal evolution within the Fabaceae angiosperm family.

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