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Heterochromatin distribution and comparative karyo-morphological studies in *Vigna umbellata* Thunberg, 1969 and *V. aconitifolia* Jacquin, 1969 (Fabaceae) accessions

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Abstract

Chromosome studies along with heterochromatin distribution pattern analysis have been carried out in two domesticated species of *Vigna* Savi, 1824 which grow in contrasting geo-climatic conditions of India: *Vigna umbellata* Thunberg, 1969, a legume well acclimatized to subtropical hilly regions of North-east India and *V. aconitifolia* Jacquin, 1969, a species of arid and semi-arid regions in desert plains of Western India. Karyo-morphological studies in both species reveal $2n = 22$ chromosomes without any evidence of numerical variation and the overall karyotype symmetry in chromosome morphology suggest that the diversification at intraspecific level in genus *Vigna* has occurred through structural alteration of chromosomes, rather than numerical changes. Heterochromatin distribution as revealed by fluorochrome binding pattern using CMA_3 and DAPI, confirms the occurrence of relatively more GC content in *V. aconitifolia* as compared to *V. umbellata*. However, AT content was found to be comparatively higher in *V. umbellata* which perhaps play a role in species interrelationships.

Keywords

Asymmetry index, C-heterochromatin, Fabaceae, karyotype, NOR-chromosomes, *Vigna*

Introduction

The pantropical genus *Vigna* Savi, 1824 (Fabaceae) includes 104 described species (Lewis et al. 2005). Among its subgenera, only *Ceratotropis* Marechal, 1978 is known for its rich species diversity in Asia (Verdcourt 1970, Marechal et al. 1978, Tateishi 1996). Tomooka et al. (2002) recognized 21 species in the subgenus *Ceratotropis*, out of which six species are domesticated: azuki bean (*V. angularis* Willdenow, 1969), mung bean (*V. radiata* Linnaeus, 1954), black gram (*V. mungo* Linnaeus, 1956), rice bean (*V. umbellata* Thunberg, 1969), moth bean (*V. aconitifolia* Jacquin, 1969) and creole bean (*V. reflexo-pilosa* var. *glabra* Marechal, 1911). The genetic resources and diversity in cultivated and wild forms of subgenus *Ceratotropis* occurring in Indian subcontinent are extremely rich and interesting (Bisht et al. 2005). The domesticated *V. aconitifolia* is confined only to the tropical region of India, while *V. umbellata* is widely domesticated across the South-east Asia. The origin of *V. umbellata* is considered to be Indo-China region and also to a certain extent from South-east Asia (Marechal et al. 1978, Baudoin and Marechal 1988).

The structure and morphology of the chromosomes are of vital importance when studying the origin, evolution and classification of taxa (Yang et al. 2005) as well as distance or relatedness among diverse genomes (Stace 2000, Kumar and Rao 2002). Quite a few number of reports dealing with such studies are available for *Vigna* species (Rao and Chandel 1991, Rao and Raina 2004, Shamurailatpam et al. 2012).

Chromosome location and characterization of C-heterochromatin by fluorescence staining procedures which preferentially stain GC-rich DNA and DAPI, which localised AT-rich regions has been successfully applied in a large number of Fabaceae taxa including *Cicer arietinum* Linnaeus, 1753 (Galasso et al. 1996a); *Phaseolus calcaratus* Roxburgh, 1832 (Zheng et al. 1991); *Sesbania tetraptera* Hochstetter, 1871 (Forni-Martins et al. 1994, Forni-Martins and Guerra 1999); *Vicia faba* Linnaeus, 1753 (Greilhuber 1975); *Vigna ambacensis* Welwitsch, 1978 (Galasso et al. 1996b).

A certain degree of chromosomal variation at inter-specific level of the genus *Vigna* has been documented using cytogenetic approaches by earlier workers (Rao and Chandel 1991, Shamurailatpam et al. 2012). Hence, it will be quite significant to see the extent of variation among the domesticated species of *Vigna* (*Ceratotropis*). *V. umbellata* is a species domesticated extensively in the subtropical hilly and moist regions of North-east India. On the other hand, *V. aconitifolia* has been adapted to the arid and semi-arid region of tropical Western plain of India. Analysis of karyo-morphological details in *V. umbellata* and *V. aconitifolia*, adapted to extremely contrasting environmental conditions, may ultimately help us to define their chromosome variation. Meaningful propagation programs can be developed from such information.

Materials and methods

Karyo-morphological studies were undertaken in ten accessions each of *V. umbellata* and *V. aconitifolia*. The germplasm has been obtained from Indian Council of Agricultural Research (ICAR), Baranapi, Meghalaya and also from National Bureau of Plant Genetic Resources (NBPGR), New Delhi. Actively growing root tips of about 1–2 cm long were excised from germinating seeds on moist filter paper in Petri dishes at 25 ± 2 °C, pre-treated with 0.025% colchicine (Himedia) for 3 h at room temperature (20 ± 2 °C). The root tips after pre-treatment were fixed in freshly prepared ethanol-acetic acid (v/v, 3:1) and subsequently stored at 4 °C until required. For slide preparation, the root tips were washed twice in distilled water, hydrolyzed in 1N HCl at 60 °C for 8 min and stained in Feulgen stain (leuco-basic fuchsin) for 45 min. The stained root tips were thoroughly washed and subsequently squashed in 1% acetocarmine. The microphotographs of the metaphase plates were taken from both temporary and permanent preparations. At least 10–15 clear preparations of chromosome complements of each species were analyzed. Photo-idiograms were prepared from photomicrographs by cutting out individual chromosome and arranging them in descending order of their length and matching on the basis of morphology, the chromosomes were resolved into 11pairs. The standard method of chromosome classification given by Battaglia (1955) classification of metacentric / median (V), submetacentric/ submedian (L), subtelocentric (J) and telocentric (I) based on the arm ratio of 1:1, >1:1<1.3, >1:3<1:0 and 1:0 respectively was employed for comparison. The degree of asymmetry was estimated by means of the parameters proposed by Peruzzi and Eroğlu (2013): Coefficient of Variation of Chromosome Length (CV_{CL}) and Mean Centromeric Asymmetry (M_{CA}).

For heterochromatin characterization, root-tips were digested in 2% cellulase and 20% pectinase solution for 180 min at 37°C. 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 plates. 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 slides were stained with CMA_3 (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). Slides were analyzed under Leica DM 4000 B microscope and photographs were carried out with different filter combinations using Leica CCD camera.

Results

The somatic chromosome number of all the accessions had consistently $2n = 2x = 22$ (Fig. 1). The chromosome complements were resolved into 11 pairs which formed a graded series from longest to shortest within the idiograms. A noticeable difference in

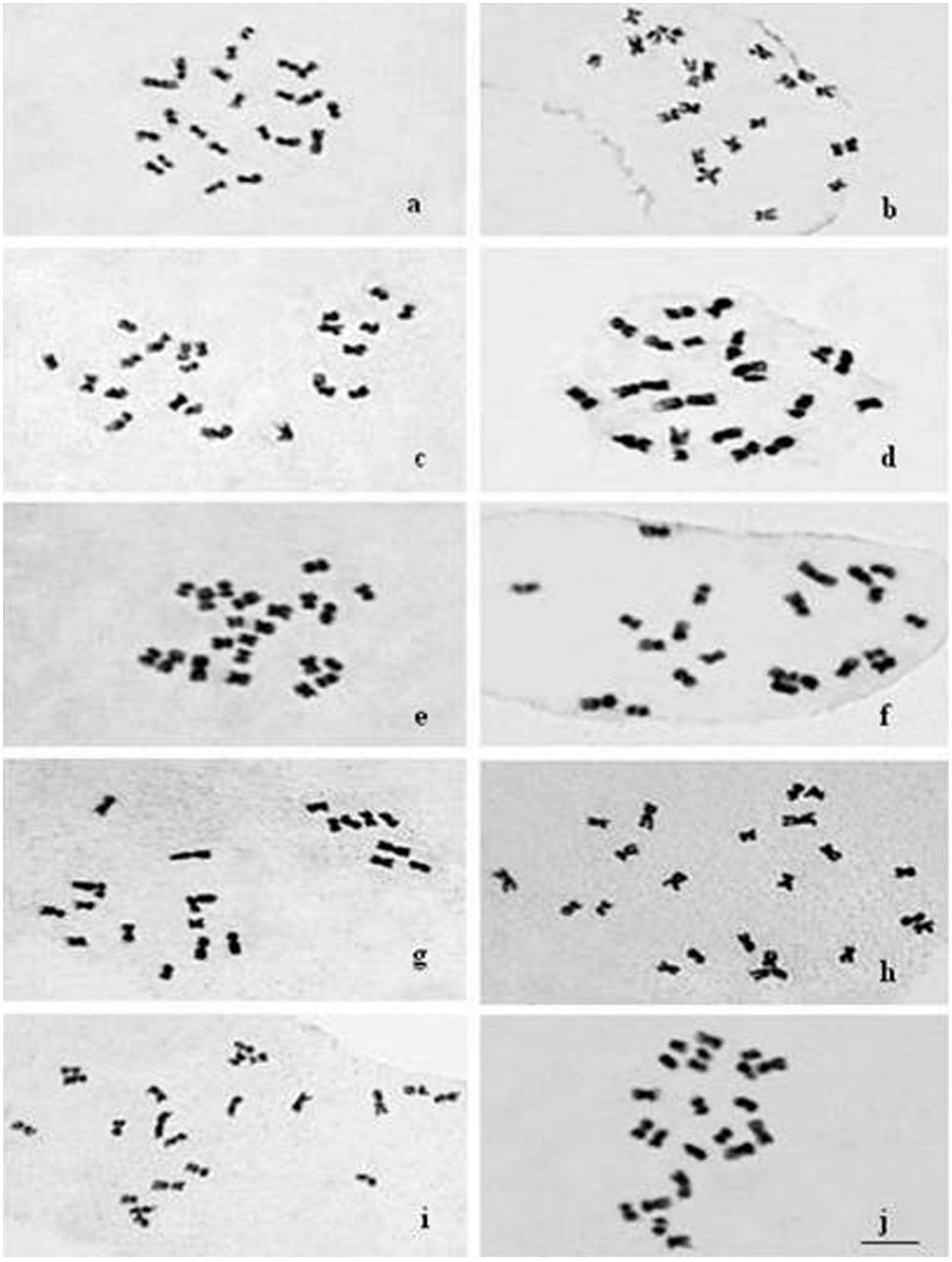

Figure 1. Mitotic complements of 10 accessions of *V. umbellata*. **a–j: a** BKSB 205 **b** TRB 160 **c** RBS 35 **d** IC 551699 **e** BKSB 192 **f** RBS 53 **g** IC 55440 **h** IC 176563 **i** EC 97882 **j** BKSB 194. Bar = 5 μm.

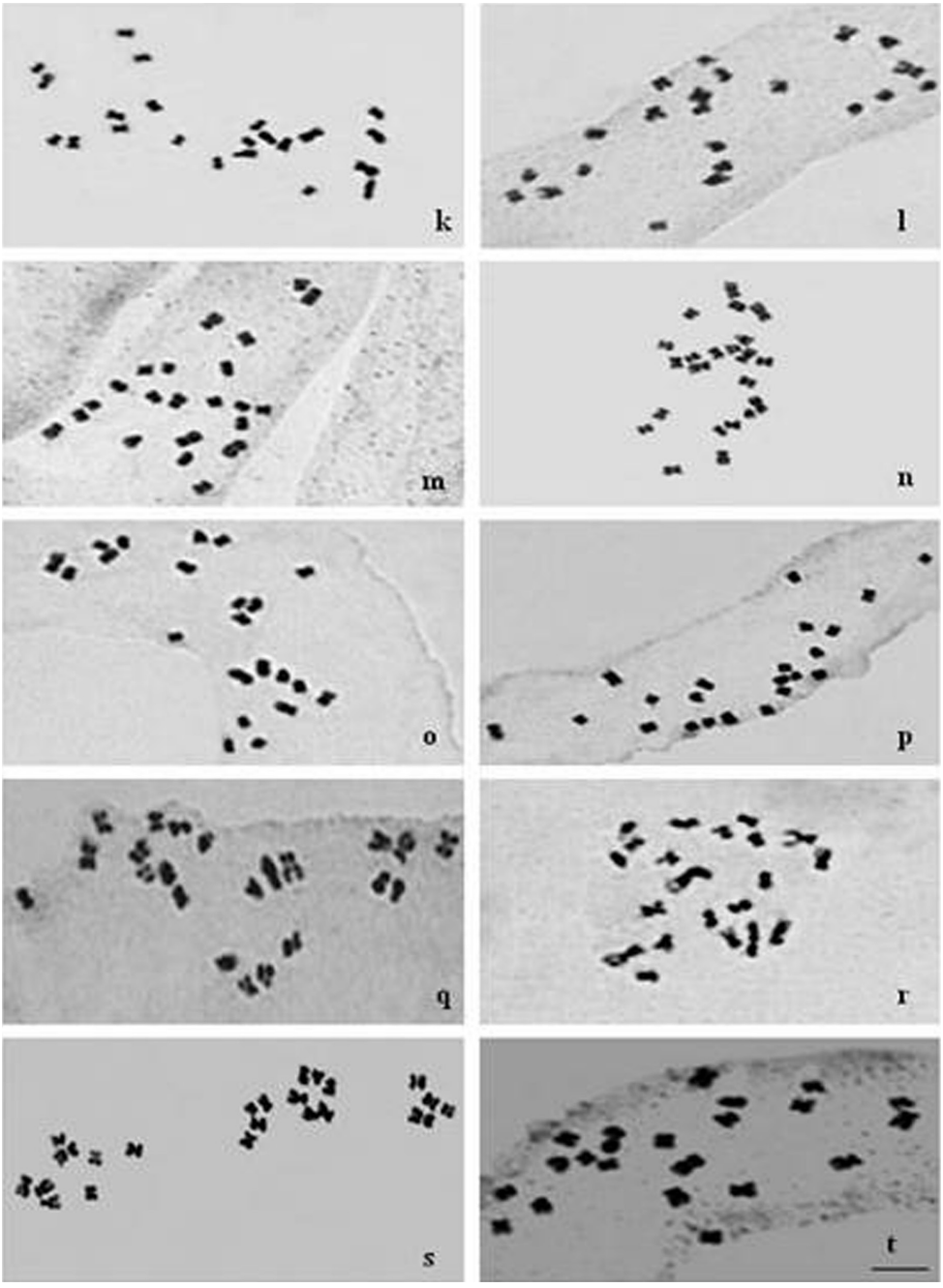

Figure 1. Continued. **k–t: k** IC 36157 **l** VDV 6175 **m** IC 472147 **n** RM 040 **o** IC 39809 **p** IC 285159 **q** IC 36592 **r** IC 472173 s IC 39713 **t** IC 36562. Bar = 5 μm.

Table 1. Karyomorphology and arm ratio in studied taxa of *Vigna*.

Sl. no.	Species	Accessions no.	2n	Chromosome arm length (L/S ratio)											Ratio of longest and shortest chromosome	Karyotype formula
				I	II	III	IV	V	VI	VII	VIII	IX	X	XI		
1	*V. umbellata*	BKSB 205	22	1.12 1.14	1.8	1.5	1.5	1.25	1	1.3	1.3	1.3	1.3	1.3	2.4	2V + 20L
2	*V. umbellata*	TRB 160	22	1.2 1.5	1.1 1.25	1.75	1.37	1	1	1	1.3	1.3	1	1	2.2	12V + 10L
3	*V. umbellata*	RBS 35	22	1.2	1.6	1	1	1	1	1	1.15	1.3	1.3	1	2.8	12V + 10L
4	*V. umbellata*	IC 551699	22	1 1	1.3	1.7	1.15	1.57	1.75	1.1	1	1	1	1	4.0	12V + 10L
5	*V. umbellata*	BKSB 192	22	1	1	1	1.25	1.12	1	1	1	1.3	1	1	2.0	16V + 6L
6	*V. umbellata*	RBS 53	22	1.2	1.1	1.25	1.25	1	1	1	1	1.3	1.3	1	2.3	12V + 10L
7	*V. umbellata*	IC 55440	22	1.57 1.42	1.2	1.35	1.12	1.12	1.12	1	1	1	1.15	1.3	3.0	6V + 16L
8	*V. umbellata*	IC 176563	22	1.14 1.28	1.6	1.1	1.25	1.6	1.3	1	1	1.6	1.3	1	2.6	8V + 14L
9	*V. umbellata*	EC 97882	22	1.7	1.47	1.2	1.37	1.2	1	1	1.65	1.3	1.3	1	2.3	8V + 14L
10	*V. umbellata*	BKSB 194	22	1	1	1	1.3	1.3	1.3	1.3	1.3	1	1	1	3.0	12V + 10L
11	*V. aconitifolia*	IC 36157	22	1.33	1.9	2	1.5	1.1 1.5	1.5	1.5	1.5	1	1	1	1.75	6V + 16L
12	*V. aconitifolia*	VDV 6175	22	1.5 1.66	2.5	1.33	2	1.5	1.5	1.5	1.5	1.25	1	1	2.5	4V + 16L + 2J
13	*V. aconitifolia*	IC 472147	22	2	2.5	2.5 2	1	1	1	1.5	1.75	1.5	1	1	2.25	10V + 12L
14	*V. aconitifolia*	RM 040	22	1.5	1.66	3 2	1.5	2	2	1.75	1.25	1.5	1.5	1	2.5	2V + 18L + 2J
15	*V. aconitifolia*	IC 39809	22	1.57	2	1.66	1.33	2	1.16	1	1.25	1.25	1.5	1	3.0	4V + 18L
16	*V. aconitifolia*	IC 285159	22	1.33	2	1.5	1.5	1	1.5	1.25	1	1	1	1	3.5	10V + 12L
17	*V. aconitifolia*	IC 36592	22	1.75 1.6	1.62	1	1	1.66	1	1	1.33	1.33	1	2	2.16	10V + 12L
18	*V. aconitifolia*	IC 472173	22	2.4 1.8	1.83 2	1.86	1.25	1.12	1	1	1	1	1	1	4.25	14V + 8L
19	*V. aconitifolia*	IC 39713	22	2	2.5	1	2	1	1.5	1.5	1.5	1.5	1.5	1	2.25	6V + 16L
20	*V. aconitifolia*	IC 36562	22	1.58	3	1.49	1.33	1	1	1	1.5	1.5	1.5	1	2.5	8V + 14L

length between the longest and the shortest chromosomes within the complement was recorded (Table 1). The longest chromosome of the haploid complement was almost 2.5 times longer than the shortest one in *V. aconitifolia* accessions, while it was 2 times longer than the shortest one in *V. umbellata* accessions. Further investigated accessions belonging to *V. umbellata* and *V. aconitifolia* had metacentric, submetacentric and subtelocentric chromosomes in their respective chromosome complements. Submetacentric chromosomes outnumbered the metacentric ones in *V. aconitifolia* accessions while metacentric chromosomes outnumbered the submetacentric chromosomes in the case of *V. umbellata* accessions.

Various accessions of these species have shown distinctive variation in the karyotype with respect to number of metacentric and submetacentric chromosomes (Fig. 2). Subtelocentric chromosomes were found in *V. aconitifolia* but not in *V. umbellata* accessions. Heteromorphic chromosome and nucleolar chromosomes are recorded in the accessions of both *V. umbellata* and *V. aconitifolia*.

Telocentric chromosomes were absent in both the taxa studied. Heteromorphic chromosomes were observed in some of the *V. umbellata* accessions: BKSB 205 (1st pair, Fig. 2a), TRB 160 (1st and 2nd pair, Fig. 2b), IC 551699 (1st pair, Fig. 2d), IC55440 (1st pair, Fig. 2g) and IC 176563 (1st pair, Fig. 2h). In *V. aconitifolia* heteromorphic chromosomes were found in IC 36157 (5th pair, Fig. 2k), VDV 6175 (1st pair, Fig. 2l), IC 472147 (3rd pair, Fig. 2m), RM040 (3rd pair, Fig. 2n), IC 36592 (1st pair, Fig. 2q) and IC 472173 (1st and 2nd pair, Fig. 2r) accessions. Nucleolar Organizing Regions (NORs), as a secondary constriction/ satellites, were observed in *V. umbellata* accessions RBS 35 (1st pair, Fig. 2c), IC 551699 (2nd pair, Fig. 2d), and EC 97882 (3rd and 4th pair, Fig. 2i). *Vigna umbellata* was characterized by the presence of both metacentric and submetacentric chromosomes and two *V. aconitifolia* accessions (VDV 6175 and RM 040) were characterized by the presence of distinct subtelocentric chromosome, though their position differed in karyotype. The remaining accessions were devoid of any subtelocentric chromosome.

According to the scatter plot obtained by CV_{CL} vs. M_{CA}, BKSB 192 (*V. umbellata*) and EC 97882 (*V. umbellata*) showed the lowest (2.81) and highest (55.07) M_{CA} respectively (Fig. 4). Furthermore IC 285159 (*V. aconitifolia*) and RM 040 (*V. aconitifolia*) showed lowest (10.8) and highest (22.88) M_{CA} values. In *V. umbellata* TRB 160 and IC 551699 exhibited lowest (19.59) and highest (37.4) CV_{CL} values. Among *V. aconitifolia* accessions IC 36157 and IC 472173 had shown lowest (19.92) and highest (42.64) CV_{CL} values.

A comparative account of heterochromatin distribution pattern within the chromosome complements in *V. umbellata* and *V. aconitifolia* has been summarized in Table 3 and the data have been illustrated in Fig. 3. The CMA_3^+ and $DAPI^+$ binding sites were found either in terminal or in interstitial regions, in both the taxa studied. *V. umbellata* had more of $DAPI^+$ sites 3.1(± 1.9) in the interstitial region of the chromosomes and the terminal binding sites were 1.8(± 0.6). The number of chromosomes showing different CMA^+ and $DAPI^+$ sites also ranged from 2–7 in this

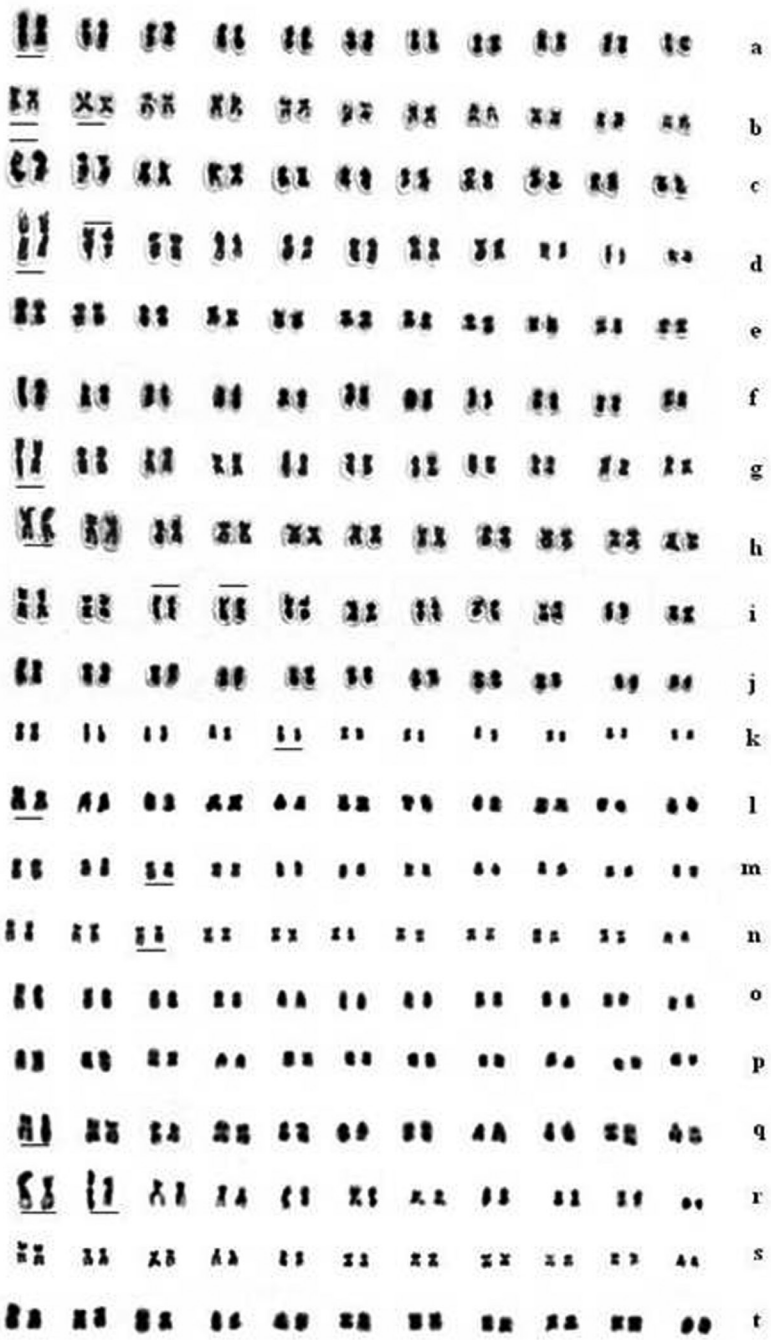

Figure 2. Photo-idiograms of **a–j** 10 accessions of *V. umbellata* **a** BKSB 205 **b** TRB 160 **c** RBS 35 **d** IC 551699 **e** BKSB 192, **f** RBS 53 **g** IC 55440 **h** IC 176563 **i** EC 97882 **j** BKSB 194 **k–t** 10 accessions of *V. aconitifolia* **k** IC 36157 **l** VDV 6175 **m** IC 472147 **n** RM 040 **o** IC 39809 **p** IC 285159 **q** IC 36592 **r** IC 472173 **s** IC 39713 **t** IC 36562. Heteromorphic groups marked above the short arm and nucleolar groups are marked below the long arm.

Table 2. Karyotype formulae and characteristics in the studied taxa of *Vigna*. SC the shortest chromosome length; LC the longest chromosome length; CL mean length of chromosome; CI mean centromeric index; SD standard deviation; CV_{CL} component expressing the relative variation in chromosome length; M_{CA} mean centromeric asymmetry.

Sl. no.	Accessions no.	2n	Range SC-LC (µm)	Ratio LC/SC	CL (µm) Mean (± SD)	CI Mean (± SD)	CV_{CL}	M_{CA}
1	BKSB 205	22	17-7	2.4	9.13 (± 2.76)	42.87 (± 3.47)	30.25	14.30
2	TRB 160	22	11-5	2.2	7.95(± 1.55)	45.22 (± 4.65)	19.59	9.09
3	RBS 35	22	17-6	2.8	9.18(± 2.95)	46.93 (± 3.85)	32.19	6.18
4	IC 551699	22	24-6	4	11.5(± 4.3)	45.59 (± 5.03)	37.4	9.06
5	BKSB 192	22	12-6	2	8.45(± 1.78)	48.57 (± 2.49)	21.16	2.81
6	RBS 53	22	14-6	2.3	8.40(± 1.74)	47.09 (± 2.91)	20.72	5.78
7	IC 55440	22	18-6	3	9.31(± 2.99)	46.55 (± 2.92)	32.13	6.49
8	IC 176563	22	16-6	2.6	9.18(± 2.66)	44.29 (± 4.65)	29.02	11.37
9	EC 97882	22	14-6	2.3	9.18(± 2.27)	44.94 (± 3.94)	24.82	55.07
10	BKSB 194	22	18-4	3	7.09(± 1.62)	46.75 (± 3.55)	22.86	6.49
11	IC 36157	22	7-4	1.75	5.22 (± 1.04)	42.44 (± 6.07)	19.92	15.32
12	VDV 6175	22	10-4	2.5	5.68 (± 1.54)	40.76 (± 7.06)	27.25	18.55
13	IC 472147	22	9-4	2.25	5.81 (± 1.36)	41.61 (± 8.54)	23.54	16.51
14	RM 040	22	10-4	2.5	6.09 (± 1.67)	38.78 (± 6.45)	27.52	22.88
15	IC 39809	22	12-4	3	6.72 (± 2.02)	42.04 (± 6.17)	30.12	16.22
16	IC 285159	22	7-2	3.5	4.68 (± 1.25)	43.63 (± 5.72)	26.85	10.8
17	IC 36592	22	11-6	2.16	7.95 (± 1.60)	43.89 (± 6.32)	20.22	12.21
18	IC 472173	22	17-4	4.25	8.59 (± 3.66)	44.99 (± 7.29)	42.64	10.14
19	IC 39713	22	9-4	2.25	5.72 (± 1.28)	40.47 (± 6.85)	22.44	19.05
20	IC 36562	22	10-4	2.5	6.22 (± 1.47)	43.34 (± 8.09)	23.68	15.13

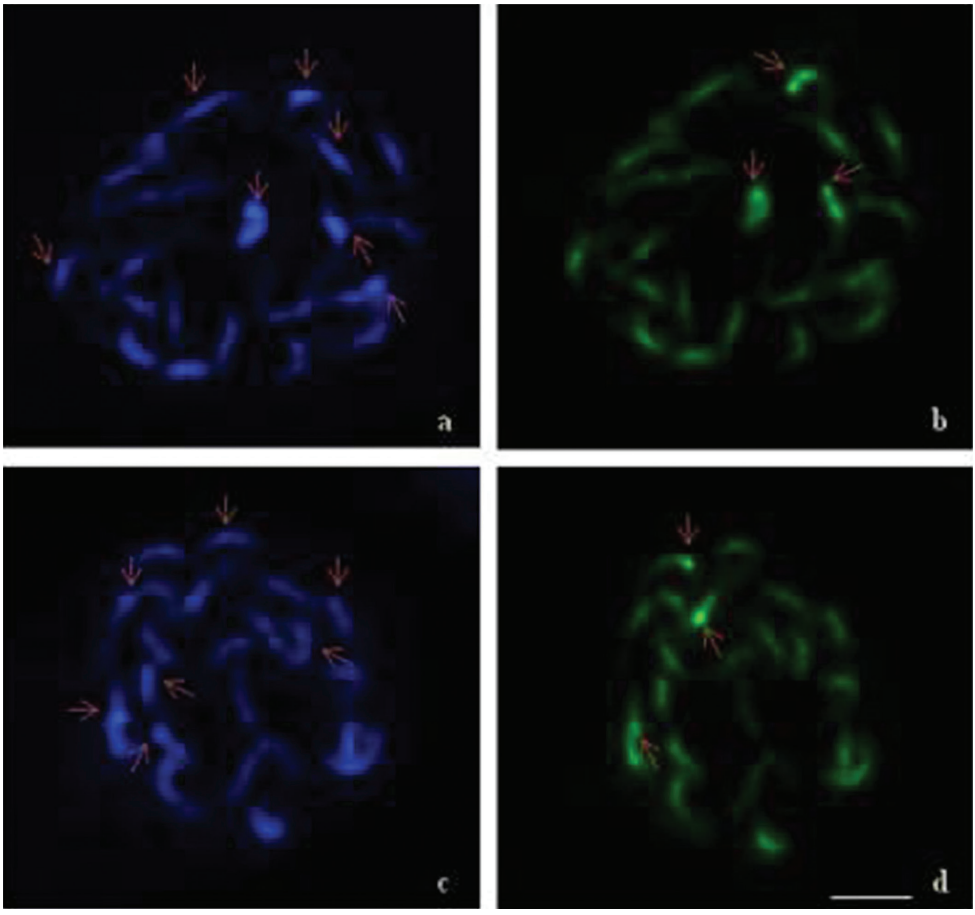

Figure 3. Differentially stained mitotic chromosomes complements **a–b** *V. umbellata* **c–d** *V. aconitifolia*. Arrows indicate CMA$^+$ and DAPI$^+$ sites. Scale bar = 5 μm in all the figures.

Table 3. Distribution of CMA$^+$ and DAPI$^+$ sites in the chromosomes of *Vigna* species.

Species	Mean± SD of CMA$^+$ sites in chromosomes		Mean± SD of DAPI$^+$ sites in chromosomes		Range of CMA$^+$ sites Terminal	Range of DAPI$^+$ sites Interstitial
	Terminal	Interstitial	Terminal	Interstitial		
V. umbellata	1.7 ± 0.8	2.1 ± 0.8	1.8 ± 0.6	3.1 ± 1.9	1.7 ± 0.8	2.1 ± 0.8
V. aconitifolia	2.9 ± 1.3	2 ± 1.2	2.7 ± 0.7	2.3 ± 0.8	2.9 ± 1.3	2 ± 1.2

species. On the other hand, in *V. aconitifolia* the heterochromatin block comprised more of CMA$^+$ binding sites 2.9(± 1.3), which were found in the terminal region of the chromosomes while 2(± 1.2) binding sites were interstitial in position. The number of chromosomes showing CMA$^+$ sites ranged from 3–7, while those showing the DAPI$^+$ sites ranged from 3–8.

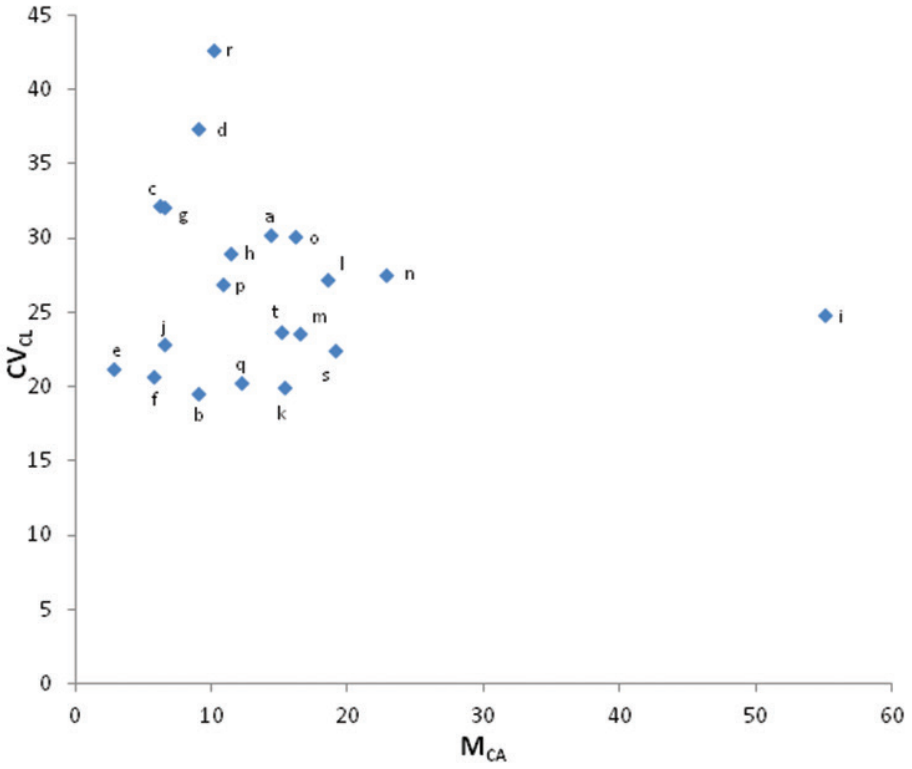

Figure 4. Scatter plot based on the karyotype parameters M_{CA} (x axis) vs. CV_{CL} (y axis) **a** BKSB 192 **b** RBS 53 **c** RBS 35 **d** BKSB 194 **e** IC 55440 **f** IC 551699 **g** TRB 160 **h** IC 472173 **i** IC 285159 **j** IC 176563 **k** IC 36592 **l** BKSB 205 **m** IC 36562 **n** IC 36157 **o** IC 39809 **p** IC 472147 **q** VDV 6175 **r** IC 39713 **s** RM 040 **t** EC 97882.

Discussion

The present data, combined with the chromosome counts available from the literature confirm the somatic chromosome number of $2n = 22$ for both species, *V. umbellata* and *V. aconitifolia*. Such observation received support from reports of Singh and Roy (1970), Rao and Chandel (1991), Rao and Raina (2004), Shamurailatpam et al. (2012). The presence of subtelocentric chromosomes in *V. aconitifolia* accessions is in agreement with the earlier report of Sinha and Roy (1979).

All the accessions of *V. umbellata* and *V. aconitifolia* have shown no deviation in somatic chromosome numbers and overall karyotype appearance. However, *V. umbellata* had a higher degree of karyotype asymmetry as compared to *V. aconitifolia*, suggesting structural rearrangements in karyotypes. Hence, the observed karyotype variation is likely to have originated by structural changes in chromosomes vs. duplication, deletions, interchanges and inversions (Stebbins 1971, Rao and Chandel 1991). Thus, structural alteration of the chromosomes involving centric fusion and centromere repositioning might have influenced the speciation in genus *Vigna*.

Due to the very small size of chromosomes accompanied by technical difficulties, the nucleolus organisers among the chromosome complements could not be clearly resolved. Other cytogenetic techniques such as silver staining and fluorescence *in situ* hybridization (FISH) can be useful in detecting NOR-loci on chromosomes.

The DAPI$^+$ binding sites in chromosomes, which are indicative of AT-rich region, were recorded in the interstitial regions of chromosomes in *V. umbellata*. However CMA$^+$ sites, found mostly in *V. aconitifolia* chromosomes, suggest that the heterochromatin blocks were rich in GC base composition at terminal regions of chromosomes. The higher distribution of AT- and GC- repetitive sequence in heterochromatin blocks is probably reflecting the processes of divergent evolution of repetitive sequences, in heterochromatin regions of *Vigna* species (Shamurailatpam et al. 2014).

In the course of evolution, most of the heterochromatin regions tend to increase (Ikeda 1988), this phenomenon is also observed in *Vigna* (Shamurailatpam et al. 2015). Certain genera such as *Vicia*, *Phaseolus*, *Sesbania*, *Cicer* and *Vigna* (Greilhuber 1975, Zheng et al. 1991, Forni-Martins et al. 1994, Galasso et al. 1996a, b, Forni-Martins and Guerra 1999) showed a heterochromatin-rich chromosome configuration, that might have been involved in diversification of this genus. *Vigna umbellata*, which is domesticated extensively in the sub tropical hilly and moist regions of North-east India, had its heterochromatin blocks rich in AT content with fewer GC base pairs. On the contrary, more GC content in heterochromatin blocks was observed in *V. aconitifolia*, which is acclimatized to the arid and semi-arid region of tropical Western plains of India, helping the species to overcome adverse climatic conditions of Indian desert. Our observations in this regard constitute a first attempt to probe the role of heterochromatin distribution pattern, if any, in species differentiation of plant groups.

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