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Comparative Cytogenetics and Fluorescent Chromosome Banding in Five Indian Species of *Dipcadi* Medik

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Abstract: The genus *Dipcadi* Medik. (Subfamily: Scilloideae) has a narrow distribution in India and several overlapping morphological traits make the genus taxonomically challenging at the species level. Cytogenetic characterization can provide additional taxonomic data and can be used to evaluate genetic diversity at the species level. We have accomplished comparative karyotype analysis and fluorescence banding patterns using 4′-6-Diamidino-2-phenylindole (DAPI) and Chromomycin A_3 (CMA) in five Indian species for the first time. The karyotypes of *D. concanense* and *D. goaense* exhibited similar fluorochrome banding profiles. However, *D. montanum*, *D. ursulae and D. erythraeum* differ distinctly in their karyotypes. In all taxa, $CMA^{+ve}/DAPI^{-ve}$ or $DAPI^{0}$ (GC-rich) constitutive heterochromatin was located at the constriction region or terminal satellite of the nucleolar chromosome. $DAPI^{+ve}/CMA^{-ve}$ or CMA^{0} (AT-rich) heterochromatin dominates in *D. montanum, D. ursulae and D. erythraeum*. However, *D. erythraeum* shows a distinct variation in fluorochrome banding pattern from all other species. The distribution of CMA and DAPI bands is a reflection of heterochromatin composition and variations acquired by different species. This characterization can be used to assess phylogenetic relationships in the understudied genus *Dipcadi* and may serve as a basis for other genomic analyses and evolutionary studies.

Keywords: *Dipcadi*; karyotype; fluorochrome banding; DAPI bands; CMA bands

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1. Introduction

The subfamily Scilloideae (family Asparagaceae) *sensu* Angiosperm Phylogeny Group, (APG III) [1] is a major group of small perennial bulbous plants, consisting of four monophyletic tribes: Hyacintheae, Ornithogaleae, Urgineeae and Oziroeeae [2–5]. Bulbous geophytes of this subfamily have long been used in traditional medicine and specialized metabolites from members of each tribe have been reported such as homoisoflavonoids and triterpenoids from Hyacintheae, bufadienolides from Urgineeae and cardenolides from Ornithogaleae [6–11]. Scilloideae is represented mainly by three genera in India viz. *Drimia* Jacq. ex Willd. (Urgineeae), *Dipcadi* Medik. (Ornithogaleae) and *Ledebouria* Roth (Hyacintheaee). Due to taxonomic disputes at interspecific levels [12], the three genera have been subjected to revision from time to time [2–4].

The genus *Dipcadi* Medik. is morphologically distinct from other genera in having tubular flowers, quadrate capsules and large discoid seeds [13]. There are confusing reports on the number of valid species of *Dipcadi* in India [12,14–16], ranging from seven to nine species [12,14,16,17]. Most of the species of *Dipcadi* in India are endemic to the Western Ghats, a biodiversity hotspot and a world heritage site [12,17]. Some species are assessed as threatened according to the red data book of Indian plants of which *D. concanense* and *D. reidii* were declared extinct but rediscovered and assessed as critically endangered [17,18]. *Dipcadi goaense* was located along the lateritic gravelly area of South Goa [19] and the species is known by a single population restricted to the type

locality [14]. *Dipcadi erythraeum* is endemic to desert areas of Rajasthan. These species have several uniform and overlapping morphological characters, making the genus taxonomically difficult at the species level [12,20] and necessitating the study of additional parameters for the thorough characterization of taxa. The importance of the Indian species of *Dipcadi* resides not only in their endemism and narrow distribution but also in their phytochemical constituents [12,15,21–24].

Detailed cytogenetic and molecular phylogenetic studies are reported to be useful for the advancement of classification at the species level [2,3,25]. Globally, chromosome counts have been reported for 14 species of *Dipcadi* [25] showing wide diversity in chromosome number [26]. Among the species occurring in India, *D. concanense, D. goaense* and *D. saxorum* show 2n = 12 chromosomes while *D. montanum* and *D. ursulae* show 2n = 20 chromosomes [14,27–29]. *Dipcadi erythraeum* is reported to have 2n = 20 [30] as well as 2n = 22 chromosomes [31]. Meiosis shows regular bivalent formation in most of the species studied [14,30–32]. Karyotype analysis in the Indian species of *Dipcadi* exhibits asymmetric bimodal karyotypes but detailed characterization is lacking [14,30–33].

Over the years, molecular cytogenetics has rejuvenated research on plant chromosomes. Chromosomes prepared through the enzymatic maceration and air drying (EMA) method followed by Giemsa staining [34,35] provides distinct chromosomal morphology in a cytoplasm-free background. Application of contrasting base-specific fluorochrome dyes 4′-6-Diamidino-2-phenylindole (DAPI) and Chromomycin A_3 (CMA) helps to identify heterochromatin blocks of repetitive DNA sequences directly on the chromosomes [36]. The present study is a continuation of our previous work on the karyological relationship and molecular phylogeny of the Indian taxa of Scilloideae [37,38]. It is evident that chromosomal evaluation and molecular phylogenetic study may complement each other to enrich existing knowledge regarding the relationship among different species of the understudied genus *Dipcadi*.

The objective of the present study was to establish fluorescent karyotypes of Indian species of *Dipcadi*, to evaluate the genetic diversity at chromosomal level in the species collected. Traditional taxonomic parameters such as vegetative and floral morphology, anatomy and pollen architecture have been reported to show very little or continuous variation in *Dipcadi* species and hence the study of additional parameters seems necessary. Molecular cytogenetic characterization providing precise knowledge on chromosome number and architecture is reported to be useful for phylogenetic studies and the advancement of classification at the species level. This characterization can be used to assess genetic diversity, provide additional taxonomic data, and serve as a basis for other genomic analyses and evolutionary studies.

2. Results

2.1. Chromosome Number and Karyotype Analysis

This karyo-morphometric analysis of the Indian species of *Dipcadi* is based on five species collected from Western Ghats (*D. concanense, D. goaense, D. montanum, D. ursulae*) and Rajasthan (*D. erythraeum*). Chromosome counts from 20–25 well-scattered metaphase plates of each population of each species (Table 1) revealed interspecific differences in the diploid chromosome number. *Dipcadi concanense* (a threatened species) and *D. goaense* (an endemic species) showed 2n = 12 chromosomes, while *D. montanum* and *D. ursulae* show 2n = 20 chromosomes and *D. erythraeum*, an endemic species from Rajasthan, showed 2n = 22 chromosomes.

The technical standardization of the methodology for EMA-based Giemsa staining allowed us to identify very clearly the numbers and the position of constrictions (primary and secondary) for the first time in all five species. The chromosomes have been categorized into three basic types [39,40] in five species: sub-median (sm), sub-terminal (st) and terminal (t). The longest chromosome pair in all the species is either with sub-terminal constriction (*D. concanense, D. goaense* and *D. erythraeum*) or with sub-median constriction (in *D. montanum* and *D. ursulae*). The smallest chromosome pair was sub-median in

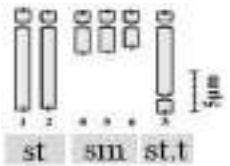
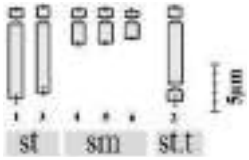
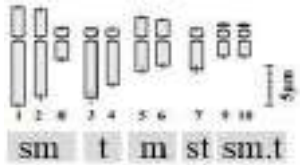
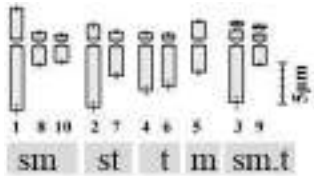
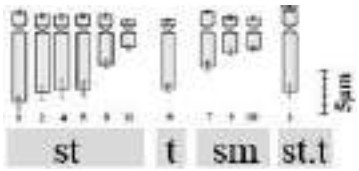
all the species except in *D. erythraeum*. Chromosomes with secondary constriction (i.e., chromosomes with two constrictions) have been identified for the first time in *Dipcadi* species in this study (Figures 1a, 2a, 3a, 4a and 5a). One pair of chromosomes with two constrictions was clearly identified in *D. concanense, D. goaense* and *D. erythraeum*, (Table 2, Figures 1a, 2a and 5a) whereas two pairs of chromosomes with secondary constrictions were identified in *D. montanum* and *D. ursulae* (Table 2, Figures 3a and 4a). These were located on the 3rd pair of chromosomes in *D. concanense*, and *D. erythraeum* and on the 2nd pair in *D. goaense*. On the other hand, in *D. montanum*, the 9th and 10th pair of chromosomes were with two constrictions. In *D. ursulae*, these were located on the 3rd and 9th pair of chromosomes as shown in the karyotype of each species (Table 2). *Dipcadi concanense* and *D. goaense* with the same chromosome number (2n = 12), showed similar karyotype (4st + 6sm + 2st.t). Additionally, in *D. concanense* and *D. goaense*, the secondary constricted chromosomes were of the same type, i.e., of the two constrictions, one is subterminal (st) and the other is terminal (t), at two ends of the long arm. *Dipcadi montanum* and *D. ursulae*, with the same chromosome number (2n = 20), differ in their karyotype (Table 2) and the type of secondary constricted chromosomes. In *D. montanum*, of the two constrictions in the 9th and 10th pair of chromosomes, one is sub-median (sm) in position and the other is terminal (t) at the distal end of the short arm. In *D. ursulae*, one is sub-terminal (st) and the other is terminal (t), at the distal end of the short arm in the 3rd and 9th pair of chromosomes. The two populations of *D. erythraeum* (2n = 22) exhibited distinctly different karyotypes from all other four species. However, the type of secondary constricted chromosome was similar to *D. ursulae* (Table 2).

Table 1. Collection details, somatic chromosome number and total chromosome Length (TCL) in different populations of Indian species of *Dipcadi*.

Species	Population	Site of Collection	Geographic Details	Chromosome Number (2n)	TCL (Mean ± µm) *
D. concanense	Dcon1	Rajapur, Maharashtra	16.6571° N, 73.5211° E	12	85.68 ± 0.53 [b]
	Dcon2	Ratnagiri, Maharashtra	16.9902° N, 73.3120° E	12	86.20 ± 0.71 [b]
D. goaense	Dgoa1	Quepem District, South Goa	15.2282° N, 74.0647° E	12	81.98 ± 0.72 [a]
D. ursulae	Durs1	Thosegar, Maharashtra	17.6031° N, 73.8478° E	20	110.58 ± 1.38 [c]
	Durs2	Panhala, Maharashtra	16.8107° N, 74.1181° E	20	117.00 ± 0.87 [f]
	Durs3	Satara, Maharashtra	17.6805° N, 74.0183° E	20	118.08 ± 1.00 [g]
D. montanum	Dmon1	Ajara, Maharashtra	16.1159° N, 74.2106° E	20	118.84 ± 0.43 [g]
	Dmon2	Badami, Karnataka	15.9186° N, 75.6761° E	20	112.00 ± 0.64 [d]
D. erythraeum	Dery1	Jaisalmer, Rajasthan	26.9157° N, 70.9083° E	22	112.24 ± 0.43 [d]
	Dery2	Jodhpur, Rajasthan	26.2389° N, 73.0243° E	22	113.30 ± 0.98 [e]

* Values followed by same letter are not significantly different; according to Duncan's test ($p = 0.05$).

Table 2. Chromosome morphometric data generated through EMA-Giemsa staining in the five Indian species of *Dipcadi*.

Species & Population		Absolute Length of Longest Chromosome (Mean ± SD in μm) *	Absolute Length of Shortest Chromosome (Mean ± SD in μm) *	ACL (Mean ± SD in μm) *	No. of SAT Chromosome & Ordering No. of SAT Bearing Pair	Diploid Karyotype Formula	Diagrammatic Representation of Karyotype (Haploid Set)
D. concanense (Dalzell) Baker 2n = 12	Dcon1	11.25 ± 0.30 ^{b}	3.10 ± 0.20 ^{u}	7.15 ± 0.40 ^{z}	2 (3rd pair)	4st + 6sm + 2st.t	
	Dcon2	11.14 ± 0.45 ^{b}	3.19 ± 0.14 ^{u}	7.18 ± 0.31 ^{z}			
D. goaense Prabhug. 2n = 12	Dgoa1	10.93 ± 1.36 ^{b}	3.05 ± 0.47 ^{u}	6.83 ± 0.81 ^{yz}	2 (2nd pair)	4st + 6sm + 2st.t	
D. montanum (Dalzell) Baker 2n = 20	Dmon1	11.79 ± 0.87 ^{b}	2.35 ± 0.18 st	5.94 ± 0.55 ^{xy}	4 (9th & 10th pair)	6sm + 4t + 4m + 2st + 4sm.t	
	Dmon2	11.66 ± 0.68 ^{b}	2.40 ± 0.15 ^{t}	5.60 ± 0.54 ^{x}			
D. ursulae Blatt. 2n = 20	Durs1	11.29 ± 1.66 ^{b}	2.01 ± 0.29 ^{qr}	5.52 ± 0.86 ^{x}	4 (3rd & 9th pair)	6sm + 4st + 4t + 2m + 4st.t	
	Durs2	11.54 ± 0.32 ^{b}	2.05 ± 0.22 ^{qr}	5.85 ± 0.92 ^{xy}			
	Durs3	11.79 ± 0.87 ^{b}	2.01 ± 0.28 ^{qr}	5.90 ± 0.74 ^{xy}			
D. erythraeum Webb & Berthel. 2n = 22	Dery1	7.88 ± 1.02 ^{a}	1.66 ± 0.20 ^{p}	5.10 ± 1.05 ^{x}	2 (3rd pair)	12st + 2t + 6sm + 2st.t	
	Dery2	7.93 ± 1.58 ^{a}	1.72 ± 0.16 ^{pq}	5.15 ± 0.75 ^{x}			

* Values followed by the same letter are not significantly different; according to Duncan's test ($p = 0.05$).

The interspecific and intraspecific variation in total chromatin length (TCL) was determined for all populations (Table 1). Inter-specific variation in TCL was observed between all species and between populations of *D. montanum*, *D. ursulae, D. erythraeum* (Table 1). The range of chromosome size found in these species indicated the bimodal nature of their karyotype (Table 2). The Average Chromosome Length (ACL) of the different populations in five species ranged between 5.10–5.15 µm (*D. erythraeum*) and 7.15–7.18 µm (*D. concanense*). The karyotypes of all species studied, were asymmetrical considering the centromeric position and chromosome size variation.

2.2. Fluorochrome Banding Pattern

Fluorescent banding with DAPI and CMA led to a diversified, scorable and species-specific fluorescent banding pattern in species of *Dipcadi*. 0.1 mg mL^{-1} of CMA solution required 60 min. to induce scorable bands, while, 0.1 µg mL^{-1} of DAPI solution took 25 min to induce clearly visible bands. A minimum of 15 metaphase plates for each species stained with DAPI and CMA were considered for the analysis of band patterns (Figures 1–6). Considering the high preferential nature of CMA and DAPI in GC- and AT-rich sequences, as suggested by Barros e Silva and Guerra [41], we identified different types of heterochromatic signals/bands as GC-rich ($CMA^{+ve}/DAPI^{-ve}$), AT-rich ($DAPI^{+ve}/CMA^{-ve}$) or as AT/GC-neutral ($DAPI^{0}/CMA^{0}$) in different species of *Dipcadi* (Table 3). In chromosomes showing $CMA^{+ve}/DAPI^{-ve}$ banding pattern (type B, Table 3), DAPI staining resulted in a clear gap ($DAPI^{-ve}$ bands) corresponding to the CMA^{+ve} signal/band. On the other hand, in chromosomes showing $DAPI^{+ve}/CMA^{-ve}$ banding pattern (type D, Table 3), CMA staining resulted in a clear gap (CMA^{-ve} bands) corresponding to the $DAPI^{+ve}$ signal/band.

In *D. concanense and D. goaense* with 2n = 12, only four $CMA^{+ve}/DAPI^{-ve}$ bands (type B, Tables 3 and 4, Figures 1, 2 and 6) were located on two pairs of chromosomes. It is noteworthy that no $DAPI^{+ve}$ bands were identified in these two species. In *D. montanum* and *D. ursulae* (2n = 20), four and six $CMA^{+ve}/DAPI^{-ve}$ bands (type B, Tables 3 and 4, Figures 3 and 4) were observed, respectively. The distinctive feature of both species was the presence of $DAPI^{+ve}/CMA^{-ve}$ bands (type D, Table 3, Figures 3, 4 and 6) on the chromosomes. Sixteen type D bands were located on four pairs of chromosomes in *D. montanum* while seven bands on three chromosomes were located in *D. ursulae* (Table 4). *Dipcadi erythraeum* (2n = 22), differed from all other species in the banding type, showing two $CMA^{+ve}/DAPI^{0}$ bands (type C, Tables 3 and 4, Figure 5) and 26 $DAPI^{+ve}/CMA^{0}$ bands (type E, Tables 3 and 4, Figures 5 and 6). The $DAPI^{+ve}/CMA^{0}$ bands were interstitial in position, located on the short arm (type E1) or long arm (E3) or on both arms (E4) of chromosomes. Thus, the fluorochrome karyotype showed significant differences in the type of bands and in the number of bands between the five species. The fluorochrome karyotypes of five species reveal similarities and distinct differences between species (Figure 6).

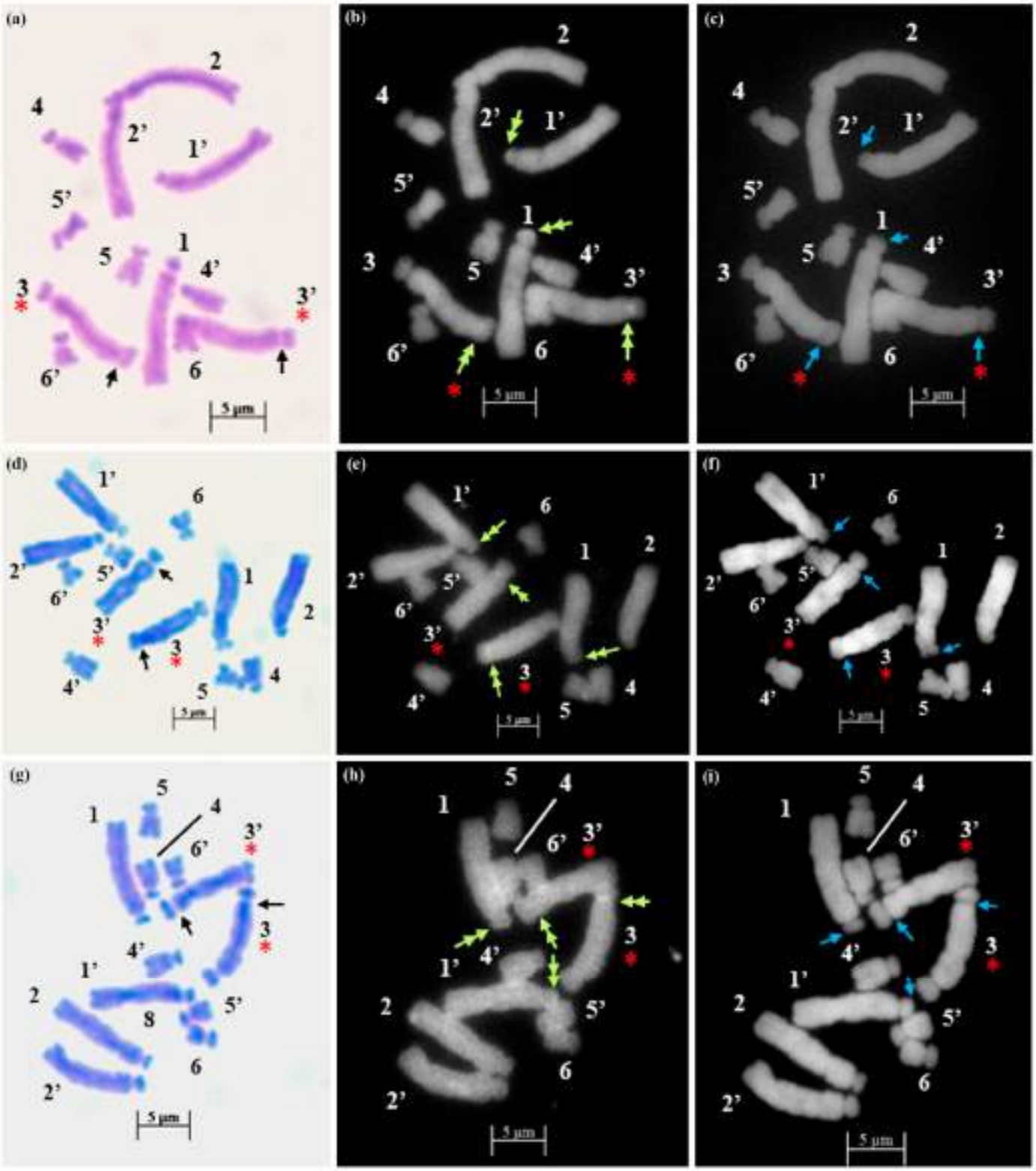


Figure 1. Somatic metaphase plates of *Dipcadi concanense* (Dalzell) Baker with 2n = 12 chromosomes stained with Giemsa (**a**,**d**,**g**), CMA (**b**,**e**,**h**) and DAPI (**c**,**f**,**i**)**.** Black arrows indicate position of secondary constrictions in Giemsa-stained plates (**a**,**d**,**g**). Double yellow arrows indicate CMA^{+ve} bands (**b**,**e**,**h**). Single blue arrows indicate the $DAPI^{-ve}$ band/gap (**c**,**f**,**i**). Red asterisks mark chromosomes with secondary constrictions in all the plates. Bars 5 µm.

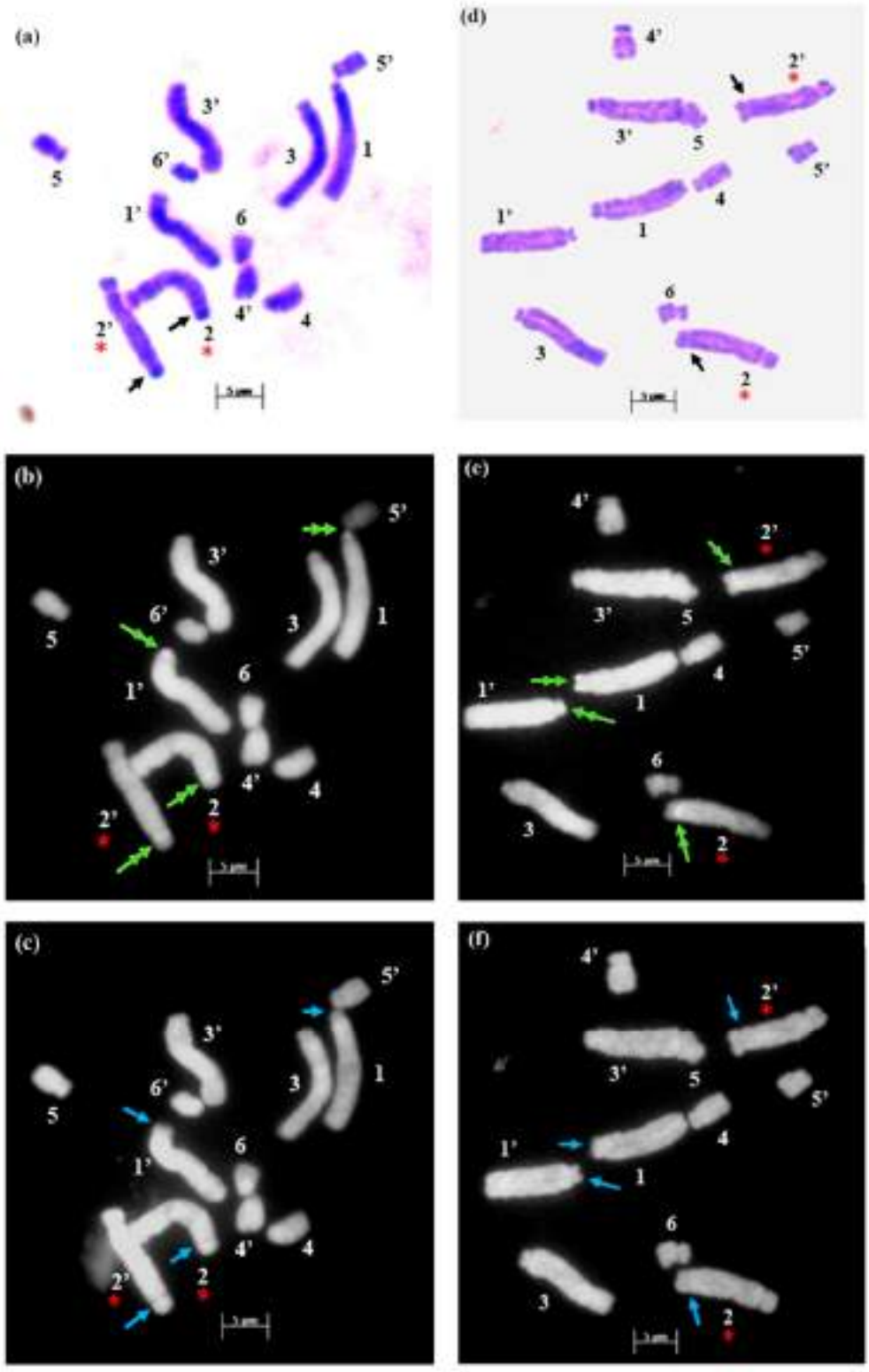


Figure 2. Somatic metaphase plates of *Dipcadi goaense* Prabhug. with 2n = 12 chromosomes stained with Giemsa (**a**,**d**), CMA (**b**,**e**) and DAPI (**c**,**f**). Black arrows indicate position of secondary constrictions in Giemsa-stained plates (**a**,**d**). Double yellow arrows indicate CMA^{+ve} bands (**b**,**e**). Single blue arrows mark the $DAPI^{-ve}$ gaps (**c**,**f**). Red asterisks mark chromosomes with secondary constrictions in all the plates. (**d**–**f**); one chromosome less, out of field). Bars 5 µm.

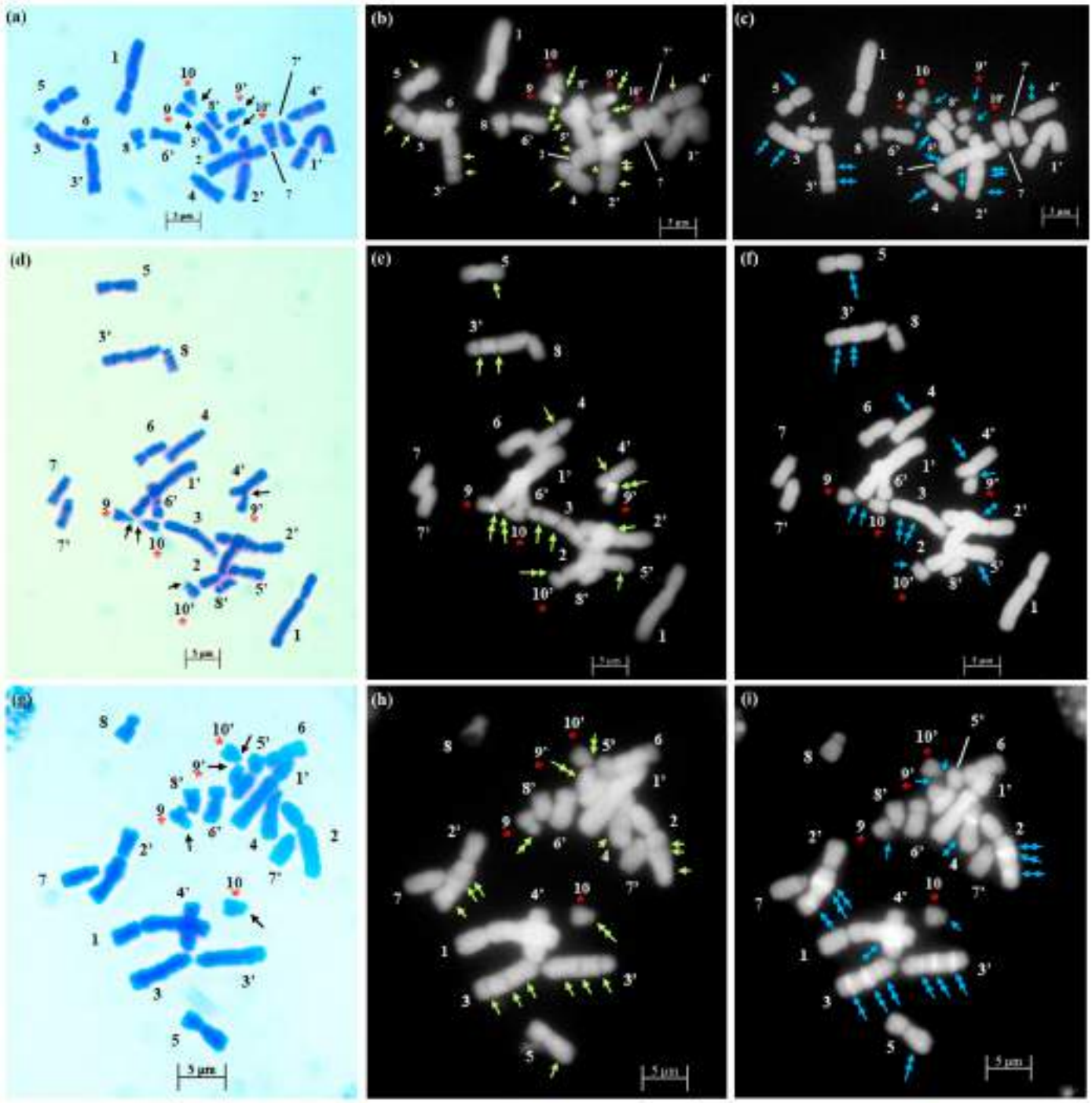


Figure 3. Somatic metaphase plates of *Dipcadi montanum* (Dalzell) Baker with 2n = 20 chromosomes stained with Giemsa (**a**,**d**,**g**), CMA (**b**,**e**,**h**) and DAPI (**c**,**f**,**i**)**.** Black arrows indicate position of secondary constrictions in Giemsa-stained plates (**a**,**d**,**g**). Double yellow arrows indicate CMA^{+ve} signals and single yellow arrows indicate CMA^{-ve} gaps (**b**,**e**,**h**). Double blue arrows indicate $DAPI^{+ve}$ signals and single blue arrows mark the $DAPI^{-ve}$ gaps (**c**,**f**,**i**). Red asterisks indicate chromosomes with secondary constrictions in all the plates. Bars 5 µm.

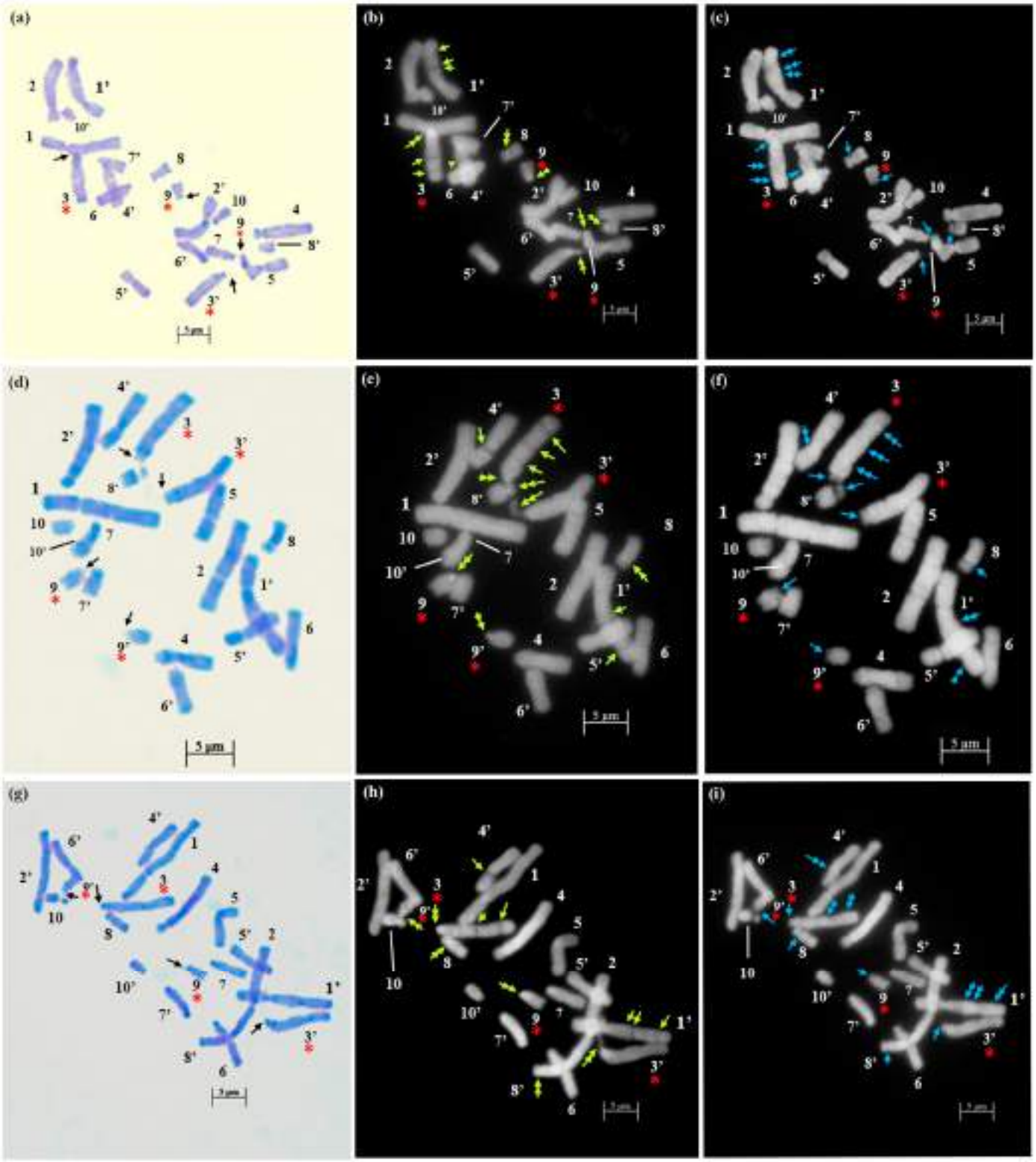


Figure 4. Somatic metaphase plates of *Dipcadi ursulae* Blatt. with 2n = 20 chromosomes stained with Giemsa (**a**,**d**,**g**), CMA (**b**,**e**,**h**) and DAPI (**c**,**f**,**i**). Black arrows indicate position of secondary constrictions in Giemsa-stained plates (**a**,**d**,**g**). Double and single yellow arrows indicate bands of CMA^{+ve} signals and CMA^{-ve} gaps (**b**,**e**,**h**). Double blue arrows indicate $DAPI^{+ve}$ signals and single blue arrows mark the $DAPI^{-ve}$ gaps (**c**,**f**,**i**). Red asterisks indicate chromosomes with secondary constrictions in all the plates. Bars 5 µm.

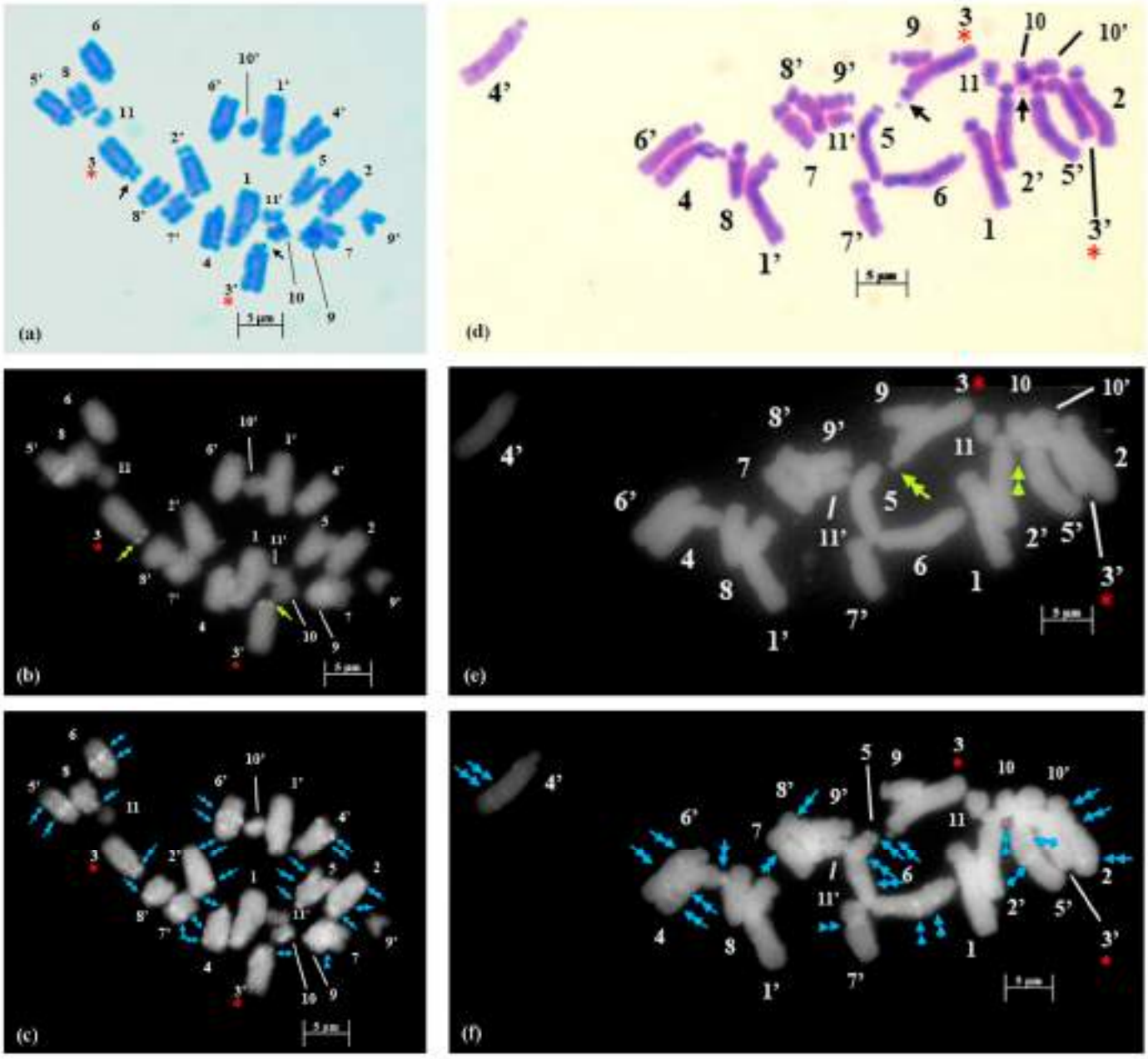


Figure 5. Somatic metaphase plates of *Dipcadi erythraeum* Webb & Berthel. with 2n = 22 chromosomes stained with Giemsa (**a**,**d**), CMA (**b**,**e**) and DAPI (**c**,**f**). Black arrows indicate position of secondary constrictions in Giemsa-stained plates (**a**,**d**). Double yellow arrows indicate bands of CMA^{+ve} signals in CMA-stained plates (**b**,**e**). Double blue arrows showing clear and bright $DAPI^{+ve}$ signals (**c**,**f**). Red asterisks in all the three stained plates indicate chromosomes with secondary constrictions and an associated unusual $CMA^{+ve}/DAPI^{0}$ signal. Bars 5 µm.

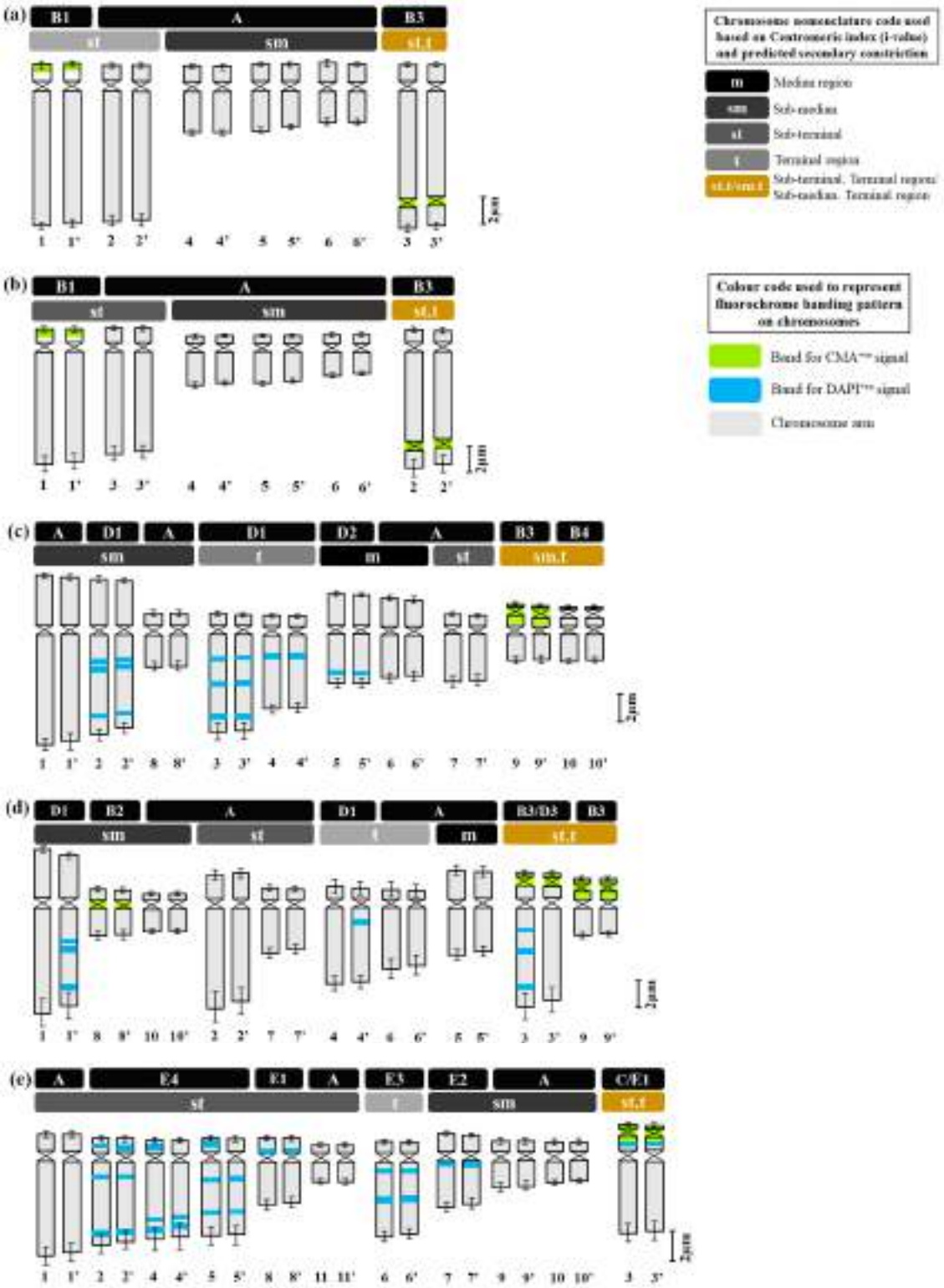
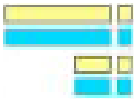
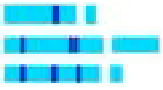
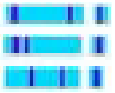


Figure 6. Comparative ideograms of the five species of *Dipcadi* ((**a**) *D. concanense*, (**b**) *D. goaense*, (**c**) *D. montanum*, (**d**) *D. ursulae*, (**e**) *D. erythraeum)* showing CMA^{+ve} and $DAPI^{+ve}$ banding patterns. (The upper two bars of each ideogram mention the types of CMA^{+ve} and $DAPI^{+ve}$ signals observed on chromosome arms and category of chromosomes based on centromeric index, respectively. The numbers in the lower panel represent the numerical sequence of the chromosomes in the karyotype based on their average absolute length. Bar scale: 2 µm). Colour code used for centromeric index based chromosomal nomenclature and CMA^{+ve} and $DAPI^{+ve}$ signals (or, bands) are described in the top-right corner of the figure.

Table 3. A brief typification of CMA and DAPI fluorescent bands observed in somatic chromosomes of five Indian species of *Dipcadi*.

Major Band/Signal Types Based on CMA/DAPI Staining	Sub-Types Based on Position of Bands/Signals on Chromosomes	Position of Bands/Signal(s) on Chromosome Arms	Diagrammatic Representation	Species Name
Type A $CMA^{0}/DAPI^{0}$	A	No distinct signal		In all species
Type B $CMA^{+ve}/DAPI^{-ve}$	B1	Short arm, distal to constriction		*D. concanense*, *D. goaense*
	B2	Centromeric region		*D. ursulae*
	B3	Nucleolar		*D. concanense*, *D. goaense*
		Nucleolar, extended to terminal satellite		*D. ursulae*
		Nucleolar, extended to short arm and terminal satellite		*D. montanum*, *D. ursulae*
	B4	Short arm and terminal satellite		*D. montanum*
Type C $CMA^{+ve}/DAPI^{0}$	C	Nucleolar, extended to short arm and terminal satellite		*D. erythraeum* *
Type D $DAPI^{+ve}/CMA^{-ve}$	D1	Long arm, interstitial, 1–3 in number		*D. montanum*, *D. ursulae*
	D2	Long arm, Distal		*D. montanum*
Type E $DAPI^{+ve}/CMA^{0}$	E1	Short arm, interstitial		*D. erythraeum* *
	E2	Long arm, proximal to constriction		*D. erythraeum*
	E3	Long arm, interstitial, 2 in number (2 bands)		*D. erythraeum*
	E4	Both arms, interstitial, 3 in number		*D. erythraeum*

[Light yellow and light blue line diagrams represent CMA and DAPI-stained chromosome arms, respectively. The fluorescent green bands indicate CMA^{+ve} signals on CMA-stained chromosomes. The dark blue bands indicate $DAPI^{+ve}$ signals on DAPI-stained chromosome arms, respectively. * Chromosome pair in *D. erythraeum* showing both CMA^{+ve} (C type) and $DAPI^{+ve}$ (E1 type) signals].

Table 4. CMA and DAPI fluorescent banding patterns somatic chromosomes of five Indian species of *Dipcadi*.

Sl. No.	Species & Chromosome Number	Order of Nucleolar Pair	CMA^{+ve} Bands			DAPI^{+ve} Bands			Total No. CMA^{+ve} & DAPI^{+ve} Bands/2n	Fluorescent Karyotype (2n) *
			No.	Chromosome Pair (p)/Single (s)	Type	No.	Chromosome Pair (p)/Single (s)	Type		
1.	*D. concanense* (2n = 12)	3rd	2 2	1st (p) 3rd (p)	B1 B3	Nil	--	--	4	8A + 4B
2.	*D. goaense* (2n = 12)	2nd	2 2	1st (p) 2nd (p)	B1 B3	Nil	--	--	4	8A + 4B
3.	*D. montanum* (2n = 20)	9th 10th	2 2	9th (p) 10th (p)	B3 B4	6 6 2 2	2nd (p) 3rd (p) 4th (p) 5th (p)	D1 D1 D1 D2	20	8A + 4B + 8D
4.	*D. ursulae* (2n = 20)	3rd 9th	2 2 2	3rd (p) 8th (p) 9th (p)	B3 B2 B3	3 3 1	1st (s) 3rd (s) 4th (s)	D1 D3 D1	13	10A + 4B + 2B/D + 4D
5.	*D. erythraeum* (2n = 22)	3rd	2	3rd (p)	C	6 2 4 + 1 4 + 1 4 2 2	2nd (p) 3rd (p) 4th (2p + 1s) 5th (2p + 1s) 6th (p) 7th (p) 8th (p)	E4 E1 E4 E4 E3 E2 E1	28	8A + 2C/E+12E

* Type A (CMA^{0}/DAPI^{0}), Type B (CMA^{+ve}/DAPI^{-ve}), Type C (CMA^{+ve}/DAPI^{0}), Type D (DAPI^{+ve}/CMA^{-ve}), and Type E (DAPI^{+ve}/CMA^{0}).

3. Discussion

Although traditional karyotype analysis can be considered obsolete in the genomic era, it is in fact quite contrary as basic karyotype information, chromosome number, genome size, and position of landmarks including repetitive DNA, will remain important for necessary data interpretation [42–48]. Chromosome features used in cytotaxonomy may present a continuous variation (average chromosome length, mean arm ratio or r index, symmetry indices) or a discontinuous variation (chromosome number, heterochromatic bands, number of rDNA sites) [46,49]. Measurements of chromosome arms for the identification of chromosomes in a karyotype are useful to quantify differences or similarities among karyotypes. The symbols used in the present study to describe karyotypes correspond to those coined by Levan et al. [39], as described by Mitrenina et al. [40]. Although most publications in plant cytogenetics followed the nomenclature for chromosome morphology as reviewed by Levan et al. [39], some variations in nomenclature [50] and development of R scripts for the determination of standardized karyotype have also been reported [45,48].

In this study, we have presented a karyotype analysis of five endemics, threatened Indian species of *Dipcadi*, which was reported as a disappearing genus in India [51]. *Dipcadi* with 41 species distributed in the Mediterranean region, Africa and Southeast Asia [13,20], is poorly defined taxonomically because of overlapping morphological characters [12]. Consistent with the general trend in Scilloideae, continuous variation is prevalent in most Indian taxa requiring detailed taxonomic characterization, including molecular and cytogenetic characterization. Molecular phylogenetic studies in the tribe Ornithogaloideae (Subfamily Scilloideae) recognized nineteen monophyletic genera, including *Dipcadi* [5].

Somatic chromosome counts available globally for about 14 species of *Dipcadi* [25] have revealed wide diversity in chromosome number (2n = 6, 8, 16, 20, 22, 24, 32, 40). Since *D. serotinum*, with a broad distribution (Europe and Northern Africa to the Arabian Peninsula and India) shows 2n = 2x = 8 chromosomes with n = 4 [33], a base chromosome number [49,52] of x = 4 has been suggested [30]. Thus, considering x = 4, taxa with 2n = 12, 20, 22 in the present study may be polyploid derivatives. However, the probable base chromosome number of x = 6 has also been proposed for the genus *Dipcadi* [26] according to which in the present study, species with 2n = 12 are diploids (*D. concanense* and *D. goaense*) while species with 2n = 20, 2n = 22 (*D. ursulae*, *D. montanum*, *D. erythraeum*) presumably resulted from descending dysploidy and subsequent polyploidisation, and are thus probable hypotetraploids.

In the present study, the somatic chromosome number of 2n = 12 was observed in *D. concanense*, and *D. goaense* reconfirms earlier chromosome number reports in the two species [19,28,29]. Chromosome numbers of *D. montanum* and *D. ursulae* were observed to be 2n = 20, while *D. erythraeum* showed 2n = 22. Although Mahabale and Cheenavariah [27] reported 2n=20 for *D. montanum*, Naik [32] reported two cytological races for *D. montanum* collected from Aurangabad showing 2n = 10 and 2n = 12 in somatic metaphases. In this study, we reconfirm 2n = 20 for *D. montanum*. Jakhi et al. [31] first reported 2n = 22 and n = 11 in *D. erythraeum* collected from Rajasthan, reconfirmed by Jehan et al. [12], although 2n = 20 has also been reported in the species [30]. We confirm 2n = 22 in *D. erythraeum* in two populations from Rajasthan.

Meiotic analysis in species reported revealed regular bivalent formation in pollen mother cells [14,30–33]. However, in some species, low pollen fertility [31], the occurrence of univalents [30] and abnormalities during meiosis have also been reported [33]. Hybridization followed by polyploidisation may have resulted in ascending dysploid series of chromosome numbers in the genus *Dipcadi*. Dysploid variation is caused by complex mechanisms [52] and further analyses in a large number of species are a prerequisite to suggest the trend of karyotype evolution in the genus *Dipcadi*.

Previous reports of karyotype analysis on Indian species of *Dipcadi* are few and have not revealed distinct morphology of the karyotype with respective to a position of centromere and secondary constriction. In *D. erythraeum*, Jakhi et al. [31] described

chromosome morphology revealing one pair of long and two pairs of short chromosomes with sub-terminal constrictions, while the remaining chromosomes were sub-median. Rawat et al. [30] determined the karyotype formula revealing the majority of telocentric chromosomes followed by sub-metacentrics. They also stated that the analysis of chromosomes with secondary constrictions could not be obtained due to technical difficulties. The karyotype of *D. goaense* was reported to be similar to *D. concanense* [14,27].

This is the first report of EMA-Giemsa-based karyotype analysis establishing the modal karyotypes of five endemic or threatened Indian species of *Dipcadi* showing the presence of chromosomes with secondary constrictions in each species, varying in type and number. The karyotypes are characterized by the predominance of acrocentric/telocentric chromosomes with distinctly bimodal or graded chromosome complement.

In most plant species, the centromere or primary constriction is present in all the chromosomes, while on some chromosomes, a secondary constriction at the nucleolar organizer region (NOR) has been identified from the earliest microscopy [53,54]. At metaphase, NORs are often visible as secondary constrictions as the arrays of genes active at the previous metaphase remain decondensed [54]. Chromosomes with secondary constrictions are considered landmark chromosomes in karyotype analysis. In *D. concanense*, *D. goaense* and *D. erythraeum* one pair of chromosomes with secondary constrictions were identified while in *D. montanum* and *D. ursulae*, two pairs of chromosomes with secondary constrictions were identified.

The EMA-based Giemsa-stained karyotypes of two populations of *D. concanense and D. goaense* (from type locality), were similar in number and morphology including the type of nucleolar chromosomes. On the other hand, *D. montanum*, *D. ursulae* and *D. erythraeum* differ distinctly in their karyotypes including the number and type of nucleolar chromosomes. Fluorescent banding, particularly with CMA and DAPI, has been frequently used in a wide range of plant species to characterize individual chromosomes and delineate heterochromatic regions comprised of repetitive DNA sequences at different locations in a chromosome [36,55]. Chromosomal CMA^{+ve} bands imply the prevalence of heterochromatic GC elements mainly surrounding the NORs [55,56] whereas the $DAPI^{+ve}$ bands reflect a type of condensed heterochromatin occupied by AT elements since DAPI is specific for AT-rich DNA stretches [36,57]. The same fluorochromes may also negatively stain AT-poor ($DAPI^{-ve}$) or GC-poor (CMA^{-ve}) heterochromatin blocks [55].

It is apparent from the fluorochrome band profiles in the present study that all five species of *Dipcadi* exhibited the CMA^{+ve} band in one of the constriction regions or terminal satellites of the nucleolar chromosome. These CMA^{+ve} bands were $DAPI^{-ve}$, showing a clear gap corresponding to the CMA^{+ve} bands in all species except in *D. erythraeum*. It is noteworthy that by using fluorochrome banding with base-specific fluorochromes [42], GC-rich heterochromatin has been identified in all species of *Dipcadi*, mostly in the nucleolar chromosomes. The majority of heterochromatic bands [42] have been reported to be AT-rich and are usually at interstitial regions in species with medium and large chromosomes. 35S rRNA genes often have been found to coincide with GC-rich bands [55]. The distal $CMA^{+ve}/DAPI^{-ve}$ bands in the short arm of long chromosomes in *D. concanence* and *D. goaense* has not been observed in any other species in the present study. The CMA^{+ve} signals are generally considered to represent GC-rich heterochromatin found mostly at NORs and also at proximal positions, coinciding with DAPI-negativity in the majority of plants reported [55]. It is now known that secondary constrictions represent only the expression of rRNA genes that were active during the last interphase [58]. Other functional sites may not form secondary constrictions if located at the terminal end of chromosomes [59].

AT-specific $DAPI^{+ve}$ banding profile revealed unique species-specific characteristic features for the first time in *Dipcadi*. $DAPI^{+ve}$ bands were distributed in the different interstitial regions of the long arm and short arm of the chromosomes in all species except in *D. concanence* and *D. goaense*. No $DAPI^{+ve}/CMA^{-ve}$ or $DAPI^{+ve}/CMA^{0}$ signals/bands were obtained in *D. concanence* and *D. goaense. Dipcadi montanum* and *D. ursulae* showed dis-

tinctive DAPI^{+ve}/CMA^{-ve} bands in the chromosomes of the diploid complement (2n = 20). However, the two species differ in the number and occurrence of DAPI^{+ve}/CMA^{-ve} bands in the homologous chromosomes. In *D. montanum*, sixteen DAPI^{+ve}/CMA^{-ve} bands occur in four chromosome pairs. While in *D. ursulae*, seven DAPI^{+ve}/CMA^{-ve} bands were observed in three chromosomes, but not in the corresponding homologous pair. Thus, in *D. ursulae*, three out of ten pair of chromosomes show heteromorphism in homologous chromosomes with respect to DAPI^{+ve}/CMA^{-ve} banding patterns.

Dipcadi erythraeum with 2n = 22, shows distinct variation in banding type and pattern from all other species of *Dipcadi* studied. CMA^{+ve}/DAPI^{0} signals in one of the constriction regions (in the chromosome pair with secondary constriction or nucleolar chromosome) extended to the short arm and terminal satellite. No CMA^{+ve}/DAPI^{-ve} bands were observed in any of the chromosomes. DAPI^{+ve}/CMA^{0} signals were observed in seven chromosome pairs including one pair of nucleolar chromosomes. A total of 26 DAPI^{+ve}/CMA^{0} bands were found on the seven chromosomes. Thus, in *D. erythraeum* two out of seven pair of chromosomes show heteromorphism in homologous chromosomes. Rawat et al. [30] suggested an amphidiploid origin for *D. erythraeum*. Jehan et al. [12] based on studies using molecular markers found *D. erythraeum* from Rajasthan to be distinctly different from the other species of Western Ghats. The heteromorphism in the banding pattern (DAPI^{+ve}) supports both studies. Thus, in *D. ursulae* and *D. erythraeum*, CMA^{+ve}/DAPI^{+ve} signals in different regions of the same chromosome indicate the heterochromatin variation acquired by the species. The distribution of CMA and DAPI bands is a reflection of heterochromatin composition and variations acquired by different species [60–62]. AT-specific DAPI^{+ve} banding pattern obtained in the present study revealed unique species-specific characteristic features for the first time.

In the subfamily Scilloideae, fluorochrome banding and fluorescence in situ hybridization (FISH) has been reported in some species under the tribe Hyacintheae. In *Bellevalia*, CMA^{+ve} signals were associated with nucleolar chromosomes and rDNA probes colocalized with CMA^{+ve} signals [63], although some variations have been reported in *B. romana* [64]. In *Muscari*, with bimodal karyotype, CMA^{+ve} signals were located at NOR and rDNA probes colocalized with CMA^{+ve} signals [65,66]. Interspecific variation in the distribution of DAPI^{+ve} signals [65,67] and CMA^{+ve} signals [67] have been reported in *Muscari*. Varied distribution of DAPI^{+ve} signals has also been reported in species of *Lachenalia* [68,69]. In *Drimia* (tribe Urgineeae), CMA^{+ve}/DAPI^{-ve} signals were associated with nucleolar chromosomes, with some interspecific variations in additional signals [37]. In *Albuca bracteata*, (tribe Ornithogaleae), CMA^{+ve} signals were detected at NOR regions, colocalized with rDNA signals while DAPI^{+ve} signals were also reported at the centromeric or intercalary regions in the species [70].

Deshpande et al. [20] investigated the phylogenetic relationship between the two endemic and critically endangered Indian species of *Dipcadi*, *D. concanense* and *D. goaense*, using a plastid (*mat*K) and ITS sequences. This study [20] revealed that *D. concanense* and *D. goaense*, were not only morphologically similar [19], with the same chromosome number [14], but they were also phylogenetically closely related species. In the present study, the karyotype analysis based on chromosome morphometric data as well as the fluorochrome banding pattern of *D. concanense* and *D. goaense*, were found to be very similar, and in agreement with findings based on molecular phylogenetic data [20]. Jehan et al. [12] studied in detail genetic diversity among the three genera, *Drimia*, *Dipcadi* and *Ledebouria* of subfamily Scilloideaea in India, using RAPD and SRAP markers. The study resolved the three genera into monophyletic groups corresponding to three subfamilies (now subtribes); *Urginoideae*, *Hyacinthoideae* and *Ornithogaloideae*. Among the Indian species of *Dipcadi* (excluding *D. goaense*), studied by Jehan et al. [12], *D. concanense* was found to be very distinct from other species of Western Ghats and *D. erythraeum* was also found to be a genetically distinct species in this study. The species from the Western Ghats formed a well distinct group, "whereas, northern Indian species, *D. erythraeum* from Rajasthan and *Dipcadi serotinum* from Delhi stood out as well differentiated taxa". Jehan et al. [12]

suggested that *Dipcadi serotinum* may have been introduced from Europe, as the flowering time differs from the Indian species. Thus, the unique fluorochrome banding patterns of *D. montanum* and *D. ursulae*, reveal that though the two species share the same chromosome number, the species are distinctly different as observed by Jehan et al. [12].

The application of fluorescent banding for comparative analysis of karyotypes has enriched present-day cytogenetics enormously as an integrative approach to solving the issues of systematics and phylogeny [71–73]. The present EMA-based Giemsa and fluorescent banding karyotype in five Indian *Dipcadi* species have confirmed distinct patterns and diversity of landmark nucleolar chromosomes. The result has revealed a diverse number of species-specific AT-rich, DAPI-positive repetitive sequences (0–26 in number) for the first time in the genus *Dipcadi*. These may be considered useful molecular markers for analyzing genetic diversity and studying genome evolution in other species. To resolve the interspecific phylogenetic and evolutionary relationships more molecular cytogenetic-based chromosome analysis using fluorescent banding and fluorescence in situ hybridization (FISH) deserves attention.

4. Materials and Methods

4.1. Plant Materials and Their Collection

Collection of different populations of *Dipcadi* species was possible as part of this study under the guidance of Professor SR Yadav and Dr. MM Lekhak, Shivaji University, Kolhapur, Maharashtra and with the help of Professor NS Shekhawat, Jodhpur University, Rajasthan. We could not collect/obtain any other Indian species for this study. Herbarium vouchers were prepared for each species, identified and deposited to the Shivaji University Herbarium, Kolhapur as well as Calcutta University Herbarium. Brief information on the place of collection of these five species is given in Table 1. Bulbs of each species were grown in pots and maintained in the experimental garden of the Department of Botany, University of Calcutta.

4.2. Mitotic Chromosome Preparation and Giemsa Staining

Ten to fifteen actively growing root tips from bulbs of each species were harvested during the months of June to August and pre-treated with 0.5% colchicine for 4 to 4.5 h at 14–16 °C [74]. The pre-treated root tips were fixed in a 3:1 methanol-acetic acid solution overnight and stored at −20 °C. The chromosome preparations were performed through standardization of the basic EMA technique following our earlier protocol [37,74] with modifications required. Fixed root tips were placed in water and kept at 4 °C for 3 h. One to two mm root tips were excised and carefully placed inside a microtube containing a cocktail enzyme mixture containing 0.15% Pectolyase (Y-23) plus 0.75% Macerozyme (R-10) and 1% Cellulose (Onozuka RS) along with 1mM EDTA. Root tips were incubated at 37 °C for 80–90 min. Enzyme-digested root tips were washed with distilled water and macerated in freshly prepared acetic methanol (1:3) solution on glass slides. Air-dried slides were stained in 2% Giemsa solution (Giemsa azure eosin methylene blue solution, Merck, Darmstadt, Germany) in 1/15th phosphate buffer (2.390 g Na_2HPO_4 and 0.900 g KH_2PO_4 in 100 mL distilled water) for 20–25 min at room temperature. A staining period of 25 min with Giemsa was found optimum for *Dipcadi* species. Giemsa-stained slides were screened under Axio Lab. A1 Carl Zeiss microscope to assess the quality of cytological preparations. Data sheets for individual species were prepared for selected well-scattered metaphase plates. Photomicrographs were taken under Axio Lab. A1 microscope fitted with a CCD camera and computer.

4.3. Karyotype Analysis

Somatic chromosome numbers and karyotypes were determined from 20–25 well-scattered Giemsa-stained metaphase plates of each population of species. The software Axiovision L.E 4 (Carl Zeiss, Jena, Germany), was used for chromosome morphometric data. A minimum of 10 well-scattered metaphase plates were selected for each popula-

tion/species and analysed using this software for the estimation of short arm length (s), long arm length (l), arm ratios (r = l/s), chromosome length (CL), and total chromosome length (TCL). The centromeric index (i-value) was determined following Levan et al. [39] and Mitrenina et al. [40]. Absolute and relative chromosome lengths were calculated [75]. Karyotype formulae and the respective ideogram for each of the individual species were generated using the chromosome morphometric data [76].

4.4. Fluorochrome Staining of Somatic Chromosomes

Giemsa-stained slides were de-stained with 70% methanol for 45 min, air dried and further used twice for two separate fluorochrome staining with 4′-6-Diamidino-2-phenylindole (DAPI) and Chromomycin A_3 (CMA) following the protocol described earlier [74,76], with minor modifications. Slides were incubated in McIlvaine buffer I (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 7.0) for 30 min and stained with 0.1 µg/mL DAPI solution for 25–30 min. Slides were washed in the same buffer and counterstained with Actinomycin D (0.25 mg/mL) for 15 min. Slides were air-dried and mounted in non-fluorescent glycerol. The slides were kept overnight at 4 °C for maturation and were examined under the microscope with a UV filter cassette and images were captured with a CCD camera. For CMA staining, the slides were de-stained in 70% Methanol and air-dried. Slides were incubated in McIlvaine buffer I (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 7.0) for 30 min and then in McIlvaine buffer II (with 5 mM $MgCl_2 \cdot 6H_2O$) for 15 min. The slides were then flooded with 0.1 mg/mL CMA for 55–70 min. Excess stain was washed off in McIlvaine buffer II, air dried and mounted in non-fluorescent glycerol and kept at 4 °C refrigerator for 48 hrs. The observations were made by fluorescence microscopy with a BV filter cassette and the images were captured with a CCD camera.

4.5. Statistical Analysis

Data from at least ten different scattered metaphase plates were taken for determination of all the karyo-morphometric data, and data for each karyo-morphometric parameter was expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyse the data in order to find statistically significant variations in the mean values among the five species of *Dipcadi*. Using SPSS statistic software (IBM®, Armand, NY, USA) version 17.0, a descriptive post hoc mean separation analysis for the karyo-morphometric data set was carried out using Duncan's multiple range test (DMRT) at the 5% probability level.

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Abstract	Lentil is an annual protein rich valuable edible crop with only one cultivated and six wild taxa. Keeping in mind its narrow gene pool, the genus deserves critical assessment of genomic diversity at the chromosomal level. Genetic diversity represents the heritable variation within and between populations of organisms. Over the decades classical and molecular cytogenetics have played an immense role in the field of crop improvement. Lentil, though grown in different countries, country-wise chromosomal information is inadequate. Critical evaluation of more than seven decades chromosomal information has revealed unique karyotype diversity within the landraces of different countries. Application of fluorescent banding and fluorescent in situ hybridization (FISH) has helped to segregate cultivars based on cultivar specific chromosomal markers and landmarks. Selection of cultivated and wild cultivars based on qualitative and diseases related morpho-traits and new information from this critical review especially on molecular cytogenetics may provide more options for crop improvement. More research in the field of molecular cytogenetics from country specific species and cultivars are needed to enrich the repository of gene pool. Alien gene introgression from extended gene pool through the advanced genomics and biotechnological tools could facilitate the path of sustainable improvement of this crop.	
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REVIEW

Critical review on karyotype diversity in lentil based on classical and molecular cytogenetics

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Abstract

Lentil is an annual protein rich valuable edible crop with only one cultivated and six wild taxa. Keeping in mind its narrow gene pool, the genus deserves critical assessment of genomic diversity at the chromosomal level. Genetic diversity represents the heritable variation within and between populations of organisms. Over the decades classical and molecular cytogenetics have played an immense role in the field of crop improvement. Lentil, though grown in different countries, country-wise chromosomal information is inadequate. Critical evaluation of more than seven decades chromosomal information has revealed unique karyotype diversity within the landraces of different countries. Application of fluorescent banding and fluorescent in situ hybridization (FISH) has helped to segregate cultivars based on cultivar specific chromosomal markers and landmarks. Selection of cultivated and wild cultivars based on qualitative and diseases related morpho-traits and new information from this critical review especially on molecular cytogenetics may provide more options for crop improvement. More research in the field of molecular cytogenetics from country specific species and cultivars are needed to enrich the repository of gene pool. Alien gene introgression from extended gene pool through the advanced genomics and biotechnological tools could facilitate the path of sustainable improvement of this crop.

Keywords Lentil · Karyotype diversity · Classical and molecular cytogenetics

Introduction

The genus Lentil is an annual domesticated nitrogen fixing and self-pollinated seasonal pulse crop in the family Fabaceae (Leguminosae). Each domesticated crop comprises a number of cultivated and wild relatives. More than nine decades ago a classification of *Lens esculenta (*now culinaris*)* with seven varieties was first proposed by Alefeld was the consensus [1]. However, the said classification failed to convince Barulina as from different parts of the globe she collected large number of samples and noted enormous diversity within the cultivated species and even mentioned that 66 types of *L.culinaris* were available in India [2]. Barulina [1] divided all forms of lentil mopo-geographically into two vast groups on seed morphology; macrosperma and microsperma and accommodated five species within the genus namely *L.lenticula* Alef., *L. kotschyana* Alef. *L. esculenta* (now culinaris), *L.orientalis* and *L.nigricans. Lens culinaris* remained as the only cultivated species and rest four were wild, but *L.orientalis* remains as close relative to *L.culinaris*. Debate over taxonomic relationships is still going on and the above classification was completely revised with five species genera; *Lens culinaris, Lens orientalis, Lens ervoides, Lens nigricans, and Lens montbretii* [3]. The very next year *L. montbretii* was removed from the list [4]. In subsequent years Ladizinsky [5] added a new species *L.odemensis*. However, considering the morphological, cytological and crossability factors among the species, Ladizinsky [6] suggested subspecies status of cultivated *culinaris* and its wild progenitor *orientalis* under the species *L.culinaris*. Another new species *L. lamottei* was recognized long back by Czefranova [7] but its acceptance by others took long time. In 1997 *L.tomentosus* was added with the list [8]. Revising all the earlier classification a new classification of lentil with one cultivated and six wild taxa was proposed by the same group [9]. This classification was contradicted and an alternative classification was proposed based on morphological, cytological, biochemical and molecular

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parameters [10]. The classification recognized four groups within the genus namely,

(a) *L. culinaris*, ssp. *culinaris*, ssp. *orientalis*, ssp. *tomentosus*, and ssp. *odemensis*
(b) *L. ervoides*
(c) *L. nigricans*
(d) *L. lamottei*

It was again altered by Hancock [11] based on morphological, molecular and intercrossable factors to,

(i) *L. culinaris,* ssp. *culinaris,* ssp. *orientalis and* ssp. *odemensis.*
(ii) *L. nigricans, L. ervoides* and *L. lamottei.*
(iii) *L. tomentosus*

It appears that with time and diversity parameters debating lentil groupings may continue but this cannot alter the urgency of crop improvement and importance of *Lens culinaris* as "poor man's meat". Lentil is becoming an important pulse crop in international and domestic market with gradually increasing demand. To reduce the gap between demand and supply improvement of cultivated species deserves adequate attention of the lentil breeders and genome researchers. Despite the fact that lentil is cultivated in many countries, as a major producer of lentil the pressure is mounting primarily on Canada, India, Turkey, Australia, USA, Nepal, China, and Ethiopia [12]. Global Lentil production in 2021 has touched 5.73 million metric tons [13].

Lentil scientists have noted that *Lens culinaris* has large number of country specific cultivars and considerable genetic diversity within cultivated cultivars for agro-morphological and phenological characteristics [14–18]. The narrow gene pool of lentil due to self-pollinating nature and low out-crossing abilities are the primary hurdles in their improvement against climatic changes, biotic and abiotic stresses. The expansion of the genetic base and application of new breeding technologies are prerequisite for this valuable pulse crop. Application of new breeding technologies in any crop demands foundational and advanced knowledge on genetics. Similar to other major crops, wild relatives of lentil played an important role in the process of evolution and domestication. Wild relatives are actually the untapped genetic repository but remain mostly uneatable. They have faced extreme climatic conditions along with biotic and abiotic stresses in the past and accordingly modified their genomic configuration to grow in different soil types and to confront disease causing organisms [19]. In all crop plants nature itself and breeders have played an important role in evolving the cultivated species from their wild relatives for human consumption. And gradually a reproductive barrier developed within the groups. Our attention was shifted from wild relatives for many decades. Nowadays, plant breeding through gene transfer and gene editing is quite possible with the advanced scientific knowledge and technology in the field of genetics and biotechnology to fight against the problems like explosion of human population, reduction of cultivable land, per capita potable water, climatic changes and hunger. In this endeavor related wild species could be significant. Cumulative information from all sources may help us to achieve our goal "hunger free world". Considering the food value and inadequate gene pool of cultivated lentil, researchers from various countries have been deeply involved in analyzing cultivated and wild lentils on morphological, chromosomal and molecular parameters. Adequate studies have been made in the field of morphological and molecular parameters. [12, 20–27]. But, foundational genetic studies through chromosome analysis are far from complete in the genus lentil. Thus, the compilation and critical assessment of available chromosomal diversity and further harnessing the diversity present in wild relatives now available in different countries over many decades deserves adequate attention. Chromosomes and genes represent different levels of genetic information. Chromosomes are the first visible genomic structures remain free from environmental influences and through which ploidy level of any species/cultivars can be confirmed. Skilled processing, short time period and cost-effectivity can help direct visualization of numerical and unique morphology of chromosomes pointing to the positions and type of primary and landmark secondary constrictions within and between the species members. It also helps to check the adaptation variability within the chromosome complements in cultivated and wild species conserved in different countries. For many decades cytogenetics has enormously enriched taxonomy, phylogeny, evolution, genetics, breeding and biotechnology. Applications of different genome based molecular techniques including next generation sequencing (NGS) have benefitted crop breeding programmes. The methods are costly and reacquiring chromosomal information from the NGS data are difficult. While combination of chromosomal information with genomics will help correct data interpretation and framing a new template for future crop improvement.

Quality plant chromosome preparation is the utmost objective of plant cytogenetics. Since the early part of the twentieth century plant cytogeneticists have been deeply involved in developing the methods and techniques for good chromosome preparation. They were aware of the fact that plant cell wall and cytoplasmic contents are challenges to good chromosome preparations. They have managed and still managing the basic limitations of classical cytogenetics skillfully. Overall contribution of plant chromosome studies is enormous. Stagnancy in classical cytogenetics between 1950 and 1970 was apparent [28] and many opined that 'cytogenetics is a dead science' and that it was no longer

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needed because of advances in other disciplines of biology. However, with the advanced knowledge of chromosome structure, combination of cytogenetic techniques with molecular techniques and how DNA is arranged in chromosomes, classical cytogenetics overcome its stagnancy and entered in a new era of molecular cytogenetics. The basics of molecular cytogenetics rely on the process of good chromosome preparation. Standardization of enzymatic maceration and air drying (EMA) method in plants has opened a new door for plant chromosome research. The method has helped the removal of cell wall and cytoplasmic contents, the basic constrains of classical cytogenetics. EMA method has rejuvenated plant cytogenetics. Excellent clarity can be achieved in chromosome morphology with this method and uses of the same slide for different experiments make the process more acceptable for plant chromosome research. Giemsa, which was used previously for animal chromosomes is now used for plant chromosomes. Like animal chromosome banding, use of Giemsa has helped developing plant chromosome banding. [29–32]. Schweizer [33] started application of DNA base specific fluorochrome dyes in plant system. Banding with nonfluorescent and fluorescent dyes was efficient to reveal the locations of constitutive heterochromatin (highly repetitive DNA sequences) directly on the chromosomes. Distribution of heterochromatins in chromosome and species-specific locations helped in identification of individual chromosome within a karyotype [34]). Banding studies have significantly enhanced the role of chromosome studies in new plant breeding programmes. Further advancement in molecular cytogenetics is the application of fluorescent in situ hybridization (FISH) in plants. FISH has switched on a new analytic door for rDNA and repetitive DNA sequence sites directly on the chromosomes [35, 36].

Keeping in mind the above noted considerations and present role of this protein rich crop in human welfare, compilation and assessment of genetic diversity of the small genus lentil at the chromosomal level was undertaken. The review has compiled and attempted to highlight critically the chromosomal information achieved in lentils over many decades through conventional and molecular cytogenetics and has suggested future trends of research. In the genomic era, the information may be useful for lentil breeders and genome researchers in formulating new strategies for crop improvement.

Lentil cytogenetics: past and present

Classical cytogenetics: (i) somatic chromosome analysis

Chromosomal analysis through conventional methods in the genus lentil was initiated in India [37]. Since then, a large number of researchers from few counties have reported chromosome analysis in both cultivated and wild relatives of lentil. Detailed country wise report is presented in Table 1. AQ4 It appears from the table that all the species under this small genus contain 2n = 14 chromosomes with almost similar morphology. However, the compilation of all the reports revealed that authors from different countries have failed to produce uniform karyotypic features within the cultivated and wild species members. Disagreement persists on the basic foundational chromosomal features like total chromatin length, chromosome types as well as number and actual position of secondary constrictions (Table 1).

Globally, Canada is the highest lentil producer and exporter country, but assessment of genetic diversity within the Canadian cultivars through chromosomal analysis has not received adequate attention. Chromosomal analysis conducted by Sindhu et al. (Table 1), on *L. culinaris*, *L. orientalis, L. nigricans, L. ervoides* showed significant variation in TCL, and similar karyotype formula with three submetacentric (one of them with a secondary constriction), one metacentric and three acrocentric chromosome pairs in all the species. The longest and shortest chromosomes were reported in *L. orientalis* and *L. ervoides* respectively. In another experiment the same authors have reported similar characteristics with or without secondary constriction in eleven cultivars of *L. orientalis* (Table 1) [38, 39].

India is the second highest producer and biggest consumer of lentil and accounts for nearly 50% of the world's lentil consumption. Despite the fact that India has a vast genetic diversity of lentils in the form of cultivated and wild species and the chromosome analysis was initiated in India, the country has not focused on developing a comprehensive chromosomal database on lentil. The chromosomal studies reported so far have mostly been confined to some certified / uncertified cultivars (Table 1). The results have reported various karyotypic diversities like number and position of secondary constrictions, total chromatin length (TCL) and karyotype formula [37, 40–46]. Twelve certified Indian and three Russian cultivars of *L.culinaris* were subjected to chromosome analysis [41]. Karyotypic formula for nine Indian cultivars was mentioned as 4 M + 1Sm sat + 2Sm with one pair intercalary secondary constriction. However, for rest of the cultivars karyotypic formula was reverse type 3 M + 4Sm/4 M + 3Sm and without any secondary constrictions [41] (Table 1).

Israel was never listed as a primary lentil producing country but has made significant contribution in the field of chromosomal research. A group of scientists led by Ladizinsky [4–6, 8, 20] initiated a project for collection of lentils from inside and outside of their country. They started documentation and identification using morphological, cytological and molecular parameters for species delimitation as well as to assert the potential wild gene pool of the cultivated

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Table 1 Karyotype diversity in cultivated and wild Lentils based on conventional and EMA based chromosome analysis

Sl. No	Name of the species	Cultivar	TCL (μm) (mean ± S.D)	Karyotype formula	No. of s. cons., Position of Sec. cons. & Chromosome bearing pair	Method of chromosome analysis	Country	References
1	*L. esculenta* Moenck	Microsperma	NM	NM	4, NM, NM	Orcein squash	India	Bhattacharjee [37]
2	*L. esculenta* Moenck	NM	NM	NM	2, NM, NM	Orcein squash	India	Sharma and Muhkopadhyay [40]
3	*L. culinaris* (Macro.)	NP-1	45	4 M + 1Sm.sat + 2Sm	2, Intercalary, NM	Carmine squash	India	Sinha and Acharia [41]
		NP-2	42					
		NP-3	39					
		NP-4	28.2					
		NP-7	35					
		NP-8	33					
		NP-10	32					
		NP-16	40					
		NP-45	72.5					
		EC-36676	49	4 M + 3Sm	NM, NM, NM			
		EC-27161	NM					
		EC-27656	NM					
4	*L. culinaris* (Macro.)	K 1895	55	3 M + 4Sm	NM, NM, NM	Carmine squash	Russia	Sinha and Acharia [41]
		K 1995	52.5					
		K 1289	57.5					
5	*L.esculenta* Moenck	NM	NM	NM	2, NM, NM	Orcein squash	India	Naithani and Sarbhoy [42]
6	*L. culinaris*	Pant L 369	NM	NM	2, NM, NM	Orcein squash		Gupta and Singh [43]
7	*L. culinaris* *L. orientalis* *L. nigricans* *L.ervoides*	NM	NM	3Sm (sat included) + 1 m + 3St	2, NM, NM	Orcein squash	Canada and other countries	Sindhu et al. [38, 39]
8	*L. culinaris*	3847	NM	7Sm	2, NM, NM	Orcein squash	India	Lavania and Lavania [44]
9	*L. culinaris*	Barimasur-2	107.05	8 m + 6Sm	2, NM, NM	Orcein squash	Bangladesh	Khandaker et al. [48]
		Barimasur-3	97.83	1 m + 13Sm				
		Barimasur-4	135.39	8 m + 7Sm				

Table 1 (continued)

Sl. No	Name of the species	Cultivar	TCL (µm) (mean ± S.D)	Karyotype formula	No. of s. cons., Position of Sec. cons. & Chromosome bearing pair	Method of chromosome analysis	Country	References
10	*L. culinaris*	Flip-92 15L I116002 I116468 I117523 I116206 Khalkhal Koraeim Ardebil Moghan Ahar	NM	4 M + 3Sm	2, Intercalary, 4th	Aceto-Iron-Hematoxilin	Iran	Namazi et al. [47]
11	*L. culinaris*	NM	NM	3 M + 1 M.sat + 3Acro	2, NM, NM	NM	Israel, Ethiopia, Chile	Ladizinsky [20]
12	*L. tomentosus* Ladizinsky	NM	NM	3 M + 3Acro + 1St.sat	2, Intercalary, 7th	NM	South-east Turkey	Ladizinsky [8]
13	*L. ervoides*	NM	42.9	3 Sm + 1Sm + 3Acro 3 M + 1 M.sat + 3Acro	2, Intercalary, 5th	NM	Ethiopia	Sindhu et al. [38, 39] Ladizinsky et al. (1992)
14	*L. nigricans*	NM	NM	3 M + 1 M.sat + 3Acro	2, NM, NM	NM	Turkey	Ladizinsky [20]
15	*L. orientalis*	NM	NM	3 M + 1 M.sat + 3Acro	2, NM, NM	NM	Israel	Ladizinsky [20]
16	*L. odemensis*	ILWL- 35	103.71 ± 0.38	3 m + 1 m. sat + 2Sm + 1St	2, Intercalary, 4th	Enzymatic maceration & Air drying (EMA) followed by Giemsa	India	Jha et al. [52, 64]
17	*L. orientalis*	ILWL- 365	108.27 ± 0.66					
18	*L. orientalis*	ILWL-248	84.0 ± 0.06a					
19	*L. nigricans*	ILWL- 19	92.64 ± 0.81	1 M + 4 m + 1 + m. sat + 1St	2, Terminal, 6th			
20	*L. lamottei*	ILWL- 14	85.05 ± 0.84	5 m + 1St.sat + 1St	2, Terminal, 5th			
21	*L. ervoides*	ILWL -61	NM	5 m + 1St.sat + 1St	2, Terminal, 6th			

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Table 1 (continued)

Sl. No	Name of the species	Cultivar	TCL (μm) (mean ± S.D)	Karyotype formula	No. of s. cons., Position of Sec. cons. & Chromosome bearing pair	Method of chromosome analysis	Country	References
22	*L. culinaris*	IPL 81	89.6 ± 0.06	3 m + 1 m. sat + 2Sm + 1St	2, Intercalary, 4th	Enzymatic maceration & Air drying (EMA) followed by Giemsa	India	Jha et al. [56, 57]
		IPL 316	96.0 ± 0.03					
		JL-1	109.6 ± 0.01					
		PL 1 406	104.4 ± 0.01					
		KLS 210	121.2 ± 0.12					
		EC 70,403	107.6 ± 0.04					
		EC 78,455	104 ± 0.01					
		EC 267,877	89.0 ± 0.05					
		Micro (Bankura)	106.4 ± 0.06					
		Macro (Barasat)	53.6 ± 0.06					
		DPL-15	90.00 ± 3.05					
		DPL-62	83.49 ± 5.93					
		IPL-526	125.48 ± 5.29					
		EC-70306	100.33 ± 1.57					
		EC-70404	101.32 ± 6.25					
		EC-78410	74.77 ± 1.34					
		EC-78451A	74.53 ± 0.47					
		EC-78452	114.34 ± 5.71					
		EC-78476	106.29 ± 4.09					
		EC-223188	96.19 ± 0.49					
		EC-255491	111.96 ± 3.08					

Table 1 (continued)

Sl. No	Name of the species	Cultivar	TCL (μm) (mean ± S.D)	Karyotype formula	No. of s. cons., Position of Sec. cons. & Chromosome bearing pair	Method of chromosome analysis	Country	References
22	*L. culinaris*	Macro (U.P)	100.53 ± 3.10	3 m + 1 m. sat + 2Sm + 1St	2, Intercalary, 4th	Enzymatic maceration & Air drying (EMA) followed by Giemsa	India	Jha et al. [56, 57]
		EC-78461	69.18 ± 1.22					
		EC-78473	107.39 ± 4.45					
		EC-267590	117.9 ± 1.90					
		EC-78542A	84.81 ± 4.09	3 m + 1 m. sat + 2Sm + 1St	2, Intercalary, 2nd			
		EC-67569A	113.40 ± 0.65	3 m + 1 m. sat + 2Sm + 1St	2, Intercalary, 3rd			
		EC-78475	137.24 ± 0.17					
		EC 78,498	70.8 ± 0.04					
		Micro (Barasat)	100.8 ± 0.01					
		HUL-57	85.92 ± 0.14					
		IPL-406	90.67 ± 0.44					
		EC-70394	76.06 ± 5.49	3 m + 1 m. sat + 2Sm + 1St	2, Intercalary, 5th			

**NM* not mentioned

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lentil from their collections [6]. Their detailed chromosomal analysis on different species (Table 1) for the first time demonstrated more or less similar karyotypic features in all the species. Karyotype comprises three pairs of metacentric, or submetacentric chromosomes, a pair of metacentric chromosomes with a secondary constriction very close to the centromere (apparently intercalary), and three pairs of acrocentric chromosomes [4–6, 8, 20] (Table 1). Ladizinsky for the first time reported considerable magnitude of intra and inter chromosomal rearrangements involving metacentric and acrocentric chromosomes in cultivated and wild lentils. Intra chromosomal rearrangement was noted at the time of recognition of *L.tomemtosus* as a new species. [6, 8].

Aceto-iron-hematoxilin staining and giemsa C banding was performed in 10 Iranian cultivars of *L.culinaris* and reported 4 m + 3sm karyotype formula with 4th chromosome pair bearing the secondary constriction near centromeric region [47] (Table 1).

Bangladesh is another important grower of lentil but chromosome analysis is reported only in three cultivars of lentil [48]. They obtained TCL diversity but failed to locate sat chromosomes in lentil cultivars.

Australia is now listed as a major lentil growing country and currently contribute to approximately 10% of global trade [49]. The country initiated molecular diversity analysis of sixteen Australian cultivated landraces and some other lentil species using RAPD and 5S rRNA gene analysis [50]. In another studies genetic diversity of 505 lentil cultivars and landraces preserved in the Australian Grains Gene bank (AGG), using 384 SNP markers was assessed (Lombardi et al. (2014). However, chromosomal analysis of Australian lentils is not available.

Turkey stands fourth in terms of lentil production. It has large number of available landraces. They have used AFLP and ISSR molecular markers to evaluate the genetic diversity of large number of landraces in their country [51] unfortunately; they have not undertaken any chromosomal analysis. Analysis of karyotype diversity deserves attention.

(ii) Gametic chromosome analysis

Gametic or meiotic analysis is a traditional cost-effective method to observe chromosome behaviour inside a gametic cell. In plant breeding programmes inter- and intraspecific crosses are aimed for introgression of desirable traits to generate variability in crop plants. Meiotic analysis is time saving method for the selection of stable hybrids. The lone cultivated and six wild species of lentil contains 2n = 14 chromosomes. Meiotic analysis revealed seven bivalents formation in *L,culinaris* and two wild relatives *L.lamottei* and *L.nigricans*. No chromosomal anomaly was recorded in *L.culinaris* and *L. lamottei*. However late separation and sticky bridges in small number of pollen mother cells were noted in *L. nigricans* [52]. Ladizinsky and his group carried out a number of intra and interspecific hybridization experiment within the genus lentil to assess their compatibility and genetic distance [6, 20, 53–55]. Their important observations are listed below.

(i) inter-varietal hybrids are normal in cultivated lentil.
(ii) Chromosome rearrangements were rare in cultivated lentils but relatively common in wild lentils. Three chromosomal interchanges were noted in crosses between *L. culinaris* and *L. nigricans*, and only one between the *L. culinaris* and *L. orientalis*.
(iii) Hybridization between *L.culinaris* and some cultivars of *L.orientalis* resulted in fertile tertiary hybrids plants (2n = 15 chromosomes) but observed embryo abortion in others. Resultant hybrids arise through reciprocal translocation between a submetacentric and acrocentric chromosomes and heteromorphic quadrivalent was detected.
(iv) Trisomics between *L. culinaris* and *L. ervoides* was possible only through embryo culture.
(v) Intraspecific hybrids of *L.ervoides* are possible but countrywise diversity within the germplasms yielded different types of plant.

The above noted somatic and meiotic chromosome analysis revealed that chromosomal analysis has unlocked diverse foundational information within the genus lentil and has proved its utility as an important tool for assessing genetic diversity. Chromosomal diversity has not been adequately assessed from major lentil growing countries, on the other hand morphological, biochemical or molecular diversity has been reported from many countries. Looking at the genetic information presented in Table 1, it can be opined that blueprint karyotype of the genus lentil comprises three types of chromosomes; metacentric, submetacentric and acro / sub telocentric with one pair of secondary constrictions [56, 57]. Intra and inter specific natural chromosomal rearrangements particularly in wild species justify the existence of country wise genetic diversity in Lentil. These diversity parameters need further reassessment through molecular cytogenetics.

Molecular cytogenetics

(i) EMA based chromosome analysis

Kurata and Omura [58] applied the enzymatic maceration and flame drying method and giemsa staining in rice for the identification of individual chromosomes for the first AQ5 time. Since then, many workers from different countries have applied this method for chromosome analysis along with fluorescent banding and FISH [34, 52, 57–65]. Standardization

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of EMA method and detailed chromosomal analysis in cultivated and five wild lentil species is available only from India [52, 56, 57]. Analysis of large number of cytoplasm free metaphase plates from more than thirty cultivated cultivars of *L.culinaris* and five wild species (Table 1) have unlocked some important karyotypic features like (i) all cultivars contain 2n = 14 chromosomes except some monosomic cells (2n – 1) with 2n = 13 chromosomes in some cultivars (Table 1, IPL 81, Macro Barasat, EC 78,410 NBPGR, and EC 78,498 NBPGR), (ii) diversity in TCL from 53.6 ± 0.06 to 121.2 ± 0.12 µm, (iii) a pair of interstitial sat chromosomes in all the cultivated cultivars, (iv) sat chromosomes in cultivated cultivars are predominantly present on the 4th pair of chromosomes but some cultivars carry it on the 5th, 3rd and 2nd pair of median chromosomes, (v) similar karyotypic formula; 3 m + 1 m.sat + 2Sm + 1St (Table 1/2), (vi) Two wild species *L.orientalis and L.odemensis* contains a pair of interstitial sat chromosomes on 4th pair of chromosomes with similar karyotypic formula like *L.culinaris*. On the other hand (vii) one pair terminal sat chromosome was documented in three wild species namely *L.nigricans, L.lamottei* and *L.ervoides* on 6th, 5th and 6th pair of chromosomes, (viii) different karyotypic formula for *L.nigricans*—1 M + 4 m + 1 St (sat on 5th pair) + 1St, *L.lamottei*—5 m + 1St (sat on 6th pair) + 1St and *L.ervoides* 5 m + 1St (sat on 5th pair) + 1St (Table 1).

(ii) Fluorescent banding and FISH- rDNA analysis

Molecular cytogenetics provides new possibilities in the study of chromosomal evolution and genome organization [66]. Identification of individual chromosomes was further strengthened with the introduction of fluorescent banding and FISH which stems from the foundational information that heterochromatin in each species, seemed to be diversely distributed in proximal, interstitial and telomeric regions of a chromosome [67]. It was also revealed that constitutive heterochromatic regions are chromosome and species-specificity can be used to mark a particular chromosome within the karyotype. In majority of plant species, they are made up of highly repetitive AT or GC DNA sequences [68].

Over the decades many authors have used two contrasting DNA base specific fluorochrome dyes Chromomycin A3 and DAPI (4,6-diamidino-2phenylindole) for characterization of individual chromosomes and confirmation of the sat chromosome site in NOR-HC [33, 34, 59–62, 64–66, 69–72]. On the other hand, FISH, another widely used molecular cytogenetic tool [73, 74] primarily targets constitutive heterochromatin and rRNA genes within the chromosomes. 18S -5.8S-26S and 5S are the two families highly conserved rRNA genes present on chromosomes. These gene sites are termed chromosomal landmark in molecular cytogenetics. 18S -5.8S-26S gene site is part of nucleolar organizing region (NOR), also called secondary constriction region on chromosomes. Guerra [75] reported that NOR-heterochromatins are different from others as they contain rRNA genes along with other repeated sequences not related with NOR. Analysis of rDNA site distribution by FISH shows that they are always present in the NOR and often in other regions where secondary constrictions have not been located. Thus, FISH is extremely useful in locating rDNA genes, centromeric, sub-telomeric, and telomeric genes directly on the chromosome [75–80].

Landraces are the most diverse populations of cultivated plants. They are well adapted in natural environments and genetic diversity among and within the landraces make them a resourceful potential gene donor for any future breeding programme [81, 82]. Country wise wide genetic diversity has been unearthed in cultivated and wild lentils so (Table 1), application of fluorescent banding and FISH will help to segregate cultivars based on cultivar specific chromosomal markers and landmarks for future breeding programme.

Literature review revealed that fluorescent banding was not attempted in lentil over a long period of time. However, AQ6 detailed fluorescent banding with CMA and DAPI in more than thirty cultivated cultivar of *L.culinaris* and another five wild species has been reported [64, 65]. Detailed results are presented in Table 2. The results for the first time have generated many new information for future breeding programmes which are summarized below.

EMA based nonfluorescent Giemsa and contrasting fluorescent dye CMA and DAPI have confirmed intercalary secondary constriction in all the cultivars of *L.culinaris* and two wild species *L.orientalis* and *L.odemensis*. In *L.orientalis, L.odemensis* and majority of *L.culinaris*, secondary constrictions are present on the 3rd and 4th chromosome pairs, while in two cultivars it was noted on the 2nd and 5th chromosome pairs (Table 2).

Terminal secondary constriction was reported earlier in three wild species namely *L. nigricans*, *L. lamottei* and *L. ervoides* [53] (Table 1) has now been confirmed through fluorescent banding (Table 2). These three species carry their secondary constrictions on the 6th, 5th and 6th acrocentric chromosome pairs. Secondary constrictions are considered landmark chromosomes and may be used as chromosomal marker in cross breeding programmes.

Diversity in karyotype formula within the cultivated landraces of different countries and their wild relatives confirms the earlier view that exchange of genetic materials within and between the species is a continuous evolutionary process operating in the genus lentil [6, 8].

Fluorescent banding has revealed interesting molecular features of lentil chromosomes. These are the new tools for unlocking gene sequences for future crop improvement. NOR region of all the investigated wild lentil

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Table 2 Karyotype diversity through Fluorescent banding & FISH for rDNA loci in cultivated and wild Lentils

Name of the species	Indian Cultivars	Chromosome pair for NOR	Number of CMA and DAPI fluorescent bands on chromosome pairs				References
			CMA^+	$DAPI^+CMA^+$	$DAPI^+$	Total no	
L. culinaris	IPL 81	4th	2 (4th)	0	–	2	Jha et al. [64]
	IPL 316	**4th**	**14 (4th, 1st-3rd, 5th -7th)**	**0**	**–**	**14**	
	JL-1	4th	2 (4th)	0	–	2	
	IPL 406	3rd	2 (3rd)	0	2 (6th)	4	
	KLS 210	4th	2 (4th)	0	–	2	
	EC 70,403	4th	2 (4th)	2(4th)	2 (2nd)	6	
	EC 78,455	4th	2 (4th)	0	–	2	
	EC 78,498	4th	2 (4th)	0	–	2	
	EC 267,877	4th	4 (4th, 5th)	2 (4th)	–	6	
	DPL-15	4th	2 (4th)	0	–	2	
	DPL-62	4th	2 (4th)	0	–	2	
	EC-267526	4th	2 (4th)	2 (4th)	4 (1st,3rd)	8	
	HUL-57	3rd	2 (3rd)	2 (3rd)	2 (2nd)	6	
	EC-70306	4th	2 (4th)	0	–	2	
	EC-70394	5th	4 (5th, 4th)	0	–	4	
	EC-70404	4th	2 (4th)	2 (4th)		4	
	EC-78410	**4th**	**2 (4th)**	**2(4th)**	**12 (1st-3rd, 5th -7th)**	**16**	
	EC-78451A	4th	2 (4th)	0	–	2	
	EC-78452	4th	2 (4th)	0	–	2	
	EC-78475	3rd	2 (3rd)	0	–	2	
	EC-78476	4th	2 (4th)	0	–	2	
	EC-78542A	2nd	2 (2nd)	0	–	2	
	EC-223188	4th	2 (4th)	0	–	2	
	EC-255491	4th	2 (4th)	0	–	2	
	EC-267590	4th	2 (4th)	0	–	2	
	EC 67569A	3rd	2 (3rd)	2 (3rd)	–	4	
	EC-78461	4th	2 (4th)	0	–	2	
	EC-78473	4th	2 (4th)	0	–	2	
	Micro (Bankura)	4th	2(4th)	2(4th)	9 additional	13	
	Macro (Barasat)	4th	2 (4th)	0	2(3rd)	4	
	Micro (Barasat)	3rd	2 (3rd)	2(3rd)	–	2	
	Macro (U.P)	4th	2 (4th)	0	2 (4th)	4	
L. odemensis	**ILWL- 35**	**3rd**	**2 (3rd)**	**2 (3rd)**	**4 (1st, 2nd)**	**8**	
L. orientalis	**ILWL- 365**	**4th**	**2 (4th)**	**2 (4th)**	**4 (1st, 6th)**	**8**	
L. nigricans	**ILWL- 19**	**6th**	**2 (6th)**	**2 (6th)**	**6 (1st, 3rd, 5th)**	**10**	
L. lamottei	**ILWL- 14**	**5th**	**2 (5th)**	**2 (5th)**	**4 (1st, 2nd)**	**8**	
L. ervoides	**ILWL -61**	**6th**	**2 (6th)**	**2 (6th)**	**16 (All pairs)**	**20**	

Name of the species	Cultivar	No and position of rDNA sites		Country	References
		18S-5.8S-25S	5S rDNA		
L. culinaris	No. 2	On a metacentric pair	NM	Hebrew Univ Israel	Abbo et al. [83]
L. orientalis	No.133	On a acrocentric pair	NM		

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Table 2 (continued)

Name of the species	Cultivar	No and position of rDNA sites		Country	References
		18S-5.8S-25S	5S rDNA		
L. culinaris	NM	3rd pair	1st and 6th pair	Italy	Galasso et al. [84]
L. culinaris 'Eston'	CN51819	3rd pair in all		NM	Galasso [21]
L. culinaris 'Laird'	CN35415			NM	
L. orientalis	ILWL113	3rd pair in all		Turkey	
	ILWL114			Syria	
	ILWL402			Lebanon	
	PI572379			Turkey	
	PI572398			Turkey	
	PI572366			Turkey	
L. odemensis	ILWL223	3rd pair in all		Turkey	
	ILWL153			Syria	
	PI572361			Israel	
	ILWL403			Libyan	
L. ervoides	ILWL02	3rd pair in all		Turkey	
	ILWL42			Syria	
	ILWL387			Israel	
L. lamottei	ILWL14	3rd pair in all		France	
	ILWL430			Spain	
	No.73			Spain	
L. nigricans	ILWL112	6th pair in all		Turkey	
	ILWL191			Former Yugoslavia	
	PI572340				
	PI572344			Italy	
	PI572358			Spain	
L. tomentosus	ILWL120	3rd and 2nd pair 3rd pair in rest		Turkey	
	ILWL90			Turkey	
	ILWL93			Turkey	
	ILWL282			Turkey	
	ILWL308			Turkey	
	No.133			Syria	
	ILWL149			Syria	
				Russian Federation	
L. culinaris	PL641, LG60	Intercalary 3rd pair	2nd and 6th pair	India	Balyan et al. [86]
L. orientalis	166/92 IPK	Intercalary 3rd pair	1st and 6th pair	Germany	
	166/92 IPK				
L. odemensis	IG72686, IG672606 IG72558, B271/92, B893/93	Intercalary 3rd pair	1st and 7th pair	Germany	
L. ervoides	IPK	Intercalary 3rd pair	3rd and 6th pair	ICARDA, Syria	
L. nigricans	**191/93 and B893/93 IPK**	**Terminal on 6th pair**	**2nd and 6th pair**	**Germany**	

**NM* not mentioned

AQ7 species have shown CMA^+ and $DAPI^+$ bands and appears to be high DNA-rich regions. More molecular analysis is required in this regard. Though, Jha et al. [65] could not locate CMA^+ bands besides NOR regions in wild species but have scored 4–16 additional $DAPI^+$ positive bands on different chromosome pairs of different species. Maximum 16 $DAPI^+$ bands in all the chromosomes have been reported in *L. ervoides* [65], (Fig. 1, Table 2). Intense CMA^+ bands as well as $DAPI^+$ NOR region have also been noted in some cultivated cultivars [64, 65] (Fig. 2). Two cultivated

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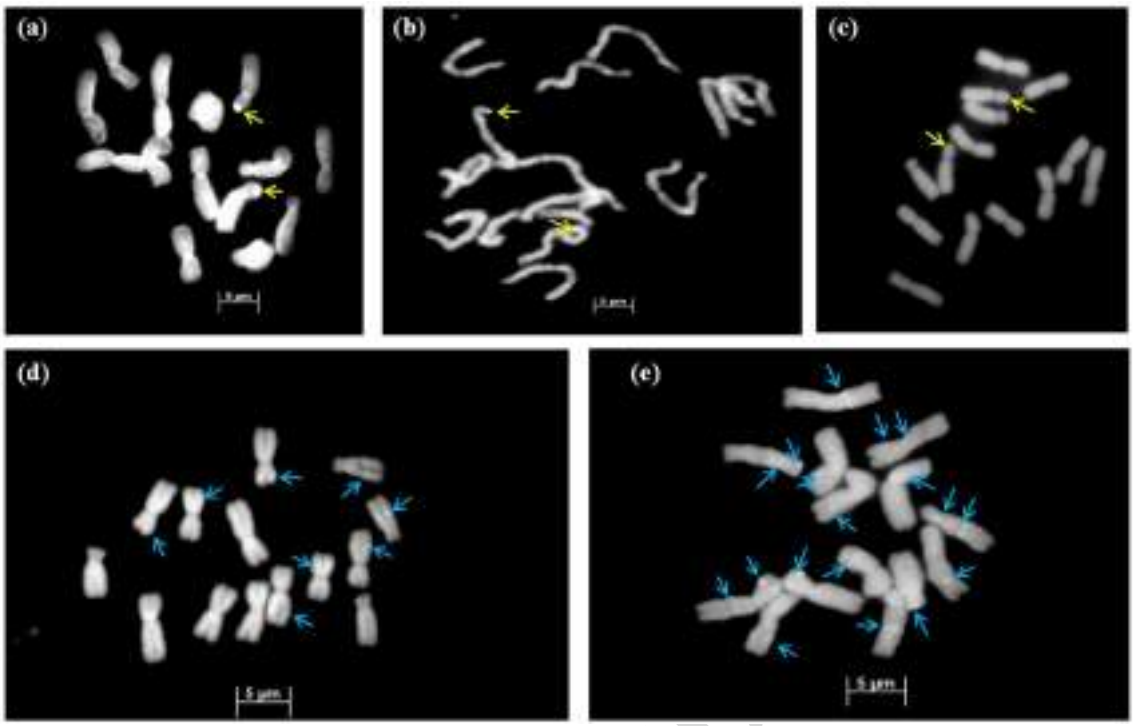

Fig. 1 Showing confirmed CMA$^+$ terminal NOR regions in three wild Lentil species along with many DAPI$^+$ bands, **a** *L.nigricans* (ILWL-19) (Table 2) terminal CMA$^+$NOR region, **b** *L.ervoides* ILWL -61(Table 2) terminal CMA$^+$NOR region, **c** *L.lamottei* ILWL-14 ((Table 2)) terminal CMA$^+$NOR region, **d** *L.nigricans* ILWL-19 (Table 2) showing many DAPI $^+$ bands, **e** *L. ervoides* (L11) ILWL -61, showing DAPI $^+$ bands in all the chromosomes [Yellow and blue arrows indicate CMA$^+$NOR bands and DAPI $^+$ bands, respectively.]

cultivars IPL 316 and EC-78410 have shown 12 additional CMA and DAPI bands on karyotypes (Table 2). While nine additional DAPI$^+$ bands have been documented in cultivated cultivar Micro (Bankura) (Fig. 2f). Diverse banding pattern on karyotypes of cultivated and wild species members may be treated as chromosomal markers for lentil breeders and genome researchers.

Nearly three decades ago Abbo et al. [83] applied FISH technique to detect 18S-5.8S-25S rDNA sites in *L.culinaris* (sample no.2) and *L.orientalis* (sample no.133). One pair of 18S-5.8S-25S rDNA sites was located in the intercalary NOR region of one metacentric chromosome pairs in sample no.2 which confirmed the earlier report of Ladizinsky [20]. However, the same site was located in terminal position of a small acrocentric chromosome pair in sample no.133. Ladizinsky [6] reported similar karyotype variation in *L.orentalis* (sample no.133,collected from Syria) and opined that chromosomally ssp. *L. orientalis* is the most variable taxon.

Galasso et al. [84] performed FISH with five probes in cultivated *L. culinaris* samples obtained from Italy. They obtained many signals on different chromosomes and were able to discriminate seven pairs of chromosomes and constructed a FISH karyotype. Probe pLc7 can be used as chromosome specific marker as it generated only one signal on chromosome pair one. Authors confirmed the earlier FISH results of Abbo et al. [83] and noted 18S-5.8S-25S rDNA site on chromosome pair 3. Interestingly, 5S rRNA gene loci was located on chromosomes pairs 1 and 6. The results confirm the earlier hypothesis that the 5S rRNA genes may be present on different chromosomes [85]. To check the distribution of 5.8S-25S rDNA and 5S rRNA gene loci on 30 lentil samples collected from different countries (Table 2), Galasso [21] initiated a new set of FISH experiment using two highly repetitive sequences probes pLc30 and pLc7 from lentil and multigene family pTa71 and pTa 794 of wheat. It was noted that two 5S rDNA sites on chromosome pairs 1 and 6 were conserved in all the species. Galasso supported the findings of Balyen et al. [86] for all the species except *L. ervoides*. (Table 2). Galasso (21) pointed out that distribution of probe pLc30 on chromosomes is chromosome- and species-specific and thus, it can be used as FISH probe. Mophological, biochemical and molecular homology between *L.culinaris* and *L.orientalis* opined by different authors was fully supported by FISH karyotype [6, 9, 10, 87]. On the other hand FISH karyotype study disagreed with the view of Fergusen et al. [10] and supported the separation of *L.tomentosus* and *L.lamottei* as independent species [6–9]. The FISH karyotype justified the separation of *L.lamottei* and *L.nigricans* despite their morphological similarity and cross compatibility [53]. Galasso [21] observed

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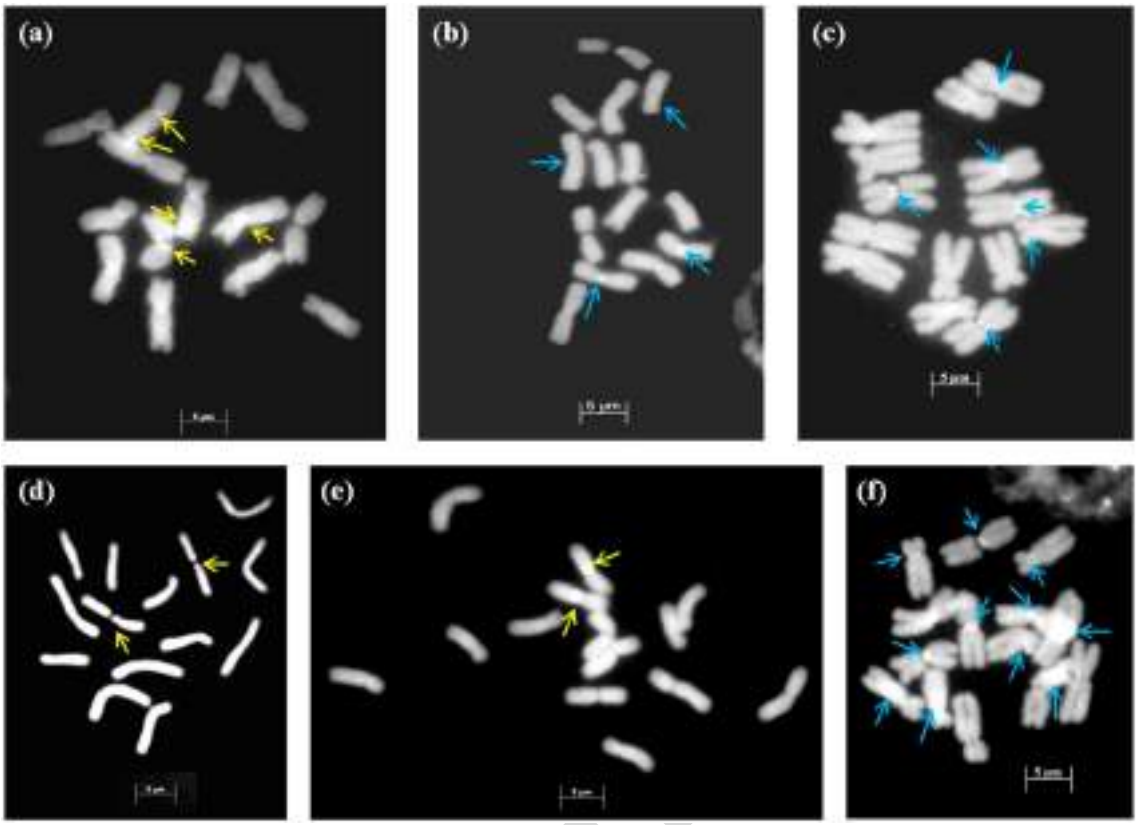

Fig. 2 Indian cultivars of *L.culinaris* showing either CMA$^+$ or DAPI$^+$ fluorescent bands as well as intense CMA$^+$ and DAPI$^+$ NOR regions. **a** IPL-316 (Table 2) with a number of CMA + bands, **b** EC-78410 (Table 2) with many DAPI$^+$ bands, **c** with intense DAPI$^+$ bands in Micro (Bankura), (Table 2), **d** intense CMA + NOR regions in Macro (U.P) (Table 2), **e** intense CMA$^+$ NOR regions in in EC-267526 (Table 2), **f** nine additional DAPI$^+$ bands in Micro (Bankura) (Table 2). [Yellow and blue arrows indicate CMA$^+$NOR bands and DAPI $^+$ bands, respectively.]

country wise diversity in 18S–5.8S–25S rDNA sites within different samples of *L.tomentosus*. In general, one pair of 18S-5.8S-25S rDNA sites were located in chromosome pair 3, but it was present on chromosome pair 6 in *L. nigricans* and one additional site on chromosome pair 2 was located in *L. tomentosus* (ILWL120).

Balyan et al. [86] also investigated FISH analysis in five lentil species namely *L. culinaris, L. orientalis, L. odemansis, L. ervoides and*, *L. nigricans* for distribution of 18S-5.8S-25S rDNA and 5S rRNA loci. One prominent signal for 18S-5.8S-25S rDNA on a single pair of chromosomes was noted in all the five species collected from various countries (Table 2). But except one uniformity in chromosome number for that site was seen. It was near terminal region of an acrocentric chromosome pair 6 in *L.nigricans* and it was near the centromere of a metacentric/ submetacentric chromosome pair 3 in all other four species. Additionally, two signals on two different pairs of chromosomes were noted for 5S rDNA loci. Surprisingly both the 18S-5.8S-25S rDNA and 5S rDNA loci were present on chromosome pair 6 and 3 in *L.nigricans* and *L. ervoides* respectively. Fernández et al. [88] carried out FISH analysis using two probes pTa71 and pTa794 to detect 18S-5.8S-25S rDNA and 5S rDNA locations in metaphase plates of four species of lentil and supported the views of the earlier authors [21, 83, 86]. Confirmation of karyotypic diversity in all the five lentil species reported by Balyan et al. [86] have ruled out the earlier report that all the Lens species have similar karyotypes [6, 89]. Giemsa staining and fluorescent banding has clearly pointed out karyotype diversity in Indian lentils [52, 56, 57]. It is believed that karyotypic diversity in lentil is a continuous process and resulted through the intra and inter specific chromosomal interchanges [6, 20, 90].

Conclusions and future outlook

In global perspective lentil is designated as a 'narrow gene pool crop' serving the humanity since the dawn of agriculture as 'poor man's meat'. Authors have reported natural ability of chromosomal interchanges within and between the lentil species growing in different agro

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climatic conditions. Genetic diversity in both cultivated and wild lentil has been carried out using morphological, DNA based molecular markers and genotyping tools based on SNP markers [49, 91, 92]. The review has compiled genetic information in cultivated and wild lentils and revealed that strong crossibility barriers have reduced crop improvements options. Critical analysis of chromosomal diversity presented in this review could be informative for the breeding as well as plant improvement and conservation programme. Molecular cytogenetic karyotype analysis has opened up large number of cultivar/species-specific band as chromosomal markers and landmarks in this genus. These CMA + and DAPI + chromosome and species-specific GC /AT rich repetitive DNA sequences and FISH generated 18S-5.8S-25S rDNA and 5S rDNA sites in cultivated and wild relatives will provide tags for authentication of genotypes and development of population specific markers. More genetic information in support of widening gene pool is one important option for lentil improvement and alien gene introgression from wild species is a prerequisite. Combination of chromosomal information with genomics can frame a new template for future lentil breeding. FISH as modern cytogenetic tool is already in use to map sequences and identify alien chromatin in new breeding lines [93]. It has been reported that the field of genomics with restricted focus on DNA sequence has largely distanced itself from cytogenetics, though the term 'genome' was originally coined to describe the study of genes and chromosomes [94]. In a critical juncture of climatic changes and promises of hunger free world requires combination of genes and chromosomes. Combination of chromosomal information with genomics will help correct data interpretation and framing a new template for future crop improvement.

All the available information will enrich lentil repository of genomic research and genomic databases to design future genome modification programmes for sustainable crop improvement programmes. The review has shown inadequacy of molecular cytogenetic work and invites more chromosomal research on country specific species and landraces of lentil.

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Studies on the effect of pesticides on the mycoflora diversity in the agricultural environments

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Studies on the effect of pesticides on the mycoflora diversity in the agricultural environments

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Plant leaves are the natural habitat for growth of many microorganisms like fungi, bacteria, actinomycetes etc. Various chemicals are sprayed time to time over the leaves of economically important crops to manage the insect pests, pathogenic fungi, nematodes, pathogenic bacteria etc. Three chemicals were tested in laboratory condition over the fungal strains isolated from phylloplane. Cildon (85% Phosphamidon) showed various degrees of inhibition on *Aspergillus ochraceus*, *Curvularia geniculata, Alternaria solani*, *A. brassicae, A. tenuissima*, *A. tenuis*, *Nigrospora sphaerica* and on other fungi tested by cup assay method. BHC caused total inhibition over *Curvularia lunata, C. geniculata, Penicillium, Stachybotrys atra*, *T. lignorum*, *Cladosporium herbarum*, *Rhizopus nigricans* and also over pathogenic types like *Drechslera, Alternaria solani*, *A. brassicae, A. tenuissima*, *A. tenuis* etc. Although a number of fungi were inhibited totally with Bavistin, a fungicide, 12 fungi were found to grow normally including plant pathogenic *Alternaria* spp. except *A. tenuis, Drechslera*, *Helminthosporium sativum* and the saprobic *Curvularia lunata*, *C. pallescens, S. atra*, *E. purpurascens*, *Brachysporium* and *Nigrospora sphaerica*. Similar results were also obtained in dry weight method with Cildon and Bavistin. BHC caused toxicity to the whole culture medium showing nil growth in almost all fungi under experiment.

Key words: Crop fields, fungi, fungicides, growth inhibition, pesticides, phytopathogens

INTRODUCTION

The airspora studies in the crop fields revealed that pathogenic and non-pathogenic fungal spores are present at different growth stages (Uddin 2004, 2005, 2007). These are coming out mainly from the crop plants itself acting as the immediate source. Besides fungi and bacteria, crop plants mostly suffer from the attack of insect pests damaging the plants. Consequently, the farmers usually use pesticides and fungicides to cope up with the problem of diseases. The chemicals are mostly sprayed on the foliar parts. Hence, studies were made to find out the effect of the chemicals on the phylloplane mycoflora which in turn affects the local airspora to some extent. The effects of pesticides were studied by Abd-Alla *et al.* (2000) on VAM fungi; on fungal and bacterial population by Pandey and Singh (2004), and on the pathogenic fungi by Olajire and Fawole (2009).

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The impact of pesticides were also studied by Johnsen *et al*. (2001) on bacterial diversity, on microbial community by Lo (2010), on phytopathogenic bacteria by Patyka *et al*. (2016). Flores *et al*. (2014) studied the effect of imazalil (fungicide) and Diazinon (insecticide) on the stream fungi associated with litter breakdown; on soil mycoflora (Rajbonshi *et al*. 2014; Saramanda and Kaparapu, 2017); on *Aspergillus niger* from agricultural soil with three fungicides, three insecticides, three herbicides and two biopesticides (Geetha *et al*. 2016); on fungal phytopathogens (Mohan *et al*. 2017); on bacterial and fungal populations in Ecuadorian tomato cultivated soils (Srinivasulu and Ortiz, 2017); on soil microorganisms (Mehjin *et al*. 2019; Singh *et al*. 2019; Meena *et al*. 2020; and Kremer, 2021). Fiedler and Sosnowska (2017) showed the side effects of fungicides and insecticides on entomopathogenic fungi while Koladar *et al*. (2018) studied the *in vitro* evaluation of non-systemic fungicides, at different concentrations on Turmeric Anthracnose caused by *Colletotricum capsici.* Very

recently the effects of pesticides on the diversity of endophytic fungi in tea plants caused by *Colletotrichum camelliae* were studied by Win *et al*. 2021.

MATERIALS AND METHODS

Cildon (85% Phosphamidon) and Benzene hexachloride *i.e*. BHC (50%) as pesticides and Bavistin (Carbendazim compound) as fungicide are frequently applied by the farmers to protect the existing crops from the severe damages due to insect pests and fungal pathogens respectively. These three compounds were tested against 28 phylloplane fungi at field doses using cup assay and dry weight methods.

Cup Assay Method

Spore suspension was prepared from a fresh 14 days old pure culture of the test fungi. One ml of spore suspension was distributed in each of the sterile petridishes (four replicates for each concentration against each fungus), to which sterile but cold nutrient medium was poured thickly and rotated several times to mix the spores evenly with the medium. After solidification, two cups of 6 mm diameter were made with a cork borer in each plate. Solutions at field doses were prepared from Cildon (*i.e*. 0.06% concentration), BHC (*i.e.* 0.5% concentration) and Bavistin (*i.e.* 1% concentration) with sterile distilled water. Requisite amount of solutions were poured very carefully to the cups with a micropipette. Control set using sterile distilled water without chemicals was also maintained. The plates were incubated at 28°C for 24 hrs and examined every 24 hours. The inhibition zone in diameter if any, was measured and recorded. Similar agar diffusion method was also followed by Geetha *et al*. (2016), Saramanda and Kaparapu (2017) and the inhibition zone was measured.

Dry Weight Method

For this method 25 ml of nutrient liquid medium with the requisite amount of pesticides or fungicide were taken in 100 ml conical flask. To these one ml of spore suspension of each fungus was added. Four replicates for each test were maintained. Control sets (4 flasks) containing only 25 ml broth and one ml of fungal suspension were always maintained. After 14 days of growth, the mycelial mats were harvested on Whatman's filter paper No. 42 (weighed previously) and were kept in hot air oven at 60°C for 72 hrs. for complete drying. The dry weight of mats were taken and mean was calculated. The dry weight method was also followed by Olajire and Fawole (2009).

RESULTS AND DISCUSSION

The mean readings of four replicates of inhibition measured by the cup assay method are represented in Table 1. Cildon caused inhibition in variable degrees on *Aspergillus ochraceus, Curvularia geniculata, Trichoderma lignorum, Fusarium* sp., *Drechslera* sp*., Helminthosporium oryzae, Nigrospora sphaerica, Alternaria humicola*, *A. solani, A. brassicae, A. tenuissima, A. tenuis* and *Helminthosporium sativum.* No inhibition was recorded on the rest (15 in number) fungi tested which included different species of *Aspergillus* excepting *A. ochraceus, Curvularia lunata, C. pallescens, Penicillium* spp., *Stachybotyrs atra, Epicoccum purpurascens, Cladosporium herbarum, Rhizopus nigricans, Brachysporium* sp., *Cercospora* sp. *and Chaetomium homopilatum.*

With BHC, 15 fungi (namely *Curvularia lunata*, *C. geniculata, Penicillium* sp., *Stachybotrys atra*, *T. lignorum*, *C. herbarum*, *Rhizopus nigricans, Drechslera*, *Alternaria solani*, *A. brassicae*, *A. tenuissima*, *A. tenuis*, *Chaetomium homopilatum, N. sphaerica* and *H. sativum*) were totally inhibited and 8 fungi (*viz. Aspergillus parasiticus, A. fumigatus, A. ochraceus, C. pallescens, Fusarium*, *H. oryzae, Alternaria humicola* and *Brachysporium*) were inhibited partially. No inhibitory effect was obtained in fungi like *A. niger, A. terreus*, *P. funiculosum*, *E. purpurascens* and *Cercospora* sp. adopting the cup assay method.

With the fungicide Bavistin, a number of fungi were found to be totally inhibited. Remarkable (45 mm diameter) inhibition was recorded for *A. niger*, *A. ochraceus*, *Penicillium* sp*., T. lignorum, C. herbarum* and *Chaetomium homopilatum. Penicillium funiculosum* (43 mm), *A. parasiticus* (42 mm), *Rhizopus nigricans* (42 mm), *A. terreus* (40 mm) and *Cercospora sp.* (40 mm) showed a good inhibition while moderate (28-35 mm) inhibition was recorded for the fungi*, A. fumigatus, Fusarium*, *C. geniculata*, *H. oryza*e and *A. tenuis.* Surprisingly, twelve fungi were found to grow normally in presence of Bavistin which included different species of plant pathogenic *Alternaria* (excepting

A. tenuis), *Drechslera*, *H*. *sativum* and saprobic *C*. *lunata, C. pallescens*, *S. atra*, *E. purpurascens, Brachysporium* and *Nigrospora sphaerica.*

In dry weight method (Table 2), no growth was recorded in broth containing Cildon for fungi like *C. geniculata, A. solani*, *A. tenuissima*, *A*. *tenuis* and *N. sphaerica*; negligible growth was recorded in *Drechslera* and *A. brassicae*, and reduced growth was obtained in *H. oryzae, A. ochraceus, C. pallescens*, *T. lignorum, Fusarium*, *A*. *humicola*, *C. herbarum*, *R*. *nigricans* and in *H*. *sativum*. However, growth similar to control was measured in *A. parasiticus*, *A. fumigatus, A*. *terreus, Penicillium* sp., *P. funiculosum*, *S. atra*, *E. purpurascens, Brachysporium* and *Chaetomium homopilatum*. Hyphal growth was found to be accelerated in presence of Cildon in cases of fungi like *A. niger, C. lunata* and *Cercospora* sp.

The broth with BHC at 0.5% concentration was found to be most toxic where no growth was recorded for all fungi tested except the very negligible growth of *C. pallescens.* A total of 13 fungi (*viz*. 5 species of *Aspergillus*, 2 species of *Penicillium*, *C. geniculata*, *T. lignorum, Fusarium* sp.*, A. tenuis*, *C*. *homopilatum* and *Cercospora* sp.) were inhibited totally with Bavistin. Significant inhibition was found in *C. herbarum* and *H*. *oryzae* where very less amount of dry mycelia was obtained in comparison to control. *Rhizopus nigricans* and *Drechslera* were fairly inhibited. Almost similar amount of dry mycelia as control was recorded in *C. lunata*, *C. pallescens*, *S*. *atra, E. purpurascens, Brachysporium, N. sphaerica* and even pathogenic *A*. *solani*, *A. tenuissima* and *H. sativum*. Mycelial growth of *A*. *humicola* and *A. brassicae* was found to be accelerated with the presence of fungicide Bavistin.

Pesticides include insecticides, herbicides, fungicides and rhodenticides. In the field, pesticides especially insecticides are applied to crops time to time to control insect pests which may affect the non-target mycoflora of the crop foliage. Fungicides are applied during severe attack of fungal pathogens to eradicate the disease. Fungal population generally decline due to fungicide treatment which are gradually recovered with time (Rajbonshi *et al.* 2014). Indiscriminate use of insecticides and fungicides may cause severe threat in the growth of some beneficial fungi, and also on other microbes (Srinivasulu and Ortiz, 2017); and even may have harmful effect on human beings and endangered species. Singh *et al.* (2019) explained that pesticides which are applied continuously in different growth phases of crop, vegetables and fruits may get deposited to some extent in the fruits, crops etc. as chemical residues and consumption of these pesticide residues showed mutagenic, carcinogenic, cytotoxic, genotoxic effects and also a range of health related issues in the human beings.

Cildon *i.e.* 85% Phosphamidon caused the death of non-pathogenic fungi (*Aspergillus ochraceus, Curvularia geniculata*) as well as pathogenic *Altemaria solani*, *A. brassicae*, *A. tenuissima, A. tenuis*; partially affected the growth in *Trichoderma lignorum*, *Fusarium, Drechslera, Helminthosporium oryzae*, *H. sativum* and *Altemaria humicola*. Thus, during the application of Cildon in the field, there would be significant reduction in the fungal level in the agricultural environment. However, some fungi were left unaffected. Similarly, Lo (2010) observed that some pesticides stimulate the growth of microorganisms, but other pesticides have depressive effects or no effects on microorganisms. Application of Karate (Pyrethroids compound), an insecticide significantly reduced mycelial growth in the three fungi, *viz*. *Aspergillus flavus*, *Fusarium moniliforme* and *Fusarium oxysporum* when compared with control (Olajire and Fawole, 2009).

BHC, an effective pesticide caused growth inhibition or killing of a number of fungi tested in the laboratory. *Aspergillus niger*, *A. terreus, Penicillium funiculosum*, *Epicoccum purpurascens* and *Cercospora* sp. showed nil inhibition (*i.e.* normal growth as control) and a number of fungi showed partial inhibition with BHC in cup assay method; on the contrary showed total inhibition with dry weight method. The reason may be due to the non-permeability of BHC from the suspension placed in the cups towards the semisolid growth media; as BHC is partially soluble in water. The dry weight method showed the toxic efficacy of BHC on the fungi tested. Abd-Alla *et al*. (2000) reported that application of various pesticides inhibits the growth of cowpea, bean and lupin due to inhibition of arbuscular mycorrhizal root colonization. Geetha *et al*. (2016) reported from their results that fungicides, insecticides, herbicides and biopesticides caused drastic reduction in

Table 1: Effect of different pesticides on radial growth of microorganisms

Fungal organism	Control (Without Chemical)		Cildon (0.06%)		50% BHC (0.5%)		Bavistin (1.0%)	
	Nature of Inhibition	Inhibition Zone (mm)	Nature of Inhibition	Inhibition Zone (mm)	Nature of Inhibition	Inhibition Zone(mm)	Nature of Inhibition	Inhibition Zone(mm)
Aspergillus niger	Nil	0	Nil	0	Nil	0	Total	45
A. parasiticus	Nil	0	Nil	0	Partial	16	Total	42
A. fumigatus	Nil	0	Nil	0	Partial	12	Total	35
A. terreus	Nil	0	Nil	0	Nil	0	Total	40
A. ochraceus	Nil	0	Total	14	Partial	18	Total	45
Curvularia lunata	Nil	0	Nil	0	Total	25	Nil	0
C. geniculata	Nil	0	Total	30	Total	33	Total	30
C.pallescens	Nil	0	Nil	0	Partial	20	Nil	0
Penicillium sp.	Nil	0	Nil	0	Total	16	Total	45
P. funiculosum	Nil	0	Nil	0	Nil	0	Total	43
Stachybotrys atra	Nil	0	Nil	0	Total	30	Nil	0
Epicoccum purpurascens	Nil	0	Nil	0	Nil	0	Nil	0
Trichoderma lignorum	Nil	0	Partial	22	Total	45	Total	45
Cladosporium herbarum	Nil	0	Nil	0	Total	30	Total	45
Rhizopus nigricans	Nil	0	Nil	0	Total	20	Total	42
Fusarium sp.	Nil	0	Partial	30	Partial	27	Total	32
Drechslera sp.	Nil	0	Partial	19	Total	25	Nil	0
Helminthosporium oryzae	Nil	0	Partial	24	Partial	18	Total	30
Alternaria humicola	Nil	0	Partial	20	Partial	16	Nil	0
A. solani	Nil	0	Total	28	Total	24	Nil	0
A. brassicae	Nil	0	Total	40	Total	23	Nil	0
A. tenuissima	Nil	0	Total	30	Total	38	Nil	0
A. tenuis	Nil	0	Total	32	Total	27	Total	28
Brachysporium sp.	Nil	0	Nil	0	Partial	18	Nil	0
Cercospora sp.	Nil	0	Nil	0	Nil	0	Total	40
Chaetomium homopilatum	Nil	0	Nil	0	Total	45	Total	45
Nigrospora sphaerica	Nil	0	Total	45	Total	45	Nil	0
Helminthosporium sativum	Nil	0	Partial	18	Total	24	Nil	0

Aspergillus niger population in the soil, which agree with the present investigation. Fungicides and herbicides were proved to be more destructive on *Aspergillus* sp. as compared with insecticides, where maximum zone of inhibition was seen in *Aspergillus* spp. with three fungicides (Saramanda and Kaparapu, 2017) which is coordinated with the present findings.

The fungicide, Bavistin showed total inhibition in majority of the fungal isolates. There were some types including pathogens showing no inhibition at all, even the growth was found to be accelerated in cases of some fungi including phytopathogens. In the present investigation, total inhibition was not recorded in all fungi with Bavistin; whereas thiocarbamate fungicides demonstrated significant inhibitory action on phytopathogens (Patyka *et al*. 2016). Koladar *et*. *al.* (2018) reported that highest percentage of inhibition was obtained by thiram to control *Colletotrichum capsici* followed by ziram and other fungicides. Among the five fungicides,

Table 2: Effect of different pesticides on mycelial dry wt. of the different microorganisms

Fungal organism	Control	Treatment		
	(mg)	Cildon (0.06%) (mg)	50% BHC (mg)	Bavistin (1.0%) (mg)
Aspergillus niger	262	306.5	0.0	0.0
A. parasiticus	252	234.0	0.0	0.0
A. fumigatus	268	232.0	0.0	0.0
A. terreus	105	98.0	0.0	0.0
A. ochraceus	201	90.0	0.0	0.0
Curvularia lunata	244	260.0	0.0	222.5
C. geniculata	213	0.0	0.0	0.0
C. pallescens	178	89.5	4.5	148.5
Penicillium sp.	211	188.5	0.0	0.0
P. funiculosum	132	129.5	0.0	0.0
Stachybotrys atra	218	189.0	0.0	192.5
Epicoccum purpurascens	188	181.5	0.0	162.5
Trichoderma lignorum	152	99.0	0.0	0.0
Cladosporium herbarum	239	187.0	0.0	31.5
Rhizopus nigricans	156	113.0	0.0	66.5
Fusarium sp.	112	77.5	0.0	0.0
Drechslera sp.	105	16.0	0.0	74.5
Helminthosporium oryzae	223	54.0	0.0	45.0
Alternaria humicola	103	80.0	0.0	154.5
A. solani	168	0.0	0.0	138.5
A. brassicae	121	12.5	0.0	136.5
A. tenuissima	204	0.0	0.0	150.0
A. tenuis	71	0.0	0.0	0.0
Brachysporium sp.	366	241.0	0.0	313.0
Cercospora sp.	214	262.0	0.0	0.0
Chaetomium homopilatum	154	151.5	0.0	0.0
Nigrospora sphaerica	209	0.0	0.0	177.5
Helminthosporium sativum	81	28.5	0.0	71.0

Hexaconazole had highest inhibitory effect on the growth and population of fungi whereas Bavistin showed least inhibitory effect over the dominant genera, *viz*. *Aspergillus* sp., *Penicillium* sp., *Curvularia* sp. *Alternaria* sp. and *Trichoderma* sp. (Rajbonshi *et al*. 2014). Insecticides and fungicides showed variation in reduction of growth in three fungi namely *Metarhizium anisopliae*, *Beauveria basiana* and *Acremonium* sp. as observed by Fiedler and Sosnowska (2018). Use of various agrochemicals lowered the infection rate of fungal endophytic community especially *Colletotrichum camelliae* in the leaf tissues of tea plants (Win *et al*. 2021).

Different species of *Alternaria* (excepting *A. tenuis*) were found to be unaffected with Bavistin and even Bavistin caused acceleration of growth in *A. humicola* and *A. brassicae*. The abundance, diversity and function of soil microbiota were disrupted with the use of agricultural pesticides (Meena *et al*. 2020; Kremer 2021). The *Fusarium* sp., in the present study was inhibited totally by Bavistin and BHC; while slight inhibition was also observed with the Cildon. Similarly, growth of two *Fusarium* spp., *i.e. F. oxysporum* and *F. moniliforme* was completely inhibited by Benomyl at 500 mg/L concentration (Olajire and Fawole, 2009). Herbicides and insecticides caused decrease in the microbial activities and counts of soil bacteria, fungi and actinomycetes and the effects were inversely proportional to the concentration of the pesticides (Mehjin *et al*. 2019). In the present investigation, Cildon caused growth acceleration in *A. niger*, *C. lunata* and *Cercospora* sp. as measured in dry weight, while Bavistin showed acceleration in *Alternaria humicola* and *A. brassicae*, as also observed by Lo (2010) where some pesticides stimulated the growth of microorganisms, although the fungicide tebuconazole affected greatly the growth of *Cladosporium tenuissimum*.

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Unravelling the genetic diversity and phylogenetic relationships of Indian Capsicum *through fluorescent banding*

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RESEARCH ARTICLE

Unravelling the genetic diversity and phylogenetic relationships of Indian *Capsicum* through fluorescent banding

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Abstract Hot chilli species of *Capsicum* are one of the most significant spice crops in India having several genetically distinct cultivars. The present study was targeted to upgrade chromosomal database of 15 cultivars belonging to *C. annuum* L., *C. frutescens* L. and *C. chinense* Jacq. EMA-based fluorochrome banding with GC-specific stain Chromomycin A3 (CMA) was elemental to reveal specialization in karyotypes that could not be traced out by Giemsa-stained preparations. CMA banding pattern was combined with karyomorphometric indices to statistically evaluate chromosomal relationship among the cultivars. The marker chromosome pairs 11 and 12 containing nucleolar CMA bands could be suggested as the evolutionary landmarks in '*C. annuum* complex'. We found direct correspondence between fruit morphotype variation and CMA banding pattern to demonstrate maximum genetic diversity within *C. annuum*, followed by *C. frutescens* while Bhut jolokia of *C. chinense* displayed genetic uniformity. UPGMA phenogram upheld distinct species separation, cultivar diversity and relationships within and among the species. Out of nine *C. annuum* cultivars, at least two cultivars showed some sort of genetic relationship with *C. chinense*, particularly the Ghee cultivar shares more proximity with *C. chinense*. The fluorescent karyotype database reliably symbolized uniqueness of *Capsicum* germplasm of India, especially from the NEH sector that grows Bhut jolokia. Our attempt is believed to complement genomic investigation in the popular and exotic chilli cultivars of India and interest breeders in search of alternative genetic resources.

Keywords *Capsicum annuum* · *Capsicum frutescens* · *Capsicum chinense* · Fluorescent karyotypes · CMA bands · NORs

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Introduction

Diverse agro-climatic conditions of India have helped the country to become a repository of huge plant genetic resources. Since ancient time, capsicum or chilli is an important and valuable cultivated commercial crop in our country. The crop belongs to the family Solanaceae and contains five cultivated and nearly 30 wild species (Carrizo García et al. 2013). *Capsicum* is worldwide valued as food, flavouring substance and a beneficiary condiment for human health (Milla 2006). However, basic genetic characterization of *Capsicum* species and cultivars growing

in India are far from complete. In addition, reassessment of genetic diversity in the era of climatic changes is equally important and can play a pivotal role in new plant breeding programmes.

Though India ranked high in terms of production and consumption of capsicums, only three cultivated species with large number of cultivars are reported from the country (Govt. of India 2009, 2018). Hilly terrain with distinct geographical positioning and diverse agro-climatic conditions has made West Bengal and North Eastern Himalayan states (NEH) a repository of *Capsicum* diversity. Two important discoveries that enriched our repository have further necessitated assessment of genetic diversity of many unattended cultivars. The first one was world's hottest chilli Bhut jolokia/Naga jolokia/King chilli/Tezpur chilli cultivars under the species *Capsicum chinense* reported from NEH regions of India (Reddy et al. 2014) and the other one was allopolyploid cultivar of *Capsicum annuum* from hilly terrain of West Bengal and Sikkim, India (Jha et al. 2012). Besides the above, different less known cultivars of *C. frutescens* and *C. annuum* are also available in various parts of India. Local people or farmers are preserving these unattended resources in their own garden. Collection, documentation and characterization of these additional genetic resources could be utilized by the breeders and genome researchers as supplementary gene pool for crop improvement and conservation (Baral and Bosland 2002).

The concept of "*C. annuum* complex" comprises primarily the three cultivated species *C. annuum* L., *C. frutescens* L. and *C. chinense* Jacq. based on similar floral morphology, isozyme patterns and DNA sequence characteristics (Pickersgill 1988). However, we have observed remarkable diversity in floral traits within the members of the three Indian *Capsicum* species (Jha et al. 2017). Thus we consider to put more genomic information of Indian capsicums through fluorescent chromosome analysis to strengthen the unresolved phylogenetic relationship within this large "*C. annuum* complex". Chromosomes are the earliest genomic structures and have played an important role for conservation of genetic diversity and improvement of crops. It is "the quickest, cheapest, and easiest way to get any substantial and foundational pieces of genomic information of any species which is not possible by any other methods" (Guerra 2008; Soltis et al. 2014). Chromosome analysis can exclusively prove the ploidy level of any plant in a cost effective manner. It can indicate whether additional variation originates over a long period of time against climatic, biotic and abiotic stresses (Jha et al. 2015). Chromosomal analysis on Indian *Capsicum* was restricted only on *C. annuum* cultivars using conventional orcein staining by various authors (Dixit 1931; Raghavan and Venkatasubban 1940; Chennaveeraiah and Habib 1966). Although enzymatic maceration and air drying method (EMA) is an improved version of plant chromosome analysis introduced long back by Kurata and Omura (1978) in rice, was not attempted by anyone in India over the decades on capsicums. The method has rejuvenated plant chromosome research and opened a new field of molecular cytogenetics. Besides Giemsa staining of the EMA slides, they can also be stained with nucleic acid base specific fluorescent dyes to identify heterochromatin rich segments directly on metaphase chromosomes. The method is precise, repeatable and can differentiate morphologically similar chromosomes (Schweizer 1976; Hizume et al. 1989; Fukui 1996; Moscone et al. 1996; Yamamoto 2012). Considering the significance of chromosome characterization in this prime spice crop, scientists have made several attempts from countries other than India (Moscone et al. 1995, 1996, 2007; Scaldaferro et al. 2013, 2016; Barboza et al. 2019; Scaldaferro and Moscone 2019; Zhou et al. 2019). In our earlier reports, we have detailed standardization of EMA based Giemsa staining and karyomorphometric analysis on Indian capsicums (Jha and Saha 2017; Jha et al. 2017). Keeping in mind the importance of fluorescence banding in assessment of genetic diversity within and between the species members, the objective of the present paper is to unravel the genetic diversity of 15 Indian *Capsicum* cultivars through fluorescence banding for the first time. Our findings will conserve new genetic information of Indian germplasms in one hand and help to utilize the data in new breeding programmes for crop improvement.

Materials and methods

Collection of plant samples

For fluorescence banding studies we have considered 15 different cultivars collected from different parts of

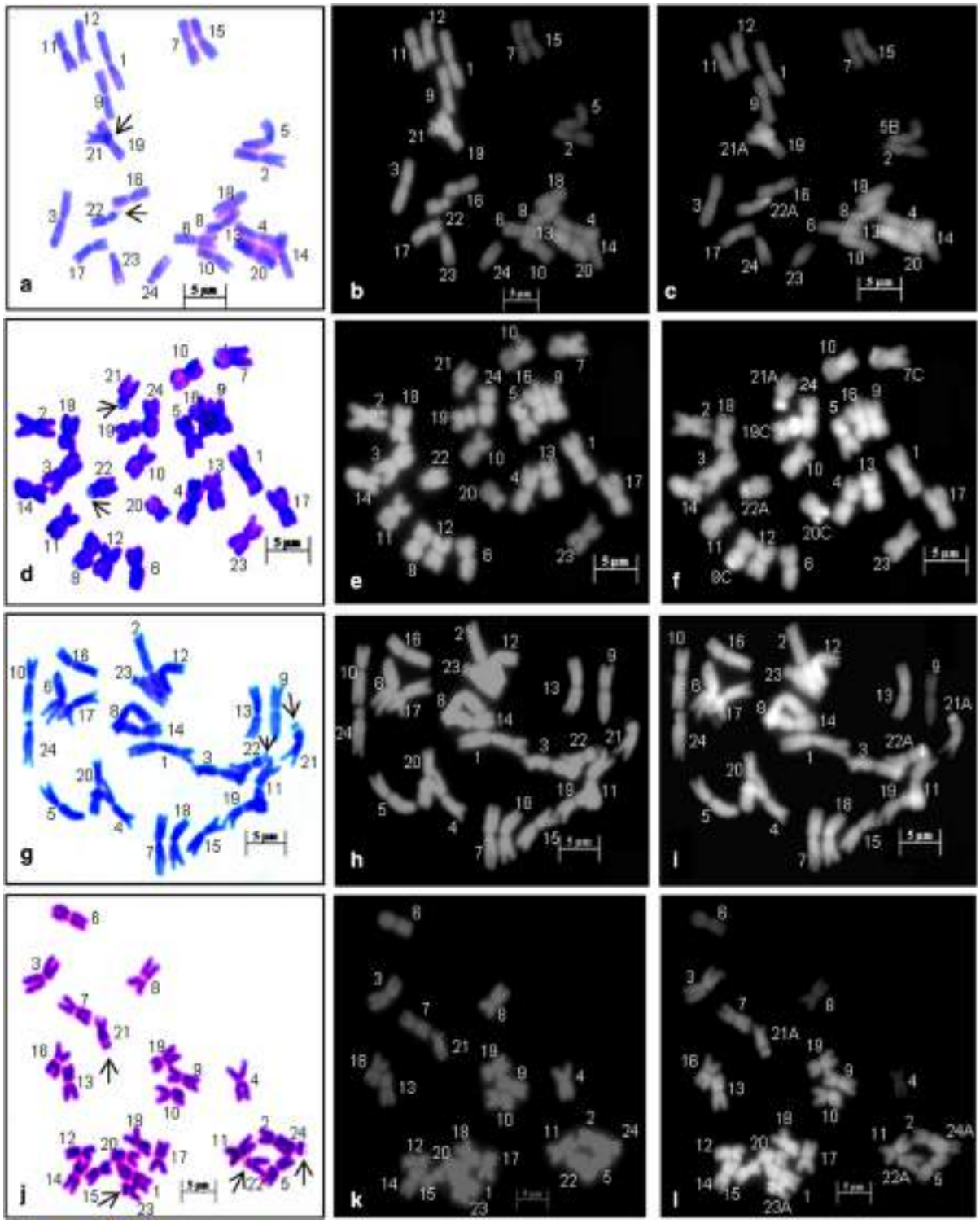
West Bengal, Assam, Manipur, Meghalaya and Kerala belonging to the three *Capsicum* species namely *C. annuum*, *C. frutescens* and *C. chinense*. We have previously reported detailed EMA based Giemsa karyotype analysis in nine different cultivars under three *Capsicum* species (Jha and Saha 2017). In the present study, we have added 6 cultivars of *C. annuum*, one under Akashi (CA-1), three under Bullet (CA-4, CA-5, CA-6), one under Kul (CA-3) and one under Sada (White, CA-7) from different geographical locations as detailed in Table1. Among these, Kul (CA-3) and Sada (White, CA-7) cultivars were not studied earlier and are completely different from other *C. annuum* cultivars. They were collected from the home gardens of Hooghly districts of West Bengal (Table1) and are new addition in this report. The Kul cultivar produces white pendant flowers/node with 5–6 petals, green round shaped fruits that turn red when ripe (Fig. 5c). On the other hand, the Sada (white) cultivar produces pale green white erect flowers/node with 6 petals that develop completely white fruits at the beginning and turns orange when ripe (Fig. 5g). Minimum 20 seeds from mature fruits of each cultivar were germinated in dark on moist filter papers to produce roots and also to grow into plants.

Chromosome preparation

Following our earlier method of EMA based chromosome preparation (Jha et al. 2012; Jha and Saha 2017), fresh slides were prepared from each of the cultivars included in present study (Table 1). Fixed roots were digested in enzyme cocktail [1% Cellulose (Onozuka RS), 0.75% Macerozyme (R-10), 0.15% Pectolyase (Y-23), 1 mM EDTA]. Enzyme digested root tips were uniformly macerated with 1:3 acetic methanol on clean slides and air dried. Chromosome preparations were stained with 2% Giemsa solution (Merck, Germany) in 1/15 phosphate buffer for 10–12 min. Slides were then air-dried, mounted with xylene for observation, making data sheet of well scattered metaphase plates and imaging under Axio Lab. A1 microscope fitted with CCD camera and computer.

Table 1 Collection details of Indian *Capsicum* species for present study

Species	Cultivar's popular name	Cultivar's code	Place of collection	Latitude- longitude	Cultivation status
C. annuum L.	Akashi	CA-1	Guwahati, Assam	26.1445° N, 91.7362° E	Wide cultivation
	Karenga	CA-2	Haldibari, West Bengal	26.3345° N, 88.7807° E	Limited cultivation
	Kul	CA-3	Hooghly, West Bengal	22.8963° N, 88.2461° E	Limited cultivation
	Bullet type I	CA-4	Kolkata, West Bengal	22.5726° N, 88.3639° E	Wide cultivation
	Bullet type II	CA-5	Ghatsila, Jharkhand	23.6102° N, 85.2799° E	Wide cultivation
	Bullet type III	CA-6	Tawang, Arunachal	28.2180° N, 94.7278° E	Wide cultivation
	Sada (white)	CA-7	Hooghly, West Bengal	22.8963° N, 88.2461° E	Limited cultivation
	Kalo (black)	CA-8	Siliguri, West Bengal	26.7271° N, 88.3953° E	Limited cultivation
	Ghee	CA-9	North 24 Pgs, West Bengal	22.6168° N, 88.4029° E	Limited cultivation
C. frutescens L.	Dhani type I	CF-1	Hooghly, West Bengal	22.8963° N, 88.2461° E	Limited cultivation
	Dhani type II	CF-2	Jalpaiguri, West Bengal	26.5215° N, 88.7196° E	Limited cultivation
	Dhani type III	CF-3	Shillong, Meghalaya	25.5788° N, 91.8933° E	Limited cultivation
	Dhani type IV	CF-4	Thekkady, Kerala	9.6031° N, 77.1615° E	Limited cultivation
C. chinense Jacq.	Bhut jolokia type I	CC-1	Shillong, Meghalaya	25.5788° N, 91.8933° E	Restricted cultivation
	Bhut jolokia type II	CC-2	Imphal, Manipur	27.4728° N, 94.9120° E	Restricted cultivation



◀**Fig. 1** Somatic metaphase chromosomes of *C. annuum* cultivars stained with Giemsa (**a, d, g, j**), DAPI (**b, e, h, k**) and CMA (**c, f, i, l**). **a–c** CA-1 (Akashi), **d–f** CA-2 (Karenga), **g–i** CA-3 (Kul), **j–l** CA-4 (Bullet type I). Black arrows indicate satellite bearing chromosomes in Giemsa plates, types of chromosomes with CMA bands (A, B, C) are indicated with block letters beside chromosome numbers in CMA plates. Bars 5 μm

Fluorochrome staining of somatic metaphase chromosomes

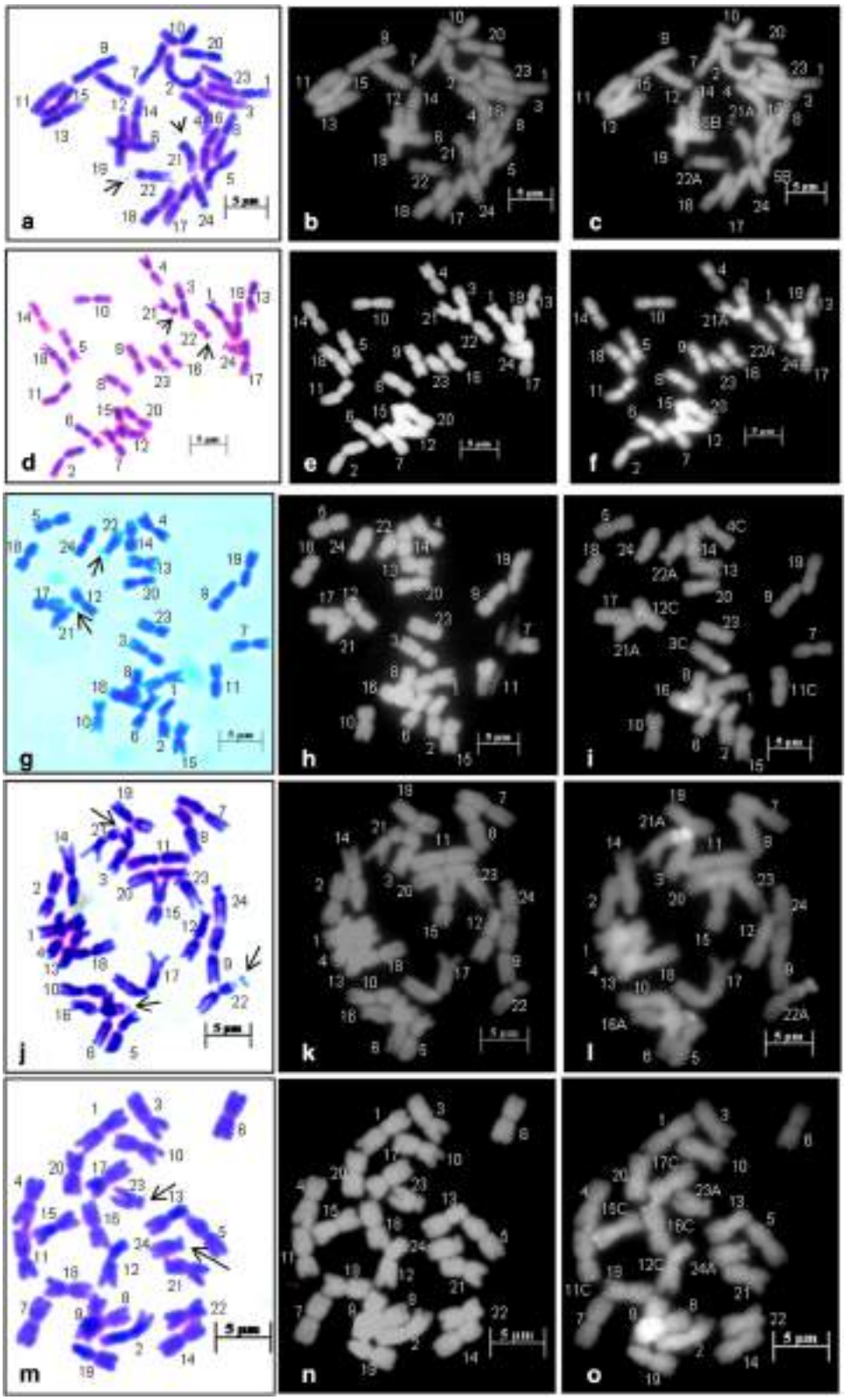
Giemsa slides were destained in 70% methanol for 45 min and air dried for staining with DAPI and CMA following our previous protocols (Jha and Yamamoto 2012; Bhowmick and Jha 2019) with required modifications. Slides were incubated in McIlvaine buffer I (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 7.0) for 30 min and stained with 0.1 μg mL^{-1} DAPI solution for 25 min in dark. Excess stain was washed off from slides in the same buffer and counterstained in Actinomycin D (0.25 mg/ml) for 15 min in dark. After washing off excess stain, slides were blow dried and mounted in non-fluorescent glycerol. DAPI stained chromosomes were observed under the same microscope with a UV filter cassette and images were captured with CCD camera. The slides were destained again and air dried. For CMA staining, slides were incubated in McIlvaine buffer I (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 7.0) for 30 min and then in McIlvaine buffer II (with, 5 mM $MgCl_2.6H_2O$) for 15–20 min. After blowing out buffer, slides were stained with 0.1 mg mL^{-1} CMA for 1 h in dark. Excess stain was washed off in McIlvaine buffer II, blow dried and mounted in non-fluorescent glycerol. Slides were kept in 4 °C refrigerator for maturation. Observations were taken under the same fluorescence microscope with BV filter cassette and images captured with CCD camera.

Karyotype analysis

Minimally 10 metaphase plates from each cultivar were selected for karyotype study and assessment of karyomorphometric parameters. Chromosome morphometric measurements were taken in Axiovision L.E 4 software for determination of long arm length (l), short arm length (s), chromosome length (CL) and total diploid chromosome length (TCL). Chromosomes were categorized based on centromere positions on the basis of arm ratios (r value = l/s) following the nomenclature of Levan et al. (1964). The chromosomes were arranged according to decreasing order of length and grouped according to arm ratio for presenting the ideograms of each cultivar. Inter- and intra-chromosomal asymmetry/symmetry in the karyotypes were evaluated after calculating values of about 9 well known indices viz. intrachromosomal asymmetry index (A1) and interchromosomal asymmetry index (A2) (Zarco 1986), total form percentage (TF%) (Huziwara 1962), asymmetry index of karyotype (AsK%) (Arano 1963), coefficients of variation of chromosome length (CV_{CL}), centromeric index (CV_{CI}) and arm ratio (CV_r) (Paszko 2006), asymmetry index (AI) (Paszko 2006) and Stebbins asymmetry categories (Stebbins 1971). At least 25 metaphase plates were analysed to count the number of distinct CMA positive and DAPI negative fluorescent bands in karyotype of each cultivar. Similarly we have identified the chromosome type and number bearing the fluorescent bands in each karyotype and idiogram of 15 cultivars.

Statistical analysis of karyotype relations

Chromosome morphometric data were analyzed by Descriptive statistics including means and standard deviations. One-way analysis of variance (ANOVA) was performed to detect significant differences ($p \leq 0.05$) in the mean (Rohlf 1998), if any. Duncan's multiple range test (DMRT) was used for post hoc analyses. All analyses were conducted at 0.05 probability level using IBM SPSS v16.0 statistical package. Karyotype relations among the cultivars was assessed with the help of cluster analysis for data matrix normalization by unweighted pair group method with arithmetic averages (UPGMA) based on Euclidean distance using Infostat 2017d (free version). Here we considered the values of standard karyotype symmetry/asymmetry indices (A1, A2, TF%, AsK%, CV_{CL}, CV_{CI}, CV_r, and AI). Since the present investigation focuses on chromosome banding



◀**Fig. 2** Somatic metaphase chromosomes of *C. annuum* cultivars stained with Giemsa (**a, d, g, j, m**), DAPI (**b, e, h, k, n**) and CMA (**c, f, i, l, o**). **a–c** CA-5 (Bullet type II), **d–f** CA-6 (Bullet type III), **g–i** CA-7 (Sada), **j–l** CA-8 (Kalo), **m–o** CA-9 (Ghee). Black arrows indicate satellite bearing chromosomes in Giemsa plates, types of chromosomes with CMA bands (A, B, C) are indicated with block letters beside chromosome numbers in CMA plates. Bars 5 µm

pattern, we have improved the procedure of statistical evaluation by including additional numerical parameters like the number of NORs/diploid complement *vis-a-vis* number of CMA bands (type A/B/C) per diploid complement of each cultivar.

Results

Karyotype analysis

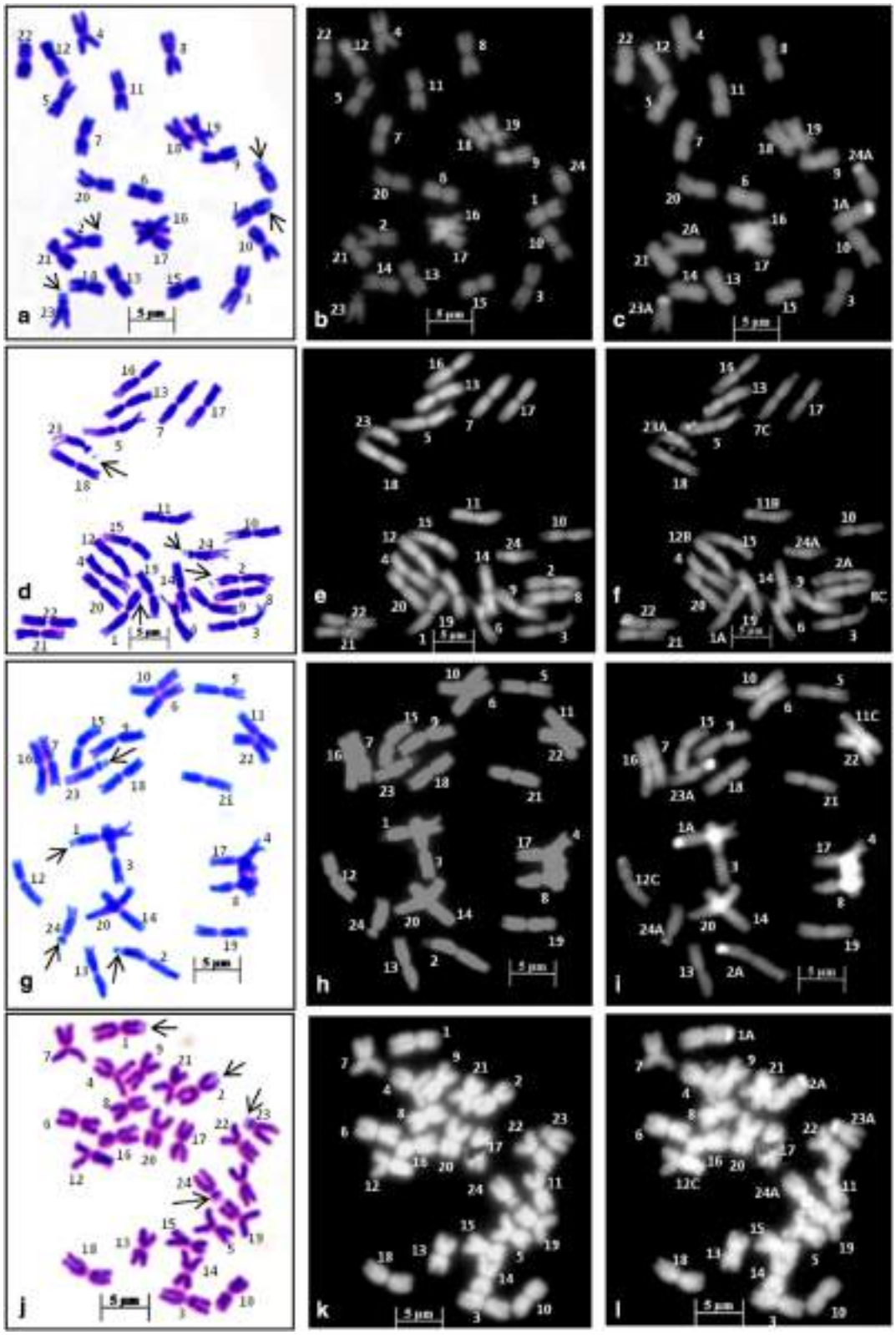
The presented *Capsicum* species and cultivars contain 2n = 24 chromosomes in their diploid complement (Figs. 1, 2, 3 and 4). However, variant chromosome numbers of 2n = 23 and 22 have been noted at a very low frequency (2–3%). For each cultivar, minimum 10 metaphase plates were considered for karyotype analysis. The karyotypes of all cultivars showed a prevalence of nearly metacentric chromosomes. However, considerable variation was noted in average chromosome length (ACL) that correlated directly with the variation in total diploid chromatin length (TCL) in all cultivars studied at present. Among the cultivars of *C. annuum* (CA-1 to 9), ACL ranged from 5.87 ± 0.74 to 3.88 ± 0.55 µm and the TCL ranged from 95.18 ± 3.14 to 141.10 ± 2.39 µm (Table 2). In addition to the nearly metacentric and metacentric chromosomes in the cultivars (Figs. 1a, d, g, j and 2a, d, g, j, m), an acrocentric pair was detected in CA-1 (Fig. 1a), CA-3 (Fig. 1g), CA-5 (Fig. 2a), CA-6 (Fig. 2d) and CA-8 (Fig. 2j) while CA7 showed the presence of a submetacentric pair (Fig. 2g) (Table 2). CA-3 showed highest ACL (5.87 ± 0.74 µm) and TCL (141.10 ± 2.39 µm) compared to the other CA cultivars (Table 2) and also other *Capsicum* species. The karyotype of CA-1, CA-3 and CA-5 was 18 m + 2 M + 2st + 2sm.sat (Table 2) while the other cultivars had slight differences in karyotypes (Table 2). A detailed karyotype analysis showed that the 11th pair bears satellite according to the order of chromosome length in the *C. annuum* cultivars except CA-9. The satellite bearing chromosome was the 12th pair in the CA-9 cultivar (Table 2). Additionally, the CA-4 cultivar contained two satellite bearing pairs (11th and 12th) and CA-8 contained three satellite bearing chromosomes (11th pair and chromosome no. 16) (Table 2). However, some cells showed the presence of one satellite bearing pair in CA-8 like in our earlier investigation (Jha and Saha 2017).

The cultivars of *C. frutescens* (CF-1 to 4) showed ACL ranging from 3.51 ± 0.40 to 4.77 ± 2.10 µm and the TCL ranged from 84.42 ± 2.14 to 114.51 ± 1.34 µm (Table 2). CF-4 cultivar showed highest ACL and TCL among the *C. frutescens* cultivars. Chromosome complement of all cultivars had nearly metacentric and metacentric chromosomes with two nucleolar pairs (Fig. 3a, d, g and j, Table 2) and the karyotype was determined as 18 m + 2 M + 2 m.sat + 2st.sat. One of nucleolar pairs (m.sat) was the longest pair (1st) in the complement while the other (st.sat) was the shortest (12th) in terms of chromosome length.

In case of *C. chinense*, ACL ranged from 3.85 ± 0.44 to 4.84 ± 1.02 µm and the range of TCL was 92.47 ± 1.28 to116.18 ± 1.25 µm (Table 2). The karyotype of the cultivars had nearly metacentric chromosomes and a pair of satellite bearing chromosomes (Fig. 4a, d) and the karyotype was 22 m + 2st.sat (Table 2). The satellite baring pair was the shortest (12th) pair in the complement in terms of chromosome length.

Fluorochrome banding pattern

Application of fluorochrome staining resulted in appearance of distinct and scorable CMA^{+ve} bands in the chromosomes of *Capsicum* cultivars (Figs. 1, 2, 3 and 4). DAPI positive signals were not observed in any of the cultivars. However, distinct $DAPI^{-ve}$ sites were observed corresponding with CMA^{+ve} signals. The $CMA^{+ve}/DAPI^{-ve}$ bands followed a differential pattern of distribution in the cultivars studied (Figs. 5 and 6). The number and position of the $CMA^{+ve}/DAPI^{-ve}$ bands were considered to be the parameters for chromosome identification and characterization. We had considered the pattern of fluorochrome banding in at least 25 metaphase spreads of each cultivar for determination of the types of signals. For convenience of data interpretation in the cultivars,



◀**Fig. 3** Somatic metaphase chromosomes of *C. frutescens* cultivars stained with Giemsa (**a, d, g, j**), DAPI (**b, e, h, k**) and CMA (**c, f, i, l**). **a–c** CF-1 (Dhani type I), **d–f** CF-2 (Dhani type II), **g–i** CF-3 (Dhani type III), **j–l** CF-4 (Dhani type IV) Black arrows indicate satellite bearing chromosomes in Giemsa plates, types of chromosomes with CMA bands (A, B, C) are indicated with block letters beside chromosome numbers in CMA plates. Bars 5 μm

CMA^{+ve}/ $DAPI^{-ve}$ bands appearing at the nucleolar regions of satellite bearing chromosomes were typified as 'A', CMA^{+ve}/ $DAPI^{-ve}$ bands found at intercalary region of chromosomes as 'B' and those occurring at telomeric (distal) region of non-nucleolar chromosomes were typed as 'C'. Chromosomes with no CMA bands were named type D. The metaphase chromosomes with CMA^{+ve}/$DAPI^{-ve}$ bands are presented along with Giemsa stained plates (Figs. 1, 2, 3 and 4) for convenience of ideogram analysis following the respective CMA banding pattern (Figs. 5 and 6) as mentioned above.

C. annuum

Three types of CMA^{+ve}/ $DAPI^{-ve}$ bands were found to occur in the different cultivars (Table 2). In CA-1 cultivar, two CMA^{+ve}/ $DAPI^{-ve}$ bands (A) appeared at the satellite region of the nucleolar chromosomes (Fig. 1a–c, Table 2). In addition, one intercalary CMA^{+ve} band (B) was observed in one of the nearly metacentric chromosomes in the third pair (Fig. 5a). CMA banding pattern in CA-1 was 2A + 1B + 21D. The CMA^{+ve} bands were moderately intense in this cultivar (Fig. 1a–c). The CA-2 cultivar showed the presence of 6 CMA signals in the somatic chromosome complement (Table 2). Apart from type A bands in the pair of nucleolar chromosomes, 4 distal CMA^{+ve}/ $DAPI^{-ve}$ bands (type C) were observed in two nearly metacentric pairs (Table 2, Figs. 1d–f, 5b). The CMA^{+ve} bands were fairly intense in CA-2. CMA banding pattern in this cultivar was determined as 2A + 4C + 18D. In CA-3, CMA^{+ve}/ $DAPI^{-ve}$ bands were found at nucleolar regions (type A) (Table 2, Figs. 1g–i, 5c). The CMA bands in CA-3 were fairly intense. Chromosome banding pattern in this cultivar was 2A + 22D. In case of CA-4, nucleolar (type A) CMA bands had been observed in the two pairs of satellited chromosomes (Table 2, Figs. 1j–l, 5d). The signals were moderately intense. CMA banding pattern was determined as 4A + 20D in CA-4. The presence of two nucleolar pairs of chromosomes could be confirmed by the co-localized type A CMA positive bands in this cultivar. The CA-5 cultivar had 4 CMA bands of which type A bands were found in the pair of satellite bearing chromosomes (Table 2, Figs. 2a–c, 5e). However, the intensity of type A bands were very poor. Two intercalary CMA^{+ve}/ $DAPI^{-ve}$ bands (type B) were detected in one pair of nearly metacentric chromosomes (Table 2, Figs. 2a–c, 5e). CMA banding pattern in this cultivar was 2A + 2B + 20D. Type B bands were slightly brighter than type A bands in CA-5. In case of CA-6, two moderately intense type A bands were detected in a pair of nucleolar chromosomes (Table 2, Figs. 2d–f, 5f). CMA banding pattern in CA-6 was 2A + 22D. In CA-7, there were six fairly intense CMA signals (Table 2, Fig. 2g–i). Two of the bands were nucleolar (type A) and rest were distal (type C) (Fig. 5g). The CMA banding pattern was 2A + 4C + 18D. In case of CA-8, the nucleolar chromosomes showed intense CMA^{+ve}/ $DAPI^{-ve}$ bands at satellite regions. The number of such signals was found to be 3, corresponding with 3 NORs detected in somatic complement (Table 2, Figs. 2j–l, 5h). The CMA banding pattern in CA-8 was 3A + 21D. We observed highest number of CMA signals in the CA-9 cultivar (Table 2, Fig. 2m–o). There were two intense type A bands in the satellited pair of chromosomes (Figs. 2m–o, 5i). Additionally, we found distal CMA bands (type C) in 5 chromosomes (Table 2, Figs. 2m–o, 5i). The CMA banding pattern was 2A + 5C + 17D in CA-9 cultivar.

C. frutescens

Three types of CMA^{+ve}/ $DAPI^{-ve}$ bands (types A, B and C) were found in the different cultivars (Table 2). However, the number of type A bands was consistently found to be 4 per somatic chromosome complement in all cultivars studied (Fig. 3). In CF-1, there were four CMA^{+ve}/ $DAPI^{-ve}$ bands (type A) occurring at the nucleolar regions of the largest and smallest satellite bearing chromosome pairs (Table 2, Fig. 3a–c). Intensity of the CMA^{+ve}/ $DAPI^{-ve}$ bands was considerably fair in CF-1. In case of the longest pair of nucleolar chromosomes, one of the chromosomes showed greater intensity and size of type A (nucleolar) CMA^{+ve} band compared to its homologue (Figs. 3b, c, 6a). CMA banding pattern in CF-1 was

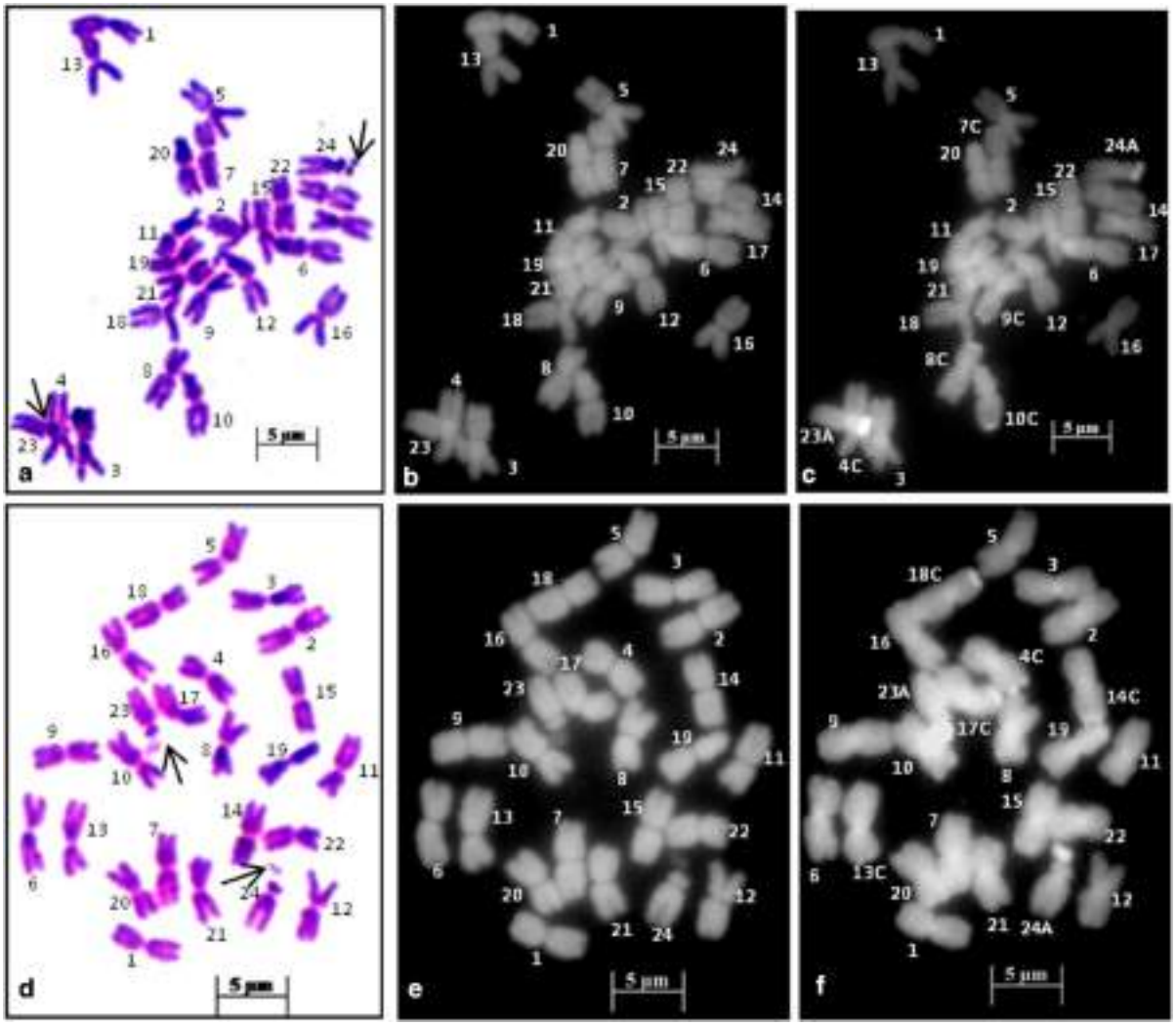


Fig. 4 Somatic metaphase chromosomes of *C. chinense* cultivars stained with Giemsa (**a, d**), DAPI (**b, e**) and CMA (**c, f**). **a–c** CC-1 (Bhut jolokia type I), **d–f** CC-2 (Bhut jolokia type II). Black arrows indicate satellite bearing chromosomes in Giemsa plates, types of chromosomes with CMA bands (A, B, C) are indicated with block letters beside chromosome numbers in CMA plates. Bars 5 µm

4A + 20D. In CF-2, eight CMA^{+ve} bands were found in the diploid chromosome complement (Table 2, Figs. 3d–f, 6b). The intensity of CMA^{+ve} signals was quite low compared to CF-1. Type A nucleolar CMA^{+ve} bands were found in the two satellite bearing pairs. In addition, intercalary and distal CMA^{+ve} bands (types B and C, respectively) were scored in two different pairs of nearly metacentric chromosomes (Table 2, Figs. 3e–f, 6b). CMA banding pattern in CF-2 was 4A + 2B + 2C + 16D. In CF-3, the 6 bright CMA^{+ve}/ $DAPI^{-ve}$ bands were observed (Table 2, Fig. 3g–i). Out of them, four type A bands were found in the nulceolar regions. However, intensity and size of the type A nucleolar band was different in the two chromosomes of the smallest satellite bearing pair (st.sat) in this cultivar (Figs. 3h–i, 6c). Additionally, distal type C band was observed in a nearly metacentric pair (Table 2, Figs. 3h–i, 6c). CMA banding pattern in this cultivar was 4A + 2C + 18D. The CF-4 cultivar had 5 fairly intense CMA^{+ve}/$DAPI^{-ve}$ bands in the diploid complement (Table 2, Figs. 3j–l, 6d). Among them, the four nucleolar CMA^{+ve}/ $DAPI^{-ve}$ bands (type A) were detected in the satellite bearing chromosomes along with one prominent

Table 2 Chromosome measurements and CMA banding pattern in *Capsicum* cultivars

Species	Cultivar's code	CSR (μm)		TCL (μm) (mean ± S.D.)	ACL (μm) (mean ± S.D.)	Karyotype	No. of CMA bands/ 2n	Chromosomes showing bands		CMA
		Absolute (mean ± S.D.)	Relative (mean ± S.D.)					Chromosome pair/ chromosome no	Position of band	Type
C. annuum	CA-1	7.20 ± 0.24^{e} – 3.6 ± 0.02^{ab}	5.89 ± 0.57^{c}– 2.93 ± 0.54^{a}	122.05 ± 2.00^{c}	4.35 ± 0.07^{b}	18 m + 2 M + 2st + 2sm.sat	3	11th (sm.sat)	Nucleolar	A
								5 (m)	Intercalary	B
	CA-2	5.87 ± 0.14^{bc}– 3.26 ± 0.10^{ab}	5.83 ± 0.45^{c}– 3.25 ± 0.33^{a}	100.91 ± 6.16^{b}	4.20 ± 0.66^{b}	20 m + 2 M + 2st.sat	6	11th (st.sat)	Nucleolar	A
								4th (m), 10th (m)	Distal	C
	CA-3	8.5 ± 0.12^{f}– 4.25 ± 0.47^{c}	6.02 ± 0.50^{d}– 3.01 ± 0.36^{a}	141.10 ± 2.39^{e}	5.87 ± 0.74^{d}	18 m + 2 M + 2st + 2 m.sat	2	11th (m.sat)	Nucleolar	A
	CA-4	5.05 ± 0.54^{b}– 3.05 ± 0.27^{a}	6.04 ± 0.05^{d}– 3.20 ± 0.39^{a}	95.18 ± 3.14^{a}	3.88 ± 0.55^{a}	16 m + 4 M + 2st.sat + 2sm.sat	4	11th (st.sat), 12th (sm.sat)	Nucleolar	A
	CA-5	8.06 ± 0.66^{f}– 4.02 ± 0.81^{c}	5.85 ± 0.20^{c}– 2.95 ± 0.45^{a}	137.38 ± 3.05^{d}	5.73 ± 0.40^{cd}	18 m + 2 M + 2st + 2sm.sat	4	11th (sm.sat)	Nucleolar	A
								3rd (m)	Intecalary	B
	CA-6	6.00 ± 0.35^{c}– 3.00 ± 0.00^{a}	5.51 ± 0.10^{bc}– $.75 \pm 0.05^{a}$	105.60 ± 4.03^{b}	4.40 ± 0.16^{b}	16 m + 4 M + 2st + 2 m.sat	2	11th (m.sat)	Nucleolar	A
	CA-7	4.75 ± 0.22^{a}– 3.00 ± 0.07^{a}	4.85 ± 0.33^{a}– 3.06 ± 0.20^{a}	97.93 ± 1.88^{ab}	3.91 ± 0.67^{a}	18 m + 2 M + 2sm + 2st.sat	6	11th (st.sat)	Nucleolar	A
								2nd (m), 6th (m)	Distal	C
	CA-8	6.76 ± 0.50^{d}– 4.15 ± 2.00^{c}	5.88 ± 0.22^{c}– 3.65 ± 0.11^{b}	115.29 ± 2.41^{c}	4.86 ± 0.30^{bc}	15 m + 4 M + 2st + 1 m.sat + 2sm.sat	3	11th (sm.sat), 16 (m.sat)	Nucleolar	A
	CA-9	5.92 ± 0.21^{bc}– 4.22 ± 0.00^{c}	4.72 ± 0.07^{a}– 3.37 ± 0.17^{b}	125.35 ± 6.54^{c}	5.22 ± 0.43^{c}	22 m + 2st.sat	7	12th (st.sat)	Nucleolar	A
								6th (m), 8th (m), 17 (m)	Distal	C

Table 2 continued

Species	Cultivar's code	CSR (μm)		TCL (μm) (mean ± S.D.)	ACL (μm) (mean ± S.D.)	Karyotype	No. of CMA bands/ 2n	Chromosomes showing bands		CMA
		Absolute (mean ± S.D.)	Relative (mean ± S.D.)					Chromosome pair/ chromosome no	Position of band	Type
C. frutescens	CF-1	5.15 ± 0.20^{b}–3.60 ± 0.71^{b}	5.08 ± 0.26^{a}–3.55 ± 0.20^{ab}	101.36 ± 2.01^{b}	4.22 ± 0.60^{b}	18 m + 2 M + 2 m.sat + 2st.sat	4	1st (m.sat), 12th (st.sat)	Nucleolar	A
	CF-2	4.23 ± 0.26^{a}–2.89 ± 0.14^{a}	5.00 ± 0.19^{a}–3.42 ± 0.08^{a}	84.42 ± 2.14^{a}	3.51 ± 0.40^{a}	18 m + 2 M + 2 m.sat + 2st.sat	8	1st (m.sat), 12th (st.sat)	Nucleolar	A
								6th (m)	Intercalary	B
								4th (m)	Distal	C
	CF-3	4.39 ± 0.07^{a}–2.88 ± 0.13^{a}	5.13 ± 0.19^{b}–3.37 ± 0.22^{a}	85.45 ± 1.79^{a}	3.56 ± 0.40^{a}	18 m + 2 M + 2 m.sat + 2st.sat	6	1st (m.sat), 12th (st.sat)	Nucleolar	A
								6th (m)	Distal	C
	CF-4	6.00 ± 0.14^{c}–3.80 ± 0.00^{b}	5.23 ± 0.30^{b}–3.31 ± 0.07^{a}	114.51 ± 1.34^{c}	4.77 ± 2.10^{bc}	18 m + 2 M + 2 m.sat + 2st.sat	5	1st (m.sat), 12th (st.sat)	Nucleolar	A
								12 (m)	Distal	C
C. chinense	CC-1	4.66 ± 0.20^{a}–3.02 ± 0.07^{a}	5.04 ± 0.15^{a}–3.26 ± 0.03^{a}	92.47 ± 1.28^{a}	3.85 ± 0.44^{a}	22 m + 2st.sat	7	12th (st.sat)	Nucleolar	A
								4 (m), 4th (m), 5th (m)	Distal	C
	CC-2	5.95 ± 1.34^{b}–3.70 ± 0.98^{b}	5.12 ± 1.05^{a}–3.18 ± 0.77^{a}	116.18 ± 1.25^{b}	4.84 ± 1.02^{b}	22 m + 2st.sat	7	12th (st.sat)	Nucleolar	A
								4 (m), 7th (m), 9th (m)	Distal	C

Values followed by same superscript letter are not significantly different according to Duncan's multiple range test ($p = 0.05$)

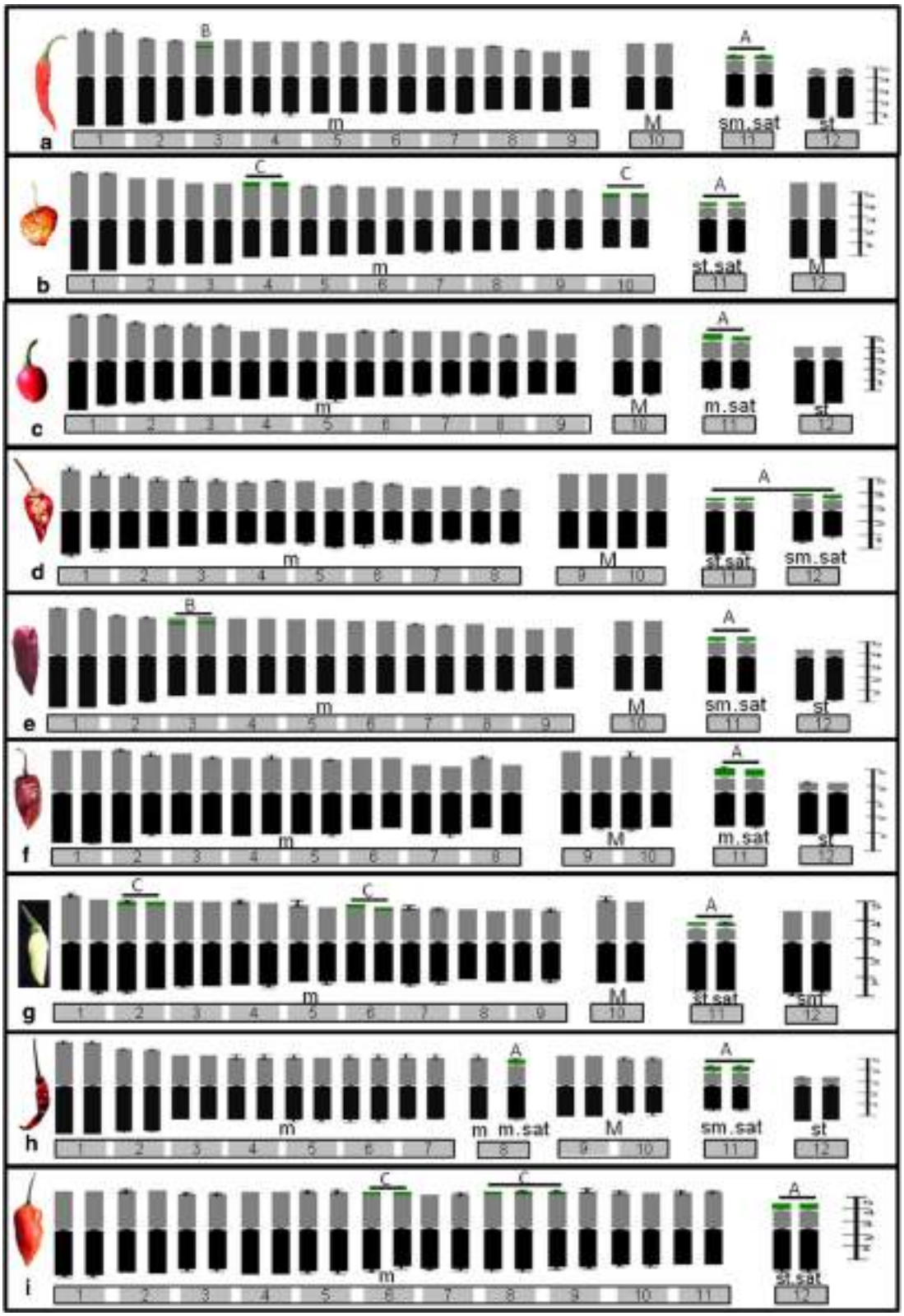
CSR chromosome size range, *TCL* total diploid chromatin length, *ACL* average chromosome length

Table 3 Comparative analysis of karyotype asymmetry/symmetry in *Capsicum* cultivars

Parameters	*C. annuum*									*C. frutescens*				*C. chinense*	
	CA-1	CA-2	CA-3	CA-4	CA-5	CA-6	CA-7	CA-8	CA-9	CF-1	CF-2	CF-3	CF-4	CC-1	CC-2
A1	0.782^{a}	0.787^{b}	0.783^{a}	0.780^{a}	0.785^{a}	0.781^{a}	0.790^{b}	0.787^{b}	0.798^{c}	0.791^{b}	0.814^{c}	0.813^{c}	0.798^{c}	0.795^{c}	0.791^{b}
A2	0.016^{a}	0.061^{g}	0.029^{d}	0.018^{ab}	0.018^{ab}	0.032^{d}	0.031^{d}	0.037^{e}	0.052^{f}	0.021^{b}	0.025^{c}	0.021^{b}	0.020^{b}	0.013^{a}	0.018^{ab}
TF%	42.812^{b}	43.620^{g}	44.570^{jk}	43.188^{c}	43.353^{e}	43.580^{f}	42.301^{a}	43.645^{g}	44.017^{i}	44.420^{j}	43.952^{h}	44.035^{i}	43.233^{d}	43.975^{h}	44.171^{ij}
AsK%	57.185^{e}	55.625^{bc}	58.344^{f}	56.524^{de}	56.372^{d}	56.204^{d}	54.630^{a}	55.712^{c}	55.391^{b}	55.793^{c}	54.435^{a}	54.840^{ab}	56.671^{de}	55.419^{b}	55.163^{b}
CV_{CL}	16.931^{ef}	16.147^{e}	16.391^{e}	18.203^{f}	18.521^{f}	18.532^{f}	11.367^{c}	18.845^{f}	8.332^{a}	10.656^{b}	11.439^{c}	11.321^{c}	13.176^{d}	11.675^{c}	11.507^{c}
CV_{CI}	37.582^{h}	16.569^{b}	29.470^{f}	44.068^{j}	25.654^{e}	32.621^{fg}	31.521^{fg}	16.448^{b}	18.090^{d}	32.530^{fg}	17.650^{c}	14.550^{a}	41.653^{i}	17.708^{c}	17.216^{c}
CV_{r}	54.433^{e}	50.532^{d}	55.277^{e}	60.756^{g}	55.261^{e}	58.906^{fg}	49.005^{d}	45.882^{b}	64.033^{h}	50.033^{d}	57.590^{f}	39.234^{a}	47.091^{c}	60.797^{g}	57.341^{f}
AI	6.362^{h}	2.675^{c}	4.835^{f}	8.020^{i}	4.752^{f}	6.061^{gh}	3.586^{e}	3.099^{d}	1.507^{a}	3.466^{e}	2.019^{ab}	1.647^{a}	5.483^{g}	2.067^{ab}	1.981^{ab}
Stebbins group	1A	1A	1A	1A	1A	1A	1A	1A	1A	1A	1A	1A	1A	1A	1A

Values followed by same superscript letter are not significantly different according to Duncan's multiple range test ($p = 0.05$)

CA-1: Akashi, CA-2: Karenga, CA-3: Kul,CA-4: Bullet type I, CA-5: Bullet type II, CA-6: Bullet type III, CA-7: Sada, CA-8: Kalo, CA-9: Ghee, CF-1: Dhani type-I, CF-2: Dhani type II, CF-3: Dhani type III, CF-4: Dhani type IV, CC-1: Bhut jolokia type I, CC-1: Bhut jolokia type II. A1: intra-chromosomal asymmetry index (Zarco 1986), A2: inter-chromosomal asymmetry index (Zarco 1986), TF%: total form percent (Huziwara 1962), AsK%: asymmetric karyotype percent (Arano 1963), CV_{CL}: coefficient of variation of chromosome length (Paszko 2006), CV_{CI}: coefficient of variation of centromeric index (Paszko 2006), C_{Vr}: coefficient of variation of arm ratio(Paszko 2006), AI: asymmetry index (Paszko 2006), Stebbins group: Stebbins asymmetry categories (Stebbins 1971)



◀**Fig. 5** Ideograms of *C. annuum* cultivars based on CMA^{+ve}/$DAPI^{-ve}$ banding pattern presented with respective fruit morphotypes. **a** CA-1 (Akashi), **b** CA-2 (Karenga), **c** CA-3 (Kul), **d** CA-4 (Bullet type I), **e** CA-5 (Bullet type II), **f** CA-6 (Bullet type III), **g** CA-7 (Sada), **h** CA-8 (Kalo), **i** CA-9 (Ghee). Pairs are numbered in grey boxes below chromosome diagrams. Types of CMA bands (green bands) are written above the chromosome diagrams as A: nucleolar CMA^{+ve}/$DAPI^{-ve}$ bands, B: intercalary CMA^{+ve}/$DAPI^{-ve}$ bands and C: telomeric (distal) CMA^{+ve}/$DAPI^{-ve}$ bands. Bars 5 μm

CMA^{+ve} band in the distal part (type C) of one nearly metacentric chromosome of the 6th pair (Figs. 3k–l, 6d). The CMA banding pattern in CF-4 was 4A + 1C + 19D.

C. chinense

Two types of CMA^{+ve}/$DAPI^{-ve}$ bands (types A and C) were seen in the *C. chinense* cultivars. All CMA bands were fairly intense. Type A CMA^{+ve}/ $DAPI^{-ve}$ bands were found in the nucleolar regions of the satellite bearing pair (12th, st.sat) in the cultivars (Table 2, Fig. 4a–c, d–f). Five distal type C CMA^{+ve}/ $DAPI^{-ve}$ bands were observed in the nearly metacentric chromosomes of both the cultivars (Table 2, Fig. 4). The chromosomes showing distal bands were found to be different in the CC-1 and CC-2 cultivars which is represented in the ideograms (Figs. 4, 6e, f). The total number of CMA bands was 7 in the somatic complement and CMA banding pattern of *C. chinense* was 2A + 5C + 17D.

Comparative statistical evaluation of karyomorphometric parameters

We have performed comparative analysis of karyomorphometric features in all of the 15 *Capsicum* cultivars. The values of different parameters were found to corroborate to our previous report for those accessions studied before (Jha and Saha 2017). Considering intra-chromosomal asymmetry, the A2 values showed greatest variation in between the *C. annuum* cultivars ranging from 0.016- 0.061 while the *C. frutescence* and *C. chinense* cultivars had less variation (0.020–0.025 and 0.013–0.018, respectively) (Table 3). The CV_{CL} values also have much variation in CA cultivars (8.332–18.845) than the CF (10.65–13.17) and CC (11.507–11.675) cultivars (Table 3). Greater difference in chromosome size among CA cultivars resulted in much difference in A2 and CV_{CL} values. Considering the intra-chromosomal asymmetry, the A1 values were slightly greater in most of the CF (0.791–0.814) and CC (0.791–0.795) cultivars than the CA cultivars (0.780–0.798), indicating little trend of asymmetry in CF and CC cultivars (Table 3). Values of the other intra-chromosomal asymmetry parameters (TF%, AsK%, CV_{CI}, CV_r and AI) were found to specifically vary from one to the other cultivar (Table 3). However, the Stebbins category 1A was consistent among the cultivars that indicated a general tendency for symmetric karyotypes, corresponding to our previous study (Jha and Saha 2017).

Statistical evaluation of karyotype relations among the three species and their cultivars was done using Euclidean distance matrix using the 8 numerical parameters along with the number of NORs and CMA bands of different types (A, B and C). The UPGMA phenogram highlighted relative karyotype affinities and distances at inter-specific and intra-specific levels with a cophenetic correlation of 0.861 as a good fit between the cophenetic value matrix and the average Euclidean distance matrix (Fig. 7). The cultivars of *C. annuum*, *C. frutescens* and *C. chinense* were well separated into the clusters as per our previous study. The present statistical analysis has been advanced by incorporation of CMA banding and inclusion of more diverse accessions. Within the *C. annuum* cluster, we could observe that the Akashi (CA-1) and the Bullet accessions (CA-4, CA-5 and CA-6) formed a cluster along with Kul (CA-3) distinct from the other CA cultivars (Fig. 7), corroborating to our previous study (Jha and Saha 2017). The second cluster of *C. annuum* consisted of Karenga (CA-2), Kalo (Black) (CA-8) followed by Sada (White) (CA-7) cultivars (Fig. 7). The Karenga and Kalo cultivars were found to have karyotype affinity previously (Jha and Saha 2017) which has been strengthened in present analysis. However, Sada (CA-7) is showing some affinity with the cluster of *C. chinense* cultivars. The Ghee cultivar in our earlier analysis showed its distance from rest of the CA cultivars (Jha and Saha 2017). Presently, we also obtained similar distancing results of Ghee (CA-9) from other *C. annuum* cultivars and noted some proximity with *C. chinense* in the phenogram (Fig. 7). It appears from the phenogram that *C. chinense* group arose from a single node. And

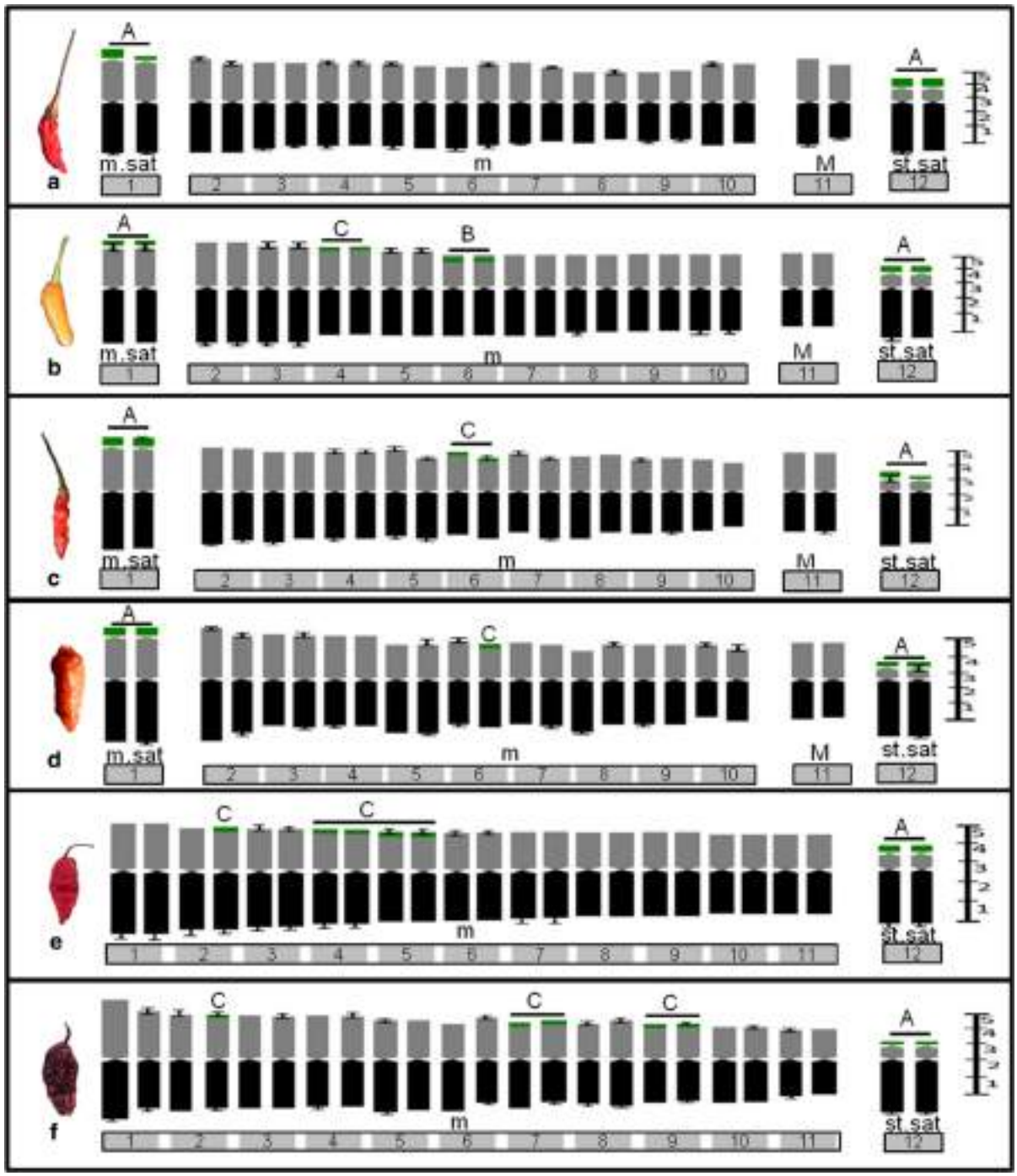


Fig. 6 Ideograms of *C. frutescens* and *C. chinense* cultivars based on $CMA^{+ve}/DAPI^{-ve}$ banding pattern presented with respective fruit morphotypes. **a** CF-1 (Dhani type I), **b** CF-2 (Dhani type II), **c** CF-3 (Dhani type III), **d** CF-4 (Dhani type IV), **e** CC-1 (Bhut jolokia type I), **f** CC-2 (Bhut jolokia type II). Pairs are numbered in grey boxes below chromosome diagrams. Types of CMA bands (green bands) are indicated above the chromosome diagrams as A: nucleolar $CMA^{+ve}/DAPI^{-ve}$ bands, B: intercalary $CMA^{+ve}/DAPI^{-ve}$ bands and C: telomeric (distal) $CMA^{+ve}/DAPI^{-ve}$ bands. Bars 5 μm

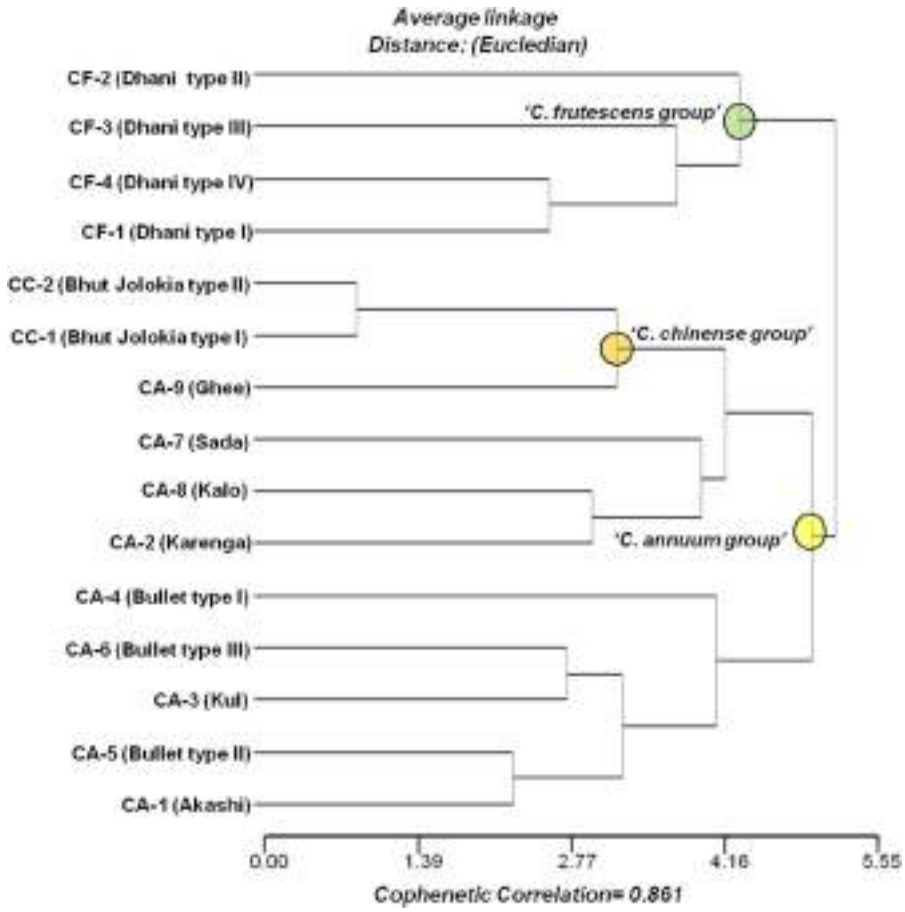


Fig. 7 UPGMA dendrogram derived from average Euclidean distance among 15 *Capsicum* cultivars using CMA banding patterns and karyomorphometric parameters

C. frutescens cultivars (CF-1- CF-4) also emerged as a distinct cluster from a single node in the phenogram (Fig. 7). The cultivars of *C. frutescens* had affinity among each other in a group which was quite separable from rest of the cultivars in terms of the karyotype relationships.

Discussion

Conspecific nature of *C. annuum*, *C. frutescens* and *C. chinense* as a part of '*C. annuum*' complex was supported historically by researchers because of overlapping morphological parameters, DNA sequence similarities and absence of strict crossing barriers (Pickersgill 1991; Walsh and Hoot 2001). The NEH regions of India have been renowned worldwide for being the centre of diversity of *Capsicum* species including the hottest cultivars of *C. chinense* (Bosland and Baral 2007). Immense genetic variation in the cultivars of *C. annuum* and *C. frutescens* is also noted in West Bengal and other states of India. Although genome sequencing of the Mexican *Capsicum annuum* and wild *C. chinense* has been reported (Kim et al. 2014), chromosome analysis is fundamentally germane to address diversity of unexplored cultivars of India's own germplasm. We could come over the limitations of conventional squash method to gain better visibility of chromosome morphology by EMA method (Jha and Saha 2017) compared to the earlier published karyotype reports on Indian capsicums (Cheema and Pant 2013). We had targeted chromosome characterization that starts with the right choice of roots, pretreatment and fixation as the general prerequisite for squash based or alternative methods for accurate karyotyping. In our present approach,

EMA-based fluorochrome staining has been done for the first time in Indian *Capsicum* species. We have established the *Capsicum* chromosome catalogue as the foundational set of genomic information on cultivars belonging to different agro-climatic zones of India.

Capsicum species show a general consistency in chromosome number (2n = 24) which corresponds to previous studies (Huskins and La-Cour 1930; Dixit 1931; Sugiura 1936; Raghavan and Venkatasubban 1940; Sinha 1950; Darlington and Wylie 1955; Emboden 1961; Ohta 1962; Chennaveeraiah and Habib 1966; Datta 1966; Pickersgill 1971; Moscone et al. 1996; Pozzobon et al. 2006; Cheema and Pant 2013; Jha and Saha 2017) in spite of diverse morpho-ecological variations. At the level of karyotype, the cultivars have a dominance of nearly metacentric chromosomes though there are differences in karyomorphometric parameters, corroborating to our previous findings (Jha and Saha 2017). All of the *C. annuum* cultivars considered in our study have the satellited chromosome in the 11th pair except Ghee (CA-9) where the satellited chromosome is in the 12th pair. The 12th pair of chromosomes bears satellite part in all the cultivars of *C. frutescens* and *C. chinense*. We could validate our observation with the nucleolar CMA bands in the 11th and 12th pair of chromosomes in *Capsicum* species. Previously, the 11th and 12th pairs were reported to contain NOR in Brazilian cultivars of *C. annuum* while 12th pair was reported to be nucleolar in *C. frutescens* and *C. chinense* (Moscone et al. 1996). Thus, the 11th and 12th pairs of chromosomes appear to be evolutionarily conserved in the *C. annuum* complex and serve as the marker chromosomes for species characterization. Additionally, the longest chromosome pair (1st) invariably contains satellite in all *C. frutescens* cultivars which is confirmed in our present study. Hence, the 1st nucleolar pair also becomes a species specific marker in Indian *C. frutescens*, corroborating with previous reports (Moscone et al. 1996). The cultivars of *C. annuum*, *C. frutescens* and *C. chinense* were well separated into different clusters in UPGMA dendrogram based on CMA banding pattern and other karyotype symmetry/asymmetry parameters, corroborating to our previous study (Jha and Saha 2017). However, we confirmed deviation of the Ghee cultivar from other *C. annuum* cultivars, a trend observed in earlier report (Jha and Saha 2017). The karyotype, pattern of nucleolar and non-nucleolar CMA bands suggested proximity of Ghee with *C. chinense*. The affinity is also reflected in morphological appearance of the fruit. We could statistically justify our observation from clustering of Ghee in a group with *C. chinense* in the UPGMA phenogram. Interspecific hybridization had been formerly suggested in Bhut jolokia cultivar (Bosland and Baral 2007). Hence, there might be a possibility of inter-specific hybridization between Bhut jolokia of NEH region and certain cultivar of *C. annuum*, giving rise to the deviant Ghee cultivar in the plains. Apart from Ghee, existence of additional satellite bearing pairs in one type of Bullet (type I) and Kalo cultivars point out to diversity in the NORs among cultivars of *C. annuum*. Again, Bullet and Kalo retain the marker nucleolar chromosome (11th), typical of *C. annuum*. The Bullet types collected from different locations indeed had prominent differences in CMA banding pattern. The type II cultivar had least intense nucleolar CMA^{+ve} bands. The intercalary B type CMA band in the 3rd chromosome pair of this cultivar was brighter than the nucleolar bands, pointing out to a pronounced chromosomal distinction. Considering the apparent intensity, CMA bands were fairly intense in *C. chinense*, *C. frutescens* and Ghee cultivar of *C. annuum* compared to the moderately intense bands in the CA cultivars. Intensity and size of nucleolar bands was found to differ between the homologues of the longest pair of nucleolar chromosomes in case of Dhani type I while the same pattern was observed for the 12th pair of nucleolar chromosomes in Dhani type III of *C. frutescens*. Previous reports of NOR heterogeneity in *Capsicum* was suggested to result from differences in copy number of rDNA genes or distinct condensation of chromatin at NORs (Moscone et al. 1996; Scaldaferro et al. 2016). However, our finding has elucidated heterogeneity in NORs which adds to the distinctiveness found within Indian cultivars of *C. frutescens*. Although nucleolar CMA bands grossly highlight inter-specific diversity, intercalary and distal CMA bands further ascertained infra-specific diversity within cultivars because of their differential pattern of distribution. Maximum number of distal CMA bands (5) was found in Ghee of *C. annuum* and the hottest chilli (Bhut jolokia) cultivars of *C. chinense*, as evident from their affinity. The number of distal bands was less in CF and CA cultivars though Karenga and Sada of *C. annuum* showed 4 distal bands, establishing

chromosomal distinction. Apart from Bullet type II, intercalary CMA bands were detected in CA-1 (Akashi) of *C. annuum* and Dhani type II of *C. frutescens*. Earlier, two chromosomes with intercalary CMA bands were consistently found in the Brazilian cytotypes of *C. annuum*, *C. frutescens* and *C. chinense* (Moscone et al. 1996). Moscone et al. (1996) also confirmed the presence of distal bands in eight chromosomes of *C. frutescens* and four chromosomes of *C. chinense* in the karyotypes. We could not assign any marker chromosome pair with intercalary bands for the three species under Indian *C. annuum* complex. Again, each of the investigated *Capsicum* species shows difference in distal CMA bands than the Brazilian cytotypes, suggesting uniqueness in the Indian germplasm. The diversity in CMA banding pattern, NORs and karyomorphpmetric parameters was maximum in *C. annuum*, corresponding with fruit morphotype variations. Our observation could seem puzzling karyotype interrelationships but a statistical evaluation of CMA banding pattern and karyomorphometric parameters clarified relations among cultivars. Our study is the first kind of detailed cultivar level analysis of chromosomal relationship of *Capsicum*, especially *C. annuum*. Coincidentally, diversity in *C. annuum* is well known in other parts of the world (Baral and Bosland 2002). Presently, the Akashi and the Bullet accessions along with Kul were found more related in the UPGMA cluster while Karenga, Kalo and Sada cultivar formed a distinct group. Earlier, karyotype affinity was evident between Akashi and Bullet and between the Karenga and Kalo cultivars based on karyomorphometric values (Jha and Saha 2017). Hence, we could validate a trend of delineation between the widely and less cultivated accessions of *C. annuum* based on CMA banding pattern. Noticeably, the Ghee cultivar of *C. annuum* was found to be more closely related to Bhut jolokia of *C. chinense* as apparent in the UPGMA phenogram. Furthermore, the phenogram shows some kind of relationship of Sada cultivar of *C. annuum* with *C. chinense* group. Hence, the genetic or phylogenetic relationship of Ghee and Sada cultivars of *C. annuum* with *C. chinense* remain to be a subject of further study. Regarding *C. frutescens*, the cultivars have minor differences in karyotype features and CMA banding pattern (differences mainly in the non-nucleolar bands). Our current approach revealed affinity within CF cultivars (Fig. 7). The data gains support from the gross similarity in fruit morphotypes and limited cultivation of the CF cultivars in spite of wide geographical variations (Table 1). The limited cultivation may have led the species to escape higher variability in fruit morphotypes and chromosomal features, a situation fairly reverse in *C. annuum*. Earlier, three cultivated species under *C. annuum* complex were found to have proximity among themselves compared to the other *Capsicum* species in molecular phylogenetic studies where *C. chinense* had often been found intermediate between *C. annuum* and *C. frutescens* (Walsh and Hoot 2001; Bosland and Baral 2007; Carvalho et al. 2014; Carrizo García et al. 2016). Although we found correspondence in placement of *C. chinense* in between *C. annuum* and *C. chinense* in the UPGMA cluster, our analysis elucidated detailed understanding of intra-specific diversity. The variation might result from difference in eco-climatic conditions, cultivation practice or hybridization frequency among the cultivars. Moreover, chromosomal distinction was also prominent in the cultivars. One of the chromosomes of the second pair contained single distal band in both of the Bhut jolokia cultivars. This was also prominent in Akashi (intercalary band in one chromosome of the third pair), Ghee (distal band in chromosome 17) and Dhani type IV (distal band in chromosome 12). Further analysis based on in situ hybridization would help to substantiate our findings on distinction between homologous chromosomes at nulcleolar and non-nucleolar parts as indicated by heterochromatic GC-rich CMA bands.

Scientists have previously attempted to link superior agronomic qualities or stress tolerance with molecular markers (Zhang et al. 2016; Haralayya and Asha 2017) in order to promote marker assisted breeding in capsicums. Genetic diversity in Indian hot chilli species is one of the most important standards to meet food security in climatic fluctuations and decreasing land resources. Base specific fluorochromes have been useful for chromosome analysis and understanding of karyotype relations among taxa (Sinclair and Brown 1971; Guerra et al. 2000; Hizume 2015; Bhowmick and Jha 2019). We have demonstrated the efficacy of this approach to constitute reliable landmarks on chromosomes otherwise impossible with Giemsa staining. The evolutionary divergence observed at inter-specific and infraspecific levels might correlate with fruit shape variations in capsicums. Our chromosomal database would

complement genomic or genetic evaluation of cultivars and further provide a platform for molecular cytogenetic analyses of Indian *Capsicums*.

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Authors contribution TBJ and BKB have contributed equally. Both the authors read and approved the final manuscript.

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Availability of data and material All data generated or analysed during this study are included in this published article.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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REVIEW

Conservation of floral, fruit and chromosomal diversity: a review on diploid and polyploid *Capsicum annuum* complex in India

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Abstract

Capsicum as a spice crop, has wild and cultivated forms admired globally, including Indian subcontinent with vast climatic ranges. Systematic representation of the Indian *Capsicum* is required to address species relationships and sustainable agriculture, in face of unpredictable climatic conditions. We have updated the catalogue of Indian '*C. annuum* complex' with 28 landraces and populations from different agro-climatic regions. The agro-climatic influence on the origin of stable chili landraces in India is remarkable, especially in the North East. The floral and fruit morphotype standards and chromosomal attributes have been considered for four distinct '*C. annuum* complex' members under three species. The highlights of study are: (1) comparative profiling of Indian *Capsicum* species revealing less infraspecific variation within *C. frutescens* and *C. chinense* than *C. annuum*, at par with cultivation status, (2) karyotype analysis of some unique diploid landraces of *C. annuum*, (3) karyotypic confirmation of the polyploid Dalle Khursani landraces exclusive to India. To obtain more information, we attempted to correlate diversity of fruit and floral morphotype with chromosomal diversity. Existence of elite and rare germplasm found in the regional pockets offer great scope for enriching the agricultural tradition. The present dataset may serve as a template to be continuously upgraded by taxonomists, genomicists and breeders.

Keyword Bhut jolokia · *Capsicum* · Chromosome · Dalle Khursani · EMA-Karyotype · Polyploid

Introduction

Capsicum or chili pepper is a highly valued crop and acclaimed worldwide as a nutritionally enriched vegetable, spice, natural colorant, essential oil source and as ingredient in pharmaceuticals due to the presence of the highly pungent capsaicin [1]. The genus belongs to the family Solanaceae and originated in the Andes Mountains, in north-western South America. It is reported that humans were using wild chili peppers as early as 8000–10,000 years ago [2, 3] while its dispersal, domestication and diversification in different parts of world have been documented over 6000 years ago [2–8]. The crop was first introduced in India by the Portuguese towards the end of the fifteenth century and to north eastern India by Christian missionaries [9, 10]. Different agro climatic zones within our country has helped adaptation and diversification of *Capsicum*, building a repository of many unique, well and less bred landraces, particularly in the North Eastern Himalayan states.

With the passage of time, list of wild *Capsicum* species has increased from 20 to 25 to 37, while the number of cultivated species remains only five all along [2, 5, 7, 11, 12]. The five cultivated species are *Capsicum annuum* L., *C. chinense* Jacq., *C. frutescens* L., *C. baccatum* L. and *C. pubescens* Ruiz et Pav. of which the first three are grouped in a taxonomic complex known as '*C. annuum* complex' or '*C. annuum- frutescens- chinense* complex' (Fig. 1). The three domesticated species within the complex are more divergent than their wild progenitors [2, 13–17]. The members within the complex also share floral morphology, cytogenetic features, DNA sequence characteristics and ancestral gene pool (Fig. 1) [18, 19]. Out of the five cultivated species, *C. annuum* quickly spread around the world most remarkably. However, domestication and continuous selection pressure has resulted in huge number of stable and unique landraces in India and elsewhere with different phenotypic traits [20,

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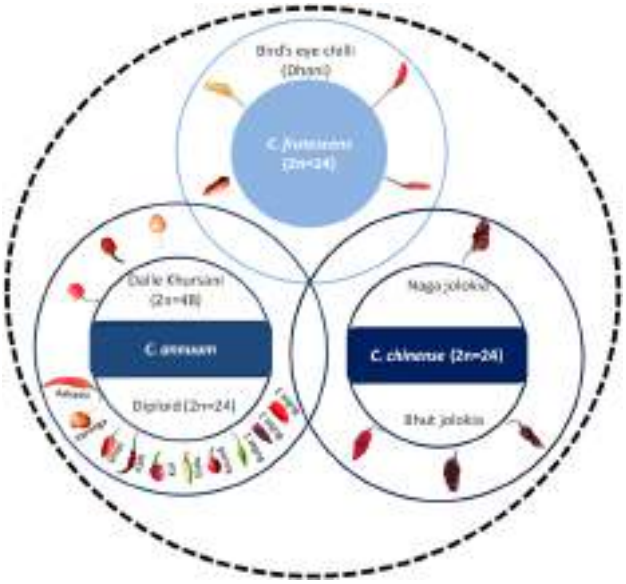


Fig. 1 The '*Capsicum annuum* complex' species of India with diverse fruit morphotypes under the primary gene pool (indicated by dotted line)

21]. Today, India holds a significant position in production, consumption and beneficial usage of *Capsicum* [22, 23].

Since 1950s, scientists are engaged in evolving methods for identification and characterization of *Capsicum* species. Though morphological descriptors have some limitations and are not free from environmental influences, the floral and fruit morphotypes of *Capsicum* have received considerable attention of scientists [24–28]. The diversity in color and shape of premature and mature chili fruits has been the most attractive trait for breeders and consumers across the world. The germplasm resources gain credibility by complementing morphological diversity with accurate and stable genetic information which has been tirelessly attempted over the decades [29, 30]. The use of advanced molecular marker systems [31–33] has been appreciated for accelerating germplasm conservation in *Capsicum* while genome-wide association study has elucidated genomic footprints for the historical domestication in *C. annuum*, *C. baccatum*, *C. frutescens* and *C. chinense* [34].

Analysis of chromosomes, the condensed genomic structures and bearer of DNA/genes within the cell nucleus provides first genetic identity of any organism, free from environmental influences and unaltered during developmental transitions of plants. The implication of classical cytogenetics remains fundamentally significant, simple and cost effective to confirm ploidy, karyotype and codes the overall blueprint of the genome to substantiate species relationships [35–39]. Classical cytogenetics may be upgraded with the innovation of newer methods for overcoming difficulties in chromosome preparation, especially for plant cells. The chromosome numerical and morphological records are a prerequisite for initiation and correct data interpretation in next-generation sequencing [40, 41]. Cytogenetics has thus become an integral part of genome analysis and no more a 'dead discipline' as was thought during the 1970s [42, 43]. Chromosome analysis of *Capsicum* species has been given special importance for conservation of genetic diversity [30, 33, 44–53], taxonomic works [12] and global compendium [3].

Compilation and conservation of entire floral and genetic diversity of Indian *Capsicum* has remained understudied in spite of being one of the leading commercial crops of the country. It appears from the literature review that distinct geographical positions with diverse agro climatic conditions have favored primary cultivation of *C. annuum* almost throughout the country. Discovery of Bhut jolokia and Naga jolokia, the highest pungent chilies under *C. chinense* from the state of Assam [54] has enriched India's repository. It is felt that collection and documentation of valuable plant genetic resources from different locations followed by analysis of valuable information deserves more attention in the era of biodiversity depletion, habitat loss, climatic changes and population explosion as stated in United Nations 2030 Agenda, Objective 15. Conservation of the species/population specific chromosomal landmarks is a prerequisite to identify and bring additional gene stocks for crop improvement in hostile climatic conditions [20, 55, 56]. Keeping these considerations in mind, the review is aimed to compile and conserve floral, fruit and chromosomal diversity of twenty eight diploid and polyploid landraces under Indian '*Capsicum annum* complex' as an aid to future crop improvement programs.

Floral and fruit diversity in *Capsicum*

Three domesticated *Capsicum* species were recognized to be growing in India [21]. However, today we have numerous landraces of *Capsicum* differing in shape, size, colour and heat level. Considering the worldwide importance of *Capsicum*, the International Plant Genetic Resources Institute (IPGRI) [57] has published Descriptors for *Capsicum* species which are highly heritable traits easily seen by the eye and are equally expressed in all environments. Floral morphology includes flower color, calyx constriction and number of flowers per axis while fruit morphology may be erect to pendent (deflexed) with extensive diversity in shape, size, color and pungency [9, 57], notable within the members of '*Capsicum annuum* complex'. *Capsicum* researchers gave importance to these traits for conservation and characterization [24–28].

Unique diversity of *Capsicum* flora under '*C. annuum* complex' namely *C. annuum* L., *C. frutescens* L. and *C.*

chinense Jacq are reported from India. Considering the scarcity of data, we have collected a total of twenty eight diploid and polyploid hot chili landraces of *Capsicum*, including some unique and less attended germplasms primarily from West Bengal and North Eastern Himalayan (NEH) states, grown them for documentation (voucher specimens were prepared for all the collected samples and preserved in the department) and conservation of their floral and fruit morphotypes (Table 1). The most widely cultivated diploid *C. annuum* is annual in nature, has amazing diversity in shapes, sizes, colors and pungency of fruits as well as other traits (Online resources 1a–f). The Aakashi and Bullet are highly cultivated landraces across the state of West Bengal and throughout India while we also identified less known diploid landraces of unique nature [28]. On the contrary, polyploid *Capsicum*, though placed under *C. annuum*, are perennial and can grow upto six feet (Online resource 2) with extra-large leaf size (Online resource 2e–g). The morphological features are unwavering over eight polyploid Dalle Khursani populations collected from Sikkim and West Bengal (Darjeeling district and adjoining regions) [58, 59], making them unique and distinct than diploid *C. annuum*, *C. chinense* and *C. frutescens* (Table 1).

The cultivars of *Capsicum frutescens* are mainly known as bird's eye chili worldwide which is represented in India by the Dhani landraces in West Bengal and also other places, having limited distribution and recognition in markets. *Capsicum frutescens* is diploid in nature, bear very small sized but highly pungent fruits and they also can survive more than a year. Almost no variations were recorded in morphological features among the populations of *C. frutescens* collected from distinct geographical locations [28] (Table 1, Online resource 1g). *Capsicum chinense* in India is represented by the hottest chilies of the world, namely Bhut jolokia and Naga jolokia with very restricted distribution in the North East. *Capsicum chinense* is a diploid species, can survive more than a year and develop unique fruit morphology. The populations of Bhut jolokia share many common morphological features within and also with the Naga jolokia [28] (Table 1, Online resource 1h).

Fruits are the most important parameter for breeding advancements in *Capsicum* [60]. Though the morph characters are suggested to be influenced by environmental or agro-climatic factors [61], QTLs [34] and single gene based expression [29] of fruit shape, size or color point out to the existence of genetic diversity of *Capsicum*. Genetically, *Capsicum* species are usually self-compatible, partially self-pollinating and cross-pollination may range from 2 to 90% [44, 62–65]. Sister species of '*C. annuum* complex' are morphologically distinguishable but are inter-fertile, constituting the primary gene pool [30] (Fig. 1). The genetic screening thus becomes important to address extent of fruit diversity and investigate the basis of variations which is still unattended in India. We found fruit and also the floral morphotype features (as per IPGRI 1995) to be specific to the landraces and not fleeting as such. Gonzalez and Bosland [66] also reported the need for collection and characterization of unattended germplasms for additional genetic resources. In addition to previous morpho-cytological studies [28, 58, 59], the present review has brought new insights for additional Aakashi, Bullet, Sada, Kul and notably the Round landrace of *C. annuum*. The comparative morphotype analysis between the diploid *C. annuum* and polyploid Dalle Khursani (Table 1) becomes a simple and effective choice for deducing prominent differences existing in *C. annuum*. High pungency and characteristic flavor of Indian Dalle Khursani has been nurtured for years by the locals of Sikkim and parts of West Bengal (Darjeeling and adjoining areas) who enjoy the culinary and medicinal benefits of this particular chili on a regular basis. In addition to the Dalle Khursani populations, comparative catalogue of other elite landraces of *C. chinense* and *C. frutescens* is also reported [58, 59]. In general, the features of *C. annuum*, *C. frutescens* and *C. chinense* are congruent with previous reports [15] while the present study provides attributes of the exotic Indian landraces afresh. The current list (Table 1) is not exhaustive and any future addition of less known *Capsicum* will enrich our national repository.

Cytogenetics in *Capsicum*: past and present

An overview of chromosome studies in *Capsicum*

Capsicum cytogenetics is almost nine decades old. Chromosome number in *C. annuum* was first reported by Huskins and La Cour [67] and from India by Dixit in 1931 as n = 12 [68]. Since then many authors have worked on *Capsicum* chromosomes using conventional orcein staining method and classified domesticated and wild species into two groups, one with $2n = 2\times = 24$ chromosomes and another with $2n = 2\times = 26$ chromosomes. Domesticated species belonged to the first group and wild species in the second group [25, 48, 49]. Mexico is the center of domestication and diversification of *Capsicum* and has world's famous 'Chilli Pepper Institute'. The institute is carrying out various projects for conservation and characterization of cultivated and wild *Capsicum* species at various levels [69]. Pepper germplasms, namely *Capsicum frutescens*, *C. annuum* and *C. chinense* were analyzed for the first time from Southern Mexico, revealing great genetic diversity [70, 71]. However, researchers particularly from Argentina, Brazil and Spain have carried out extensive chromosome analysis on cultivated, semi domesticated and wild species using conventional and molecular cytogenetic methods for conservation of genetic diversity [24, 26,

Table 1 A kit for floral and fruit morphotype diversity in Indian *Capsicum annuum* complex

Sl. no.	Landraces/populations and collection details	Floral and fruit characters with cultivation status	Flower image[a]	Fruit image[a]
C. annuum **(Diploid, 2n = 24)**				
1	Aakashi type I (Haldibari, West Bengal, 26.3345° N, 88.7807° E)	Flower: 17–22 mm, pendent; Pedicel: 1/node; Sepals: 5–6, intermediate margin; Petals: 5–6, adnate near base, milky white; Stamens: 5–6, anthers bluish; Fruit: 6–10 cm (variable), long, light or dark green when immature and red at maturity, medium pungent. Wide cultivation		
2	Aakashi type II (Guwahati, Assam, 26.1445° N, 91.7362° E)	Flower: 17–22 mm, pendent; Pedicel: 1/node; Sepals: 5–6, intermediate margin; Petals: 5–6, adnate near base; Stamens: 5–6, anthers bluish; Fruit: 6–10 cm (variable), long, light or dark green when immature and red at maturity, medium pungent. Wide cultivation		
3	Karenga (Haldibari, West Bengal, 26.3345° N, 88.7807° E)	Flower: 17–22 mm, pendent; Pedicel: 1/node; Sepals: 5, intermediate margin; Petals: 5, adnate near base, white, slightly thick; Stamens: 5, anthers bluish; Fruit: small (~ 2 cm), almost round, dark green when immature and red wrinkled at maturity, medium pungent. Limited cultivation		
4	Ghee (N. 24 Pgs., West Bengal, 22.8565° N, 88.7449° E)	Flower: 17–22 mm, pendent; Pedicel: 1/node; Sepals: 5–6, intermediate margin; Petals: 5–6, adnate near base, purple; Stamens: 5–6, anthers dark purple; Fruit: 3-4 cm (variable), elongate, wrinkled, dark green when immature, orange red at maturity, medium/ highly pungent. Limited cultivation		
5	Kalo (Siliguri, West Bengal, 26.7084° N, 88.4269° E)	Flower: 17–22 mm, pendent; Pedicel: 1/node; Sepals: 5–6, intermediate margin; Petals: 5–6, adnate near base, purple; Stamens: 5–6, anthers dark purple; Fruit: 3-4 cm (variable), elongate, dark purple when immature, blackish red at maturity, medium pungent. Limited cultivation		
6	Kul (Hooghly, West Bengal, 22.8963° N, 88.2461° E)	Flower: 15–20 mm, erect; Pedicel: 1/node; Sepals: 5, intermediate margin; Petals: 5, adnate to the middle, white; Stamens: 5; Fruit: 1–1.5 cm, slightly oval, dark green when immature, deep red at maturity, medium pungent. Limited cultivation		
7	Sada (Hooghly, West Bengal, 22.8963° N, 88.2461° E)	Flower: 17–22 mm, erect; Pedicel: 1/node; Sepals: 5–6, intermediate margin; Petals: 5–6, adnate to the middle, slightly greenish white with angular tips; Stamens: 5–6; Fruit: 2-3 cm, semi long, complete white when immature, deep orange at maturity, medium pungent. Limited cultivation		
8	Round (Shillong, Meghalaya, 25.5788° N, 91.8933° E)	Flower: 15–18 mm, erect; Pedicel: 1/node; Sepals: 5–6, intermediate margin; Petals: 5–6, adnate to the middle, white; Stamens: 5–6, anthers with pale purple tip; Fruit: 1.5-2 cm, round, dark green and brown when immature, deep red at maturity, medium/ highly pungent. Restricted cultivation		

Table 1 (continued)

Sl. no.	Landraces/populations and collection details	Floral and fruit characters with cultivation status	Flower image[a]	Fruit image[a]
9	Bullet type I (Barasat, West Bengal, 22.7228° N, 88.4806° E)	Flower: 17–22 mm, pendent; Pedicel: 1/node; Sepals: 5–6, intermediate margin; Petals: 5–6, adnate to the middle, white; Stamens: 5–6, anthers bluish; Fruit: 4-5 cm (variable), oblate, blunt, green when immature, red at maturity, medium pungent. Wide cultivation		
10	Bullet type II (Kolkata, West Bengal, 22.5726° N, 88.3639° E)	Flower: 17–22 mm, pendent; Pedicel: 1/node; Sepals: 5, intermediate margin; Petals: 5, adnate near base, white; Stamens: 5, anthers bluish; Fruit: 4-5 cm (variable), Oblate, blunt, green when immature, red at maturity, medium pungent. Wide cultivation		
11	Bullet type III (Tawang, Arunachal, 27.5861° N, 91.8594° E)	Flower: 17–22 mm, pendent; Pedicel: 1/node; Sepals: 5, intermediate margin; Petals: 5, adnate to the middle, white; Stamens: 5, anthers bluish; Fruit: 5–7 cm, wider than type I and II oblate, blunt, green when immature, dark red at maturity, medium pungent. Wide cultivation		
12	Bullet type IV (Ghatsila, Jharkhand, 23.6102° N, 85.2799° E)	Flower: 17–22 mm, pendant; Sepals: 5, intermediate margin; Petals: 5, adnate to the middle, white; Stamens: 5; Fruit: 6–8 cm (variable), oblate, green when immature dark, red at maturity, medium pungent. Wide cultivation		
C. annuum **(Polyploid, 2n = 48)**				
13	Dalle Khursani type I (Darjeeling, West Bengal, 27.0360° N, 88.2627° E)	Flower: 10–12 mm, pendent; Pedicel: 1–2/node, Sepals: 5, dentate margin, annular constriction at the base; Petals: 5, adnate at base, thick, greenish white with purple pigment at the junction of anther and filament; Stamens: 5; Fruit: ~ 3 cm, oval, dark green when immature and red at maturity, highly pungent. Limited but locally well cultivated		
14	Dalle Khursani type II (Kalimpong West Bengal, 27.0594° N, 88.4695° E)	Flower: 10–12 mm, pendent; Pedicel: 1–2/node; Sepals: 5, dentate margin, annular constriction at the base; Petals: 5, adnate at base, thick, greenish white with purple pigment at the junction of anther and filament; Stamens: 5; Fruit: 2.5 cm, oval, dark green when immature and orange/red at maturity, highly pungent. Limited but locally well cultivated		
15	Dalle Khursani type III (Mungpoo, West Bengal, 26.9740° N, 88.3397° E)	Flower: 10–12 mm, erect; Pedicel: 1–2/node; Sepals: 5, dentate margin, annular constriction at the base; Petals: 5, adnate to the middle, thick, greenish white, purple pigment at the junction of anther and filament, Stamens:5, Fruit: ~ 2 cm, oval, dark green when immature and orange /red at maturity, highly pungent. Limited but locally well cultivated		
16	Dalle Khursani type IV (Kolakham, West Bengal, 27.1024° N, 88.6763° E)	Flower: 10–12 mm, pendent; Pedicel: 1–2/node; Sepals: 5, dentate margin, annular constriction at the base; Petals: 5, adnate to the middle, thick, greenish white, purple pigment at the junction of anther and filament; Stamens: 5; Fruit: ~ 2.5 cm, oval, dark green when immature and red at maturity, highly pungent. Limited but locally well cultivated		

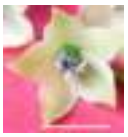
Table 1 (continued)

Sl. no.	Landraces/populations and collection details	Floral and fruit characters with cultivation status	Flower image[a]	Fruit image[a]
17	Dalle Khursani type V (Takdah, West Bengal, 27.0375° N, 88.3604° E)	Flower: 10–12 mm, pendent; Pedicel: 1–2/node; Sepals: 5, dentate margin, annular constriction at the base; Petals: 5, adnate to the middle, thick, greenish white, purple pigment at the junction of anther and filament; Stamens: 5; Fruit: ~ 2.5 cm, oval, dark green when immature and red at maturity. Limited but locally well cultivated		
18	Dalle Khursani type VI (Ramdhura, West Bengal, 27.036° N, 88.2627° E)	Flower: 10–12 mm, pendent; Pedicel: 1–2/node; Sepals: 5, dentate margin, annular constriction at the base; Petals: 5, adnate to the middle, thick, greenish white, purple pigment at the junction of anther and filament; Stamens: 5; Fruit: ~ 2.5 cm, oval, dark green when immature and orange/ red at maturity, highly pungent. Limited but locally well cultivated		
19	Dalle Khursani type VII (Jungi, Jalpaiguri, West Bengal, 26.6835° N, 88.7689° E)	Flower: 10–12 mm, erect and semi pendent; Pedicel: 1–2/node; Sepals: 5, dentate margin, annular constriction at the base; Petals: 5, adnate near base, thick, greenish white, purple pigment at the junction of anther and filament; Stamens: 5; Fruit: ~ 2 cm, oval, dark green when immature and red at maturity, highly pungent. Limited but locally well cultivated		
20	Dalle Khursani type VIII (Ravangla, Sikkim, 27.3065 N, 88.3639 E)	Flower: 10–12 mm, erect or intermediate; Pedicel: 1–2/node; Sepals: 5, dentate margin, annular constriction at the base; Petals: 5, adnate at base, thick, greenish white, purple pigment at the junction of anther and filament; Stamens: 5; Fruit: ~ 2 cm, oval, dark green when immature and red at maturity, highly pungent. Limited but locally well cultivated		
C. frutescens **(Diploid, 2n = 24)**				
21	Dhani type I (Jalpaiguri, West Bengal, 26.5657° N, 88.8186° E)	Flower: 10–12 mm, 2 pedicels/node, erect; Sepals: 5, entire margin, annular constriction at the base; Petals: 5, adnate to the middle, greenish white; Stamens: 5, anthers bluish; Fruit: very small (~ 2 cm), conical to elongate, dark green when immature, orange/red at maturity, highly pungent. Limited cultivation		
22	Dhani type II (Hooghly, West Bengal, 22.8963° N, 88.2461° E)	Flower: 10–12 mm, 2 pedicels/ node, erect; Sepals: 5, entire margin, annular constriction at the base; Petals: 5, adnate to the middle, greenish white; Stamens: 5, anthers bluish; Fruit: very small (~ 2 cm), conical to elongate, dark green when immature, red at maturity, highly pungent. Limited cultivation		

44–53, 72–81]. Moscone et al. [25, 46] for the first time applied fluorescent chromosome banding in the five cultivated species from *Capsicum* growing countries except India. Grabiele et al. [82] analyzed GC rich heterochromatin in chilli peppers. Recently, Scaldaferro and Moscone [53] provided an updated report on molecular cytogenetics of *Capsicum* in their book chapter.

In spite of the diversity of Indian *Capsicum*, considerable discontinuation in chromosome research is noted for nearly three decades. A concise summary of available cytogenetic literature in the *Capsicum* species of India has been provided in Table 2. Very few researchers have shown their interest in unraveling genetic or chromosomal diversity of Indian *Capsicum*, using conventional method of chromosome analysis (Table 2). The ancient as well as modern research reports reveal that chromosome analyses by previous Indian workers were mainly restricted to the cultivars of *C. annuum*. Some of the scientists were also

Table 1 (continued)

Sl. no.	Landraces/populations and collection details	Floral and fruit characters with cultivation status	Flower image[a]	Fruit image[a]
23	Dhani type III (Shillong, Meghalaya 25.5788° N, 91.8933° E)	Flower: 10–12 mm, 2 pedicels/ node, erect; Sepals: 5–6, entire margin, annular constriction at the base; Petals: 5–6, adnate to the middle, greenish white; Stamens: 5–6, bluish; Fruit: small in size (~3 cm), conical to elongate, green when immature, orange / red at maturity, highly pungent. Limited cultivation		
24	Dhani type IV (Thekkady, Kerala, 9.6031° N, 77.1615° E)	Flower: 10–12 mm, 2 pedicels/ node, erect; Sepals: 5, entire margin, annular constriction at the base; Petals: 5, adnate to the middle, greenish white; Stamens: 5, anthers bluish; Fruit: very small (~2 cm), conical to elongate, dark green when immature, orange/red at maturity, highly pungent. Limited cultivation		
***C. chinense* (Diploid, 2n = 24)**				
25	Bhut jolokia type I (Guwahati, Assam, 26.1445° N, 91.7362° E)	Flower: 7–10 mm, 1–3 pedicels/node, intermediate; Sepals: 5–6, entire margin, annular constriction at the base; Petals: 5–6, adnate near the middle, pale greenish white and thick; Stamens: 5–6, bluish; Fruit: 4–6 cm, campanulate, wrinkled, medium to long, dark green when immature, orange at maturity, extremely pungent. Restricted cultivation		
26	Bhut jolokia type II (Shillong, Meghalaya, 25.5788° N, 91.8933° E)	Flower: 7–10 mm, 1–3 pedicels/node, intermediate; Sepals: 5–6, entire margin, annular constriction at the base; Petals: 5–6, adnate to the middle, pale greenish white and thick; Stamens: 5–6, bluish; Fruit: 4–6 cm, campanulate, wrinkled, medium to long, dark green when immature, red/orange at maturity, extremely pungent. Restricted cultivation		
27	Bhut jolokia type III (Imphal, Manipur, 27.4728° N, 94.9120° E)	Flower: 7–10 mm, 1–3 pedicels/node, intermediate; Sepals: 5–6, entire margin, annular constriction at the base; Petals: 5–6, adnate below middle, pale greenish white and thick; Stamens: 5–6, bluish; Fruit: 4–6 cm, campanulate, wrinkled, medium to long, dark green when immature, red at maturity, extremely pungent. Restricted cultivation		
28	Naga jolokia type I (Dibrugarh, Assam, 27.4728° N, 94.9120° E)	Flower: 7–10 mm, 1–3 pedicels/node, intermediate; Sepals: 5–6, entire margin, annular constriction at the base; Petals: 5–6, adnate to the middle, pale greenish white and thick; Stamens: 5–6, bluish; Fruit: medium in size (~4 cm), campanulate, wrinkled, dark green when immature, red/orange at maturity, extremely pungent. Restricted cultivation		

[a]Bars 1 cm

eager to test cross-compatibility of the species by cytological analyses, e.g. karyomorphological analysis in hybrids of *Capsicum annuum* and *C. frutescens* conducted by Phulari and Dixit [93].

Present revision of chromosomal characteristics in Indian *Capsicum*

The procedure for collection and analysis has helped us identify the polyploid *C. annuum* from the state of Sikkim and subsequently from different locations of West Bengal [58, 59] for the first time through chromosome analysis. These landmark observations have encouraged us to make a travel schedule over the years for collection and documentation of well and less attended germplasms primarily from West Bengal and Eastern Himalayan States. The entire region looks like a hidden repository for Indian *Capsicum* and deserves sincere attention. It has been possible to present documentary evidences of some important home grown unattended and less cultivated plant genetic resources of chili pepper (Table 1).

Table 2 Summary of cytogenetic studies in Indian *Capsicum* species

Species	Varieties/cultivars studied	Method of study	Chromosomal characteristics				References[a]
			Zygotic number (2n)	Ploidy, karyotypes and banding	HCL, TCL, CSR (µm)	Gametic number (n)	
C. annuum	9 varieties[1]; 1 variety[2]; 6 varieties[3]; 3 varieties[4]; 7 cultivars[5]; 1cultivar[6]; 5 cultivars[7]; 7 Dalle Khursani cultivars[8]; 9 cultivars[9]	Conventional[10]; Conventional and EMA[11]; EMA-Giemsa and fluorochrome banding[12]	24[13], 36[14], 48[15]	Diploid[16]; triploid[17]; polyploid[18]; aneuploid[19]; Predominantly metacentric to nearly metacentric chromosomes[20]; one to two pairs of submetacentric to subtelocentric chromosomes[21]; one[22]/ two[23] satellited pairs; three such pairs (triploid)[24]; nucleolar[25]; distal[26] and intercalary[27] CMA^{+ve} signals	HCL: 40.35–60.35[28]; TCL: 48.26- 62.96[29], 66.32–106.78[30], 85.07[31], 87.34[32], 95.18–141.10[33], 100.91- 125.35[34], 134.24- 147[35]; CSR: 1.90–6.84[36], 1.72–2.097[37], 2.32–4.56[39], 2.88–5.22[40], 3.0–8.5[41]	12[42], 24[43]	[69][10,13,16]; [83][10,13,16]; [84][10,13,16]; [85][1,10,13,16]; [86][10,13,14,16,17,24,42]; [87][2,10,16,42]; [88][3,10,13,16,22,28]; [89][4,10,13,16,19,20,21,22,35,42]; [58][11,15,18,20,21,23,31,39,24]; [90][5,10,13,16,20,21,22,23,29,37,42]; [91][6,10,13,16,20,22,32]; [28][7,11,13,16,20,21,22,34,40,42]; [59][8,11,15,18,20,21,23,25,26,30,36,43]; [92][9,12,13,16,20,21,22,23,25,26,27, 33, 41
C. chinense	2 cultivars (Bhut jolokia[1], Naga jolokia[2])	Conventional[3]; Conventional and EMA[4]; EMA-Giemsa and fluorochrome banding[5]	24[6]	Diploid[7]; Predominantly nearly metacentric chromosomes[8]; one satellited pair[9]; nucleolar and distal CMA^{+ve} signals[10]	TCL: 60.59[11], 91.37-92.47[12], 92.47—116.18[13]; CSR:2.89-4.66[14], 4.04–6.22[15], 3.02–5.95[16]	12[17]	[92][1,3,6,7,8,11,15]; [28][1,2,4,6,7,8,9,12,14,17]; [92][1,5,6,7,8,9,10,13,16]
C. frutescens	2 Dhani cultivars[1], 4 Dhani cultivars[2]	Conventional and EMA[3]; EMA-Giemsa and fluorochrome banding[4]	24[5]	Diploid[6]; predominantly nearly metacentric chromosomes[7], two satellited pairs[8]; nucleolar, intercalary and distal CMA^{+ve} signals[9]	TCL: 84.42- 85.45[10], 84.42- 114.51[11]; CSR: 2.88–4.39[12], 2.88- 6.00[13]	12[14]	[28][1,3,5,6,7,10,12,14]; [92][2,4,5,6,7,8,9,11,13]

HCL total chromatin length of haploid set, *TCL* total chromatin length of diploid set, *CSR* chromosome size range

[a]Numbers in superscripts correspond to references

Enzymatic maceration and air drying or EMA is a useful value addition to classical cytogenetics research, enabling distinct chromosome morphology in a cytoplasm-free background, ideal for precise karyotype analysis for plants with small chromosomes [94]. Although the method started rolling in plant system more than four decades ago and many researchers have applied the technique for plant chromosome analysis throughout the world [82, 95–103], no researcher in India had attempted the method to characterize genetic diversity of the country's own hot pepper species and populations. Since 2012, EMA method of chromosome analysis in Indian *Capsicum* was adopted by our group for a systematic and recurring analysis of somatic chromosomes (Table 2). The same chromosome analysis method was extended to fluorescent banding in fifteen Indian '*Capsicum annuum* complex' members with two contrasting DNA base specific fluorochrome dyes namely chromomycin A3 (CMA3) and 4′,6-diamidino-2-phenylindole (DAPI) to mark GC and AT rich heterochromatic segments on chromosomes, respectively [92]. Currently, chromosome analysis was repeated in all the studied materials. A minimum of 5–10 somatic chromosome plates were considered for karyotype analysis. Chromosome measurements were taken in Axiovision L.E 4 software for determination of long arm length (l), short arm length (s), chromosome length (CL) and total diploid chromosome length (TCL). Chromosomes were classified on the basis of arm ratios (r value = l/s) following the nomenclature of Levan et al. [104]. The chromosomes were arranged according to decreasing order of length and grouped according to arm ratio for construction of haploid idiograms. On the other hand, meiotic chromosome counts provide a direct estimate of genetic variability and gives information to help plant breeding programs and agricultural productivity [105, 106]. In order to reconfirm the chromosome counts in the Dalle Khursani landrace in comparison to the diploid species, we have conducted fresh aceto-carmine staining of the pollen mother cells following Sharma and Sharma [107]. The chromosome number of the Indian *Capsicum* species is confirmed as 2n = 24 (n = 12) for diploids and 2n = 48 (n = 24) for polyploid Dalle Khursani. A predominance of nearly metacentric (or metacentric) chromosomes in the Indian *Capsicum* has been noted throughout the studies. A comprehensive karyotype profile of Indian *Capsicum* collected from diverse agro-climatic zones of the country has been outlined in the following paragraphs.

Capsicum annuum

Within the diploid landraces of *C. annuum*, Aakashi and Bullet are the most widely cultivated ones compared to the others. Apart from the nearly metacentric chromosomes predominantly found in *C. annuum* (Figs. 2, 3), one subtelocentric pair is present in Aakashi (Fig. 2a, b, g, h), Kalo (Fig. 2e, k), Kul (Fig. 2f, l) and Round (Fig. 3b, h) (Table 3). The Sada landrace has one submetacentric pair (Fig. 3a, g) and two of the Bullet populations have one subtelocentric pair (Fig. 3e, f, k, l). The 11th pair invariably has secondary constriction except Ghee landrace (12th pair satellited, Fig. 2d, j; Table 3). The Round (Fig. 3b, h) and Bullet type II (Fig. 3d, j) contain two satellite bearing pairs (11th and 12th) while Kalo (Fig. 2e, k) bears one additional satellite bearing chromosome besides the 11th pair (Table 3). Hence the 11th pair was constantly seen as the marker chromosome for diploid *C. annuum*. The satellite region of all landraces has been confirmed as the nucleolar constriction by our early fluorescent banding analysis [92], opening scope for confirmation of two satellite bearing pairs of Round in future study. The distinction of Ghee from rest of the *C. annuum* is evident in the karyotype, coming close to *C. chinense* [28, 92].

Comparing the extent of cultivation, Aakashi populations are more stable in karyotypes than the diverse karyotype profile of Bullets, corroborating with widespread distribution and morphotypic variation (Table 1). The other less cultivated chilies have their unique distribution in India (Table 1) and distinctive karyotype signatures, enabling differentiation. *Capsicum annuum* diploid landraces with one and two satellite bearing chromosome pairs were formerly classified as cytotypes 1 and 2, respectively, in the Brazilian germplasm [25, 46]. On the basis of nucleolar chromosomes, at least these two cytotypes can be observed among the Indian *Capsicum annuum* complex. Within the widely cultivated *Capsicum annuum* species, Bullet consists of cytotype 1 (Bullet type I, III and IV) and cytotype 2 (Bullet type II, Table 3) while Aakashi landrace is cytotype 1 (Table 3). Among the less cultivated landraces, Round is cytotype 2. In addition to these cytotypes, we suggest the polyploid *C. annuum* (Dalle Khursani) landrace to be the new polyploid type (cytotype 3) in Indian *Capsicum*. On the contrary, all *C. chinense* landraces are cytotype 1 having single pair of nucleolar chromosomes while *C. frutescens* is cytotype 2, showing two pairs of nucleolar chromosomes. Indian *C. annuum* complex thus points out to different extent of cytotype diversity which is greatest in *C. annuum*. Interestingly, the Round landrace is studied for the first time, having fruit morphology and distribution comparable to Dalle Khursani populations (Table 1). The Round landrace is often sold in the markets mistakenly as Dalle Khursani. It is hereby confirmed for the first time as the diploid landrace of *C. annuum*, having chromosome number and karyotype readily distinguishing it from rest of the diploid landraces and certainly from the Dalle Khursani.

Long ago, Pickersgill [108] reported natural tetraploidy in a wild accession of *C. annuum* with 2n = 4 × = 48 and since then polyploidy in *Capsicum* was considered a rarity. However, primarily in the two hilly states of India

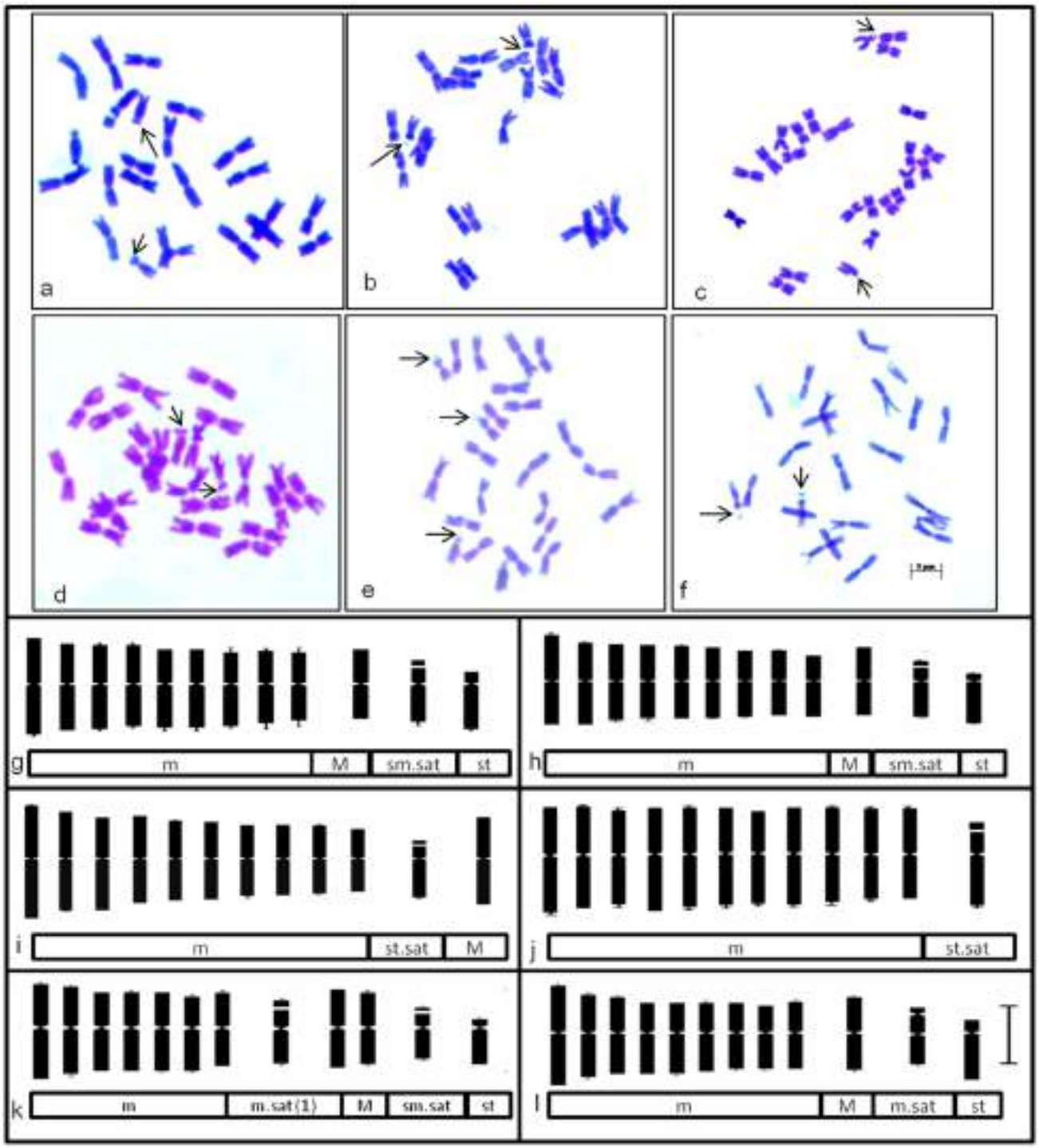


Fig. 2 **a**–**f** EMA-Giemsa plates of somatic metaphase chromosomes of diploid *C. annuum* landraces (2n = 24), **a** Aakashi type I (Haldibari), **b** Aakashi type II (Guwahati), **c** Karenga (Haldibari), **d** Ghee (N. 24 Pgs.), **e** Kalo (Siliguri), **f** Kul (Hooghly), arrows indicate satellite bearing chromosomes. **g**–**l** Corresponding somatic idiograms (haploid set), **g** Aakashi type I (Haldibari), **h** Aakashi type II (Guwahati), **i** Karenga (Haldibari), **j** Ghee (N. 24 Pgs.), **k** Kalo (Siliguri), **l** Kul (Hooghly). Bar 5 µm

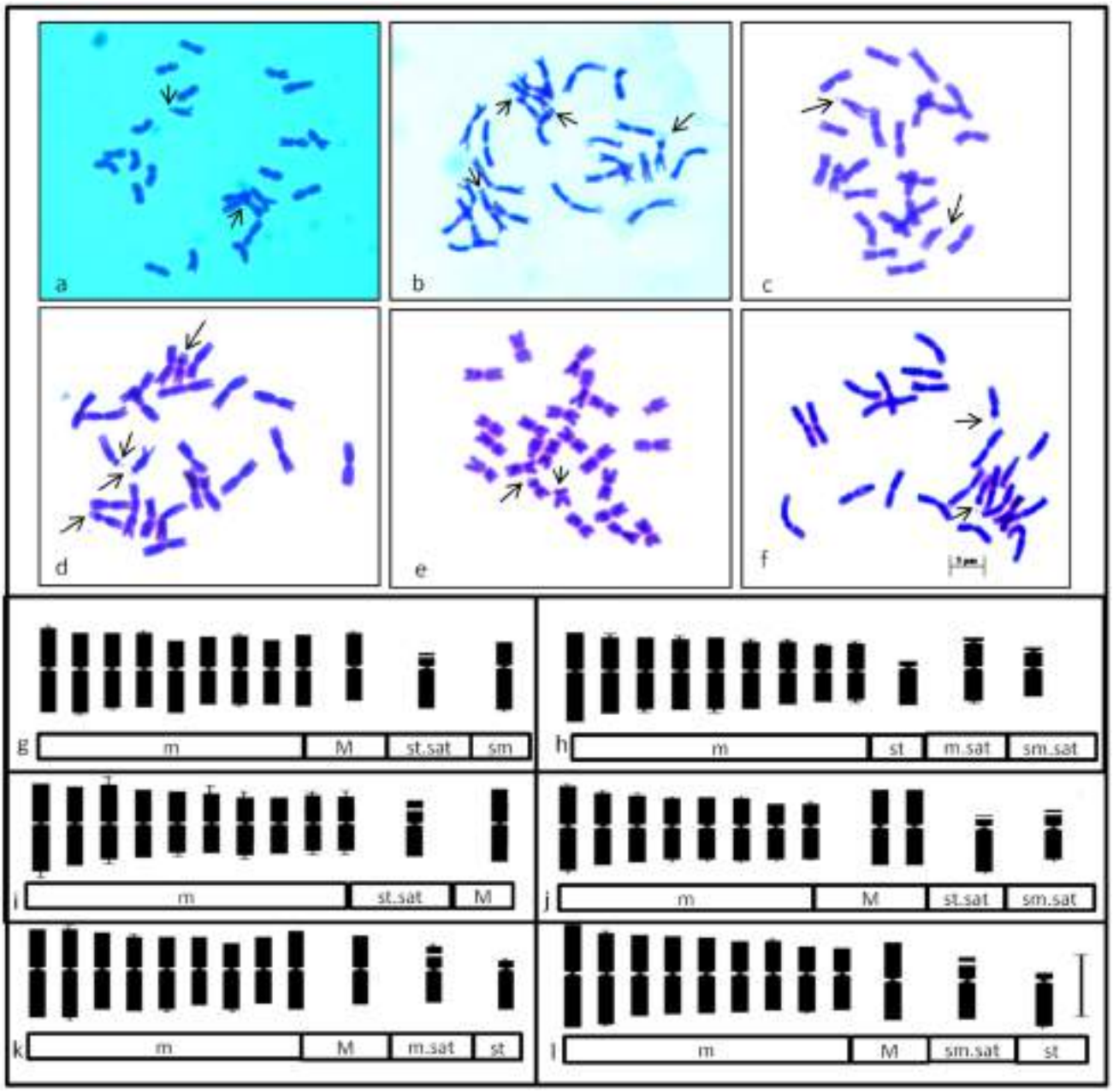


Fig. 3 **a**–**f** EMA-Giemsa plates of somatic metaphase chromosomes of diploid *C. annuum* landraces (2n = 24), **a** Sada (Hooghly), **b** Round (Shillong), **c** Bullet type I (Barasat), **d** Bullet type II (Kolkata), **e** Bullet type III (Tawang), **f** Bullet type IV (Ghatsila), arrows indicate satellite bearing chromosomes. **g**–**l** Corresponding somatic idiograms (haploid set), **g** Sada (Hooghly), **h** Round (Shillong), **i** Bullet type I (Barasat), **j** Bullet type II (Kolkata), **k** Bullet type III (Tawang), **l** Bullet type IV (Ghatsila). Bars 5 µm

(West Bengal and Sikkim), we have identified a locally grown polyploid cultivar which is known as the Dalle Khursani for decades, containing 2n = 4 × = 48 chromosomes. Subsequently many more polyploid populations were collected, documented and analyzed for future conservation [58, 59]. EMA based karyotype analysis of eight different populations of Dalle Khursani (Fig. 4a–p; Table 3) revealed 23rd and 24th chromosome pairs carrying secondary constrictions. Majority of the chromosomes are nearly metacentric and one submetacentric or subtelocentric pair was found within the populations of Dalle Khursani (Fig. 4i–p; Table 3).

Table 3 Karyotype profile of Indian *Capsicum annuum* complex

Sl. No	Landraces/populations and ploidy	TCL (mean ± SD µm)	Karyotype formulae (n)[a]	Chromosome pair(s) bearing sat
C. annuum				
1	Aakashi, type I, Diploid (2n = 24)	120.19 ± 6.26	9 m + 1 M + 1st + 1sm.sat	11th
2	Aakashi type II, Diploid (2n = 24)	122.05 ± 2.00	9 m + 1 M + 1st + 1sm.sat	11th
3	Karenga, Diploid (2n = 24)	100.91 ± 6.24	10 m + 1 M + 1st.sat	11th
4	Ghee, Diploid (2n = 24)	125.35 ± 6.54	11 m + 1st.sat	12th
5	Kalo, Diploid (2n = 24)	115.37 ± 4.36	7 m + 2 M + 1st + 1 m.sat + 1sm.sat	11th + 1 chromosome of 8th pair (m.sat)
6	Kul, Diploid (2n = 24)	141.10 ± 2.39	9 m + 1 M + 1st + 1 m.sat	11th
7	Sada, Diploid (2n = 24)	97.93 ± 1.88	9 m + 1 M + 1sm + 1st.sat	11th
8	Round, Diploid (2n = 24)	151.00 ± 2.02	9 m + 1st + 1 m.sat + 1sm.sat	11th, 12th
9	Bullet type I, Diploid (2n = 24)	122.54 ± 4.46	10 m + 1 M + 1st.sat	11th
10	Bullet type II, Diploid (2n = 24)	95.18 ± 3.14	8 m + 2 M + 1st.sat + 1sm.sat	11th, 12th
11	Bullet type III, Diploid (2n = 24)	105.60 ± 4.03	8 m + 2 M + 1st + 1 m.sat	11th
12	Bullet type IV, Diploid (2n = 24)	137.38 ± 3.05	9 m + 1 M + 1st + 1sm.sat	11th
13	Dalle Khursani type I, Polyploid (2n = 48)	77.58 ± 2.57	15 m + 6 M + 1m + 1 m.sat + 1 m.sat	23rd, 24th
14	Dalle Khursani type II, Polyploid (2n = 48)	101.27 ± 1.39	18 m + 4 M + 1 sm.sat + 1 m.sat	23rd, 24th
15	Dalle Khursani type III, Polyploid (2n = 48)	103.55 ± 3.58	18 m + 3 M + 1 sm.sat + 1 m.sat + 1st	22nd, 23rd
16	Dalle Khursani type IV, Polyploid (2n = 48)	84.17 ± 2.00	4 M + 16 m + 1 M.sat + 2sm + 1 m.sat	21st, 24th
17	Dalle Khursani type V, Polyploid (2n = 48)	106.78 ± 1.49	9 M + 12 m + 1 m.sat + 1sm.sat + 1sm	22nd, 23rd
18	Dalle Khursani type VI, Polyploid (2n = 48)	66.32 ± 3.22	8 M + 13 m + 2 m.sat + 1sm	23rd
19	Dalle Khursani type VII, Polyploid (2n = 48)	72.49 ± 1.67	5 M + 16 m + 2 m.sat + 1sm	23rd
20	Dalle Khursani type VIII, Polyploid (2n = 48)	85.07 ± 2.40	6 M + 12 m + 2sm + 2st + 2sm.sat	23rd, 24th
C. frutescens				
21	Dhani type I, Diploid (2n = 24)	84.42 ± 2.14	9 m + 1 M + 1 m.sat + 1st.sat	1st, 12th
22	Dhani type II, Diploid (2n = 24)	101.36 ± 2.01	9 m + 1 M + 1 m.sat + 1st.sat	1st, 12th
23	Dhani type III, Diploid (2n = 24)	85.45 ± 1.79	9 m + 1 M + 1 m.sat + 1st.sat	1st, 12th
24	Dhani type IV, Diploid (2n = 24)	114.51 ± 1.34	9 m + 1 M + 1 m.sat + 1st.sat	1st, 12th
C. chinense				
25	Bhut jolokia, type I, Diploid (2n = 24)	122.5 ± 1.70	11 m + 1st.sat	12th
26	Bhut jolokia, type II, Diploid (2n = 24)	92.47 ± 1.28	11 m + 1st.sat	12th
27	Bhut jolokia, type III, Diploid (2n = 24)	116.18 ± 1.25	11 m + 1st.sat	12th
28	Naga jolokia type I, Diploid (2n = 24)	91.37 ± 1.65	11 m + 1st.sat	12th

TCL total chromatin length of diploid set

[a]Chromosome types: *M* metacentric, *m* nearly metacentric, *sm* submetacentric, *st* subtelocentric, *sat* satellite[104]

Capsicum frutescens

The *C. frutescens* populations are collected from different parts of India yet showing consistent chromosome numbers (2n = 24). The karyotype features are also similar in all of the populations (Fig. 5a–h; Table 3). The 1st and 12th pairs are the marker satellite bearing chromosomes of the Dhani landraces (Table 3; Fig. 5). Our finding corroborates to the observation of two satellite bearing chromosome pairs previously documented in the Brazilian *C. frutescens* [46].

Capsicum chinense

The hottest chili cultivars of India are again an example of stable diploid landraces, evident from consistent

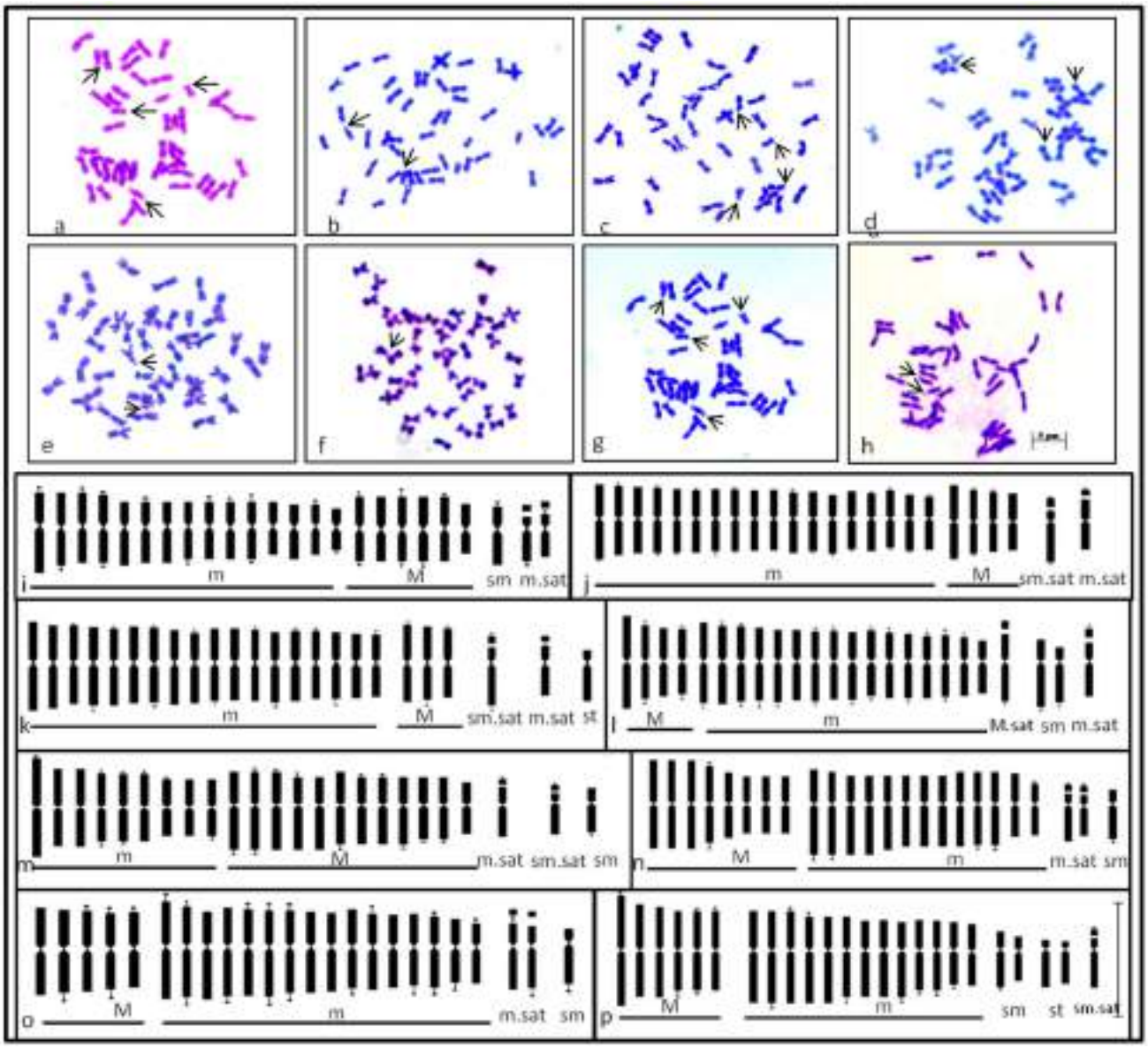


Fig. 4 **a**–**h** EMA-Giemsa plates of somatic metaphase chromosomes of polyploid *C. annuum* Dalle Khursani (2n = 48), **a** type I (Darjeeling), **b** type II (Kalimpong), **c** type III (Mungpoo), **d** type IV (Kolakham), **e** type V (Takdah), **f** type VI (Ramdhura), **g** type VII (Jungi), **h** type VIII (Ravangla), arrows indicate satellite bearing chromosomes. **i**–**p** Corresponding somatic idiograms (haploid set), **i** type I (Darjeeling), **j** type II (Kalimpong), **k** type III (Mungpoo), **l** type IV (Kolakham), **m** type V (Takdah), **n** type VI (Ramdhura), **o** type VII (Jungi), **p** type VIII (Ravangla). Bars 5 µm

chromosome numbers (2n = 24) and karyotypes. The karyotypes of both Bhut jolokia and Naga jolokia are same, composed of all nearly metacentric pairs and one subtelocentric pair bearing satellite (Fig. 5i–p; Table 3). The karyotype constitution is same as that of Ghee, holding the 12th pair as the marker nucleolar chromosome, confirmed by our previous fluorochrome banding analysis [92]. The Indian *C. chinense* also has notable karyotype similarity with the Brazilian *C. chinense* [46], having the 12th nucleolar chromosome pair conserved in this species.

The total chromatin length (TCL) of diploid set has been found to be overlapping among the diverse landraces of three *Capsicum* species. Among the Indian members of '*Capsicum annuum* complex', lowest TCL (66.32 µm) was recorded in one polyploid (2n = 48) Dalle Khursani type (VI, Table 3) while highest TCL (151.00 µm) was calculated in a diploid (2n = 24) *C. annuum* (Round, Table 3). *Capsicum annuum* shows the widest variation in chromosome lengths and blurs correlation between chromosome

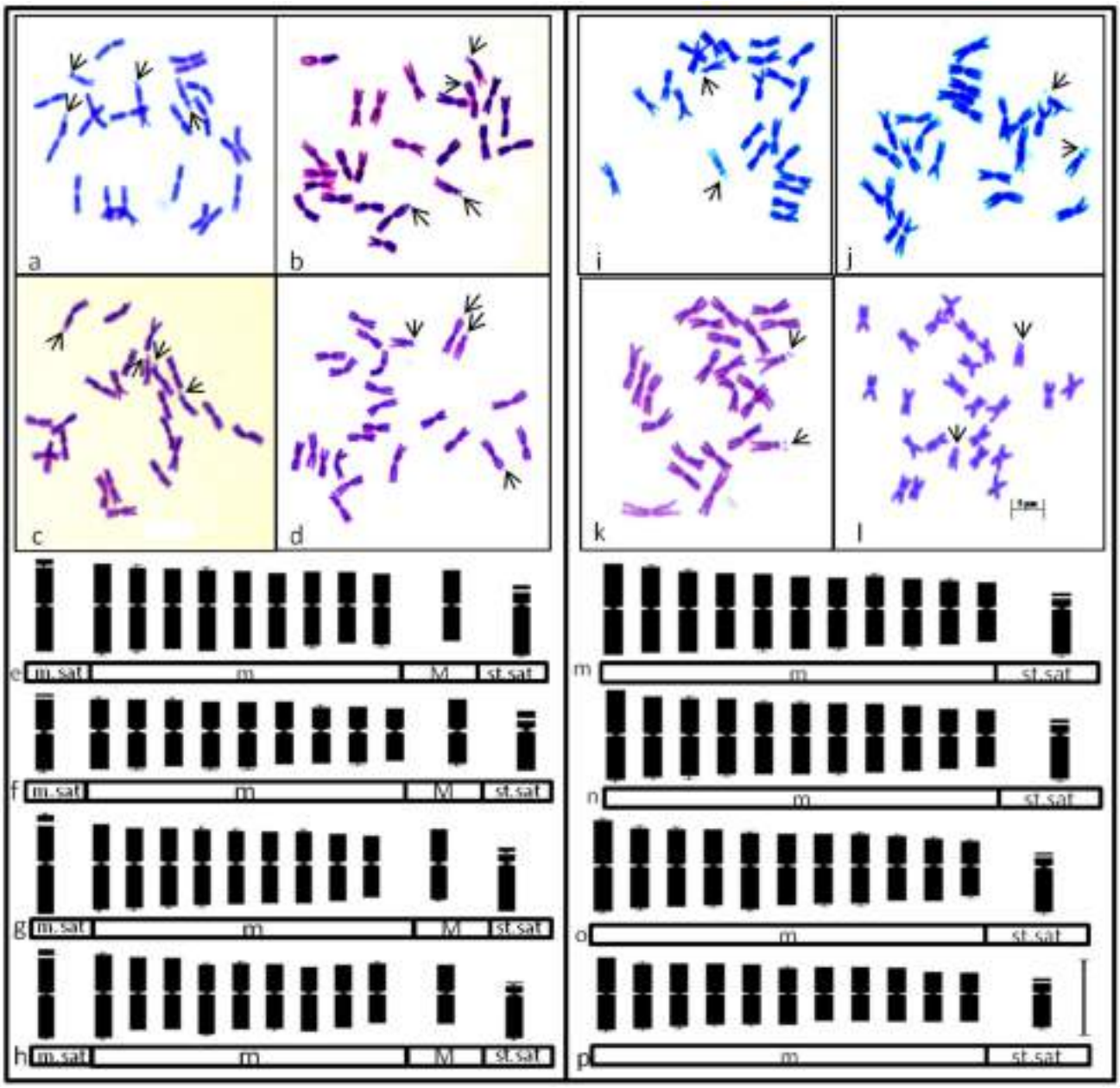


Fig. 5 **a**–**d** EMA-Giemsa plates of somatic metaphase chromosomes of diploid (2n = 24) *C. frutescens* (**a**–**d**) and *C. chinense* (**i**–**l**) landraces, **a** *C. frutescens* type I (Jalpaiguri), **b** *C. frutescens* type II (Hooghly), **c** *C. frutescens* type III (Shillong), **d** *C. frutescens* type IV (Thekkady), **i** Bhut jolokia type I (Guwahati), **j** Bhut jolokia type II (Shillong), **k** Bhut jolokia type III (Imphal), **l** Naga jolokia type I (Dibrugarh), arrows indicate satellite bearing chromosomes. **e**–**h** Corresponding somatic idiograms (haploid set) of *C. frutescens*, **e** type I (Jalpaiguri), **f** type II (Hooghly), **g** type III (Shillong), **h** type IV (Thekkady). **m**–**p** Corresponding somatic idiograms (haploid set) of *C. chinense*, **m** Bhut jolokia type I (Guwahati), **n** Bhut jolokia type II (Shillong), **o** Bhut jolokia type III (Imphal), **p** Naga jolokia type I (Dibrugarh). Bars 5 µm

number and TCL. Variation in TCL was also noted among the members of *C. chinense* (91.37–122.5 µm) and *C. frutescens* (84.42–114.51 µm) (Table 3). It is accepted that TCL and related measurements depend largely on the method for chromosome processing and cytoplasmic clarity. We have constantly followed the EMA method in all landraces and species of *Capsicum* so that the actual differences in karyotypes and TCLs are least affected by technical reasons. On the other hand, the previous TCL/HCL estimates obtained for Indian *Capsicum* species have been derived from traditional aceto-orcein preparations

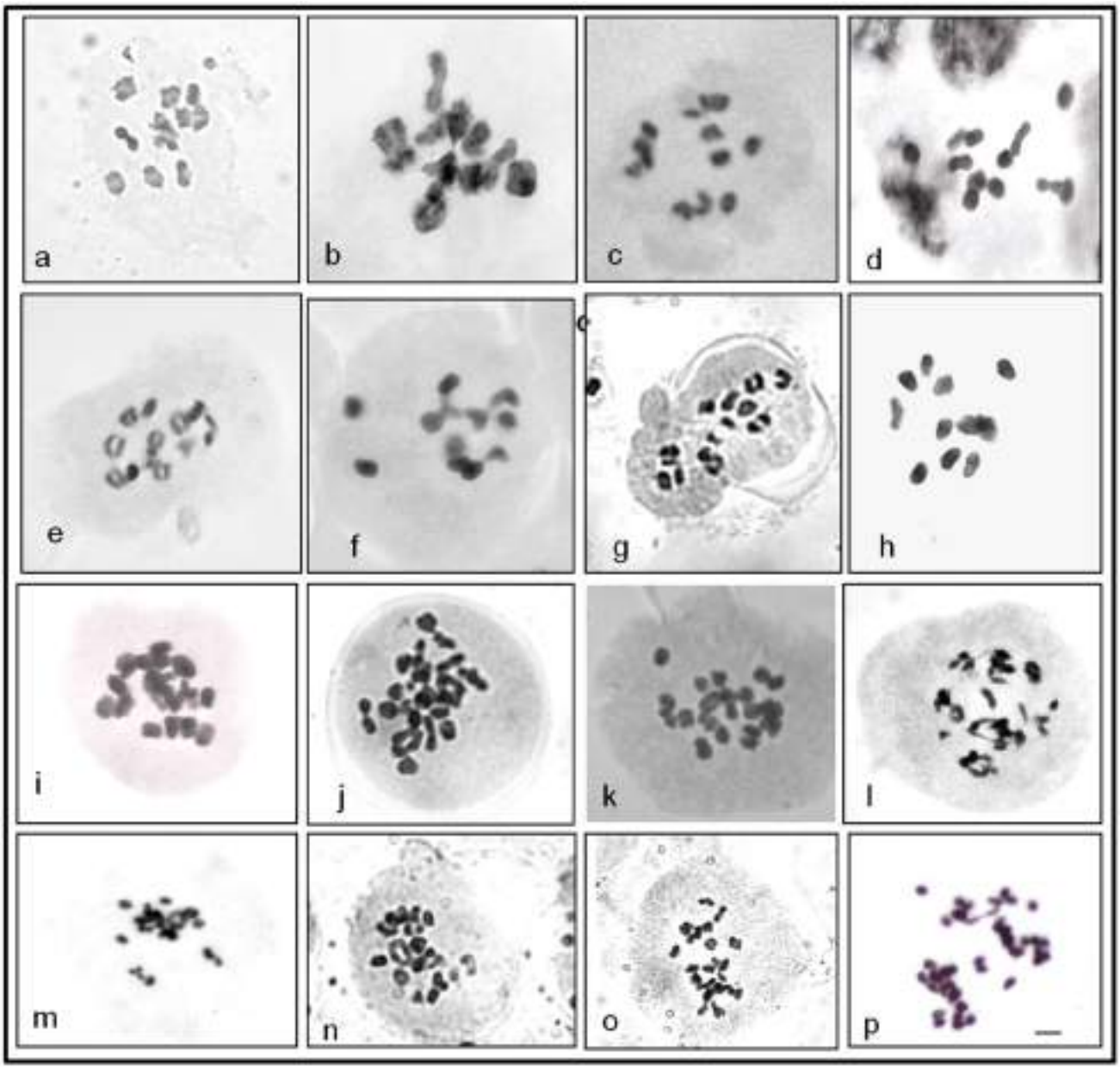


Fig. 6 **a**–**h** Meiotic metaphase I plates of diploid *Capsicum* landraces (n = 12). **a**–**e** *C. annuum*: **a** Aakashi type II (Guwahati), **b** Karenga, **c** Ghee, **d** Bullet type II (Kolkata), **e** Kalo. **f** *C. frutescens* (Dhani type I, Jalpaiguri), **g**, **h** *C. chinense*: **g** Bhut jolokia type II (Shillong), **h** Naga jolokia type I (Dibrugarh). **i**–**p** Meiotic metaphase I plates of polyploid *C. annuum* Dalle Khursani (n = 24), **i** type I (Darjeeling), **j** type II (Kalimpong), **k** type III (Mungpoo), **l** type IV (Kolakham), **m** type V (Takdah), **n** type VI (Ramdhura), **o** type VII (Jungi), **p** type VIII (Ravangla). Bar 5 µm

(Table 2), a method already known to have limitations in lucid karyotype analysis.

Regarding the gametic counts, diploid landraces of *C. annuum* and those of *C. frutescens* and *C. chinense*, have 12 bivalents in meiotic metaphases (Fig. 6a–h), congruent with previous finding [28]. The present study has further validated polyploid nature with n = 24 gametic chromosomes in all populations of Dalle Khursani (Fig. 6i–p) and indicates that a stable genetic system is operating within the Dalle Khursani populations [59].

Summary and future outlook

A systematic effort to conserve *Capsicum* genetic diversity in India was missing which was required to identify sources of genetic differentiation following both natural

and human selection. The floral and fruit morphological kit may be beneficial as a valuable genetic resource for future reference and could be utilized in transferring desirable traits into commercial cultivars. While identification of new genetic resources becomes inevitable for searching alternative genetic stocks and crop improvement, care should be taken during selection and characterization of germplasms. Genetic screening thus becomes the most reliable method for cultivar authentication. The efficacy of EMA based giemsa stained chromosomal dataset to unravel the foundational genetic information has been established. Fluorescent banding within some valuable diploid *C. annuum* complex members have been completed [92]. Our detailed update on morphotypes, ploidy, chromosome number and karyotype of Dalle Khursani has broadly introduced this elite germplasm to the world. The chromosomal identification of such a rare but stable polyploid in Indian repository has opened the door for future molecular cytogenetics research involving fluorescent in situ hybridization (FISH) and genomic in situ hybridization (GISH) in polyploid populations to unlock the parental lines much like the bread wheat story. In the light of worldwide progress in chromosomal research on *Capsicum* and to conserve and compile the genetic diversity in Indian *C. annuum* complex members, a review work was immensely felt. This article provides an updated profile of cytogenetic research on Indian *Capsicum* species. The prominent gaps in genome content analysis and rDNA-ITS sequence work are noted for the diploid and polyploid Indian *Capsicum annuum* complex members, that could advance our current understanding of inter-species relationships. Keeping in mind the distinct morphological features along with polyploid nature and chromosomal attributes, it is also proposed that 'Dalle Khursani' deserves separate species status within *C. annuum* complex. The recent GI appeal for Dalle Khursani by North Eastern Regional Agricultural Marketing Corporation Limited (NERAMAC) has been a significant value addition to Indian agro-economy (http://www.ipindia.nic.in/writereaddata/Portal/Images/pdf/Journal_140.pdf). Genetic identity of Dalle Khursani needs to be protected. Ghee cultivar under *C. annuum* is another interesting material for future studies. It has been suggested as a possible hybrid between *C. annuum* and *C. chinense* on the basis of fruit morphology which is reinforced by chromosome analysis, congruent with possibilities of inter-species hybridization within *C. annuum* complex [109]. Authors also felt the necessity to update the attributes of *C. chinense* and *C. frutescens* since few global databases have missed out India while narrating *C. chinense* distributions [110] in spite of long associations of this species with Indian subcontinent [23, 54]. The compilation of the most updated morphological and chromosomal database is believed to serve as the template for future workers interested in identifying, characterizing and conserving the Indian *Capsicum* as one of the richest genetic resource of *C. annuum* complex, in line with the contemporary global markets.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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RESEARCH ARTICLE

Evaluation of morphological traits, fluorescent banding and rDNA ITS sequences in cultivated and wild Indian lentils (*Lens* spp.)

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Abstract Lentil is a traditional nutritionally enriched crop. India is now the second-highest producer and consumer of cultivated lentils. Out of the seven species, only one is cultivated worldwide, and the remaining are wild species, which are considered valuable plant genetic resources for any future plant breeding programmes in this era of climatic changes. Distinct morphological traits, karyotype diversity assisted by fluorescent banding and ribosomal DNA ITS1-5.8S-ITS2 rDNA ITS sequence analyses are compiled in the present study. Fluorescent banding with two contrasting nucleic acid dyes, chromomycin A3 (CMA) and 4′,6-diamidino-2-phenylindole (DAPI), has been used to characterise the lentil karyotypes of two accessions of *L.culinaris* Medik (cultivated) and five wild species viz. *L.orientalis* (Boiss.) M. Popo, *L.odemensis* Ladiz, *L.nigricans* (Bieb.) Godr, *L.lamottei* Czefr. and *L.ervoides* (Brign.) Grande. This method has unravelled the diverse number and locations of GC/AT-specific heterochromatic regions on chromosomes. The present study provided rDNA ITS sequence-based phylogenetic analysis, which revealed the monophyletic origin of the Indian cultivated and wild species of *Lens* and grouped them into two possible lineages (clades I and II). Furthermore, this study provided in-depth insights on the interspecies correlation and evolutionary trends within the species of Indian lentil. Detailed knowledge of the genomic structures provides information for any valuable crop species. Thus, the correlation of morphological traits with the results of fluorescent chromosome banding and the generation of an unbiased phylogenetic tree based on rDNA sequence analysis in Indian lentil taxa might be useful in lentil crop improvement programmes.

Keywords Chromosomes · CMA and DAPI banding · ITS sequence · Cultivated and wild lentils

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Introduction

Lentil or *Lens* belongs to the family Fabaceae and comprises one cultivated and six wild species (van Oss et al. 1997; Wong et al. 2015). In both domestic and international markets, the demand for the only cultivated species *L. culinaris* Medik is increasing due to its high protein content and various health benefits. Cultivated and wild lentils are morphologically herbaceous annual plants and contain $2n = 14$ chromosomes genome-wide, self-pollinated with a low outcrossing frequency. Due to distinct differences in seed

size, *L. culinaris*is popularly divided into microsperma and macrosperma types of seed (Ferguson et al. 2000). Interestingly, the seed morphotypes of two wild species, *L. orientalis* (Boiss.) M. Popo and *L. odemensis* Ladiz, have similarities with the cultivated lentils, while the seeds of *L. nigricans* (Bieb.) Ladiz, *L. lamottei* Czefr. and *L. ervoides* (Brign.) Grande are different (Jha et al. 2015). Distinct differences have been noted in tendril formation within the wild species. Dense tendril formation was noted in *L. nigricans*, *L. lamottei* and *L. Ervoides*, while *L. orientalis*, *L. odemensis* and *L. culinaris* did not show any tendril formation.

Crop loss in cultivated lentil is severely affected by biotic and abiotic stresses. Reportedly, the wild relatives of lentils are a good source of disease resistance and agronomic traits (Gupta et al. 2006; El-Bouhssini et al. 2008; Singh et al. 2020). Considering the limited gene pool of cultivated species, the wild species of lentils could be considered valuable resources for future crop improvement programmes (Davies et al. 2007; Singh et al. 2020). Thus, inter- and intraspecific correlations within and between the species need further investigation. Barulina (1930) first observed close correlations between *L. culinaris* and *L. orientalis* and proposed *L. orientalis* as the progenitor of *L. culinaris*. Since then, several reports are focussed on the morphological, biochemical and molecular parameters to assist breeding programmes and to establish correlations within the lentil species (Zamir and Ladizinsky 1984; Tadmor et al. 1987; Havey and Muehlbauer 1989; Baldwin 1992; Sharma et al. 1996;Ferguson et al. 2000; Sonnante et al. 2003; Davies et al. 2007; Vijayan et al. 2009; Verma et al. 2014; Singh et al. 2020;Saha et al. 2015, 2017; Saha and Jha 2019).

The chromosomal analysis in lentil was initiated through a conventional method in some members of cultivated species, while a few wild species had a divergent opinion (Bhattacharjee 1951; Sharma and Muhkopadhyay 1963; Sinha and Acharia 1972; Naithani and Sorbhoy 1973; Gupta and Singh 1981; Lavania and Lavania 1983; Nandanwar and Narkhede 1991; Ladizinsky 1979, 1997;Ladizinsky et al. 1992). Detailed chromosome analysis provides a conceptual foundation for modern genomics (Figueroa et al. 2010) and supports any breeding programme. The advancement in chromosome analysis through enzymatic maceration and air drying (EMA) method (Kurata and Omura 1978) has not been applied in Indian lentils. India is the second-highest producer and consumer of lentils and has conserved about 2655 lentil accessions in the National Bureau of Plant Genetic Resources (Malhotra et al. 2019). The chromosomal database was established through the EMA-based Giemsa staining method of > 30 *L.culinaris* cultivars and four wild species of Lentil obtained from the Indian Institute of Pulses. The dataset has also added new chromosomal information for the lentil breeders and genome researchers (Jha et al. 2015, 2017; Jha and Halder 2016). To gain an in-depth insight into the chromosomes, the fluorescence banding of wild and cultivated species was carried out. The technique uses adenine–thymine (AT) base-specific 4′,6-diamidino-2-phenylindole (DAPI) and guanine-cytosine (GC) base-specific chromomycin A3 (CMA) in many crops over the decades (Kurata and Omura 1978;Fukui 1996; Moscone etal. 1996;Yamamoto 2012; Schneeweiss and Schneeweiss 2013; Scaldaferro etal. 2013; Ghosh et al. 2018;Jha 2019;Jha et al. 2020). Similarly, the use of nuclear non-coding sequences (ribosomal DNA ITS1-5.8S-ITS2) in the plant molecular phylogeny has sufficient resolution to elucidate the evolutionary trends in speciation (Baldwin 1992; Saha et al. 2015, 2017; Saha and Jha 2019). The analysis of rDNA internal transcribed spacer (ITS) sequences is useful in the phylogenetic inference of the genus *Lens* (Sonnante et al. 2003); however, the analysis in Indian lentil species has not yet been carried out. Thus, the present study conducted a morphotype analysis, fluorescent chromosome banding and rDNA ITS sequence analysis in two accessions of cultivated and five wild Indian lentil species for the first time. The present findings could be applied in any crop improvement programmes of lentil.

Materials and methods

Taxon sampling

Among the Indian species recognised by Wong et al. (2015), a total of seven accessions representing two cultivated cultivars (micro, type I and macro, type II) and five wild species of *Lens* were obtained from the Indian Institute of Pulses Research (ICAR; Kanpur, Uttar Pradesh, India). India is not the country of origin of any wild species, except the cultivars of

L.culinaris(Type-I EC-267526 and Type-II EC-267877). The studied cultivars of *L. odemensis* (ILWL-35) and *L. ervoides* (ILWL-61) are originated from Turkey (Balıkesir),*L. lamottei* (ILWL-14) originated from France (Alpes-Cote d'Azur) and *L. nigricans* (ILWL-19) originated from Spain (Singh et al. 2020); however, a specific country of origin is not known for *L.orientalis* (ILWL- 365). All the studied wild species over the decades have adapted adequately in the Indian climate (Table 1). The samples (seeds) were germinated in the dark on moist filter papers to harvest healthy root tips, then transferred in the earthen pots and maintained. The herbarium vouchers were prepared for each species, and flowers, seeds, tendrils, sensitivity to insect attack and cultivation time were studied for morphological characterisation.

Chromosome preparation for mitotic study

For mitotic chromosome preparation, a minimum of 10 root tips of each species was pretreated with saturated para-dichlorobenzene (pDB) at 14–16 °C for 4–5 h, fixed overnight in glacial acetic acid: methanol (1:3) and stored at −20 °C. Enzymatic maceration and air drying (EMA) were carried out at 37 °C following our earlier protocol (Jha et al. 2015) with specific modifications. The air-dried slides were stained with 2% Giemsa solution (Merck, Germany) in 1/15 phosphate buffer for 10–15 min at 23 ± 2 °C room temperature (Jha et al. 2017). The slides were then rinsed with ddH_2O (5 times) and air-dried. A minimum of 20 well-scattered metaphase plates of each accession was examined and analysed under a Carl Zeiss Axio Lab A1 microscope using Axiovision L. E4 software (Germany).

Fluorochrome staining of chromosomes

Giemsa-stained slides with well-scattered metaphase plates were destained with 70% methanol for 40 min, air-dried and examined by fluorochrome staining with DAPI and CMA according to the protocol of Kondo and Hizume (1982) with minor modifications (Jha and Yamamoto, 2012; Jha and Bhowmick 2021; Jha et al. 2020). For CMA staining, the slides with metaphase chromosomes were stained with 0.1 mg/mL CMA solution for different durations (from 30 to 60 min), allowed maturation for 48 h at 4 °C before observing under a fluorescence microscope (Zeiss Axioscop 2) with BV filter cassette. For DAPI stating, the slides were incubated with 0.1 µg/mL DAPI solution for10–20 min, followed by counterstaining with actinomycin D for 15 min. The slides were observed under UV filterimmediately or after 24-h maturation. A minimum of 25 metaphase plates was studied to determine the number and patterns of fluorescent signals. The images were captured and analysed using software Prog Res 2.3.3.

Genomic DNA isolation and PCR amplification of ribosomal DNA ITS1-5.8S-ITS2 region

Genomic DNA was isolated from young leaves of each of the taxa using the CTAB method (Doyle and Doyle 1987). The quality of DNA in each sample was assessed by 1.0% (w/v) agarose gel electrophoresis. The DNA concentration was measured using Eppendorf Bio Spectrophotometer. The rDNA ITS region was amplified using the universal forward primer: 5′-GGAAGTAAAAGTCGTAACAAGG-3′ and reverse primer: 5′-TCCTCCGCTTATTGATATGC-3′ (White et al. 1990) on a programmable thermal cycler (Advanced Primus 96, Peqlab, Germany) following the protocol of White et al. (1990), using the commercial kit (Genei, Bangalore, India). The 25-µL reaction consisted of 2.5 µL of 10X PCR buffer with 15 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mix, 100 ng template DNA, 0.5 µL of Taq DNA polymerase (5 U/µL) and 1 µL of each primer (4.0 pM).

DNA sequencing

The rDNA ITS PCR amplicons of *L. culinaris* (only the cultivar MicroType-I has been used), *L. nigricans, L. lamottei* and *L. ervoides* were sequenced using the Big Dye Terminator cycle sequencing method (Xcelris Labs Ltd, Gujarat, India; http://www.xcelrislabs.com). The chromatograms were analysed using the Bio-Edit.v.7.1.3 software (Ibis Biosciences, Carlsbad, CA, USA), followed by multiple sequence alignments using ClustalW (http://www.genome.jp/tools/clustalw) with gap open penalty: 15 and gap extension penalty: 6.66. All the newly generated sequences have been deposited in the NCBI GenBank database (http://www.ncbi.nim.nih.gov) under accession numbers listed in Table 1.

Table 1 List of collected Indian species of *Lens* with their chromosome characteristics, fluorescent banding patterns and NCBI GenBank accession numbers

Species & Somatic chromosome no. (2n)	Cultivar & Voucher no	Karyotype formula (n)	Ordering no. of satellite bearing pair & position of SAT on chromosome	No of CMA^{+ve} /$DAPI^{-ve}$ bands/ 2n	Chromosome pair showing CMA^{+ve} bands & position of bands	No of $DAPI^{+ve}$/ CMA^{-ve} bands	Chromosome pair(s) showing $DAPI^{+ve}$ bands	Position of $DAPI^{+ve}$ bands on chromosome	Gene Bank Accession ID (rDNA ITS sequence)*
*L. culinaris*2n = 14	Type-IEC-267526	3 m + 1 m.Sat + 2Sm + 1St	4th Intercalary	2	4thNucleolar	8	1st, 2nd, 4th and 6th	6 (centromeric) and 2 (proximal part of the short arm)	MT002736
	Type-IIEC-267877								–
*L. odemensis*2n = 14	ILWL-35	3 m + 1 m.Sat + 2Sm + 1St	3rd Intercalary	2	3rdNucleolar	6	1st, 2nd and 3rd	4 (centromeric) and 2 (proximal part of the short arm)	*EU224442.1*
*L. orientalis*2n = 14	ILWL-365	3 m + 1 m.Sat + 2Sm + 1St	4th Intercalary	2	4thNucleolar	6	1st, 4th and 6th	2 (centromeric), 2 (distal part of the short arm) and 2 (proximal part of the short arm)	*AJ441063.1*
*L. nigricans*2n = 14	ILWL-19	1 M + 4 m + 1St.Sat + 1St	6th Terminal	2	6thNucleolar	8	1st, 3rd, 5th and 6th	4 (intercalary regions of the long arm), 2 (distal part of the short arm) and 2 (nucleolar)	MT002739
*L. lamottei*2n = 14	ILWL-14	5 m + 1St.Sat + 1St	5th Terminal	2	5thNucleolar	6	1st, 2nd and 5th	2 (centromeric), 2 (distal part of the short arm) and 2 (nucleolar)	MT002738
*L. ervoides*2n = 14	ILWL - 61	5 m + 1St.Sat + 1St	6th Terminal	2	6thNucleolar	18	All pairs	2 (proximal part of the short arm), 2 (distal part of the short arm), 4 (centromeric), 8 (intercalary regions of the long arm) and 2 (nucleolar)	MT002742

*GenBank accession numbers in 'italics' have been retrieved from the NCBI public database (http://www.ncbi.nim.nih.gov)

Phylogenetic analysis using rDNA ITS1-5.8S-ITS2 sequences

Phylogenetic analysis using rDNA ITS1-5.8S-ITS2 sequences was performed using maximum likelihood (ML) and maximum parsimony (MP) methods onMEGA 6.06 (Tamura et al. 2013). The present study involved a total of eight accessions, including *L. orientalis* (accession no. EU224442.1) and *L. odemensis* (accession no. AJ441063.1), and two out group taxa viz. *Mimosa hapaloclada*(accession no.KT364054.1) and *Mimosa colombiana* (Accession no. KT386295.1). The best-fit nucleotide-substitution model was determined as T92 + G (Tamura 3-parameter model) using MEGA 6.06 (Tamura et al. 2013). Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-joining (NJ), and BioNJ algorithms applied to a matrix of pairwise distances estimated the maximum composite likelihood (MCL) and then selected the topology with a superior log-likelihood value. A discrete gamma distribution was applied to model the evolutionary rate differences across various sites (5 categories (+ G, parameter = 0.6197)). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1, wherein the initial trees were obtained by the random addition of sequences (10 replicates). The MP tree was also constructed using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). Bootstrap analyses were performed on 1000 replicates (Felsenstein 1985) in both analyses.

Statistical analysis

Descriptive statistics were analysed for all measured parameters (viz. ordering no. of satellite bearing pair, number of $CMA^{+ve}/DAPI^{-ve}$ bands, number of chromosome pairs showing CMA^{+ve} bands, number of $DAPI^{+ve}/CMA^{-ve}$ bands and number of chromosome pairs showing $DAPI^{+ve}$ bands). Duncan's multiple range test (DMRT) was used for posthoc analysis. One-way analysis of variance (ANOVA) was performed at 0.05 probability level using SPSS v 16.0 statistical package.

Results

In the present study, a comprehensive analysis of floral and vegetative morphology, karyology, fluorescent banding patterns and rDNA ITS sequence study was carried out for six Indian species of *Lens*.

L. culinaris is grown in winter as a Rabi crop (18–30 °C). It is susceptible to insect pest *Bruchus lentis* Froel, and is characterised by flattened seeds with light brown seed coats; however, the size of the seeds varied among the two cultivars (Fig. 1a and d). The *L. Culinaris* cultivar Micro showed small-sized seeds (~ 3.18 mm in diameter), while *L. Culinaris* cultivar Macro has large seeds (~ 3.5 mm in diameter); no tendril formation was noted in both cultivars (Fig. 1b and e). The flowers were large, white and identical in both cultivars (Fig. 1c and f). Each of the two cultivars of *L. culinaris* showed the $2n = 14$ somatic chromosomes. The haploid karyotype formula was 3 m + 1 m.Sat + 2Sm + 1St (Table 1). The diploid chromosome complements of each *L. culinaris* cultivar showed a single pair of chromosomes (no. 4) with intercalary satellite (Fig. 2a, c). The fluorescence staining analysis revealed the presence of both $DAPI^{+ve}$ and CMA^{+ve} bands (Table 1). The bright $CMA^{+ve}/DAPI^{-ve}$ regions were found in the nucleolar regions of the satellite bearing both cultivars (Figs. 2a, c and 4a, b). Each cultivar carried six chromosomes (chromosome pair no. 1, 2 and 6) with distinct centromeric $DAPI^{+ve}/CMA^{-ve}$ signals and two sat-bearing chromosomes (pair no. 4) with $DAPI^{+ve}/CMA^{-ve}$ signals on the proximal part of the short arm (Figs. 2b, d and 4a, b).

L. odemensis is grown in winter (18–30 °C). Also, it is susceptible to insect pest *B. lentis* Froel, seeds are large (~ 4.17 mm), flattened and light brown, tendrils are absent and flowers with whitish-purple corolla were similar to those of *L. orientalis* (Fig. 1g–i). *L. odemensis* showed 2n = 14 somatic chromosomes with the haploid karyotype formula as 3 m + 1 m.Sat + 2Sm + 1St (Table 1). Among seven pairs of chromosomes, one pair (no. 3) carried an intercalary satellite (Fig. 2e). The fluorescence staining analysis revealed two sat-bearing chromosomes with nucleolar $CMA^{+ve}/DAPI^{-ve}$ regions (Fig. 2e and Fig. 4c). On the other hand, four chromosomes (chromosome pairs no. 1 and 2) showed distinct centromeric $DAPI^{+ve}/CMA^{-ve}$ signals, and two sat-bearing chromosomes (pair no. 3) with

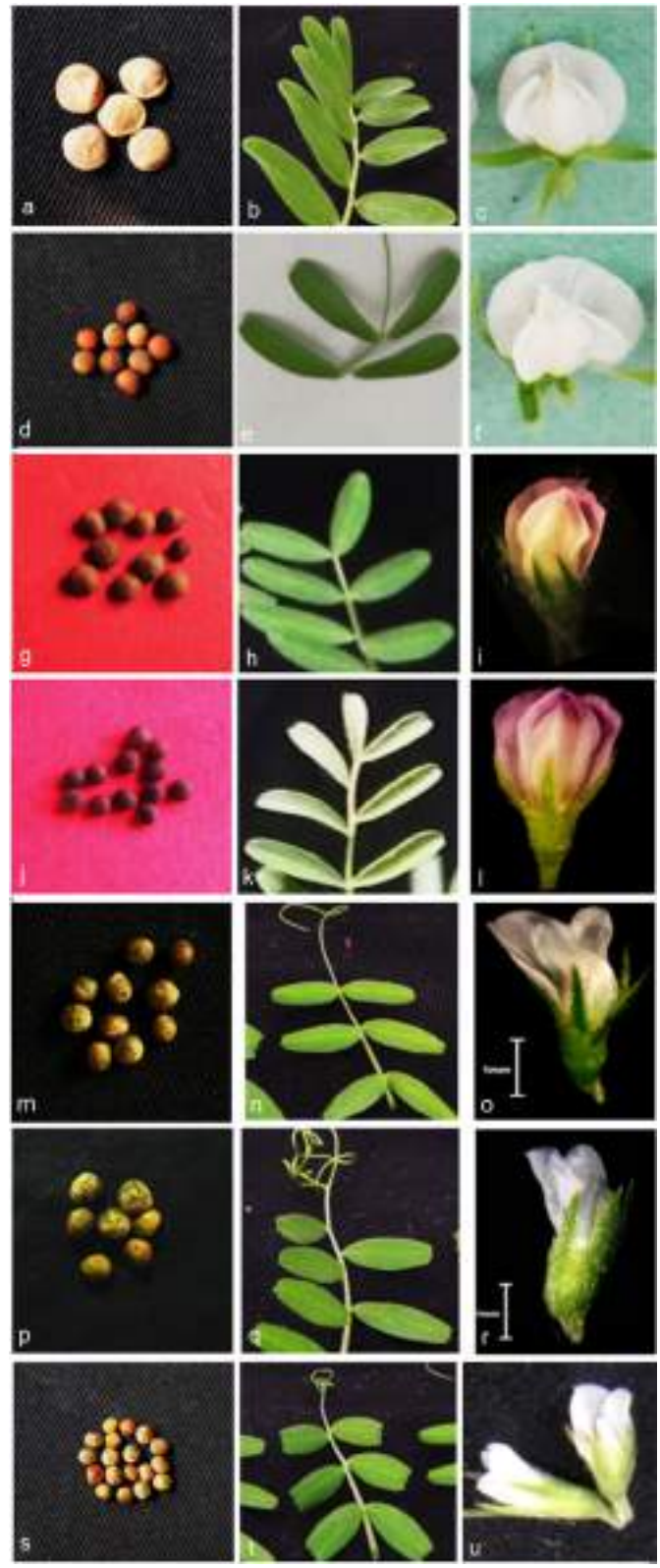

Fig. 1 Seeds, tendril habit and floral morphology of seven Indian accessions of *Lens*. **a–c** *L. culinaris* cultivar Micro; **d–f** *L. culinaris* cultivar Macro; **g–i** *L. odemensis*; **j–l** *L. orientalis*; **m–o** *L. nigricans*; **p–r** *L. lamottei* and **s–u** *L. ervoides*

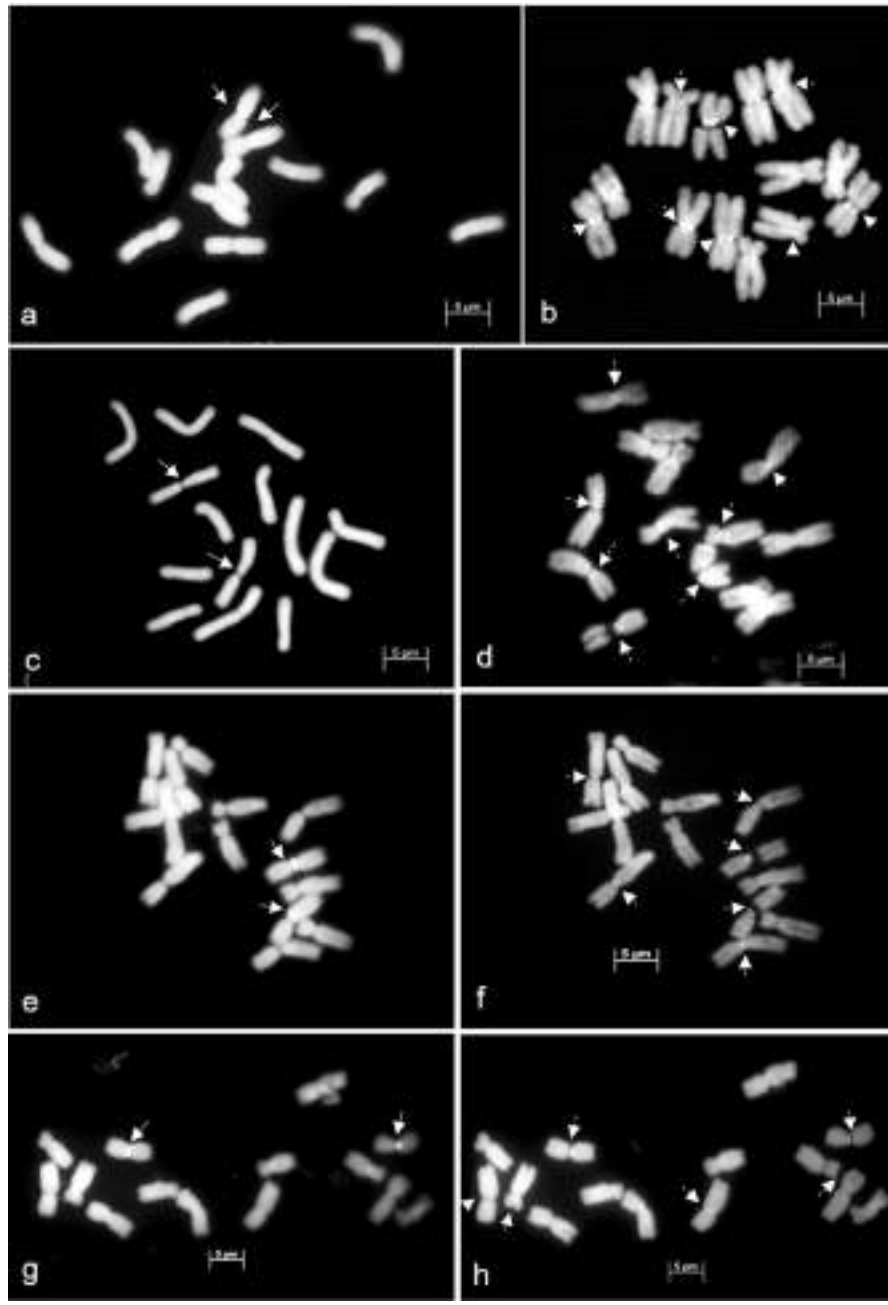

Fig. 2 Fluorescence staining (CMA: **a, c, e, g** and DAPI: **b, d, f, h** of somatic metaphase chromosomes of four accessions of *Lens.* **a, b** *L. culinaris* cultivar Micro; **c, d** *L. culinaris* cultivar Macro; **e, f** *L. odemensis*; **g, h** *L. orientalis.* Arrows indicate chromosomes with fluorochrome bands in respective plates

$DAPI^{+ve}/CMA^{-ve}$signalswere located on the proximal part of the short arm (Fig. 2f and Fig. 4c; Table 1).

L. orientalis: The cultivar was grown in winter (18–30 °C), susceptible to insect pest *B. lentil* Froel. The seeds were large, light brown and flattened in shape, tendrils were absent and flowers had whitish-purple corolla (Fig. 1j–l). The diploid chromosome complements of *L. orientalis* showed $2n = 14$ somatic chromosomes. The haploid karyotype formula was 3 m + 1 m.Sat + 2Sm + 1St (Table 1) similar to

that of *L. culinaris*. The cultivar was also characterised by a single pair of chromosomes (no. 4) with an intercalary satellite (Fig. 2g). These sat-bearing chromosomes showed bright nucleolar $CMA^{+ve}/DAPI^{-ve}$ regions (Figs. 2g and 4d), while $DAPI^{+ve}/CMA^{-ve}$ signals were located on three pairs of chromosomes (pair no. 1, 4 and 6). Among these, chromosome pair no. 1 showed two centromeric $DAPI^{+ve}/CMA^{-ve}$ signals, pair no. 6 contained two $DAPI^{+ve}/CMA^{-ve}$ signals located on the distal part of the short armand-pair no. 4 (sat-bearing chromosomes) revealed $DAPI^{+ve}/CMA^{-ve}$ signals were located on the proximal part of the short arm (Figs. 2h and 4d; Table 1).

L. nigricans was grown in winter (18–30 °C), resistant/tolerant to insect pest *B. lentis* Froel, with uniformly roundish seeds, small in size (~ 2.20 mm) with grey-blackish brown seed coats. Also, distinct tendril formation was noted from the early stage of plant development, and flowers were small in size with whitish-blue corolla (Fig. 1m–o). This is also characterised by 2n = 14 somatic chromosomes. The haploid karyotype formula was 1 M + 4 m + 1St.Sat + 1St (Table 1). The diploid chromosome complements of *L. nigricans* showed a single pair of chromosomes (no. 6) with terminal satellite (Fig. 3a). The bright CMA^{+ve} regions were found in the nucleolar regions of the satellite bearing pair (Figs. 3a and 4e), while eight chromosomes (pair no. 1, 3, 5 and 6) showed $DAPI^{+ve}$ signals (Figs. 3b and 4e). Among these, chromosome pair no. 3 and 5 showed $DAPI^{+ve}/CMA^{-ve}$ signals on the intercalary regions of the long arm, chromosome pair no. 1 showed distal $DAPI^{+ve}/CMA^{-ve}$ signals on the short arm, and pair no. 6 of the diploid complement showed distinct nucleolar $DAPI^{+ve}$ signals (Figs. 3b and 4e; Table 1).

L. lamottei: Grown in winter (18–30 °C), it is resistant/tolerant to insect pest *B. lentis* Froel. The seeds are small (~ 1.92 mm) and roundish in shape with grey-blackish brown seed coats. Thetendril formation was noted from the early stage of plant development, and flowers were small in size with whitish-blue corolla (Fig. 1p–r). Furthermore, *L. lamottei* showed 2n = 14 somatic chromosomes with the haploid karyotype formula of 5 m + 1St.Sat + 1 St (Table 1). Of the seven pairs of chromosomes, one pair (no. 5) contains a terminal satellite (Fig. 3c). The bright CMA^{+ve} signals/regions were found in the nucleolar regions of the satellite bearing pair (Figs. 3c and 4f). A total of six chromosomes (chromosome pair no. 1, 2 and 5), including two sat-bearing chromosomes, showed distinct $DAPI^{+ve}$ signals. Chromosome pair no. 1 showed centromeric $DAPI^{+ve}/CMA^{-ve}$ signals, while pair no. 2 had distal centromeric $DAPI^{+ve}/CMA^{-ve}$ signals on the short arm. Pair no. 5, i.e. the sat-bearing chromosome pair showed distinct nucleolar $DAPI^{+ve}$ signals (Figs. 3d and 4f; Table 1).

L. ervoides: Grown in winter (18–30 °C) and summer (30–36 °C), the cultivar is resistant/tolerant to insect pest *B. lentis* Froel, the seeds are small size and roundish with brownish-grey seed coats, tendril formation was observed from the early stage of plant development and flowers were small and white (Fig. 1s–u). *L. ervoides* can be grown in winter (October–February) and early summer (February–May). In the present study, the somatic chromosome number of *L. ervoides* was found to be 2n = 14 for the first time. The haploid karyotype formula was 5 m + 1St.Sat + 1St (Table 1). The diploid chromosome complements of *L. ervoides* showed a single pair of chromosomes (no. 6) with terminal satellite (Fig. 3e). The fluorescence staining analysis revealed that two sat-bearing chromosomes showed bright nucleolar CMA^{+ve} signals/regions (Figs. 3e and 4g). On the other hand, about 18 $DAPI^{+ve}$ signals/regions were differentially located on all the 14 chromosomes of the diploid complement of *L. ervoides* (Figs. 3f and 4g; Table 1).

Phylogenetic correlations among the Indian species of *Lens* based on rDNA ITS1-5.9S-ITS2 sequence.

In order to clarify the interspecies relationships among the Indian members of the genus *Lens*, rDNA ITS1-5.8S-ITS2 sequence-based phylogenetic tree was analysed using both ML and maximum parsimony (MP) methods. Both yielded identical topologies (only the ML tree is shown in Fig. 5). The trees were rooted using two closely related outgroup taxa viz. *Mimosa hapaloclada* (accession no.KT364054.1) and *Mimosa colombiana* (accession no. KT386295.1). The bootstrap support (BS) for each clade is shown below the branches (Fig. 5). All the studied Indian species of *Lens* originated from a single node and split into two distinct clades (Fig. 5). Clade I included *L. odemensis, L. culinaris* and *L. orientalis,* while clade II consisted of *L. lamottei, L. nigricans* and *L. ervoides* (Fig. 5).

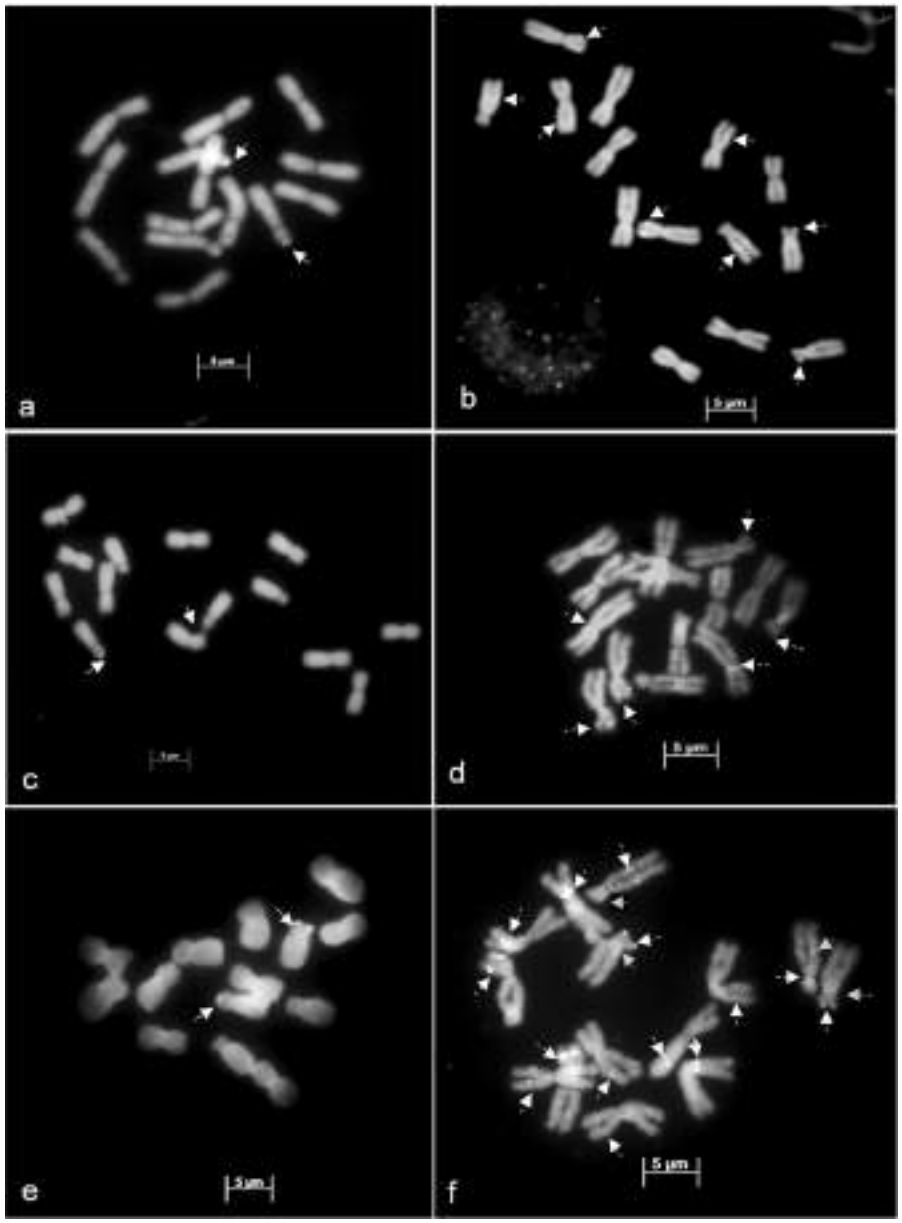

Fig. 3 Fluorescence staining (CMA: **a, c, e** and DAPI: **b, d, f**) of somatic metaphase chromosomes of three wild species of *Lens.* **a, b** *L. nigricans*; **c, d** *L. lamottei* and **e, f** *L. ervoides.* Arrows indicate chromosomes with fluorochrome bands in respective plates

Discussion

The present study demonstrated an explicit inter-species correlation among the Indian cultivated and wild lentil species based on the comprehensive analysis of morphological traits, fluorescent banding and rDNA ITS sequence-based phylogeny. All the species and cultivars of *Lens* contain $2n = 14$ somatic chromosomes. Karyotype analysis in India was mainly carried out on cultivated cultivars of lentil using the conventional aceto-orcein methods (Bhattacharjee 1951; Sharma and Mukhopadhyaya 1963; Sinha and Acharia 1972; Naithani and Sarbhoy 1973; Gupta and Singh 1981; Lavania and Lavania 1983; Nandanwar and Narkhede 1991). However, to facilitate the application of molecular cytogenetic analysis,

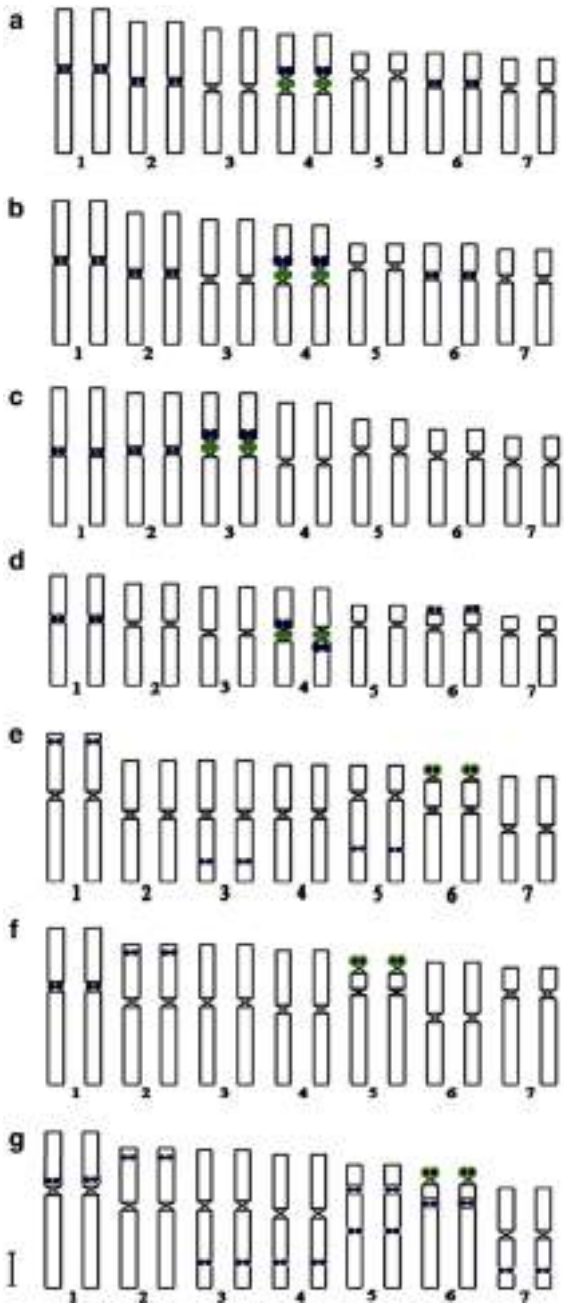

Fig. 4 **a**–**h** Ideograms of different Indian species of *Lens* based on CMA^{+ve}/ $DAPI^{-ve}$ and $DAPI^{+ve}$/CMA^{-ve} banding patterns. **a** *L. culinaris* cultivar Micro; **b** *L. culinaris* cultivar Macro; **c** *L. odemensis*; **d** *L. orientalis.* **e** *L. nigricans*; **f** *L. lamottei* and **g** *L. ervoides* (Scale bar 2 μm)

technical standardisation of chromosome preparation is a prerequisite. The systematic compilation of morphological and karyotype diversity on four wild species (except *L.ervoides*) and > 30 cultivated cultivars of Indian lentil have unravelled new information about Indian lentils through EMA-based chromosome analysis (Jha et al. 2015, 2017; Jha and Halder 2016). Balyan et al. (2002) reported the interstitial position of the nucleolar organising region (NOR) in cultivated *L. culinaris* and wild species *L. orientalis, L. odemensis* and *L. ervoides* and terminal in *L. nigricans*. In addition, this study included morphometric analysis of *L.ervoides* for the first time and documented secondary terminal constriction (sat chromosome) in *L. ervoides*similar to that of *L. nigricans* and *L. lamottei*. The fluorescent banding analysis revealed CMA^{+ve} signals in the sat-bearing chromosomes of *L. ervoides*, *L. nigricans* and *L. lamottei* and confirmed and upgraded our earlier observations. Similarly, two cultivated accessions of *L.culinaris* and two wild species, *L. orientalis* and *L. odemensis*, showed intercalary secondary constriction. The variations in the intensity of CMA^{+ve} signals within the species have been recorded. Reportedly, the NOR, the site of the secondary constriction region, is composed of higher guanine-cytosine (GC) content compared to other parts of chromosomes. The preferential staining of GC-rich regions, assessed using the CMA dyes, resulted in CMA^{+ve} signals on specific marker areas of the chromosomes. The NOR corresponds to the major sites of the 35S rDNA, consisting of a tandem repeat of a unit with the 18S-5.8S-26S rRNA genes and intergenic spacer regions (Biscotti et al. 2015). Reportedly, *L.culinaris* has two to four 5S and two 35S rDNA loci in the genome (Garcia et al. 2017). Galasso (2003) conducted fluorescence in situ hybridisation (FISH) to study the repeated DNA sequences on various species of *Lens*. Fernández et al. (2005) analysed the rDNA genome regions in some *Lens* species using a non-transcribed spacer (NTS) and FISH. However, none of the studies has yet reported about the rDNA loci in Indian wild species, which might highlight some interesting features in lentil phylogeny. CMA^{+ve} banding pattern in one cultivated and five wild species (Table 1) further confirmed the earlier proposed two-group system in Indian lentil taxa studied previously (Jha and Halder 2016). Opposite to GC-preferred CMA, DAPI is an AT-preferred nucleic acid dye used in many plant species (Guerra et al. 2000). $DAPI^{+ve}$ signals are observed on chromosomes related to AT-rich heterochromatic zones, while GC-rich regions showed $DAPI^{-ve}$ signals. Moreover,

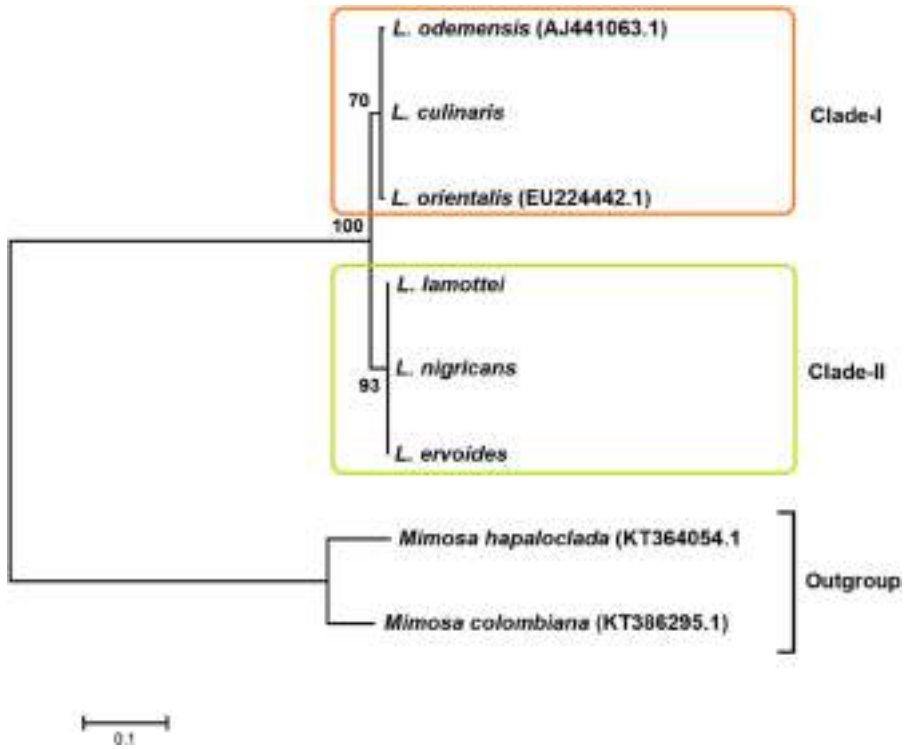

Fig. 5 ML phylogeny of the genus *Lens* based on rDNA ITS1–5.8S–ITS2 sequences data. Numbers beneath the nodes are BS indices

DAPI^{+ve} signals are frequently observed in interstitial regions on chromosomes (Guerra et al.2000), and distinct signals have been reported in many plant species (Lavania et al. 2010; Barros e Silva and Guerra 2010; Divashuk et al. 2014; Jha 2019). In some cultivars of *Lathyrus* (Fabaceae), DAPI^{+ve} bands have been reported mostly in the terminal part of the chromosomes (Akter et al. 2015). On the other hand, Rodrigues et al. (2018) failed to obtain any DAPI^{+ve} bands in another Fabaceae member *Caesalpinia*. Thus, the present findings are focussed on the genomic structure containing AT-specific DAPI^{+ve} regions in Indian lentil species. Their diversity in number (6–18) and locations on different chromosomes are not reported earlier; detailed information is presented in Table 1. A maximum of 18 DAPI^{+ve} signals distributed in the chromosomes have been recorded in *L.ervoides*, while CMA^{+ve} bands were restricted only to different sat-bearing NOR regions of the respective chromosome pairs (Table 1). In addition to the morphological and molecular cytogenetic analysis, the present rDNA ITS sequence-based phylogenetic study revealed that all the analysed Indian species of *Lens* comprise a monophyletic group and further split into two possible clades (I and II) for the first time. The study of the rDNA ITS1-5.8S-ITS2 sequence is a key attribute in the species-level phylogenetic interpretation (Saha et al. 2015, 2017; Saha and Jha 2019). Furthermore, the rDNA ITS region is highly repetitive, with nuclear copies arranged in tandem arrays. It contains variable regions flanked by conserved DNA sequences organised in NORs, potentially at more than one chromosomal locations (Alvarez and Wendel 2003; Sonnante et al. 2003). In the present phylogenetic tree, cladeI includes three taxa, *L. odemensis, L. culinaris* and *L. orientalis*, while the other three wild species, *L. lamottei, L. nigricans* and *L. ervoides*, comprised cladeII. These results were in congruence with our earlier study (Jha and Halder 2016), wherein five Indian lentil species (one cultivated and four wild) were categorised into two distinct groups based on the morphological and Giemsa analysis of the chromosomes. Thus, the results of the correlated studies presented here with two accessions of cultivated and five wild lentil species have widened the primary gene pool in a commercial crop. Interestingly, the wild

relatives of several major crops are adapted to hostile climates, adverse soil types and can tolerate many biotic and abiotic stresses constitute a repository for crop improvement (Khoury et al. 2020; Dempewolf et al. 2017). Disease resistance capacity and seeds with enriched essential minerals are crucial for the three wild species, *L. lamottei, L. nigricans* and *L. ervoides* (Kumar et al. 2018; Singh et al. 2020). Furthermore, *L. ervoides* can withstand a temperature range of 30–37 °C. The maximum number of AT-rich heterochromatic regions has also been documented in *L.ervoides*. Thus, the three wild species mentioned above, especially *L.ervoides*, deserves the focussed attention of breeders and genome researchers on the introgression of genes to the cultivated species. The introgression of the different traits for crop improvement has been achieved in several crops (Jarvis et al. 2002; Jena and Nissila 2017; Singh 2019).

In conclusion, the present study with limited samples emphasised on chromosome analysis through plant molecular cytogenetic techniques for unravelling GC-rich and diverse AT-specific heterochromatic regions on lentil chromosomes. The standardised technique of molecular cytogenetics is now open for application in other wild lentil cultivars. The process can be extended to FISH and genomic in situ hybridisation (GISH) to understand the cytogenetic mechanisms underlying the evolution of the species. The correlation of morphological parameters along with chromosomal features and generation of an unbiased phylogenetic tree through rDNA sequence analysis in Indian lentil taxa might be useful in lentil crop improvement programmes.

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Analysis of CMA-DAPI bands and preparation of fluorescent karyotypes in thirty Indian cultivars of *Lens culinaris*

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Abstract. India holds a significant rank in production and consumption of the age old protein rich crop Lentil with only one cultivated species and a large number of phenotypically similar cultivars. The need for a reliable and cost effective method of genetic characterization to unravel differences within the Lentil cultivars was felt. The present paper adopted EMA based chromosome preparation followed by staining with two contrasting fluorochrome dyes CMA and DAPI that bind directly to GC and AT rich heterochromatic segments on chromosomes. Analysis of fluorochrome banding pattern furnished a comparative account of genetic diversity within the cultivars that could not be achieved by traditional karyotyping. The marker pair of nucleolar chromosomes (4th and 3rd, majorly) occupied a pivotal position to intensify differences between cultivars in terms of banding patterns around secondary constrictions, suggestive of yet unknown variation in heterochromatin composition. Our study has strengthened genetic background and relationships of Lentil cultivars. We observed certain types of unusual fluorochrome bands that put forward the exclusivity of Indian germplasm and have questioned the mainstream heterochromatin elements of plant chromosomes captured by CMA-DAPI stains. The comprehensive fluorescent karyotypes of 30 *L. culinaris* Medik. cultivars prepared for the first time, serve as an archetype for the benefit of future breeding programmes.

Keywords: lentils, CMA-DAPI, chromosomal bands, fluorescent karyotype, heterochromatin.

INTRODUCTION

Lentil is one of the richest protein containing domesticated ancient crop with only one globally cultivated species *Lens culinaris* Medik. India is the second highest producer and biggest consumer of Lentils. The genus belongs to the largest subfamily (Papilionoideae) of Fabaceae (Azani et al. 2017), along with economically important genera producing pulses and beans. Being the single cultivated species, large number of cultivars is in cultivation in our country. The characterization of Indian germplasm is needed to

sustain conservation and programmable utilization of resources. Chromosomal characterization is a cost effective method to provide foundational information on the genome and genetic conservation for any future breeding program of particular crop plants. Cytogenetic studies of Indian Lentils through conventional method failed to provide uniformity on chromosome morphometric parameters (Bhattacharjee 1953; Sharma and Mukhopadhyay 1963; Sinha and Acharia 1972; Naithani and Sarbhoy 1973; Lavania and Lavania 1983; Nandanwar and Narkhede 1991). On the other hand, we have published detailed karyotype analysis of more than thirty *L. culinaris* cultivars obtained from the Indian Institute of Pulses (Jha et al. 2015, 2017; Jha and Halder 2016) through EMA based Giemsa staining method. Our results were found to have near similarities with the results obtained by Ladizinsky (1979). However, *Lens* chromosomes (2n=14) are nearly similar in morphology. Considering the status of research, we question i) is there any karyotype variability across cultivars beyond chromosome number, morphology and ploidy? ii) is it possible to find visible chromosomal landmarks in accordance with the germplasm diversity? and iii) whether we can step forward towards molecular kayotype database for Indian Lentils. As EMA based chromosome analysis (Fukui 1996) is the basis of molecular cytogenetics, we decided to carry forward our work with two contrasting fluorescent stains DAPI and CMA on the same cultivars. Having affinity towards specific base pairs of DNA, these fluorescent dyes reliably identify heterochromatin rich sectors on chromosomes, differentiate morphologically alike chromosomes and improve karyotype characterization (Schweizer 1976; Guerra et al. 2000; Yamamoto 2012; Weiss-Schneeweiss and Schneeweiss 2013). So, our objective is to address chromosomal behavior after application of base specific fluorochromes and compile cultivar specific fluorescent banding profiles. The present paper considers a fluorescent karyotype dataset of 30 Indian *L. culinaris* cultivars for the first time, as an important kit for Lentil breeders and genome researchers.

MATERIALS AND METHODS

Chromosome preparation and fluorochrome staining

The fluorescent karyotype analysis was carried out on 30 cultivars of *Lens culinaris* presented in Table 1. Except for two (Barasat, Micro type Barasat, Macro type, Table 1), all the cultivars of Lentil were obtained from the Indian Institute of Pulse Research (IIPR), Kanpur. Germination of seeds and chromosome processing through enzymatic maceration and air drying (EMA) was carried out as per our earlier protocol (Jha and Yamamoto 2012; Jha et al. 2015, 2017). For fluorescent staining with DAPI and CMA, we followed our protocol (Jha 2019) with required modifications. For DAPI staining, slides were kept for 30 min in McIlvaine buffer, stained with 0.1µg ml^{-1} solution of DAPI for 10 min, counterstained with 0.25mg/ml of Actinomycin D (AMD) for 15min and then mounted in non-fluorescent glycerol and observed under Carl Zeiss Axio Lab A1 fluorescence microscope using Carl Zeiss DAPI filter cassette. Chromosome images were captured with CCD camera attached with microscope. The slides were destained and air-dried. The same slides were placed in McIlvaine buffer for 30 min followed by incubation in McIlvaine buffer with 5mM $MgCl_2$ for 10 mins and then stained with 0.1mg ml^{-1} CMA solution for 45-50 mins. The slides were again washed in McIlvaine buffer with 5mM $MgCl_2$ and finally mounted with non-fluorescent glycerol and kept for maturation at 4^0C for 48-72 hrs. CMA stained slides were observed under the above-mentioned fluorescence microscope fitted with Carl Zeiss FITC filter cassette, images captured with attached CCD camera and signals were analyzed using the software Prog Res 2.3.3.

Statistical analysis of karyotype relations

Karyotype relations among the cultivars was evaluated with the help of cluster analysis for data matrix normalization by unweighted pair group method with arithmetic averages (UPGMA) based on Euclidean distance using Info Stat 2017d (free version). Here, only the fluorochrome banding pattern of the cultivars *viz*. types and numbers of CMA and DAPI bands were utilized to draw the phenogram.

RESULTS

Fluorochrome banding pattern in cultivars of L. culinaris Medik.

Somatic chromosome analysis of the 30 Lentil cultivars based on fluorescence banding patterns has provided an interesting catalogue of chromosome diversity. The chromosomes took up DAPI stain within 10 minutes of incubation while the incubation time for CMA staining was about 45-50mins. The same CMA and DAPI staining protocol was followed for all the 30 cultivars of *L. culinaris*. Interestingly, we have obtained different types of DAPI and CMA banding patterns within the studied

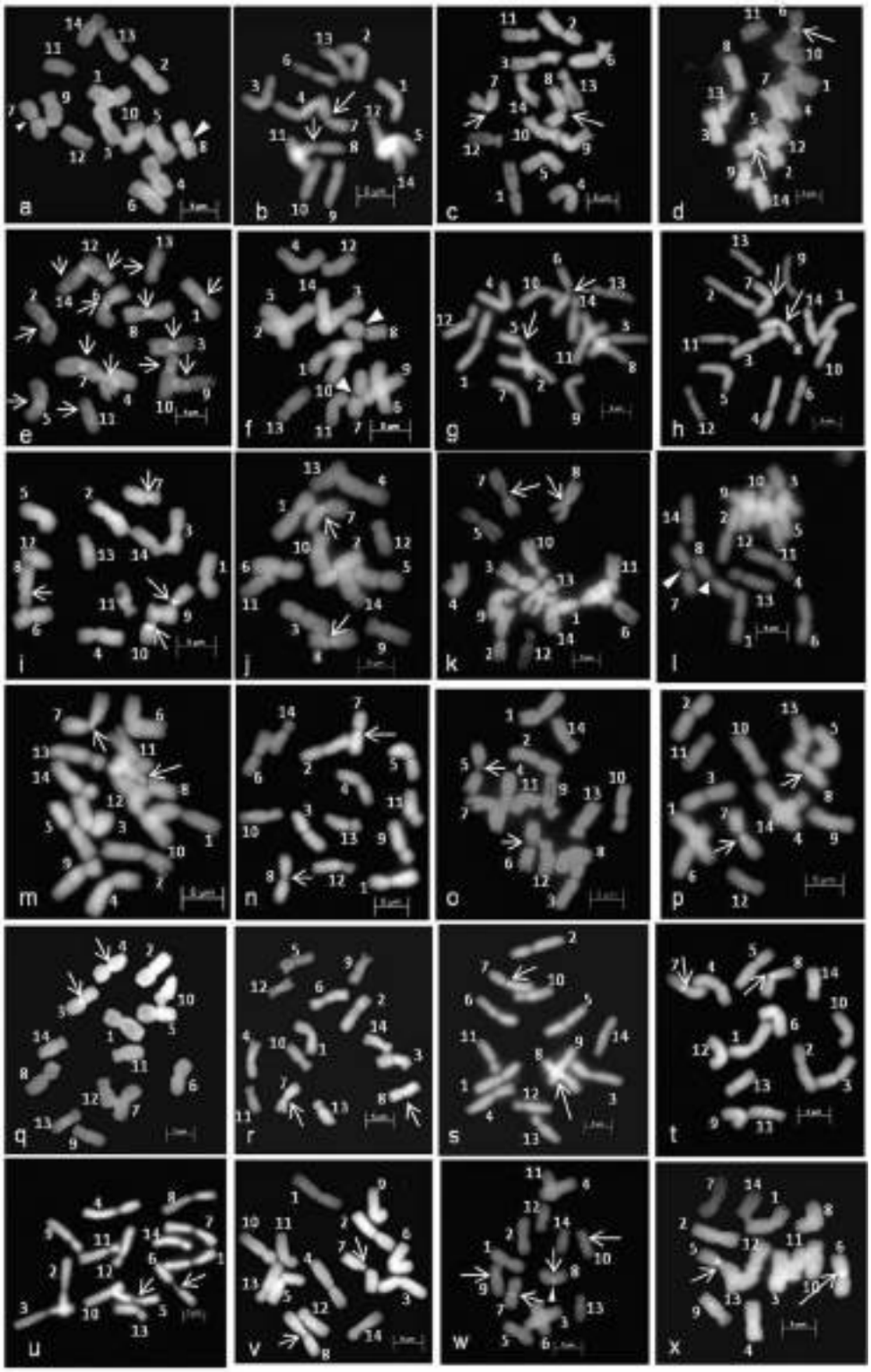

Figure 1. Somatic metaphase chromosomes of *Lens culinaris* cultivars stained with CMA: (a) DPL-15, (b) DPL-62, (c) IPL-81, (d) IPL-406, (e) IPL-316, (f) JL-1, (g) HUL-57, (h) KLS-210, (i) EC-70394, (j) EC-70403, (k) EC-70404, (l) EC-78452, (m) EC-78455, (n) EC-78461, (o) EC-78475, (p) EC-78498, (q) EC-78542-A, (r) EC–223188, (s) EC – 255491, (t) EC–267526, (u) EC-267569-A, (v) EC–267590, (w) EC–267877, (x) Barasat Micro type. White arrows indicate CMA^+ bands and arrowheads indicate CMA^0 bands. Bars 5μm.

cultivars. At least 10 plates stained with DAPI and CMA for each cultivar was considered for analysis of banding types. Secondary constriction marks the nucleolar organizing region (NOR) of most of the cultivars showed CMA^+ bands with different intensities while some NORs remained neutral and termed CMA^0 as per Barros e Silva and Guerra (2010). DAPI staining in most of the cultivars resulted a clear gap ($DAPI^-$) corresponding to CMA^+ band. However, few exceptional cultivars yielded $DAPI^+$ band in the NOR regions. Based on the CMA and DAPI fluorescent banding, we have categorized following types of somatic chromosomes–. The chromosomes with $CMA^+/DAPI^-$ band in the nucleolar region is termed type 'A'. Type 'B' has $CMA^+/DAPI^-$ nucleolar constriction followed by a $DAPI^+/CMA^0$ band below centromere. The 'C' type nucleolar chromosome has a distinct CMA^+/ $DAPI^+$ secondary constriction. The fourth type 'D' has neutral CMA band in secondary constriction. Chromosomes with centromeric $CMA^+/DAPI^0$ bands are termed type 'E' while those with centromeric $DAPI^+/CMA^0$ bands are termed type 'F'. Type 'G' chromosome contains intercalary $DAPI^+/CMA^0$ band. The chromosomes having no detectable bands were termed as type 'H'. Distribution of different types of fluorochrome bands among the cultivars is summarized in Table 1. A detailed analysis of the fluorochrome stained metaphase plates (Figures 1-3) was carried out to formulate the diagrammatic fluorescent karyotypes of the 30 cultivars under study (Figures 4 and 5).

CMA-DAPI banding pattern have revealed that in majority *L. culinaris* cultivars (Table 1) the marker secondary constrictions with CMA^+ signals are present in the 4th pair of chromosomes. However, the same in some cultivars are present in the 3rd and exceptionally 5th and 2nd pairs in two cultivars (EC-70394, EC-78542-A). The most abundant CMA^+ satellites (type A chromosomes) are found among 50% of the presently studied cultivars. In addition to CMA^+ satellites, existence of type B chromosomes is found in 8 different cultivar (HUL-57, EC-70403, EC-78542-A, EC-267526, EC – 267877, Barasat Micro type, PL -1406, EC -78410, Table 1) and type D chromosomes in 5 different cultivars (DPL15, JL-1, EC-78452, EC – 70306, EC – 78473, Table 1). Of special mention, are the two cultivars (EC-70404, EC-267569-A, Table 1) with $CMA^+/DAPI^+$ satellite (type C chromosome). Three cultivars (IPL -316, EC-70394, EC – 267877) had centromeric CMA^+ bands (type E) (Table 1). One of them (IPL -316) shows centromeric CMA^+ bands (type E) in every chromosome except the nucleolar pair (Table 1). On the other hand, EC -78410 shows intense centromeric $DAPI^+$ bands (type F) in all non-nucleolar chromosome pairs (Table 1). Centromeric $DAPI^+$ bands are consistently found in the 2nd or the 3rd pair of chromosomes in 5 cultivars (HUL-57, EC-70403, EC-267526, Barasat, Macro type, PL -1406, Table 1). Intercalary $DAPI^+$ band (type G) is seen only in IPL-406 (Table 1).

Comparative statistical assessment of fluorochrome banding pattern

Statistical evaluation of karyotype relations among the 30 Lentil cultivars was carried out using Euclidean distance matrix on the basis of CMA and DAPI bands. The UPGMA phenogram presented relative karyotype affinities and distances with a cophenetic correlation of 0.986 as a good fit between the cophenetic value matrix and the average Euclidean distance matrix (Figure 6). There are three separate groups in the UPGMA phenogram of which Group I consisted of cultivars that do not have close affinity with each other (Figure 6). Within this group, EC -78410 and IPL -316 have fluorescent banding pattern that are in contrast to each other. Also, existence of intercalary $DAPI^+$ band makes DPL-62 distinct, placed at the extreme end of the phenogram. The next noticeable cultivars are EC-70404 and EC-267569-A with $CMA^+/DAPI^+$ secondary constriction (Table 1) (Figure 6). The Group II is large, composed of three subgroups mainly differentiated by nucleolar banding pattern in their marker chromosomes. The first subgroup comprised of 5 cultivars with neutral CMA-DAPI bands in their satellites (type D) (Table 1, Figure 6). The second subgroup is largest, comprising of 13 cultivars with $CMA^+/DAPI^-$ satellite (type A). Here, two cultivars (EC-70394 and Barasat, Macro type) show little distance from rest of the cultivars, because of different types of centromeric bands (Table 1, Figure 6). The third subgroup comprises of 7 cultivars with 'B' type nucleolar chromosomes. This subgroup shows heterogeneity because of variations in centromeric bands (Table 1, Figure 6).

DISCUSSION

Cytogenetics of *L.culinaris* is traditionally acknowledged for species delimitations, crossing behavior, conservation and utilization of plant genetic resources (Ladizinsky 1979; Tadmor et al. 1987; Ladizinsky et al. 1990; Ladizinsky 1999; Mishra et al. 2007). With the present approach, we have entered the modern karyotyping system to study chromosomal specialization in Indian Lentils. The diversity of fluorescent karyotypes can be indisputably attributed to the differences in underlying chromosomal heterochromatin of the samples since i)

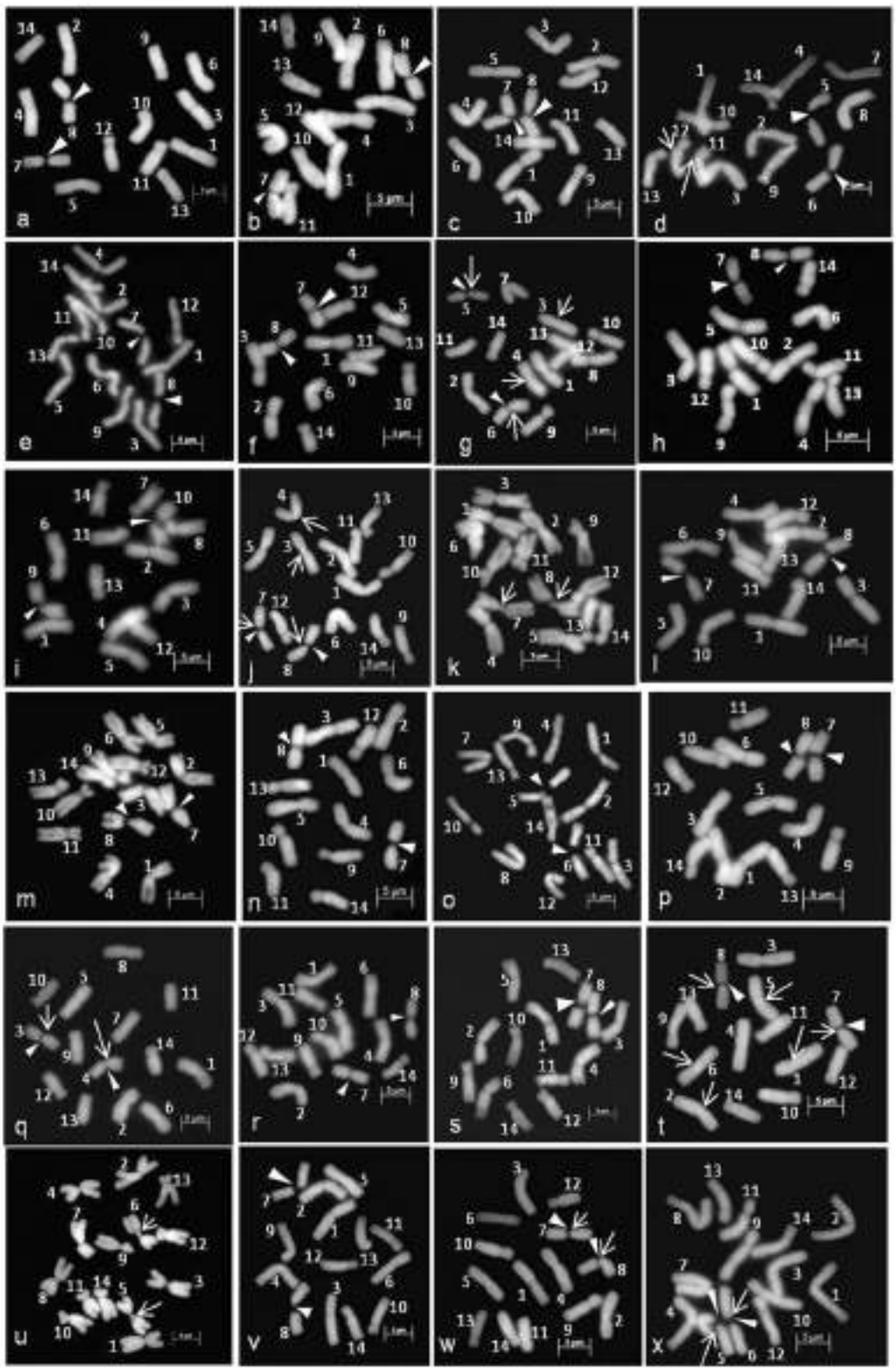

Figure 2. Somatic metaphase chromosomes of *Lens culinaris* cultivars stained with DAPI: (a) DPL-15, (b) DPL-62, (c) IPL-81, (d) IPL-406, (e) IPL-316, (f) JL-1, (g) HUL-57, (h) KLS-210, (i) EC-70394, (j) EC-70403, (k) EC-70404, (l) EC-78452, (m) EC-78455, (n) EC-78461, (o) EC-78475, (p) EC-78498, (q) EC-78542-A, (r) EC–223188, (s) EC – 255491, (t) EC–267526, (u) EC-267569-A, (v) EC–267590, (w) EC–267877, (x) Barasat Micro type. White arrows indicate $DAPI^+$ bands and arrowheads indicate $DAPI^-$ and $DAPI^0$ bands. Bars 5μm.

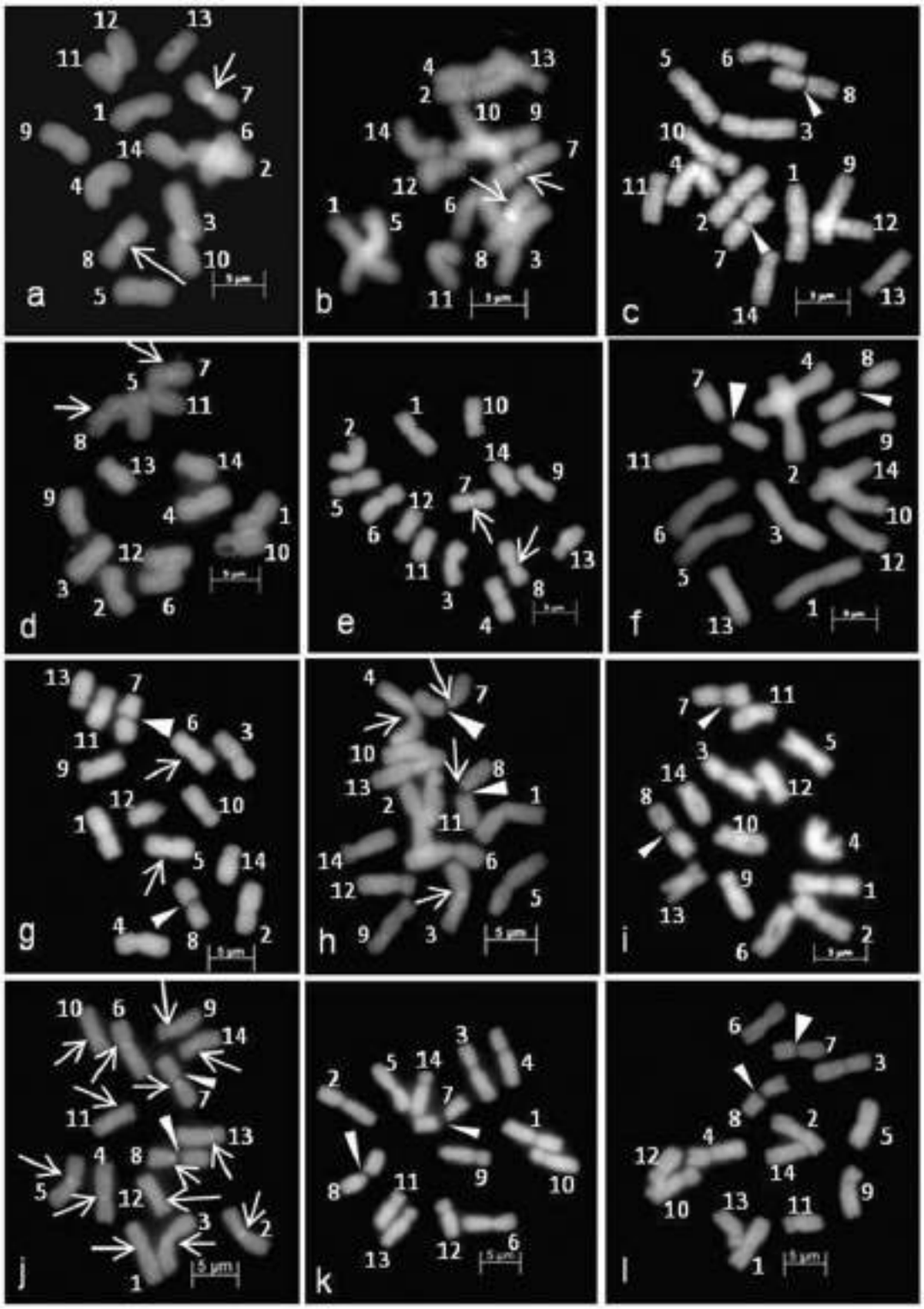

Figure 3. Somatic metaphase chromosomes of *Lens culinaris* cultivars. CMA stained plates: (a) Barasat Macro type, (b) PL-1406, (c) EC-70306, (d) EC -78410, (e) EC-78451-A, (f) EC-78473. DAPI stained plates: (g) Barasat Macro, (h) PL-1406, (i) EC-70306, (j) EC -78410, (k) EC-78451-A, (l) EC-78473. White arrows indicate CMA^{+} or $DAPI^{+}$ bands and arrowheads indicate CMA^{0} or $DAPI^{0}$ and $DAPI^{-}$ bands. Bars 5μm.

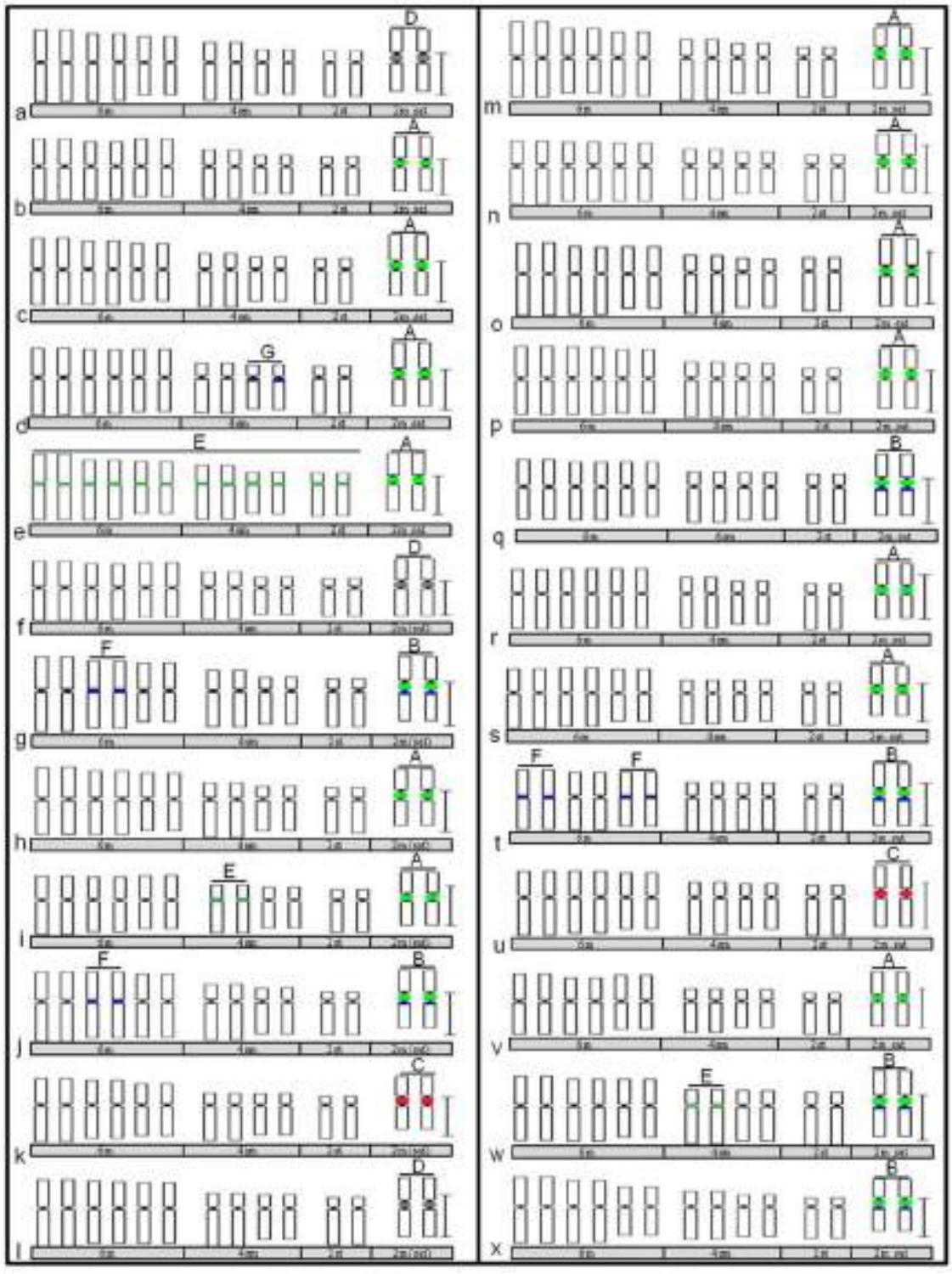

Figure 4. Fluorescent ideograms of *Lens culinaris* cultivars based on CMA/DAPI banding pattern: (a) DPL-15, (b) DPL-62, (c) IPL-81, (d) IPL-406, (e) IPL-316, (f) JL-1, (g) HUL-57, (h) KLS-210, (i) EC-70394, (j) EC-70403, (k) EC-70404, (l) EC-78452, (m) EC-78455, (n) EC-78461, (o) EC-78475, (p) EC-78498, (q) EC-78542-A, (r) EC–223188, (s) EC – 255491, (t) EC–267526, (u) EC-267569-A, (v) EC–267590, (w) EC–267877, (x) Barasat Micro type. CMA^+, $DAPI^+$, $CMA^+/DAPI^+$ and CMA^0 bands are highlighted with green, blue, red and grey colors on the chromosomes, respectively and the types are indicated above the chromosome diagrams. Bars 5µm

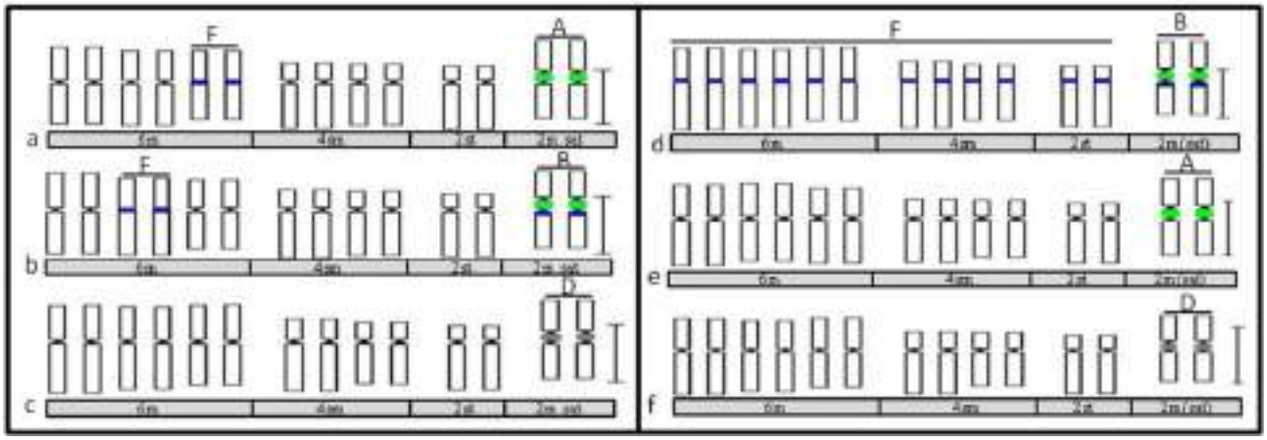

Figure 5. Fluorescent ideograms of *Lens culinaris* cultivars based on CMA/DAPI banding pattern: (a) Barasat Macro type, (b) PL-1406, (c) EC-70306, (d) EC -78410, (e) EC-78451-A, (f) EC-78473. CMA^+, $DAPI^+$, $CMA^+/DAPI^+$ and CMA^0 bands are highlighted with green, blue, red and grey colors on the chromosomes, respectively and the types are indicated above the chromosome diagrams. Bars 5μm.

we have applied the same fluorochrome staining protocol for every cultivar, ii) the method is repeated a number of times before ascertaining banding pattern in a cultivar and iii) at least 5 best metaphase plates of each cultivar with scorable signals were considered for establishing the fluorescent karyotype.

Considering the nature of nucleolar chromosomes, molecular banding technique has shed light on chromosomal landmarks and possible differences in NORs that were previously found to be similar in *Lens* (Mehra et al. 1986; Jha et al. 2015, 2017; Jha and Halder 2016). The marker nucleolar chromosomes (4^{th}, along with the 3^{rd}, 2^{nd} and 5^{th} in few cases) have been confirmed with characteristic CMA-DAPI signals, corroborating to our previous report (Jha et al. 2017). The CMA^+ signals are generally accepted as the GC heterochromatic elements of the NORs in plant groups (Guerra et al. 2000; Barros e Silva and Guerra 2010; Yamamoto 2012; Olanj et al. 2015) and so in Papilionoids such as *Vicia* (Fuchs et al. 1998), *Cicer* (Galasso et al. 1996) and *Crotalaria* (Mondin and Aguiar-Perecin 2011). Previously, 18S-5.8S-25S rDNA probes had been localised in a single pair of *L. culinaris*, near the centromere (Balyan et al. 2002), corroborating to the observation of CMA^+ signals in our present study. However, we found that the intensity of the nucleolar CMA signals (type A) varies in certain cultivars, suggesting differences in NORs that influence affinity towards the stain. Intraspecific rDNA variation has been thoroughly worked out in *Phaseolus* (Moscone et al. 1999; Pedrosa-Harand et al. 2006) and *Vigna* (Bortoleti et al. 2012; She et al. 2015, 2020) of Papilionoideae. A number of factors such as transposition, unequal crossing over, inversion or locus duplication, had been suggested to drive NOR variation in plant groups, including Papilionoideae (Moscone et al. 1999; Chung et al. 2008; Raskina et al. 2008). We consider similar possibilities in the Indian Lentils, subject to future confirmation by AgNOR staining or rDNA FISH.

The type D chromosomes have satellites that respond indifferently to the CMA stain. The CMA^0 satellites indicate GC neutral nature of heterochromatin (Barros e Silva and Guerra 2010). The type D satellites are in sharp contrast to type A bands, marking cultivar distinction. The other unusual type was the $CMA^+/DAPI^+$ satellites (type C). Previously, the $CMA^+/DAPI^+$ satellites were suggested to be a 'less common' or 'rare' type of heterochromatin (Barros e Silva and Guerra 2010), breaking the generality of GC rich composition of plant NORs (Schweizer 1976; Guerra et al. 2000). We document the occurrence of $CMA^+/DAPI^+$ satellites for the first time in *Lens* of Papilionoideae. Co-localized $CMA^+/DAPI^+$ satellites are so far reported in *Allium nigrum* (Maragheh et al. 2019) and *Cestrum* (Fernandes et al. 2009). It is difficult to ascertain the heterochromatin composition of this type. There is a possibility of having AT and GC rich segments to be placed so close that the different chromatin bands cannot be distinguished in condensed mitotic chromosomes (Maragheh et al. 2019). However, nucleolar heterochromatin composition of Indian *Lens culinaris* displays considerable variation, ~~maybe~~ due to enormous cultivation practice and artificial hybridization, which is a yet unaddressed field of study.

Cultivar specific differences were also accentuated by the non-nucleolar $DAPI^+$ and CMA^+ bands. The type E centromeric CMA bands are unique type

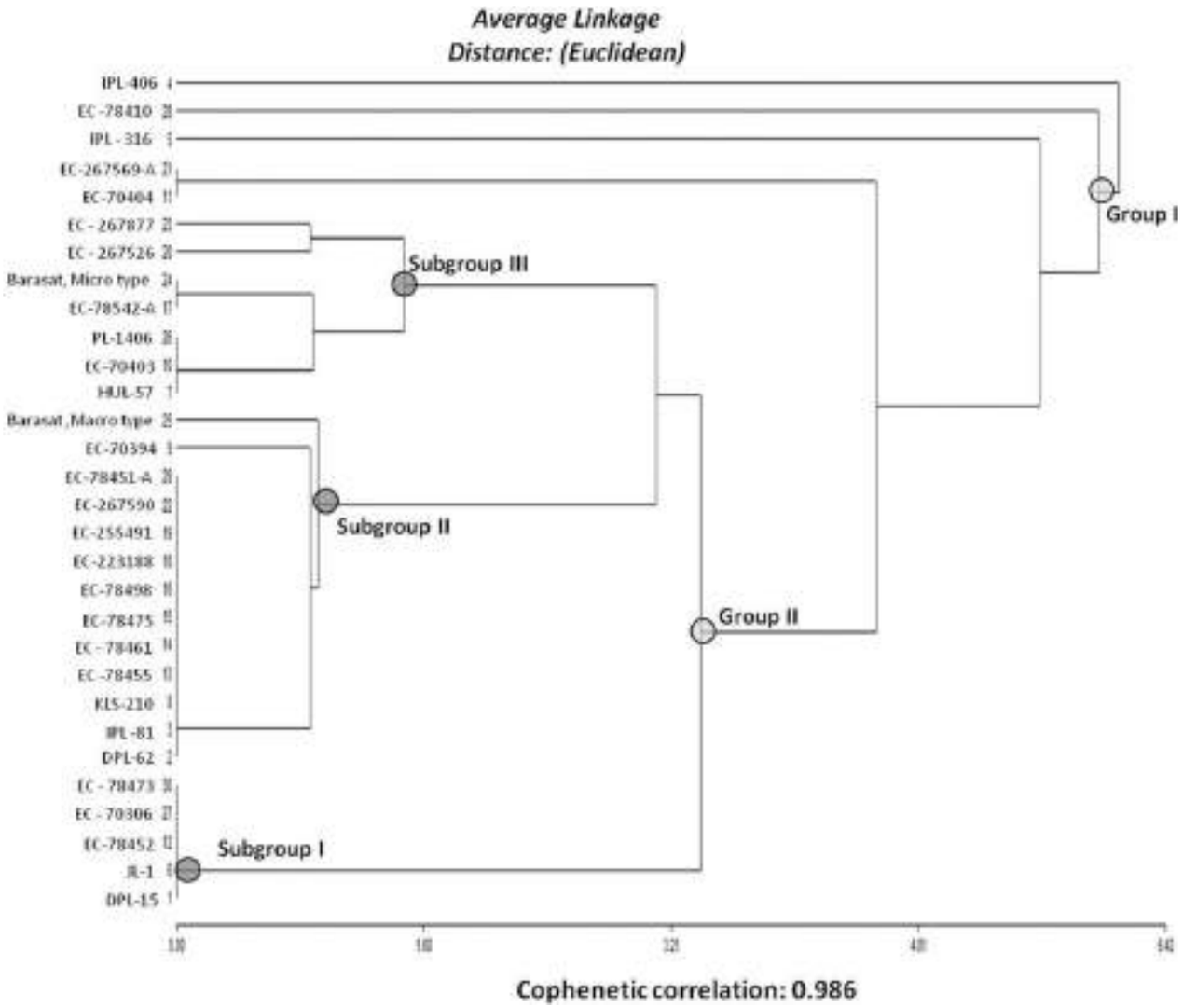

Figure 6. UPGMA dendrogram derived from average Euclidean distance based on fluorochrome banding pattern of 30 Indian Lentil cultivars, cultivar names in the left of their serial numbers.

of heterochromatin rarely reported in plants. However, non-nucleolar GC-rich heterochromatin was previously characterized in centromeric as well as pericentromeric regions of Papilionoid species belonging to *Dioclea* (Souza and Benko-Iseppon 2004), *Psophocarpus* (Chaowen et al. 2004), *Crotalaria* (Mondin and Aguiar-Perecin 2011), *Vigna* (Bortoleti et al. 2012; She et al. 2015, 2020), *Phaseolus* (Bonifácio et al. 2012), *Lablab* (She and Jiang 2015), and *Canavalia* (She et al. 2017). She et al. (2020) suggested the centromeric or pericentromeric GC- heterochromatin to be a relic of genomic evolution in the subfamily Papilionoideae. Other even rare heterochromatin blocks were the centromeric (type F), pericentromeric (type B) and intercalary (type G) DAPI bands, constituting landmarks to differentiate karyotypes of certain Lentil cultivars. Terminal or intercalary DAPI$^+$ bands were documented in few plants (Vanzela and Guerra 2000; Divashuk et al. 2014), including few species of Cucurbitaceae (Bhowmick and Jha 2015, 2019). Terminal DAPI bands are found in *Crotalaria* (Mondin and Aguiar-Perecin 2011) of Papilionoideae. Centromeric DAPI bands are yet rare to encounter. However, in case of *L. culinaris* and related species, AT heterochromatic regions were mapped by repetitive sequence probe FISH (Galasso et al. 2001; Galasso 2003). Also in Papilionoideae, AT rich heterochromatin at centromere and pericentromeric regions are reported in *Vigna* (Bortoleti et al. 2012; She et al. 2020), *Lablab* (She and Jiang 2015) and

Table 1. Analysis of CMA and DAPI fluorescent bands in thirty Indian cultivars of *Lens culinaris* (2n = 14).

Sl. No.	Cultivars	Order of nucleolar pair	CMA bands				DAPI bands				Total no. of bands/ 2n	Fluorescent Karyotype formula (2n)	Figure no.
			No.	Chromosome pair/s	Type*/ intensity	Figure no.	No.	Chromosome pair/s	Type*/ intensity	Figure no.			
1	DPL15	4th	2	4th	D/ neutral	1a	0	-	-	2a	2	2D+12H	4a
2	DPL-62	4th	2	4th	A/low	1b	0	-	-	2b	2	2A+12H	4b
3	IPL -81	4th	2	4th	A/low	1c	0	-	-	2c	2	2A+12H	4c
4	IPL-406	3rd	2	3rd	A/high	1d	2	6th	G/high	2d	4	2A+2G+10H	4d
5	IPL -316	4th	2 12	4th 1st-3rd, 5th-7th	A/high E/high	1e	0	-	-	2e	14	2A+12E	4e
6	JL-1	4th	2	4th	D/ neutral	1f	0	-	-	2f	2	2D+12H	4f
7	HUL-57	3rd	2	3rd	B/ low	1g	2 2	3rd 2nd	B/low F/high	2g	4	2B+2F+10H	4g
8	KLS-210	4th	2	4th	A/high	1h	0	-	-	2h	2	2A+12H	4h
9	EC-70394	5th	2 2	5th 4th	A/high E/high	1i	0	-	-	2i	4	2A+2E+10H	4i
10	EC-70403	4th	2	4th	B/high	1j	2 2	4th 2nd	B/high F/high	2j	4	2B+2F+10H	4j
11	EC-70404	4th	2	4th	C/high	1k	2	4th	C/low	2k	2	2C+12H	4k
12	EC-78452	4th	2	4th	D/ neutral	1l	0	-	-	2l	2	2D+12H	4l
13	EC-78455	4th	2	4th	A/high	1m	0	-	-	2m	2	2A+12H	4m
14	EC- 78461	4th	2	4th	A/high	1n	0	-	-	2n	2	2A+12H	4n
15	EC-78475	3rd	2	3rd	A/low	1o	0	-	-	2o	2	2A+12H	4o
16	EC– 78498	4th	2	4th	A/high	1p	0	-	-	2p	2	2A+12H	4p
17	EC-78542-A	2nd	2	2nd	B/high	1q	2	2nd	B/low	2q	2	2B+12H	4q
18	EC-223188	4th	2	4th	A/high	1r	0	-	-	2r	2	2A+12H	4r
19	EC-255491	4th	2	4th	A/high	1s	0	-	-	2s	2	2A+12H	4s
20	EC-267526	4th	2	4th	B/high	1t	2 4	4th 1st , 3rd	B/low F/low	2t	6	2B+4F+8H	4t
21	EC-267569-A	3rd	2	3rd	C/low	1u	2	3rd	C/low	2u	2	2C+12H	4u
22	EC-267590	4th	2	4th	A/high	1v	0	-	-	2v	2	2A+12H	4v
23	EC - 267877	4th	2 2	4th 5th	B/high E/low	1w	2	4th	B/high	2w	4	2B+2E+10H	4w
24	Barasat, Micro type	3rd	2	3rd	B/high	1x	2	3rd	B/low	2x	2	2B+12H	4x
25	Barasat, Macro type	4th	2	4th	A/high	3a	2	3rd	F/high	3g	4	2A+2F+10H	5a
26	PL -1406	4th	2	4th	B/high	3b	2 2	4th 2nd	B/low F/low	3h	4	2B+2F+10H	5b
27	EC - 70306	4th	2	4th	D/ neutral	3c	0	-	-	3i	2	2D+12H	5c
28	EC -78410	4th	2	4th	B/high	3d	2 12	4th 1st -3rd, 5th-7th	B/high F/high	3j	14	2B+12F	5d
29	EC – 78451-A	4th	2	4th	A/high	3e	0	-	-	3k	2	2A+12H	5e
30	EC - 78473	4th	2	4th	D/ neutral	3f	0	-	-	3l	2	2D+12H	5f

*Types of nucleolar bands- A: $CMA^+/DAPI^-$ satellite, B: $CMA^+/DAPI^-$ satellite and $DAPI^+/CMA^0$ band in long arm, C: $CMA^+/DAPI^+$ satellite; D: CMA^0 satellite; centromeric bands- E: $CMA^+/DAPI^0$, F: $DAPI^+/CMA^0$; intercalary band G: $DAPI^+/CMA^0$; H: no bands.

Arachis (Silvestri et al. 2020). Nonetheless, occurrence of centromeric CMA^+ or $DAPI^+$ bands along with nucleolar $CMA^+/DAPI^+$ or CMA^0 bands certainly advocate atypical heterochromatin composition in *Lens*. The non-uniform composition and rearrangements of heterochromatin had been observed repeatedly in Papilionoideae species (Moscone et al. 1999; Souza and Benko-Iseppon 2004; Pedrosa-Harand et al. 2006; Mondin and Aguiar-Perecin 2011; She et al. 2020), which becomes apparent in our study once again.

In view of the diversity in fluorochrome banding pattern, we attempted to resolve karyotype relationships by the UPGMA method. Identification of distinct subgroups have opened further scopes to complement marker assisted analysis of genetic diversity across varied range of Indian cultivars with valuable agronomic traits. Application of fluorochrome banding method has therefore helped to i) break the perception of an overall similar karyotype of cultivated Lentils as observed in Giemsa plates (Jha et al. 2015, 2017; Jha and Halder 2016) ii) serve as the chromosomal blueprint for cultivar discrimination, ii) statistically represent the status of chromosomal relationships, iii) highlight the uniqueness of certain Indian cultivars by means of unconventional banding pattern, and v) construct a fluorescent karyotype dataset of Indian Lentil cultivars.

CONCLUSION

Being a crop 'as old as agriculture' (Sandhu and Singh 2007), an exclusive chromosomal database of Lentils is essential to complement genomic research databases like Legume Information System (Dash et al. 2016) and KnowPulse (Sanderson et al. 2019). As an extension of our study involving Lentil cytogenetics, we have delved into the first molecular karyotypes of the country's native cultivars. Notably, the cultivars are hosted by world's second largest *ex situ* Lentil germplasm stock *i.e.* IIPR of NBPGR, the first being ICARDA (Muehlbauer and McPhee 2005; Coyne and McGee 2013). In future, molecular cytogenetic study of wild *Lens* species of India can be expected to strengthen the base of chromosomal evolution in Papilionoideae. In face of stern climatic changes that affect future cultivation, the Indian cultivars with interesting karyotype features and relationships can be fluently tested for performance and productivity. Thus, our findings complement traditional or marker assisted breeding and would undoubtedly bridge up the lacuna for a systematic chromosomal database of Indian Lentils.

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RESEARCH ARTICLE

Karyotype diversity in cultivated and wild Indian rice through EMA-based chromosome analysis

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Abstract. India is known for its diverse cultivated and wild rice germplasm. In today's crop improvement programmes, wild relatives are much-needed genetic repository of valuable traits. Analysis of genetic diversity at the chromosomal level is one cost-effective tool to unlock foundational information related to genetics and plant breeding. Presently, enzymatic maceration and air-drying method (EMA) has been applied for the first time in six cultivated and nine wild Indian rice (diploid and tetraploid). EMA method following Giemsa staining has yielded large numbers of cytoplasm free metaphase plates with distinct chromosome morphology. Detailed analysis has revealed karyotype diversities in terms of total chromatin length (TCL), chromosome morphology and location of sat chromosomes within and between the studied species. Most of the cultivated rice has gained additional amount in TCL during the period of domestication in comparison to their progenitor *Oryza nivara*. Morphological clarity of the small chromosomes of rice was much required and has helped to identify individual chromosomes in the diverse karyotypes. Diversity in landmark SAT chromosomes is another important observation, not reported previously in Indian rice. Present study has shown that in most of the *O. sativa* members, the 10th pair contains SAT except one where 6th pair is satellited. On the other hand, diversity of SAT in diploid and tetraplod wild species has been recorded on 5th, 7th and 8th chromosome pairs and on 9th, 12th, 22nd and 23rd chromosome pairs, respectively. Karyomorphometric indices has helped to construct dendrogram to elucidate intraspecies and interspecies relationships. Untapped genetic diversity recorded in Indian rice through chromosomal analysis will be useful to the breeders and genome researchers.

Keywords. karyotype diversity; diploid and tetraploid; cultivated and wild Indian rice; enzymatic maceration and air-drying method.

Introduction

Rice, one of the three major staple food crops consumed by nearly half of the world populations (Sasaki 2001). This crop belongs to the family Poaceae and comprises of two cultivated and 24 wild species (Brar and Khush 2018). *Oryza sativa* is the major cultivated Asian rice with two distinct ecotypes 'indica' and 'japonica' and the other one is African *O. glaberrima*. *O. sativa* is domesticated in Asia and spread all over the world while *O. glaberrima* remains confined to Africa after domestication (Vaughan 1994; Biology of *Oryza sativa* L. (Rice) Govt. of India 2011). In the process of evolution and domestication of major crops, the wild relatives have played an important role and rice is not an exception. Wild relatives of rice are actually the genetic repository of valuable traits for many agronomic and nutritional features. They contain resistance genes for pests and diseases, and are capable of confronting severe climate changes and adverse soil types (Dempewolf *et al*. 2017). The necessity of utilizing and conserving unexploited genetic material for developing new and improved varieties through the integration of classical genetics and genomics-enabled research are expected to increase in future (Shakiba and Eizenga 2014). Undeniably, analysis of genetic diversity at the chromosomal level is a prerequisite task as it is the only cost-effective method to unfold actual chromosome number of a particular plant and reveal the real ploidy status. It also provides morphological configurations and position of chromosomal landmarks which are foundational and complementary information, and difficult to retrieve from next-generation sequencing (NGS) data (Guerra 2008; Schneeweiss *et al*. 2013; Soltis 2014). Many scientists felt more

This paper is dedicated to my beloved teacher and renowned plant cytogeneticist Prof. Arun Kumar Sharma (late) for his inspiration in rice chromosome research.

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thrust on chromosome research and a closer integration of cytogenetics and genomics (Deakin *et al*. 2019).

In global perspective, India ranks first in terms of total area under rice cultivation and ranks second as producer. India is considered a repository of wild and cultivated rice germplasms (Richharia and Govindasamy 1990; Biology of Oryza *sativa* L. (Rice) Govt. of India 2011) and many places in India are considered to be centre of diversity of Asian cultigens. Chromosome analysis of cultivated rice was initiated through conventional method more than hundred years ago and confirmed to contain 2*n*=24 chromosomes (Kuwada 1910). Sharma and Mukhopadhyay (1965) attempted somatic chromosome analysis through conventional orcein method in Indian rice but they failed to obtain clear chromosome morphology. Primary difficulties included considerably small size of chromosomes and high cytoplasmic content within the cell. Further, it was reported that rice tissues are very stiff due to accumulation of silicates and stained pale with conventional acetoorcein or carmine (Fukui and Iijima 1991; Fukui 1996). Scientists also tried pachytene chromosome analysis in rice (Shastry 1964; Kurata *et al*. 1981; Khush *et al*. 1984; Cheng and Gu 1994; Kurata 2008). However, Kurata and Omura (1978) applied EMA-based chromosome analysis method for the first time in one Japanese cultivar of *O. sativa–japonica* and rendered accurate chromosome number (2*n*=2*x*=24) and morphology visible. Rice chromosome analysis has progressed further to the level of FISH and GISH (Uozu *et al*. 1997; Ohmido *et al*. 2010; Hou *et al*. 2018). Reports on chromosome number revealed that all cultivated rice members contained 2*n*=2*x*=24 chromosomes (Sharma and Mukhopadhyay 1965; Lu *et al*. 2002). While wild relatives are either diploid with 2*n*=2*x*=24 or tetraploid with 2*n*=4*x*=48 with different genomic configurations (Sanchez *et al.* 2013; Brar and Khus 2018) (table 1). Literature review over four decades has failed to provide any somatic chromosome analysis on Indian rice.

Keeping in mind that karyotype analysis is the basics of molecular cytogenetics, the present study deals with detailed karyotyping of six cultivars of *O. sativa* and nine wild relative species (both diploid and tetraploid) of Indian rice. Enzymatic maceration and air-drying (EMA) method followed by Giemsa staining has yielded cytoplasm free metaphase plates to unlock the hidden karyotype diversity and helped to present intraspecific and interspecific relationships based on karyotypic features. The untapped genetic resources of Indian rice may become very useful to the breeders and genome researchers.

Materials and methods

Taxon sampling

All the 15 studied rice species and cultivars were collected and obtained from different parts of West Bengal, Manipur and National Rice Research Institute Cuttack, Odisha, India. List of cultivated and wild rice species has been arranged following Global Rice Science Partnership (GRiSP) 2013 guidelines under *O. sativa* complex and *O. officinalis* complex with their collection details, chromosome number, genomic configuration, seed morphology and germination efficiency (table 1). A representative sample of seeds (∼60%) was stored at 4– 8°C. A minimum of 20 seeds from each cultivar was imbibed in water for 48 h and placed on moist filter papers in dark for germination and processing of chromosomes. Carl Zeiss Stemi 508 was used for photography of rice seeds.

Analysis of somatic chromosomes

Ten to 15 healthy root tips of each cultivar were collected and pretreated with 8-hydroxyquinoline (0.002 M) (BDH, India) for a period of 3½–4 h at 20°C and fixed in acetic acid: methanol (1:3) and preserved in –20°C for future use. Standardization of EMA method of rice root tips was carried out following our earlier protocol on other crops (Jha and Halder 2016; Jha and Saha 2017). The enzyme incubation time varied from 60–100 min at 37°C. Root tips were evenly macerated on glass slides with the help of a fine tip forceps in freshly prepared fixative and slides were air-dried. Slides were stained in 2% Giemsa solution (Merck, Darmstadt, Germany) mixed with 1/15th phosphate buffer solution for 20–30 min at room temperature. The air-dried slides were studied under A1 microscope Axio Lab A1 Carl Zeiss (Jena, Germany) fitted with CCD camera and computer for capturing cytoplasm free metaphase plates. Two to three roots and a minimum of 20 well-scattered metaphase plates were studied to determine the chromosome number, ploidy level and karyomorphometric analysis (table 2) using Axiovision L.E 4 software (Carl Zeiss, Jena, Germany, https:// www. zeiss. com/ microscopy/int/products/microscope-software/axiovision.html), for estimation of long arm length (l), short arm length (s), chromosome length (CL) and total diploid chromosome length (TCL). The centromeric position was determined by calculating arm ratios (r= *l*/*s*) following Levan *et al*. (1964). Chromosome morphometric data were used to prepare ideogram and karyotype formulae for individual germplasms.

Interchromosomal and intrachromosomal asymmetry/symmetry in the karyotypes were evaluated after calculating values of about nine numerical indices, namely intrachromosomal asymmetry index (A1) and interchromosomal asymmetry index (A2) (Zarco 1986), total form percentage (TF%) (Huziwara 1962), asymmetry index of karyotype (AsK%) (Arano 1963), coefficients of variation of chromosome length (CV_{CL}) (Paszko 2006), karyotype symmetry index (Syi) (Greilhuber and Speta 1976), mean centromeric index (X_{CI}) (Seijo and Fernández 2003), mean centromeric asymmetry (M_{CA}) (Peruzzi and Eroglu 2013), asymmetry index (AI) (Paszko 2006) and the qualitative

index namely Stebbins' asymmetry categories (Stebbins 1971).

Statistical analysis

Descriptive statistics including means and standard deviations were analysed for all measured parameters and one-way analysis of variance (ANOVA) was performed to detect significant differences ($P \leq 0.05$) in the mean (Sokal and Rohlf 1995). For post hoc analyses, Duncan's multiple range test (DMRT) was used. The statistical analysis was conducted at 0.05 probability level using SPSS v. 16.0 statistical package (SPSS, IBM, Chicago, http://www.spss.com.hk/corpinfo/index.htm). Three chromosome numerical data (2n, TCL and CL) and nine quantitative karyotype indices were used to construct the data matrix and to generate an unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on the average Euclidean distance in Infostat 2017d (free version) to observe intraspecies and interspecies relationships.

Results

Collection details with seed morphology and other parameters are presented in table 1. The 15 members of rice under *sativa* and *officinalis* species complex were included in karyotype analysis (figures 1, 2 and 3). Of the six *O. sativa* cultivars, five were aromatic and one was nonaromatic. Grain length of four cultivars of *O. sativa* and one wild species ranged from 8–10 mm, while in others it was less than 8 mm (table 1). Distinct awn was noted in two cultivars of *O. sativa* and eight wild species (table 1). In most *O. sativa* members, the colour of rice was white or light brown but is deep black/purple in Black grained type and white in Kalonunia (table 1) while all wild relatives exhibited brown colour rice with different sized awn. Germination efficiency was nearly 99% irrespective of cultivated and wild species (table 1).

For all studied members, enzymatic digestion for 95–100 min at 37°C and staining with 2% Giemsa solution for minimum 30 min yielded large numbers of cytoplasm free chromosome plates. Minimum 20 well-scattered metaphase plates were studied to determine the chromosome number and ploidy level of each sample. Mitotic chromosome analysis of six cultivars of *O. sativa* and two wild species, namely *O. nivara* and *O. barthii* with AA genome under *O. sativa* species complex revealed $2n=2x=24$ with very small chromosomes. On the other hand, three wild species, namely *O. officinalis*, *O. australiensis* and *O. rhizomatis* and four wild species *O. eichingeri*, *O. minuta*, *O. alta* and *O. latifolia* under *O. officinalis* species complex with different genomic configurations revealed $2n=2x=24$ and $2n=4x=48$ chromosome number, respectively (table 1). Minimum five plates of each sample were used for karyomorphometric analysis and the comparative results are presented in tables 2 and 3. Important karyometric features of each species are presented below.

O. sativa, cultivated (2n=2x=24, AA genome)

Uniform EMA based chromosome processing method has yielded very clear chromosome morphology ($2n=2x=24$) in all the six cultivated rice (figure 1a). Karyomorphometric analysis based on the position of primary constriction had revealed three chromosome types, namely metacentric, submetacentric and sub-telocentric (table 2). Variation in average length of individual chromosomes within the species has been noted which ranged from 1.91 ± 0.15 to $2.71 \pm 0.12\mu$. Minimum size of chromosomes (1.91 ± 0.15) was recorded in Sitabhog, while highest ($2.71 \pm 0.12\mu$) was recorded in Kaminibhog of *O. sativa* (table 2). It was observed that the TCL ranged from 45.88 ± 3.51 to $65.09 \pm 2.84\mu$ which was significantly correlated with the ACL of the studied *O. sativa* cultivars (table 2). One pair of secondary constrictions was distinct in all cultivars (figure 1a). The detailed karyomorphometric analysis (table 2) and ideograms showed the presence of secondary constrictions (SAT) on the 10th pair of chromosome in all the cultivars except Kaminibhog (6th pair is satellited). Six cultivars contain three different diploid karyotypes as: (i) 10m+ 10Sm+ 2St+ 2St.Sat in Sunderban, Sadanunia, Sitabhog and Black rice, (ii) 8m+ 14Sm+ 2St.Sat in Kalonunia and (iii) 12m+ 10Sm+ 2St.Sat in Kaminibhog (table 2). However, all the sat chromosomes are sub-terminal in nature (figure 4, a–f).

O. nivara, wild (2n=2x=24, AA genome)

Somatic chromosome analysis reveals $2n=2x=24$ chromosomes in this species, having very small chromosomes with distinct primary and secondary constrictions (figure 2, a–b). TCL is $45.90 \pm 2.83\mu$ and average length of individual chromosomes is $1.91 \pm 0.12\mu$. Distinct secondary constriction is marked on a subterminal 5th pair of chromosomes (figure 5a). The $2n$ karyotype formula is 8m+ 12Sm+ 2St+ 2St.Sat (table 2).

O. barthii wild (2n=2x=24, AA genome)

Chromosome counts show $2n=2x=24$ chromosomes in this species. The chromosomes are small in size with distinct primary and secondary constrictions (figure 2, c–d). TCL is $53.35 \pm 2.48\mu$ and average length of individual chromosomes is $2.22 \pm 0.10\mu$. Secondary constriction is marked on the 8th sub-telocentric pair of chromosomes (figure 5b). The $2n$ karyotype formula is 16m+ 6m+ 2St.Sat (table 2).

Table 1. Collection details, chromosome and genome status, germination efficiency and seed morphology of 15 cultivated and wild Indian Rice.

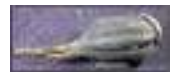
	Species	Place of collection*	Lat.-lon.	Grain length (mm)	Chromosome number and genome	Time and germination %	Seed morphology
Series *Oryza: sativa* species complex							
1	*O. sativa* L., Sundarban Nonaromatic, awnless	S.24 Parganas West Bengal	21.9497° N, 89.1833° E	8.00–10.00	2*n*=2*x*=24 AA	3–4 days, ± 99	
2	*O. sativa* L., Sitabhog Aromatic, awnless	Hooghly. West Bengal	22.8859° N, 88.3919° E	>8.00	2*n*=2*x* =24 AA	4–5 days, ± 99	
3	*O. sativa* L., Kaminibhog Aromatic, awnless	Murshidabad, West Bengal	24.1759° N, 88.2802° E	>8.00	2*n*=2*x* =24 AA	4–5 days, ± 99	
4	*O. sativa* L., Sadanunia, Aromatic, awned	Jalpaiguri West Bengal	26.5215° N, 88.7196° E	8.00–10.00	2*n*=2*x* =24 AA	4–6 days, ± 99	
5	*O. sativa* L., Kalonunia (black grained rice), Aromatic, awned	Jalpaiguri. West Bengal	26.5215° N, 88.7196° E	8.00–10.00	2*n*=2*x* =24 AA	4–6 days, ± 99	
6	*O. sativa* L. Black grained rice, aromatic, awnless	Manipur	24.6637° N, 93.9063° E	8.00–10.00	2*n*= 2*x* =24 AA	4–6 days, ± 99	
7	*O. nivara* Sharma et Shastry Wild, awned	NRRI, Cuttack, Odisha	20.4537° N, 85.9338° E	>8.00	2*n* =2*x* = 24 AA	3–4 days, ± 99	
8	*O. barthii*, A. Chev, Wild, awned	NRRI, Cuttack, Odisha	20.4537° N, 85.9338° E	10.00	2*n*=2*x* =24 AA	4–5 days, ± 99	
Series Latifolia: *officinalis* species complex							
9	*O. officinalis* Wall ex Watt Wild, awned	NRRI, Cuttack, Odisha	20.4537° N, 85.9338° E	>6.00	2*n*=2*x* =24 CC	4–5 days, ± 99	
10	*O. australiensis* Domin. Wild, awned	NRRI, Cuttack, Odisha	20.4537° N, 85.9338° E	>8.00	2*n*=2*x* =24 EE	3–4 days, ± 99	

Table 1 (*contd*)

	Species	Place of collection*	Lat.-lon.	Grain length (mm)	Chromosome number and genome	Time and germination %	Seed morphology
11	*O. rhizomatis* Vaughan Wild, rudimentary awn	NRRI, Cuttack, Odisha	20.4537° N, 85.9338° E	>7.00	2*n*=2*x*=24 CC	4–5 days, ± 99	
12	*O. eichingeri* A. Peter Wild, awnless	NRRI, Cuttack, Odisha	20.4537° N, 85.9338° E	>7.00	2*n*=4*x* = 48 CC	4–5 days, ± 99	
13	*O. minuta* J.S. Presl. ex C.B. Presl. Wild, awned	NRRI, Cuttack, Odisha	20.4537° N, 85.9338° E	>6.00	2*n*=4*x* = 48 BBCC	3–4 days, ± 99	
14	*O. alta* Swallen, Wild, awned	NRRI, Cuttack, Odisha	20.4537° N, 85.9338° E	>8.00	2*n* = 48 CC DD	4–5 days, ± 99	
15	*O. latifolia* Desv. Wild, awned	NRRI, Cuttack, Odisha	20.4537° N, 85.9338° E	>6.00	2*n*= 48 CCDD	4–5 days, ± 99	

List is prepared following the guidelines of GRiSP (2013),*NRRI (National Rice Research Institute). Bars 2 mm.

O. officinalis, wild (2n=2x=24, CC genome)

Chromosome plate contains 2*n*=24 chromosomes which are small in size, showing distinct primary constrictions while the secondary constriction could not be identified (figure 2, e–f). TCL is 50.60±0.57μ and average length of individual chromosomes is 2.11±0.02μ. The 2*n* karyotype formula is 14m+ 8Sm+ 2St (figure 5c; table 2).

O. australiensis, wild (2n=2x=24, EE genome)

This species contains 2*n*=24 chromosomes (figure 2, g–h) which are comparatively larger with distinct primary and secondary constrictions. TCL is 75.15±1.17μ and average length of individual chromosomes is 2.91±027μ which is maximum among all the studied materials. As per ideogram, secondary constriction is on 8th pair of sub-telocentric chromosomes (figure 5d). The 2*n* karyotype is 16m+ 6Sm+ 2St.sat (table 2).

O. rhizomatis, wild (2n=2x=24,CC genome)

The metaphase plate shows 2*n*=24 chromosomes that are small and have distinct primary and secondary constrictions (figure 2, i–j). TCL is 64.65±1.26μ and average length of individual chromosomes is 2.70±0.05μ. Idiogram (figure 5e) shows that one pair of secondary constriction on the 7th pair of sub-telocentric chromosome. The 2*n* karyotype formula is 10m+ 10Sm+ 2St+ 2St.Sat (table 2).

O. minuta, wild (2n=4x=48, BBCC genome)

The scattered metaphase plate without cytoplasm shows 2*n*=4*x*=48 chromosomes which are very small, having distinct primary constrictions. TCL is 85.73±1.94μ and average length of individual chromosomes is 1.79±f0.04μ. The karyotype formula (2*n*=4*x*) is 22m+ 24Sm+ 2St.Sat (table 2). One pair of secondary constriction is marked on the 9th subtelocentric pair (figures 3, a–b; 5f).

O. latifolia, wild (2n=4x=48, genome CCDD)

Cytoplasm free well-scattered metaphase plate shows 2*n*=4*x*=48 small chromosomes with distinct primary and secondary constrictions (figure 3, c–d). TCL is 99.48 ± 2.66μ and average length of individual chromosomes is 2.07 ± 0.06μ. The 2*n*=4*x* karyotype formula is 20m+ 24Sm+ 4St.Sat (table 2). Two pairs of secondary constriction are observed on 22nd and 23rd pairs of sub-telocentric chromosomes (figure 5g).

Table 2. Karyomorphometric data of 15 cultivated and wild Indian rice.

	Species and chromosome no.	Length of longest chromosome (μm)		Length of shortest chromosome (μm)		TCL (2*n*) (mean ±SD)	ACL (mean ±SD)	Number of SAT chromosome	Order of SAT bearing pair	Diploid karyotype formula
		Absolute (mean ± SD)	Relative (mean ±SD)	Absolute (mean ±SD)	Relative (mean ±SD)					
1	*O. sativa* - Sundarban, 2*n*=24	3.21 ± 0.09	5.56 ± 0.08	1.86 ± 0.02	3.22 ± 0.10	57.7 ± 0.96	2.40 ± 0.04	2	10	10m + 10Sm + 2St + 2St.Sat
2	*O. sativa* - Sadanunia, 2*n*=24	3.21 ± 0.02	5.50 ± 0.09	1.81 ± 0.01	3.11 ± 0.01	58.38 ± 0.48	2.43 ± 0.02	2	10	10m + 10Sm + 2St + 2St.Sat
3	*O.sativa* - Kalo nunia, 2*n*=24	3.07 ± 0.77	5.43 ± 0.01	1.76 ± 0.50	3.12 ± 0.13	62.40±5.04	2.60±0.21	2	10	8m +14Sm +2St.Sat
4	*O. sativa* - Sitabhog, 2*n*=24	2.77 ± 0.07	6.05 ± 0.31	1.38 ± 0.11	3.00 ± 0.00	45.88 ± 3.51	1.91 ± 0.15	2	10	10m + 10Sm + 2St + 2St.Sat
5	*O. sativa* - Kamini bhog, 2*n*=24	3.65 ± 0.28	5.60 ± 0.19	2.00 ± 0.14	3.07 ± 0.08	65.09 ± 2.84	2.71 ± 0.12	2	6	12m+ 10Sm+ 2St.Sat
6	*O. sativa* - Black rice, 2*n*=24	3.36 ± 0.22	6.01 ± 0.70	1.72 ± 0.19	3.06 ± 0.19	55.91 ± 2.81	2.33 ± 0.18	2	10	10m + 10Sm + 2St + 2St.Sat
7	*O. nivara*, 2*n*=24	2.88 ± 0.46	6.24 ± 0.62	1.40 ± 0.00	3.06 ± 0.19	45.90 ± 2.83	1.91 ± 0.12	2	5	8m+ 12Sm+ 2St + 2St. Sat
8	*O. barthii*, 2*n*=24	3.00 ± 0.29	5.62 ± 0.27	1.50 ± 0.07	2.81 ± 0.00	53.35 ± 2.48	2.22 ± 0.10	2	8	16m + 6m + 2St.Sat
9	*O.* officinalis, 2*n*=24	2.83 ± 1.56	5.58 ± 0.00	1.40 ± 0.07	2.77 ± 0.11	50.60 ± 0.57	2.11 ± 0.02	2	*	14m+ 8Sm + 2St
10	*O. australiensis*, 2*n*=24	4.9 ± 0.14	6.55 ± 0.00	2.05 ± 0.35	2.73 ± 0.40	75.15 ± 1.17	2.91 ± 027	2	8	16m+ 6Sm+ 2St.sat
11	*O. rhizomatis*, 2*n*=24	4.05 ± 0.00	6.26 ± 0.13	1.66 ± 0.04	2.56 ± 0.00	64.65 ± 1.26	2.70 ± 0.05	2	7	10m + 10Sm + 2St + 2St.Sat
12	*O. minuta*, 2*n*=4x=48	2.42 ± 0.11	2.82 ± 0.07	1.24 ± 0.04	1.44 ± 0.09	85.73 ± 1.94	1.79 ± 0.04	2	9	22m + 24Sm + 2St.Sat
13	*O. latifolia*, 2*n*=4x=48	2.95 ± 0.07	2.97 ± 0.00	1.49 ± 0.12	1.50 ± 1.50	99.48 ± 2.66	2.07 ± 0.06	4	22,23	20m+ 24Sm + 4St.Sat
14	*O. eichingeri*,2*n*=4x=48	2.45 ± 0.15	2.83 ± 0.13	1.10 ± 0.09	1.27 ± 0.13	86.36 ± 1.36	1.80 ± 0.03	4	9,12	30m+ 14Sm+ 4St.Sat
15	*O. alta*, 2*n*=4x=48	1.92 ± 0.03	3.05 ± 0.13	0.83 ± 0.04	1.32 ± 0.10	63.01 ± 1.74	1.31 ± 0.04	*	*	*

TCL, total chromatin length of diploid set; ACL, average length of chromosome; *Due to extremely small size of the chromosomes, centromeric or secondary constrictions could not be marked.

O. eichingeri, wild (2n=4x=48, genome CC)

Chromosome counts reveal that this Indian cultivar contains 2*n*=4*x*=48 very small chromosomes with distinct primary and secondary constrictions (figure 3, e–f). TCL is 86.36 ± 1.36μ and average length of individual chromosomes is 1.80 ± 0.03μ. Karyotype formula (2*n*=4*x*) is 30m+ 14Sm+ 4St.Sat (table 2). Ideogram shows two pairs of secondary constrictions on the 9th and 12th pairs of sub-telocentric chromosomes (figure 5h).

O. alta, wild (2n=4x=48, genome CCDD)

The species revealed well-scattered metaphase plate with smallest chromosomes among all the studied materials. Average length of individual chromosomes is 1.31 ± 0.04μ and TCL is 63.01 ± 1.74μ. The primary constrictions in some chromosomes along with location of SAT chromosomes could not be confirmed, impeding construction of modal karyotype in this particular species due to extremely small size of chromosomes (table 2). The presented plate (figure 3, g–h) shows 2*n*–4*x*=47 (one less) chromosomes. Ideogram based on individual chromosome length is presented (figure 5i)

Karyotype relationships in Indian rice species

The karyoevolutionary parameters were considered for all of the rice cultivars and species except *O. alta*, where karyotype could not be determined. Among the nine quantitative and one qualitative parameter examined, distinctly increased values were observed in Sitabhog (A2, CV_{CL}, AsK%, M_{CA}, AI, Stebbins' index) and Black rice (A2, CV_{CL}, TF%, AsK%, A1, AI, Stebbins' index) cultivars of diploid *O. sativa* along with *O. rhizomatis* (A2, CVCL, TF%, AsK%, A1, M_{CA}, AI), advocating higher karyotype asymmetry (table 3). *O. barthii* showed an intermediate level of asymmetric tendency since five indices (Syi%, TF%, A1, X_{CI} and Stebbins' index) had higher values while rest five indices had lower values (table 3). The other diploid rice cultivars and wild species showed different extents of karyotype asymmetry. Few of the karyoevolutionary parameters showed increased values in the rice cultivars Sundarban (AsK%, M_{CA}), Sadanunia (AsK%), Kalonunia (Syi, A1, X_{CI}), Kaminibhog (TF%, AsK%) and wild species such as *O. nivara* (A2, AsK%), *O. officinalis* (A2, CV_{CL}, X_{CI}) (table 3). *O. australiensis* had even lower extent of asymmetric tendency compared to all diploid species (table 3). Among the polyploids, increased values of karyotype analytical indices were observed for TF% (all three species), AsK% (all three species), X_{CI} (*O. eichingeri* and *O. minuta*) and Syi (*O. latifolia*) (table 3). Hence, the polyploid species have comparable values of karyotype indices and have

Table 3. Karyotype asymmetry indices of cultivated and wild diploid and tetraploid Indian rice germplasms.

	Species and chromosome number	Quantitative asymmetry parameters									
		Inter-chromosomal asymmetry indices		Intrachromosomal symmetry / asymmetry indices						Inter and intra (combined)-chromosomal asymmetry index	Qualitative asymmetry parameter
		A2[1]	CV_{CL}[2]	Syi[3]	TF%[4]	AsK%[5]	A1[6]	X_{CI}[7]	M_{CA}[8]	AI[9]	Stebbins' index[10]
1	*O. sativa* - Sundarban, 2*n*=24	0.16^{a}	16.52^{a}	49.58^{a}	35.82^{ab}	66.70^{f}	0.55^{b}	25.90^{a}	30.87^{h}	5.40^{c}	3A
2	*O. sativa* - Sadanunia, 2*n*=24	0.18^{b}	18.55^{b}	52.38^{c}	36.20^{b}	63.79^{cd}	0.57^{c}	33.50^{d}	27.58^{f}	5.26^{c}	3A
3	*O. sativa* - Kalo nunia, 2*n*=24	0.17^{a}	17.44^{ab}	56.17^{e}	37.83^{cd}	62.16^{b}	0.61^{e}	34.91^{e}	23.71^{c}	4.83^{b}	3A
4	*O. sativa* - Sitabhog, 2*n*=24	0.22^{c}	22.33^{e}	50.04^{a}	35.15^{a}	64.84^{de}	0.55^{b}	32.58^{c}	29.46^{h}	7.09^{e}	3B
5	*O. sativa* - Kamini bhog, 2*n*=24	0.17^{a}	17.99^{ab}	52.89^{c}	36.42^{b}	63.57^{c}	0.58^{cd}	33.84^{d}	27.14^{f}	5.16^{c}	3A
6	*O. sativa* - Black rice, 2*n*=24	0.23^{d}	23.24^{f}	54.16^{d}	36.98^{bc}	63.01^{c}	0.60^{d}	34.49^{e}	26.55^{e}	6.73^{d}	3B
7	*O. nivara*, 2*n*=24	0.21^{c}	21.02^{d}	49.38^{a}	34.85^{a}	65.14^{e}	0.53^{a}	32.17^{c}	30.29^{i}	5.63^{c}	3A
8	*O. barthii*, 2*n*=24	0.20^{c}	20.06^{cd}	61.58^{g}	40.08^{e}	59.98^{a}	0.66^{f}	36.65^{f}	19.96^{a}	5.69^{c}	3B
9	*O. officinalis*, 2*n*=24	0.22^{c}	22.46^{e}	59.37^{f}	37.25^{c}	62.74^{bc}	0.61^{e}	37.50^{g}	25.49^{d}	3.76^{a}	3A
10	*O. australiensis*, 2*n*=24	0.20^{c}	20.16^{cd}	56.83^{e}	38.10^{d}	61.89^{ab}	0.61^{e}	34.79^{e}	23.78^{c}	5.94^{cd}	3A
11	*O. rhizomatis*, 2*n*=24	0.23^{d}	23.26f	51.14^{ab}	35.65^{a}	64.34^{d}	0.60^{d}	33.99^{de}	28.69^{g}	8.02^{f}	3A
12	*O. minuta*, 2*n*=4*x*=48	0.19^{b}	19.88^{c}	58.26^{ef}	35.50^{a}	64.49^{d}	0.55^{b}	36.60^{f}	26.37^{e}	4.40^{b}	3A
13	*O. latifolia*, 2*n*=4*x*=48	0.17^{a}	17.37^{b}	49.46^{a}	36.40^{b}	63.59^{c}	0.55^{b}	31.18^{b}	26.21^{e}	4.86^{b}	3A
14	*O. eichingeri*, 2*n*=4*x*=48	0.16^{a}	16.85^{a}	61.04^{g}	36.29^{b}	63.70^{cd}	0.55^{b}	36.91^{f}	21.23^{b}	5.11^{c}	2A

[1]Interchromosomal asymmetry index (Romero Zarco 1986), [2]coefficient of variation of the chromosome length (Paszko 2006), [3]karyotype symmetry index (Greilhuber and Speta 1976), [4]total form percentage (Huziwara 1962), [5]karyotype asymmetry index (Arano 1963), [6]intrachromosomal asymmetry index (Romero Zarco 1986), [7]mean centromeric index (Seijo and Fernández 2003), [8]mean centromeric asymmetry (Peruzzi and Eroglu 2013), [9]karyotype asymmetry index (Paszko 2006), [10]Stebbins' index (Stebbins 1971). Values followed by same letters are not significantly different according to DMRT.

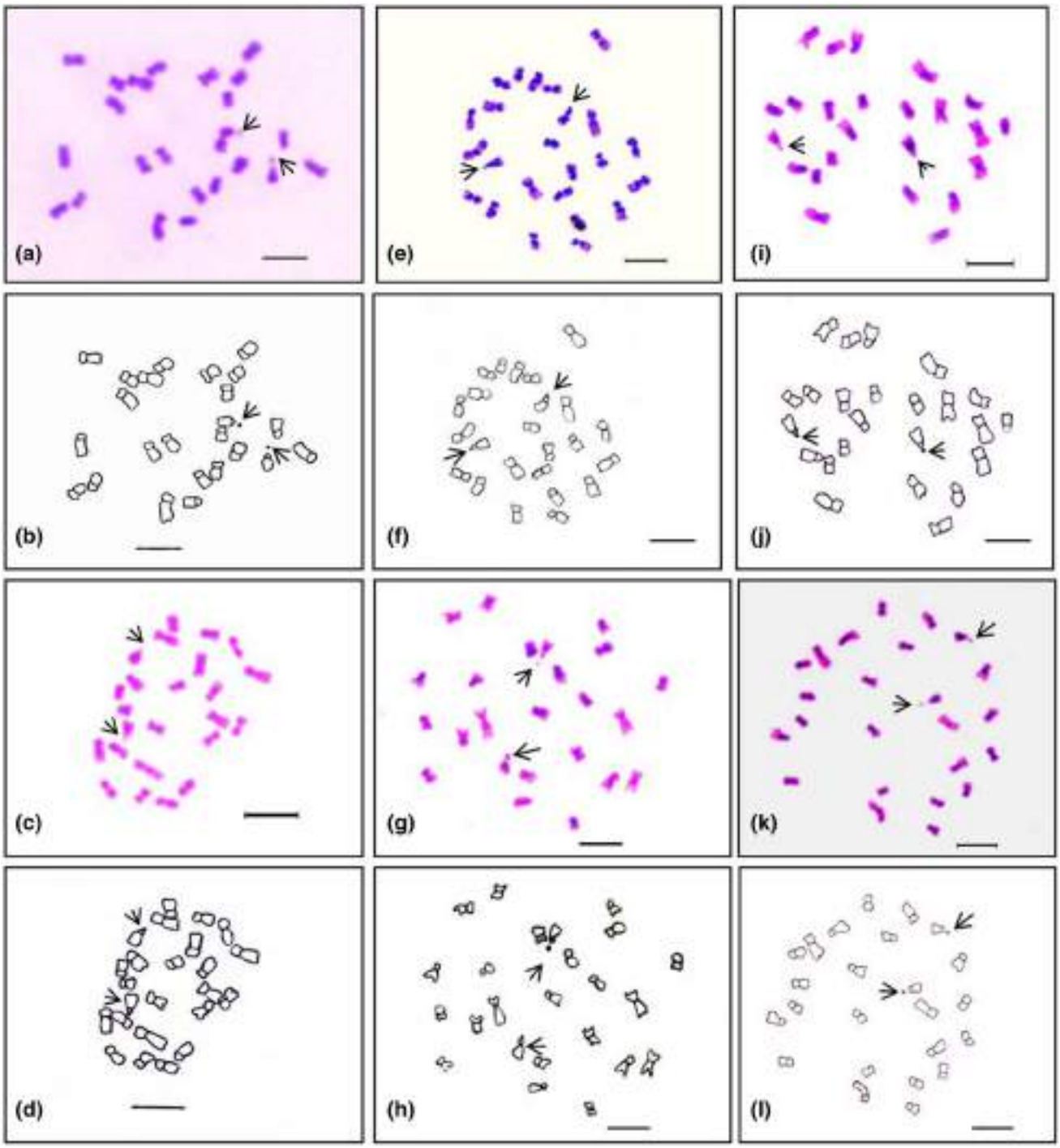


Figure 1. Giemsa stained somatic metaphase plates and corresponding hand drawings of six diploid cultivated *O. sativa* showing well scattered 2*n*=24 chromosomes. (a–b) Sundarban; (c–d) Sadanunia; (e–f) Kalonunia; (g–h) Sitabhog; (i–j) Kamini bhog; (k–l) Black rice. Arrows indicate chromosomes with two constrictions. Bars 5 μm.

lower asymmetry in karyotypes compared to diploid species. However, exact correlation between the qualitative (Stebbins' index) and quantitative indices could not be observed in terms of karyotype asymmetry tendencies of the rice species investigated here.

UPGMA dendrogram (figure 6) constructed involving the diverse karyometric features, the chromosome numbers, TCLs and mean CL of the 14 taxa revealed that the polyploid species are distinguishable as a distinct group from the diploid species. Sub-clusters in UPGMA dendrogram was observed to consist of cultivars of *O. sativa* along with the diploid wild species. However, *O. barthii* has been placed as a single and separate unit in UPGMA dendrogram (table 3; figure 6).

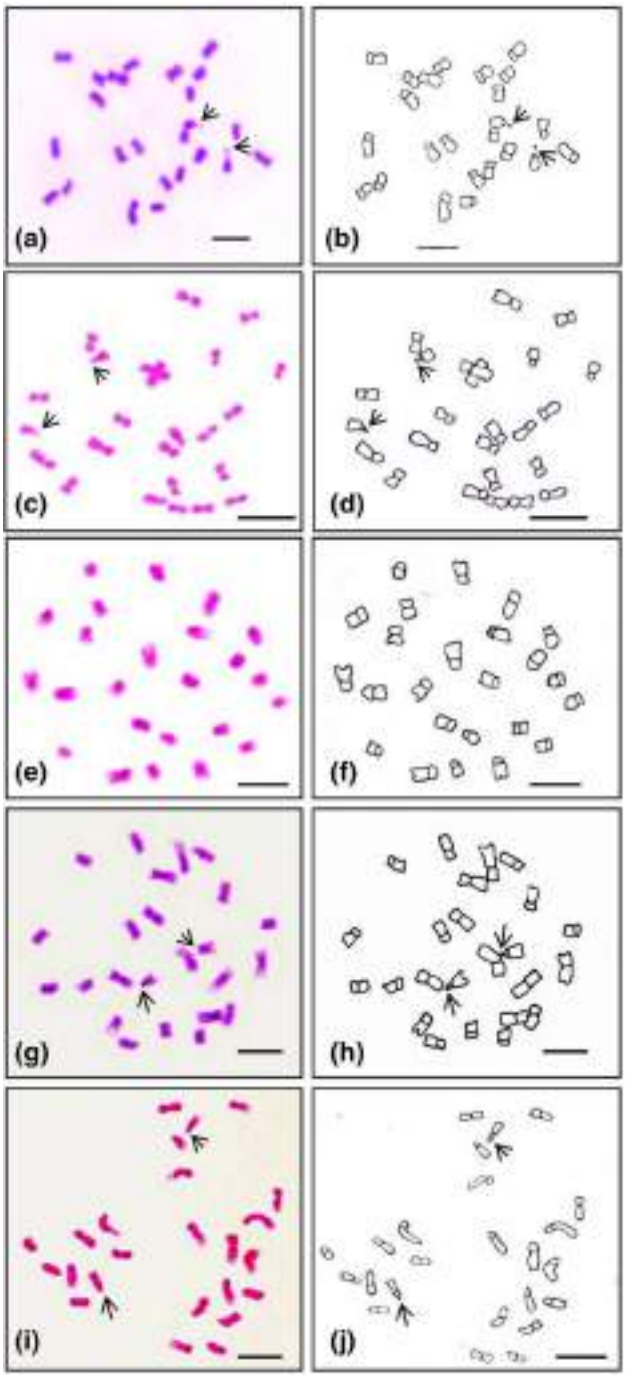


Figure 2. Giemsa stained somatic metaphase plates and corresponding hand drawings of five wild diploid rice species showing well scattered 2*n*=24 chromosomes. (a–b) *O. nivara*; (c–d) *O. barthii*; (e–f) *O. officinalis*; (g–h) *O. australiensis*; (i–j) *O. rhizomatis*. Arrows indicate the chromosomes with two constrictions. Bars 5μm

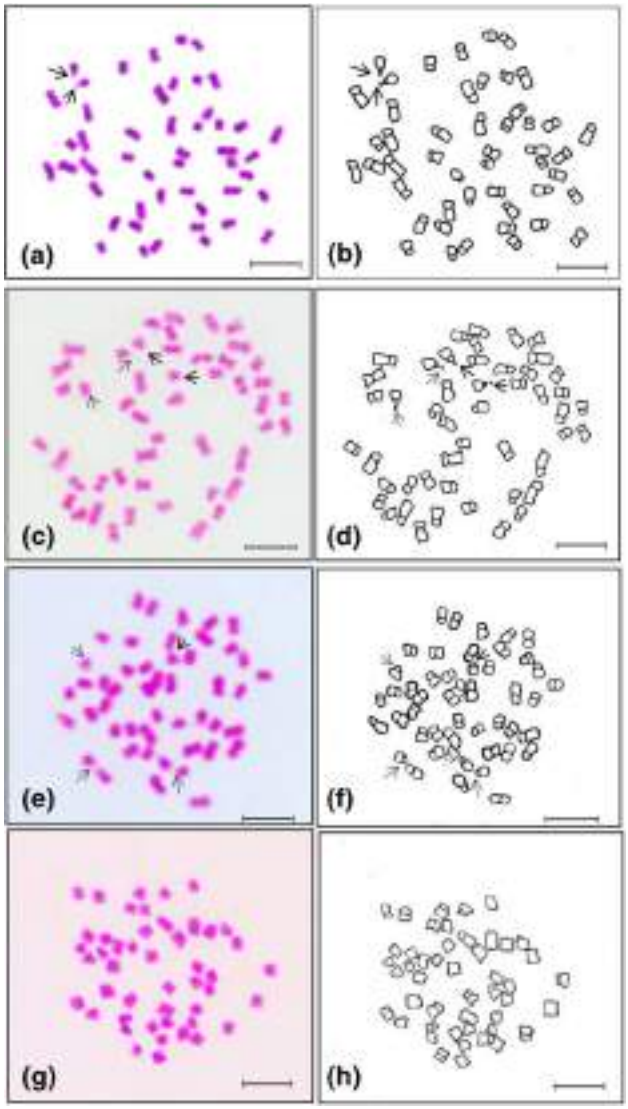


Figure 3. Giemsa stained somatic metaphase plates and corresponding hand drawings of four wild tetraploid rice species showing well scattered 2*n*=4*x*=48 and 47 (one less) chromosomes. (a–b) *O. minuta*, (c–d) *O. latifolia*, (e–f) *O.eichingeri*, (g–h) *O. alta*. Arrows indicate the chromosomes with two constrictions. Bars 5 μm.

Discussion

Improvement of rice is a continuous process and breeders and genome researchers are utilizing all possible conventional and modern methods in this regard. Wild relatives of rice are considered extra gene pool for improvement of present day rice. Genome structure analysis of rice in the form of chromosomes was initiated more than century ago (Kuwada 1910). It took more than nine decades to obtain complete genome sequencing based on individual genomic structures (Eckardt 2000). However, in spite of a repository of rice, somatic chromosome analysis has not been reported from India. Detailed chromosome analysis has always provided useful genetic information for taxonomy, phylogeny, genetics and breeding. Unlocking the new genetic resources at the chromosomal level have always been utilized by the breeders as additional gene pool for crop improvement and conservation (Baral and Bosland 2002). Thus, preparation of plant chromosomes becomes a key cytogenetic technique

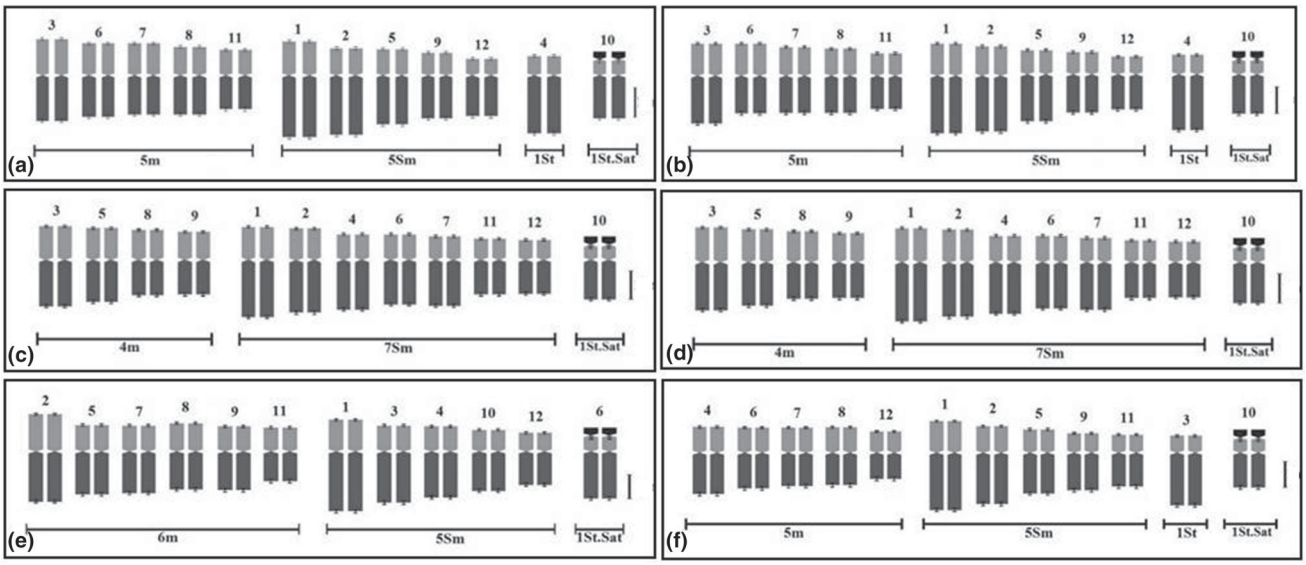


Figure 4. Comparative ideograms of six cultivated rice *O. sativa* L. (a) Sunderban; (b) Sada nunia; (c) Kalo nunia; (d) Sitabhog; (e) Kamini bhog; (f) Black rice. Bars 1 μm.

which has advanced significantly over the decades. EMA-based chromosome preparation, a basic molecular cytogenetic technique has enormously benefited plant chromosome research. Kurata and Omura (1978) have initiated EMA method in rice to overcome the earlier difficulties mentioned before. EMA-based pachytene chromosome analysis in rice is also reported (Kurata *et al*. 1981; Khush *et al*. 1984; Cheng and Gu 1994; Kurata 2008). However, misidentification of primary constrictions in pachytene chromosomes in many cases resulted errors and discrepancies in karyotyping (Cheng *et al*. 2001). The main advantage of EMA method is not only to produce cytoplasm free chromosome preparation but also to repeatedly use the same slide for possible molecular cytogenetic techniques. Plant cytogenetics research has greatly advanced, revealing unexpected details of chromosome behaviour and evolution (Guerra 2008). It is now playing more supportive role for genomics and new breeding techniques. EMA method has rejuvenated chromosome research. Irrespective of chromosome size, the method has been successfully applied in many valuable crops (Kurata and Omura 1978; Fukui 1990, 1994; Moscone *et al*. 1996; Hizume 2015; Jha and Halder 2016; Ansari *et al*. 2016; Jha and Saha 2017; Linc *et al*. 2017; Yamamoto *et al*. 2019; Bhowmick and Jha 2021; Jha and Bhowmick 2021).

EMA-based method in one Japanese rice cultivar 'Sekitori' helps chromosomewise description and identification of individual somatic rice chromosome. It becomes evident that rice karyotype comprises of five pairs of metacentrics, five pairs of sub-metacentrics and two pairs of sub-telocentric chromosomes. The landmark SAT chromosomes are present on the 10th sub-telocentric pair (Kurata and Omura 1978). The present EMA-based karyotype analysis is the maiden attempt to unlock and conserve genetic diversity of Indian rice. The present study has reconfirmed the three patterns of karyotypes reported in Japanese rice cultivar (Kurata and Omura 1978) in one hand and on the other, unravelled many novel karyotypic features in Indian rice.

Chromosome morphology (i.e., karyotype) usually shows variability involving five parameters: (i) basic number, (ii) absolute size of the chromosomes, (iii) centromere position, (iv) relative chromosome size and (v) number and position of the satellite (Stebbins 1971). The present observation has documented diversities in TCL, karyotype formula and location of the satellite landmarks on chromosomes (table 2). Irrespective of ploidy level all wild species contains lower TCL than most of the cultivated rice except *O. australiansis* (table 2). Lowest TCL content has been obtained in *O. alta* ($63.01 \pm 1.74\mu$) a wild tetraploid member. It has been noted that most of the *O. sativa* cultivars have gained additional amount of DNA (TCL) during the process of domestication in comparison to *O. nivara* ($45.90 \pm 2.83\mu$), the progenitor of *indica* type rice (table 2). TCL content has shown direct correlation with the length of metaphase chromosomes. Although highest metaphase chromosome length was obtained in wild *O. australiansis* ($2.91 \pm 0.27\mu$), most of the cultivated *O. sativa* has crossed the 2μ threshold, ahead of their wild progenitor (table 2). It is reported that correlation between genome size and metaphase chromosome length resulted from amplification of the repetitive DNA sequences during the process of domestication in the genus *Oryza* (Uozu *et al*. 1997).

Along with TCL, the present analysis has also focussed on genetic diversity in the satellited chromosomes. Secondary constriction or SAT bearing chromosomes are treated as genomic landmarks. In most of the *O. sativa* cultivars, satellite was detected on the 10th pair of chromosomes.

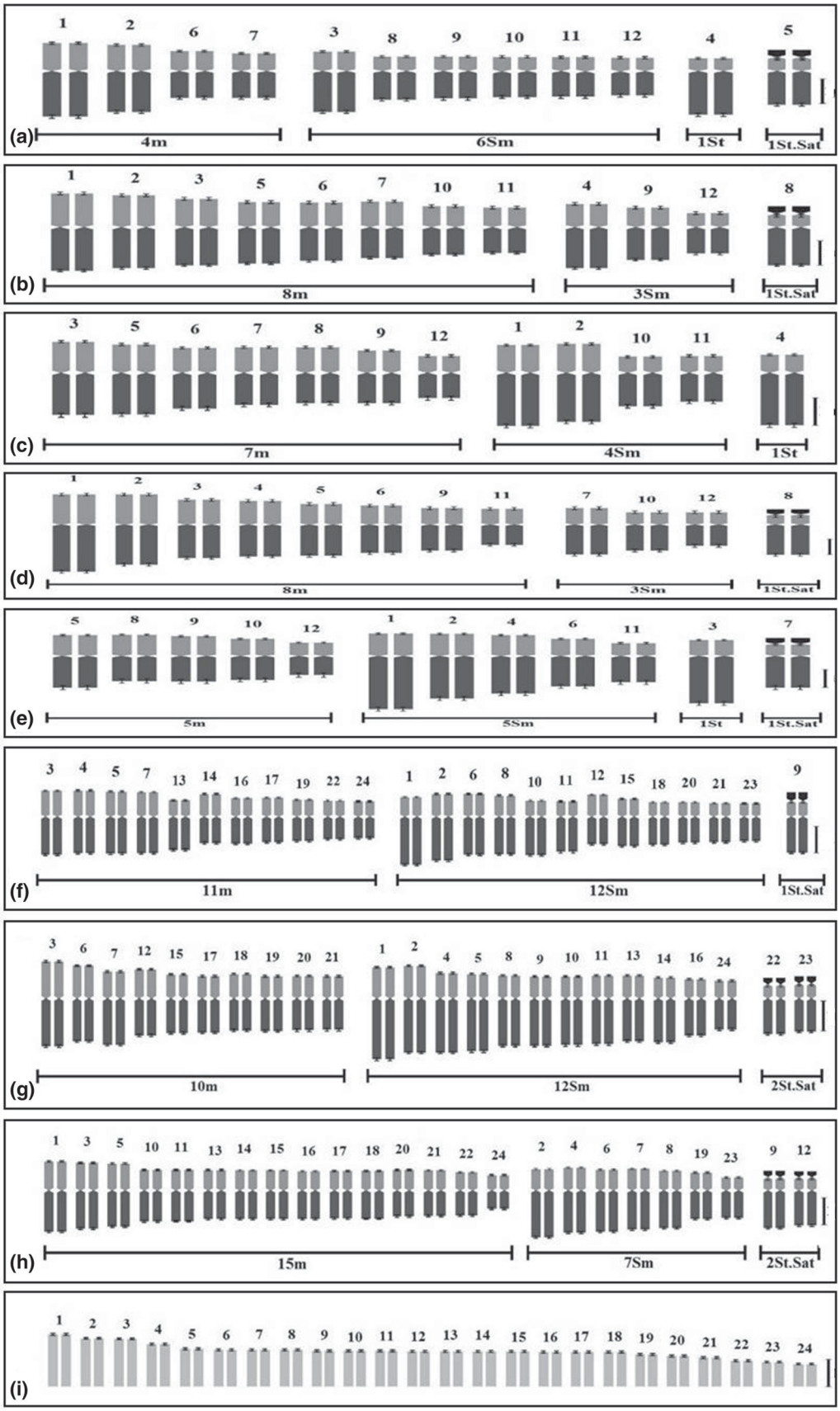


Figure 5. Comparative ideograms of five diploid (2*n*=24) and four tetraploid (2*n*=4*x*=48) wild rice. (a) *O. nivara*; (b) *O. barthii*; (c) *O. officinalis,* (d) *O. australiensis*, (e) *O. rhizomatis*, (f) *O. minuta*, (g) *O. latifolia*, (h) *O. eichingeri*, (i) *O. alta*. Bars 1 μm.

However, a clear deviation was noted in one aromatic member 'Kaminibhog' where it was distinctly present on the 6th pair of chromosomes (table 2). In five diploid wild species, secondary constriction was noted on 5th, 7th and 8th pair of chromosomes instead of 10th pair, as in most of the cultivated rice. Satellite parts were noted on 9th, 12th, 22nd and 23rd chromosome pairs in tetraploid species (table 2). Due to very small size, it was not possible to locate primary constrictions in some chromosomes and SAT bearing chromosomes in *O. alta*. Although present findings highlighted wide diversity in SAT bearing chromosomes, all SAT bearing chromosomes were characteristically sub-terminal in nature within the Indian rice (table 2). The diversity of sat chromosomes may be another evidence of ongoing intra- and inter-specific chromosomal rearrangements. An in-depth analysis of SAT bearing chromosomes is suggested to be an innovative cytomolecular line of research. The present karyomorphometric compilation clearly justifies relevance of chromosome analysis and invites more focus in this line of research.

Over the decades phylogenetic relationships within rice species has been elucidated based on morphology, isozyme, nuclear and chloroplast DNA restriction as well as single or low copy nuclear gene sequences (Ge *et al*. 1999; Saha *et al*. 2012; Choudhary *et al*. 2013; Singh *et al*. 2016). Information generated through this reports have been proved useful to the breeding programmes. The chromosomal/karyotypic parameters have so far been neglected to draw phylogenetic relationships within and between cultivated and wild rice species. Karyotype diversity is becoming relevant to address phylogenetic relationship in a statistical framework (Baltisberger and Hörandl 2016; Costa *et al*. 2020). The karyotype signatures provide the background information for identification of reproductive barriers and analysis of breeding possibilities (Levin 2002), a key question in context of wild species introgression into conventional rice cultivation in near future.

Cytological relationships and chromosomal affinities are often inferred based on the frequently used interchromosomal and intrachromosomal asymmetry/symmetry indices (Paszko 2006; Peruzzi and Eroglu 2013; Vimala *et al*. 2021). The elaborate quantitative parameters (Paszko 2006; Peruzzi and Eroglu 2013) offer scope for statistical analysis and thus, have advanced the field of karyotype evolution beyond Stebbins' index (Stebbins 1971). In case of the Indian rice, the extent of karyotype specialization and interspecific or intraspecific relationships were elucidated depending on the karyotype indices in combination with chromosome number and two fundamental morphometric parameters in UPGMA dendrogram. Within the diploid rice, the cultivars Sundarban, Sadanunia, Kaminibhog, Kalonunia of *O. sativa* formed close cluster with *O. australiensis* and *O. officinalis*, all having comparably lower asymmetric tendencies in karyotypes. The Sitabhog and Black rice cultivars form assemblage with *O. nivara* and *O. rhizomatis*, respectively, based on the overall higher trend of karyotype asymmetry. *O. barthii* has an intermediate extent of asymmetry in karyotype indices, causing separation of this species from rest of the diploids in the dendrogram. Polyploids, despite high chromosome numbers, have lower asymmetric tendency than the diploids. It should be kept in mind that the karyo-evolutionary indices in rice species provide a relative view of higher/lower tendency of asymmetry. On the basis of these data, the observed proximities between certain cultivated and wild rice species lays a foundational karyotype phylogeny which remains to be rigorously studied before any

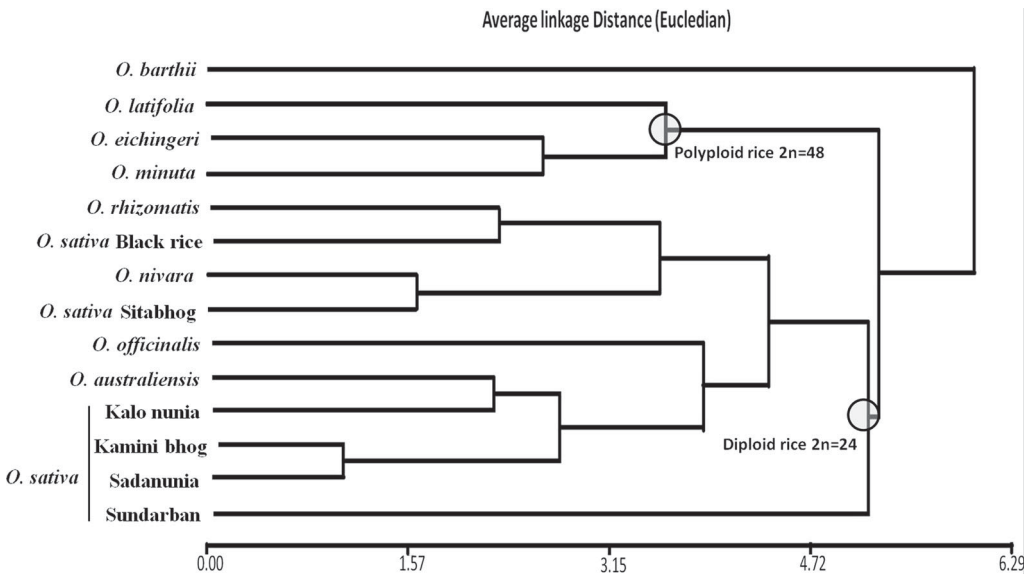


Figure 6. UPGMA dendrogram constructed from average Euclidean distance among diploid ($2n=2x=24$) and tetraploid ($2n=4x=48$) Indian rice depending on chromosome numerical and nine karyomorphometric parameters indices.

conclusive statement. Molecular phylogeny is more precise to realize species relationships and thus, interpretations may differ with karyotype phylogeny. Nevertheless, tendencies of karyotype asymmetry are indicative of ongoing advancement in chromosome constitution of a species (Stebbins 1971; Paszko 2006; Peruzzi and Eroglu 2013; Vimala *et al*. 2021). Hence, the relative karyo-evolutionary map in form of UPGMA dendrogram (figure 6) is the complementary information that might be upgraded with molecular cytogenetics or DNA sequence phylogeny in the Indian rice species context.

Taxonomical debate along with the reported chromosome numbers are still going on with the wild relatives of rice. *O. officinalis* was originally known as diploid species but a few populations of tetraploid *O. officinalis* have been reported from south India and the name of the tetraploid population has now changed to *O. malampuzhaensis* (Brar and Khush 2018). Similarly, *O. eichingeri* was reported long back (Pathak 1940) as tetraplod with $2n=4x=48$ chromosomes. Lu *et al.* (2000) confirmed the tetraploid number but also added existence of diploid members in *O. eichingeri*. Germplasms from Rice Research Institute, Cuttack, India revealed tetraploid nature of *O. eichingeri* with $2n=4x=48$ chromosomes and four distinct SATs (figure 3, e–f; table 2). No one has reported triploid wild species in rice. However, somatic chromosome number of *O. coarctata* collected from costal regions of West Bengal and Odisha has been reported as triploid of late ($2n=3x=36$) by Chowrasia *et al*. (2021). On the other hand, the same species for long period was reported as tetraploid with $2n=4x=48$ (Jena 1994; Sanchez *et al*. 2013; Brar and Khush 2018). The interesting chromosomal discrepancies deserve further attention in future through EMA-based chromosome analysis.

In the present era, close correlation between cytogenetics and genomics is stressed (Deakin *et al*. 2019). Individual chromosomes of rice received priority at the time of whole genome sequencing. Chromosome specific arsenate tolerance gene has been decoded from chromosome 6 (Dasgupta *et al*. 2004) and many disease resistance genes have been reported in chromosomes 11 and 12 (Rice Chromosomes 11 and 12 Sequencing Consortia 2005). The present karyotype analysis has added much new valuable information about Indian rice. Identification of chromosome specific genes in Indian rice may be an important future line of research.

In conclusion, it needs to be stressed that in recent times, the field of genomics has largely distanced itself from cytogenetics and these two fields were never intended to work in isolation. New era of genome biology invites a closer union among the research areas of genomics, cytogenetics, cell biology and bioinformatics (Deakin *et al*. 2019). In that context, identification of individual chromosome pairs and compilation of karyotype diversity in cultivated and wild Indian rice may be useful in understanding their genetic base and preparation of Indian rice chromosomal database for future breeding programmes.

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Exomorphological variation in *Gaultheria fragrantissima* Wall. (Ericaceae: Vaccinioideae) in India: A Micromorphological solution from leaf stomata and pollen morphology

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Abstract

Gaultheria fragrantissima Wall., a variable species in the family Ericaceae, is restricted to some south-eastern Asian countries *viz.*, India, Nepal, Bhutan, China, Myanmar, Sri Lanka, Malaysia and Vietnam. This work includes field and herbarium-based exomorphology and Light as well as Scanning Electron Microscopic studies of leaf stomata and pollen morphology of six distinct populations of *G. fragrantissima* Wall. collected from three phytogeographical regions in India including tables of comparative studies of different populations to stabilize the species delimitation based on leaf stomata and pollen morphological data.

Gaultheria fragrantissima Wall., a variable species in the family Ericaceae Juss., was first described and named by Nathalien Wallich[28] based on the plant collected from Nepal Himalaya. The etymology of the specific epithet, "*fragrantissima*" was due to its wintergreen fragrance. The species is restricted to some south-eastern Asian countries *viz.*, India, Nepal, Bhutan, China, Myanmar, Sri Lanka, Malaysia and Vietnam. In India, the species is distributed in Eastern Himalaya, North eastern India and hill tops of South western Ghats.

Detailed investigations of the genus *Gaultheria* L. were studied by workers like Clarke[4], Airy Shaw[1], Middleton[15], Kron *et al*.,[12], Ruizheng & Stevens[23], Apte *et al*.,[2], Panda[19,20], S. Panda *et al*.,[21] and Panda & Sanjappa[22]. In India, the genus is studied in detailed by Panda & Sanjappa[22] who revised the genus and described 23 species including *G. fragrantissima* Wall.

Apte *et al*.,[2] studied genetic diversity analysis in *Gaultheria fragrantissima* Wall. based on Western Ghats and Meghalayan populations using ISSR markers. They observed high gene flow within Western Ghat population, which may be enough to prevent

genetic drift, compare to very low gene flow within Meghalayan populations, while Panda[19] revised the genus *Gaultheria* L. including *G. fragrantisima* Wall. based on herbarium-based exomorphology.

The present work embodies field and herbarium-based exomorphology and Light Microscopic studies of leaf-stomata (LM) as well as Light and Scanning Electron Microscopic studies of pollen morphology (LM & SEM) of six distinct populations of *G. fragrantissima* (in case of Pollen morphology, five populations were studied due to unavailability of flower buds in one) collected from three phytogeographical regions (Eastern Himalaya, North eastern India and Western Ghats) in India to stabilize delimitation of *G. fragrantissima* Wall. based on leaf-stomata and pollen morphological data to show that exomorphological variations among these six populations are not soundly observed in micromorphological data, which may justify that all variable populations are within the circumscription of the same species, *G. fragrantissima* Wall. This work also includes tables of comparative studies of six different populations under three phytogeographical regions in India. Nair and Kothari[16] described pollen morphology of this species based on herbarium material collected only from South India (DD, no 26147). Workers like Niedenzu[17], Cox[5], Hagerup[11], Watson[29,30], Lems[13] and Stevens[27] contributed a little or no works on leaf-stomata.

Present work is the result of detailed Light (Olympus, Tokyo) as well as Scanning Electronic Microscopic (Hitachi-S530, Japan at Burdwan University Centre) studies of leaf stomata (LM) and pollen morphology (LM & SEM) of six distinct populations of *Gaultheria fragrantissima* Wall. in India. Leaf-stomatal and polliniferous materials used in this investigation were taken from total 26 dried duplicate herbarium specimens deposited in Barasat Govt. College Herbarium (BGC) as well as from live materials collected from Arunachal Pradesh, Sikkim and Meghalaya (deposited in BGC). This work was carried out partly in the Taxonomy and Biosystematics Laboratory, Post Graduate Department of Botany, Barasat Government College (first author supervised second author under PG Dissertation Project) and partly in Botany Department, Maulana Azad College, Kolkata. All measurements are given in metric system. The dimensions "D", "(d)" and "2f" corresponding to the tetrad diameter, diameter of individual pollen grains and colpi lengths respectively were measured according to Oldfield[18]. These pollen measurements are based on at least 10 grains from each specimen.

Stomatal study:

Mature leaves were obtained from duplicate herbarium specimens deposited in CAL and BGC Herbarium as well as from live collections from Arunachal Pradesh. Small cubical pieces (*c.* 1 sq.cm) were excised from the base, middle and apical regions of the blade. Several existing methods *viz*., 10% HNO_3-boiling for 10 minutes, 5% KOH overnight (12–24 hours) treatment without boiling and with boiling were done. Pieces were ringed in sterilized water until clear. After clearing, pieces were dehydrated in an ethanol series followed by staining with 10% safranin and

mounted onto microscope slide in DPX (pieces of basal, middle and apical regions in one slide). The slide was examined under Olympus (Tokyo, Japan) light microscope using 40X and 100X objectives and Camera Lucida drawings were made with the help of drawing prism. The descriptive terminology follows Metcalfe and Chalk[14], Dilcher[6], Stace[25,26], Fahn[10] and Carpenter[3].

Preparation of Pollen slides:

The method used in this study was by Erdtman[7-9]. Dry polliniferous materials (mature flower buds) were taken from duplicate herbarium specimens deposited in CAL and BGC Herbarium as well as from fresh flower buds collected from Arunachal Pradesh. The descriptive terminology follows Erdtman[9] and Sarwar *et al*.,[24].

Slide preparation for SEM :

Leaf samples for stomata and acetolysed pollen grains (following Erdtman's method[8]) were prepared for Scanning Electron Microscope observation. Pollen grains at least from 10 flowers of each species were acetolysed and studied. Observations were made with Hitachi S530 (SEM, Tokyo, Japan at Burdwan University Instrument Centre) in the high vacuum mode at an applied voltage of 15 KV. For SEM, above samples were mounted on the metallic stub using double stick tape.

Taxonomy and Exomorphological observation :

Gaultheria fragrantissima Wall., Asiat. Res. 13: 397. 1820 & Numer. List: no. 765. 1829; G. Don, Gen. Syst. 3: 840. 1834; C. B. Clarke[4] in Hook. f., Fl. Brit. India 3: 457. 1882; Hara in Hara *et al*., Enum. Fl. Pl. Nepal 3: 55. 1982; Rae in A. J. C. Grierson & D. G. Long, Fl. Bhutan 2 (1): 388. 1991; Ruizheng & P. F. Stevens[23] in Wu *et al*., Fl. China 14: 472. 2005; S. Panda, W. Kameng Ericaceae: 155. 2013; Panda & Sanjappa in Sanjappa & Sastry, Fasc. Fl. India 25: 196. 2014. Panda *et al*.,[21] Div. Gen. *Gaultheria* India: 105 – 113. 2014. *G. fragrans* D. Don, Prodr. fl. nepal.: 151. 1825. Type: Nepal, 1818, *Wallich s.n.* (K, Cibachrome image!). *Arbutus laurifolia* Buch.Ham. ex D. Don, Prodr. fl. nepal.: 151. 1825. *Gaultheria ovalifolia* Wall., Numer. List: no. 1523. 1829. *Gaultheria leschenaultii* DC., Prodr. 7 (2): 593. 1839. *Leucothoe? katagherensis* DC., Prodr. 7 (2): 606. 1839. *Andromeda katagherensis* Hook., Icon. pl. 3: t. 246. 1839. *Gaultheria forrestii* Diels, Notes Roy. Bot. Gard. Edinburgh 5 (25): 210. 1912. (Figs. 1-7). *Vernacular names*: Chanchhewaa, Dhasingare, Goenhli (Nepalese of Sikkim); Shep-Sheng (Monpas of Bomdila); Jirhapkynthai, Jirhap, Soh-lyngthrait (Khasis of Meghalaya); Kolakkaai, Moolai (Tamil of Tamil Nadu).

Stout, erect shrub, 0.3–2 (–3.5) m high, or drooping down from rock crevices. Stems always glabrous, greyish-brown to light brown, profusely branched; branchlets blood red to deep pink, glabrous, glaucous, winged or triangular. Leaves coriaceous, lamina ovate-elliptic, oblong-elliptic, ovate-lanceolate to rarely ovate or obovate, 3–12×2–4 (–6) cm, serrate to serrulate at margin, broadly cuneate at base, always mucronate at apex, deep green, glabrous above, light green, punctate beneath;

Fig. 1. Plant of ***Gaultheria fragrantissima*** Wall. : A. Bomdila, Arunachal Pradesh; B. Dodhray, Darjeeling Himalaya; C. Manipur; D. Nilgiri Hills, South Western Ghat.

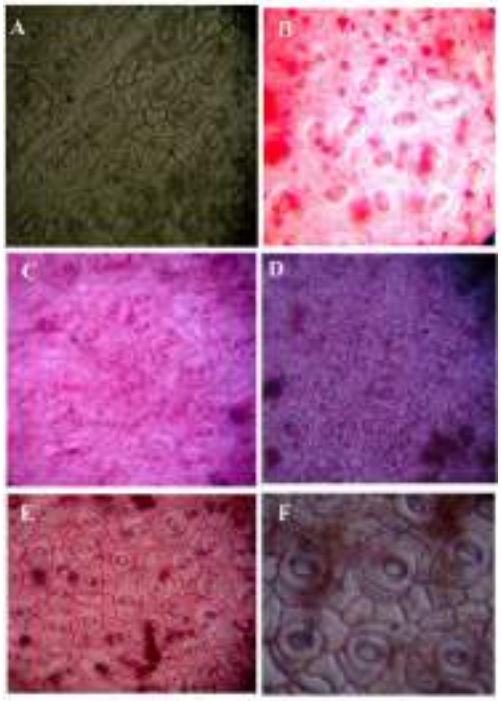


Fig. 2. Stomatal complex of ***Gaultheria fragrantissima*** Wall. (Eastern Himalayan populations): A - B (40x)- Arunachal populations (Itanagar & Walong), C - D (40X)-Sikkim populations (Yuksam-Bakhim), E-(40X) - F (100X)-Darjeeling populations.

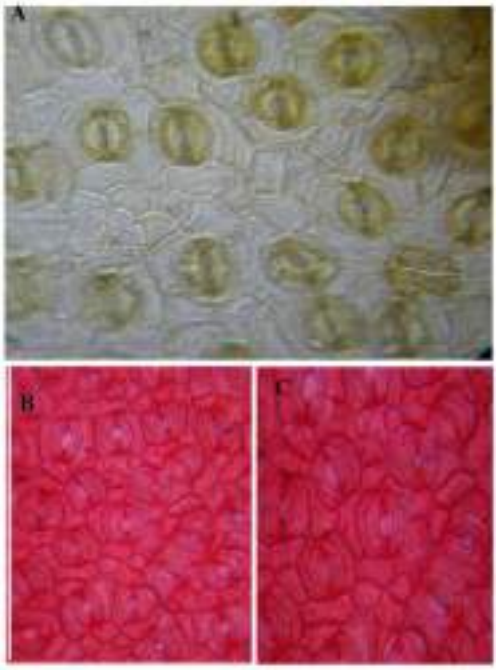


Fig. 3. Stomatal complex of ***Gaultheria fragrantissima*** Wall. (North eastern Indian populations): A (40x)- Meghalaya populations (Shillong peak), B (40X) - C (100X)- Nagaland populations (Japfu hill).

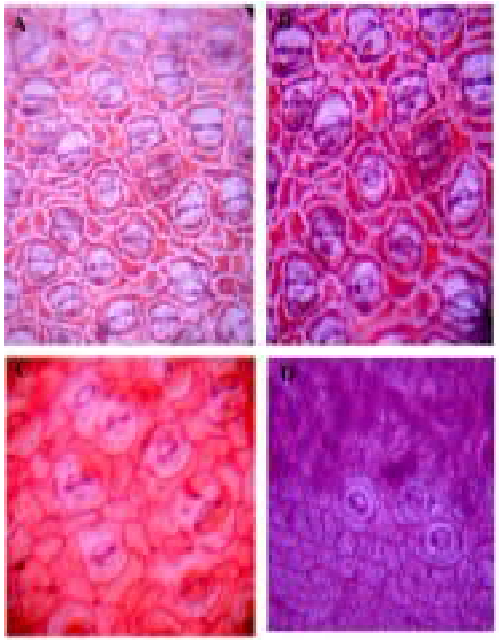


Fig. 4. Stomatal complex of ***Gaultheria fragrantissima*** Wall. (Western Ghat populations): A - C (40X)- Kerala populations (Ponmudi), D (40X)- Tamil Nadu populations (Nilgiris hill).

petioles stout, 5–11 mm long, glabrous or punctate beneath. Racemes axillary; rachis 2–6 (–10) cm long, 10–26-flowered, puberulous. Flowers always 5-merous, 8–14 mm long; pedicels greyish-white, 2–8 mm long, puberulous; bract 1, basal, light green, broadly ovate, 2–4×1.5–2.5 mm long, ciliate at margin, acute or subacute at apex, glabrous; bracteoles 2, opposite, mostly apical, rarely subapical to median on the pedicel, pink with greenish stripes, broadly ovate, 1.5–2.5×1–2 mm, ciliolate at margin, acute at apex, puberulous. Calyx lobes light pink, ovate-triangular, 2–2.5×1–1.5 mm, ciliolate at margin, acute at apex, glabrous or puberulous inside. Corolla ovoid-urceolate, greyish-white to light greenish-white, *c*. 5 mm long, 3–4 mm diameter, glabrous outside, pilose inside. Stamens 10, *c*. 3 mm long, loosely epipetalous; filaments greyish-white, *c*. 1 mm long, basally dilated; anther lobes blackish-brown, oblong, *c*. 1 mm long, each lobe with 2 apical awns of *c*. 1 mm long or minute. Pistil 2–3.5 mm long; ovary globose to subglobose, light green, 1–1.5×1.5–2 mm, tomentose; disc minutely 10-dentate; style light green, 1–2 mm long, slender, glabrous; stigma truncate. Fruit loculicidally 5-valved capsule, enclosed in a fleshy accrescent calyx, light green (immature) to deep blue or sky blue (mature), globose to subglobose, 5 – 8 × 3 – 6 mm. Seeds numerous, minute, obconical, scariose.

Distribution:

India (Eastern Himalaya: Sikkim, West Bengal and Arunachal Pradesh; NE India: Meghalaya, Nagaland and Manipur and Hill-tops of SW Ghats: Tamil Nadu, Kerala and Karnataka); Nepal; Bhutan; China; Myanmar and Sri Lanka.

Flowering: March–May; December–January. *Fruiting*: May – October.

Habitat : grown in moist rocky soil, often hanging down from rock crevices, rarely in loose humus-covered boulders or in landslide areas at altitudes ranging from 1600–2300 m.

Results (Micromorphological observation)

Leaf Stomata of *Gaultheria fragrantissima* Wall.

The study of Light Microscopic (40X, 100X) stomatal architecture includes number, form and arrangement of specialized epidermal cells associated with the stomatal guard cells. Stomata are distributed more or less evenly over the entire abaxial leaf surface in between the veins, but generally not over the finer veins and main veins.

Eastern Himalayan Population

1. Arunachal population (Walong and Bomdi-La)

Specimens studied: 7 duplicate specimens were studied (Table-1). The investigated species shows two types, amphiparacytic and brachiparacytic. *Dimensions of stomata:* The average dimension is 35.6 µm×30.8 µm in apex, middle and base. *Size of guard cells:* The average dimension is 25 µm×4.9 µm. *Size of epidermal cells:* the epidermal cells are usually isodiametric

Table-1. Source of leaf anatomy and pollen materials of *G. fragrantissima* Wall. in India

Name of species	Collector and Number	Voucher information (Herbarium acronym)
Gaultheria fragrantissima Wall. Eastern Himalayan populations 1. Arunachal Pradesh	*S. Panda* 30890; S. Panda 30762, & 30763; *T. Majumder & S. Panda* 46; *S. Panda 30826; D. K. Singh & Party* 9302 (BSD); S. Panda 30847. (=07 samples)	India: Arunachal Pradesh, Lohit district, Namti to Kibithu, 1350 m, 30.04.2003, *S. Panda* 30890 (CAL); Seru to Malinja, 2100 m, 04.05.2003, *S. Panda* 30762; Malinja Base Camp, 2100 m, 04.05.2003, *S. Panda* 30763; West Kameng district, Bomdi-La, *T. Majumder & S. Panda* 46 (Barasat Govt. College Herbarium); 3 km from Nechephu toward Jamiri, 5500 ft, 25.12.2002, *S. Panda* 30826; Dibang Valley district, Eastern bank of Mehao Lake, 26.11.2000, *D. K. Singh & Party* 9302 (BSD); Lower Subansiri district: Pange to Talle Valley, 2500 – 2800 m, 31.12.2002, *S. Panda* 30847.
2. Sikkim	*S. Panda* 29928; 30022; *R. C. Srivastava* 13162; *D. Maity* 21558. (=04 samples)	India: West Sikkim, Yuksum to Bakhim, West district, 6000 ft, 12.05.2002, *S. Panda* 29928 (CAL); Hilley to Versay, 2650 m, 27.04.2001, *S. Panda* 30022 (CAL); Phamtam, South district, 10.05.1991, *R. C. Srivastava* 13162 (BSHC); Yuksum to Bakhim, West district, 2400 m, 23.03.1999, *D. Maity* 21558 (BSHC).
North eastern Indian populations 3. Meghalaya	*S. Panda* 30708; 30701; 30703; 30712; 30822. (05 samples)	India: Meghalaya, East Khasi Hill district, Upper Shillong, 5000 ft, 17.03.2002, *S. Panda* 30708 (CAL); Jongsha Village, 4600 ft, 16.03.2002, *S. Panda* 30701; Shillong Peak, 5000 ft, 17.03.2002, *S. Panda* 30703; Mawkdak, 4500 ft, 22.03.2002, *S. Panda* 30712; Sohrareim Forest, 5000 ft, 27.11.2002, *S. Panda* 30822.
4. Nagaland	*Watt* 6873. *Dr. D. Prain s.n.*; *Dr. A. A. Mao* 106486.(03 samples)	India: Nagaland, Pegwima to Japhu Hill, 6000 ft, 19.05.1882, *G. Watt* 6873 (CAL); Pulinalodza, 7800 ft, April, 1886, *Dr. D. Prain s.n.* (CAL); Banrew Forest, 06.02.1999, *Dr. A. A. Mao* 106486 (ASSAM).
Western Ghat populations 5. Kerala	*N. C. Nair* 77213; *A. Meebold* 13337; *Ramamurthy* 66385. (03 samples)	India: Kerala, Sispara to Palghat, Palakkad district, 2000 m, 28.03.1983, *N. C. Nair* 77213 (CAL); Devicolam, Idduki district, 6000 ft, December, 1909, *A. Meebold* 13337 (CAL); Munnar to Kumili Road, Idduki district, 2000 m, 25.03.1980, *Ramamurthy* 66385 (CAL).
6. Tamil Nadu	*K. M. Sebastine* 2619; *K. Subramanyam* 2009; *K. Subramanyam* 1882; *A. Meebold* 11915. (=04 samples)	India: Tamil Nadu, Gymkhana Hills, 1766 m, 24.03.1957, *K. M. Sebastine* 2619 (CAL); Dodabetta Road, 2000 m, 07.01.1957, *K. Subramanyam* 2009 (CAL); Kotagiri, 2000 m, 04.01.1957, *K. Subramanyam* 1882 (CAL); Udagamund hills, 6000 ft, October, 1909, *A. Meebold* 11915 (CAL).

Table-2. Comparative account of field and herbarium-based exomorphological data of *Gaultheria fragrantissima* Wall. among EH, NE and WG populations

Characters	Eastern Himalayan population	North eastern Indian population	Western Ghat population
Habit	bushy erect shrub to treelet up to 3.5 m tall (usually 0.5–2 m tall).	bushy erect shrub up to 1.5 m tall (usually 0.5–1 m tall).	bushy erect shrub up to 1 m tall (rarely 1.5 m) (usually 0.4–0.7 m tall).
Branches and twigs	blood-red to greenish; trigonous to terete	blood-red; trigonous	blood-red; trigonous to terete
Leaf shape	elliptic, ovate-elliptic to ovate-lanceolate	Ovate-elliptic to elliptic	Oblong-elliptic, obovate-elliptic to obovate
Leaf size	up to14 cm long and 8 cm wide	up to 7 cm long and 5 cm wide	up to 6 cm long and 4 cm wide
Leaf apex	mucronate to shortly acuminate.	always mucronate.	mucronate to mucronulate.
Petiole	up to 12 mm long.	up to 7 mm long.	up to 5 mm long.
Inflorescence length & number of flowers per rachis	rachis up to 11 cm long; 18-30-flowered (rachis usually 4–7 cm long)	rachis up to 5 cm ong; 10-16-flowered (rachis usually 3–4 cm long)	rachis up to 4 cm long; 8-14-flowered (rachis usually 2.5–3.5 cm long)
Pedicellar bracteoles position	usually apical	apical to subapical	apical to subapical
Pedicel length	4–8 mm long	2–4 mm long	2–4 mm long
Corolla shape	ovoid-urceolate, urceolate to narrowly urceolate.	ovoid-urceolate.	ovoid-urceolate.
Ovary size	1.5 × 2 mm.	1 × 1.5 mm.	1 × 1.5 mm.
Style length	2–3 mm long.	1–2 mm long.	1–1.5 mm long.
Capsule size (maximum)	8 × 6 mm.	6 × 4 mm.	6 × 4 mm.

EH = Eastern Himalaya; NE = North eastern India; WG = Western Ghat

hexa- to polygonal. The epidermal walls in surface view are straight. *Dimensions of epidermal cells:* The average dimension is 26.9 µm ×9.6 µm (Fig. 2A–B).

2. Sikkim population (Yuksum-Bakhim)

Specimens studied: 4 duplicate specimens were studied (Table-1). The investigated species shows only one type, brachyparacytic. *Dimensions of stomata:* The average dimension is 28 µm×31.3 µm in apex, middle and base. *Size of guard cells:* The average dimension is 21.4 µm×4.9 µm. *Size of epidermal cells:* the epidermal cells are variable quadrangular, pentagonal, hexagonal to polygonal and isodiametric. The epidermal walls in surface view are straight. *Dimensions of epidermal cells:* The average dimension is 23.9 µm×13.2 µm (Fig. 2C–D).

North Eastern Indian Population :

3. Meghalaya population (Shillong peak)

Specimens studied: 5 duplicate specimens were studied (Table-1). The investigated species shows only one type, paracytic. *Dimensions of stomata:* The average dimension is 30.05 µm×26.8 µm. *Size of guard cells:* The average dimension is 20.3 µm×5.2 µm. *Size of epidermal cells:* epidermal cells are isodiametric-hexagonal to polygonal. Epidermal walls in surface view are straight. *Dimensions of epidermal cells:* The average dimension is 27.4 µm×11.3 µm (Fig. 3A).

4. Nagaland population (Japhu hill)

Specimens studied: 3 duplicate specimens were studied. The investigated species shows two types, mostly paracytic to rarely brachyparacytic. *Dimensions of stomata:* The average dimension is 24.2 µm×33.1 µm in apex, middle and base. *Size of guard cells:* The average dimension is 20.3 µm×5.7 µm. *Size of epidermal cells :* the epidermal cells are usually polygonal to occasionally quadrangular and pentagonal in shape, isodiametric. The epidermal walls in surface view are straight. *Dimensions of epidermal cells:* The average dimension is 27.2 µm×13.2 µm (Fig. 3B-C).

Western Ghat Population

5. Kerala population (Palghat)

Specimens studied: 2 duplicate specimens were studied. The investigated species shows two types, mostly amphiparacytic to rarely brachyparacytic. *Dimensions of stomata:* The average dimension is 29.4 µm×26.1 µm in apex, middle and base. *Size of guard cells:* The average dimension is 23.6 µm×4.9 µm. *Size of epidermal cells:* the epidermal cells are usually polygonal to occasionally pentagonal in shape, isodiametric. The epidermal walls in surface view are straight. *Dimensions of epidermal cells:* The average dimension is 22.3 µm×14 µm (Fig. 4A-C).

6. Tamil Nadu population (Nilgiris)

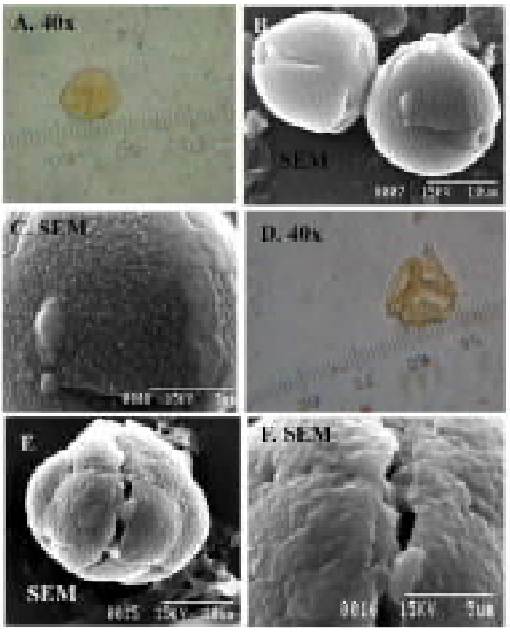
Specimens studied: 4 duplicate specimens were studied. The investigated species shows only paracytic type. *Dimensions of stomata:* The average dimension is 24.2 µm×23.1 µm in apex, middle and base. *Size of guard cells:* The average dimension is 14.9 µm×4.9 µm. *Size of epidermal cells:* the epidermal cells are usually quadrangular, penta to polygonal in shape, isodiametric. The epidermal walls in surface view are straight. *Dimensions of epidermal cells:* The average dimension is 23.9 µm×14.8 µm (Fig. 4D).

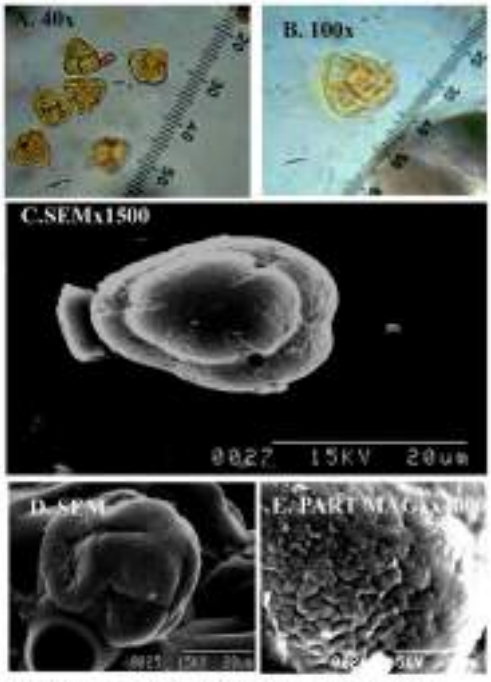
Pollen Morphology **(**for each specimen, total 10 flower buds were taken for study).

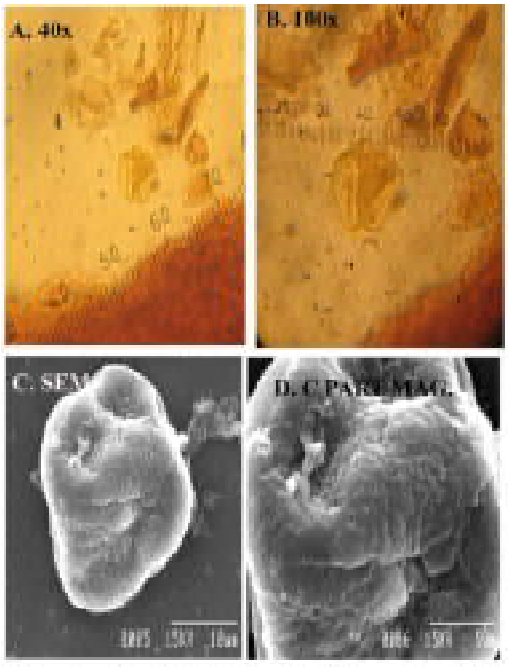
Eastern Himalayan Population

1. Arunachal population (Walong)

Pollen grains occur in tetrahedral to rhomboidal tetrads, 3-zonocolporate. *Tetrad size (D):* 23.4 µm (average) diameter. *Individual grain size (d)* variable, 11 µm–13 µm diameter. Individual grain possesses no distinct furrows. *D/d = 1.9. Exine* tectate, 3.9–5.2 µm thick, surface rugulate-psilate to faintly rugulate, the rugulae faintly striate. *Colpi* distinct, 7 µm–7.8 µm long, width 1.6 µm–1.8 µm. Ratio of colpus length (2f) to tetrad diameter (2f/D), 0.2 µm–0.3 µm, colpus margin distinct, significantly wider at middle, bluntly acute towards ends. *Septum thickness* 1.2 µm– 2.8 µm (Fig. 5A-C).







2. Sikkim population (Yuksum-Bakhim)

Pollen grains occur in tetrahedral tetrads, 3-zonocolporate. *Tetrad size (D):* 23.4 µm–24.1 µm diameter. *Individual grain size (d)* variable, 11.7 µm–12.2 µm diameter. Individual grain possesses no distinct furrows. *Exine* tectate, 3.9–4.2 µm thick, surface coarsely rugulate, the rugulae finely striate. *D/d = 1.99. Colpi* distinct, 10.8 µm–12.6 µm long, width 1.3 µm–1.6 µm. Ratio of colpus length (2f) to tetrad diameter (2f/D), 0.4 µm–0.5 µm, colpus margin distinct, significantly wider at middle, bluntly acute towards ends. *Septum thickness* 1.9 µm–2.8 µm (Fig. 5D-E).

North Eastern Indian Population

3. Meghalaya population (Shillong peak)

Pollen grains occur in tetrahedral to rhomboidal tetrads, 3-zonocolporate. *Tetrad size* (D): 20.7 µm–21.6 µm diameter. *Individual grain size (d)* variable, 7.2 µm–10.8 µm diameter. Individual grain possesses no distinct furrows. *D/d = 2.31. Exine* tectate, 2.2–2.8 µm thick, surface finely granular. *Colpi* distinct, 10.8 µm–11.7 µm long, width 0.9 µm (apex)–3.6 µm (middle). Ratio of colpus length (2f) to tetrad diameter (2f/D), 0.52 µm–0.54 µm, colpus margin distinct, significantly wider at middle, bluntly acute towards ends. *Septum thickness* 1.6 µm–1.8 µm (Fig. 6A-C).

4. Nagaland population (Japhu hill)

Pollen grains occur in tetrahedral tetrads, 3-zonocolporate. *Tetrad size (D):* 22.5–25.2 µm diameter. *Individual grain size (d)* slightly variable, 10.8–12.6 µm diameter. Individual grain possesses no distinct furrows. *D/d = 2.03. Exine* tectate, 3–3.6 µm thick, surface uneven and rugged, primary exine sculpture finely rugulate, the rugulae faintly striate. *Colpi* distinct, 10.8 µm–12.2 µm long, width 1.4 µm–1.6 µm. Ratio of colpus length (2f) to tetrad diameter (2f/D), 0.4 µm–0.5 µm, colpus margin distinct, significantly wider at middle, luntly acute towards ends. *Septum thickness* 1.2 µm–2.8 µm (Fig. 6D-F).

Western Ghat Population (sufficient and suitable flower buds not available for study for Tamil Nadu population)

5. Kerala population (Palghat)

Pollen grains occur in tetrahedral to decussate tetrads, 3-zonocolporate. *Tetrad size (D):* 21.9 µm–24.3 µm diameter. *Individual grain size (d),* 10.2 µm–11.7 µm diameter. Individual grain possesses no distinct furrows. *D/d = 2.1. Exine* tectate, 2.6–2.8 µm thick, surface uneven and rugged, primary exine sculpture finely rugulate, the rugulae faintly striate. *Colpi* distinct, 7 µm–9 µm long, width 1.4 µm–1.6 µm. Ratio of colpus length (2f) to tetrad diameter (2f/D), 0.31 µm–0.37 µm, colpus margin distinct, significantly wider at middle, bluntly acute towards ends. *Septum thickness* 1.2 µm–1.4 µm (Fig. 7A-D).

Discussion

Among three phytogeographical regions (Eastern Himalayas (EH), North eastern India (NE) and Western Ghat (WG) of *Gaultheria fragrantissima* Wall., NE populations show some minor variations in leaf-stomata and pollen morphology, while EH and

Table-3. Comparative account of leaf-stomata and pollen morphological data of *Gaultheria fragrantissima* Wall. among EH, NE and WG populations:-

Characters	Eastern Himalayan population	North eastern Indian population	Western Ghat population
Leaf-stomata			
Type	Amphi- and brachyparacytic common.	Mostly paracytic, rarely brachyparacytic.	Mostly paracytic and amphiparacytic, rarely brachyparacytic.
Dimension	26.7 µm – 39.6 µm × 22.6 µm – 36.3 µm.	22.6 µm – 31.7 µm × 25.3 µm – 36.3 µm.	22.6 µm – 36.3 µm × 16.5 µm – 29.7 µm.
Dimension of guard cell	13.2 µm – 29.7 µm × 4.9 µm – 6.6 µm.	19.8 µm – 21.4 µm × 4.9 µm – 6.6 µm.	14.9 µm – 25.8 µm × 4.9 µm.
Shape of epidermal cells (abaxial leaf)	Quadrangular, penta-, hexa- to polygonal in shape	Mostly polygonal, rarely quadrangular, penta- to hexagonal in shape	Quadrangular, penta- to polygonal in shape.
Dimension of epidermal cells	11.6 µm – 39.6 µm × 6.6 µm – 15.2 µm.	14.9 µm – 39.6 µm × 7.1 µm – 14.9 µm.	11.6 µm – 33 µm × 8.3 µm – 21.4 µm.
Abaxial epidermal wall	Straight (surface view)	Straight (Surface view)	Straight (Surface view)
Pollen morphology			
Tetrad	Tetrahedral to rhomboidal tetrads.	Tetrahedral to rhomboidal tetrads.	Tetrahedral to decussate tetrads.
Tetrad size (D)	23.4 – 24.1 µm diameter.	20.7 – 25.2 µm diameter.	21.9 µm – 24.3 µm diameter
Individual grain size (d)	11 – 13 µm.	7.2 – 12.6 µm.	10.2 – 11.7 µm.
Exine	3.9 – 5.2 µm thick; surface coarsely to faintly rugulate.	2.2 – 3.6 µm thick; surface finely rugulate to finely granular.	2.6 – 2.8 µm thick; surface finely rugulate.
D/d	1.99	2.03 – 2.31	2.1
Colpi	7 – 12.6 µm.	10.8 – 12.2 µm.	7 – 9 µm.
2f/D	0.2 – 0.5 µm	0.42 – 0.54 µm	0.31 – 0.37 µm
Septum thickness	1.2 – 2.8 µm	1.2 – 2.8 µm	1.2 – 1.4 µm

EH = Eastern Himalaya; NE = North eastern India; WG = Western Ghat

WG populations show mostly similar characters in leaf-stomata but some minor variations in pollen morphology. NE populations are characterized by lacking amphiparacytic stomata, smaller stomatal dimension (22.6 µm–31.7 µm) and polygonal epidermal cells in leaf-stomata, finely granular exine and more 2f/D (0.42–0.54 µm) value in pollen morphology. Meanwhile, WG populations are differentiated from EH and NE populations in possessing pollen decussate tetrads, smaller exine thickness (2.6–2.8 µm), shorter colpi (7–9 µm) and shorter septum thickness (1.2–1.4 µm) which are all minor quantitative data. Therefore, this work on leaf-stomata and pollen morphology of *G. fragrantissima* Wall. on six different populations from three distinct phytogeographical regions in India shows all these populations belong to the same species, *G. fragrantissima* Wall., although these populations are exomorphologically variable to some extent.

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A comparative karyo-morphometric analysis of Indian landraces of *Sesamum indicum* using EMA-giemsa and fluorochrome banding

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Abstract. *Sesamum indicum* commonly known as 'Sesame', 'Til' or 'Gingli' is an age-old high valued oil crop. With distinct seed and floral diversity and no detailed chromosomal analysis is available on Indian landraces of *S. indicum* (2n= 26). The present study demonstrates standardization of enzymatic maceration and air drying (EMA) method of chromosome preparation and comparative karyometric analysis in four Indian landraces of *S indicum.* All the landraces were characterized by very small chromosomes, length ranging from 1.24 ± 0.02 to 2.87 ± 0.09 µm. The EMA- Giemsa based karyotype analysis revealed nine pairs of chromosomes with nearly median primary constriction, three pairs were submedian and a single satellite pair in each of the studied landrace. The CMA staining of *Sesamum* chromosomes revealed the presence of distinct CMA positive (CMA^{+ve}) signals in all the studied landraces. The Black seeded til (BT) and White seeded til (WT) were characterized by six chromosomes with distal CMA^{+ve} signal on short arm, while the Dark brown seeded til (DBT) showed ten chromosomes with distal CMA^{+ve} signal on short arm. The Light brown seeded til (LBT) was characterized by eight chromosomes with distal CMA^{+ve} signal on short arm. The results obtained from the scatter plot of A1 versus A2 and PCA analysis provide a strong relationship with that of the fluorochrome banding analysis. The present research offers an explicit karyo-morphometric characterization of four Indian landraces of *S. indicum* for the first time.

Keywords: fluorochrome banding, karyotype, sesame, *Sesamum indicum*, small chromosomes, til.

INTRODUCTION

Sesamum indicum L. commonly known as 'Sesame', 'Til' or 'Gingli' is an age-old high valued oil crop. As per the Index Kewensis the genus belongs to the family Pedaliaceae and comprises 36 species. However, *S. indicum* is the only cultivated species of this genus (Nayar and Mehra 1970). Sesame seeds are also known as the 'Queen of the oil seeds' and the first oil known to be

consumed by human (Bedigian and Harlan 1986). Beneficial effects exhibited by sesame as antioxidant, antimicrobial, anti-inflammatory, antidiabetic, anticancer on human health has recently renewed the interest in this crop (Amoo *et al.* 2017, Zhang *et al.* 2013). The species was domesticated in India long back (Bedigian 2003; 2010) and now ranked first in production and export of this crop (IOPEPC Kharif 2017). Cultivated *S. indicum* has highly variable genotypes and distinct differences have been noted in floral and seed colour morphology within the cultivated landraces (Raghavan *et al.* 2010). Chromosome analysis has played an important role in genetics and plant breeding for conservation of genetic diversity and improvement of crops. It is felt that chromosome analysis still provides foundational pieces of genomic information (Soltis 2014) and considered "the quickest, cheapest, and easiest way to get any substantial information about the genome of a species which is not possible by any other methods" (Guerra 2008). Chromosome analysis in this cultivated species (2n=26) was reported long back by Morinaga *et al.* (1929), Raghavan and Krishnamurthy (1947) and Kobayashi (1949). Raghavan and Krishnamurthy (1947) reported that all 13 small chromosome pairs have terminal constrictions, while Mukherjee (1959) reported presence of five types of somatic chromosomes. However, Kobayashi (1949; 1991) in his analyses noted five pairs of median and eight pairs of sub median chromosomes. Zhang *et al.* (2013) reported three pairs median, eight pairs sub median and two pairs sub-terminal chromosomes in *S. indicum* cv. Yuzhi 11. It appears from the earlier reports that karyometric analysis of Indian sesame deserves priority as detailed chromosomal analysis is not available on *S. indicum* along with their important landraces. Thus, the present communication for the first time details the standardization of enzymatic maceration and air drying (EMA) method of chromosome preparation and comparative karyometric analysis using non-fluorescent Giemsa and fluorescent DAPI and CMA stains in four distinct Indian landraces of *S indicum*.

MATERIALS AND METHODS

Plant materials

The present study included four Indian landraces of *S. indicum* namely Black seeded till (BT), Dark brown seeded till (DBT), Light brown seeded till (LBT) and White seeded till (WT). Among these four landraces, seeds of BT, WT and LBT were collected from different parts of West Bengal and DBT was collected from Mangalore, Karnataka. All the collected seeds were germinated, grown in earthen pots and maintained under natural environment. Voucher specimens were prepared for all the collected samples.

Somatic chromosome preparation and karyo-morphometric analysis

Nearly 20- 25 seeds from each landrace were imbibed in water for overnight and germinated in dark on moist filter papers to harvest their root tips. A minimum of ten healthy root tips of each sample were pre-treated separately in saturated solution of *p*-dichlorobenzene (PDB) at 14- 16°C for 4- 5 hrs, fixed overnight in glacial acetic acid: methanol (1:3) and finally stored therein at - 20 °C. Enzymatic maceration and air-drying (EMA) method was carried out following our earlier published protocol (Jha and Yamamoto 2012; Jha *et al.* 2015; Jha and Saha 2017) with required modifications of enzyme digestion time (55 min-90 min). Completely air-dried slides were stained with 2% Giemsa solution (Merck; Germany) in 1/15th phosphate buffer solution (pH 6.8) for 10-30 min at room temperature. After 4- 5 times washing with ddH_2O, the slides were air dried, mounted with xylene and observed (a minimum of 20 well scattered metaphase plates for each landrace). They were examined and photographed under Carl Zeiss, Axio. Lab. A1 microscope fitted with CCD Camera using Axiovision L. E4 software.

For karyo-morphometric analysis, different karyological parameters viz. length of long arm (*l*) and short arm (s), absolute chromosome length (CL), relative chromosome length (RL) and total diploid chromatin length (TCL) were used. Five somatic metaphase plates were used for karyometric analysis as well as to prepare ideogram. The centromeric index (CI) was used to classify the chromosomes according to Levan *et al.* (1964) [metacentric (m) (1.00–1.70), submetacentric (Sm) (1.70–3.00), subtelocentric (St) (3.00–7.00) and telocentric (t) (7.00–α)]. The karyotype asymmetry was estimated using intra-chromosomal asymmetry index (A1) and inter-chromosomal asymmetry index (A2) (Zarco 1986), asymmetric karyotypes percent (AsK%), asymmetry Index (AI) (Paszko 2006), total form percent (TF%), coefficient of variation of chromosome length (CV_{CL}), coefficient of variation of centromeric index (CV_{CI}), coefficient of variation of arm ratio (CV_r) and categories of Stebbins (1971).

Fluorochrome staining with DAPI and CMA

Giemsa stained slides of each landrace were destained with 70% methanol for 40 min and air dried.

DAPI and CMA staining was carried out separately following the protocol of Kondo and Hizume (1982) with required modifications. For DAPI staining, slides were kept for 30 min in Mcllvaine buffer and then stained with 0.1µg ml^{-1} solution of DAPI for 10- 30 min, mounted in non-fluorescent glycerol and observed under Carl Zeiss Axio Lab A1 fluorescent microscope using carl zeiss DAPI filter cassette. For CMA staining, the same slides were de-stained air-dried and then kept in Mcllvaine buffer for 30 min followed by McIlvaine buffer with 5mM $MgCl_2$ for 10 mins. Slides were stained with 0.1mg ml^{-1} solution CMA for 30- 60 mins followed and rinsed with Mcllvaine buffer containing 5mM $MgCl_2$. Finally, slides were mounted with non-fluorescent glycerol and kept for maturation at 4^0C for 72 hrs. CMA stained chromosomes were observed under the above-mentioned fluorescent microscope fitted with Carl Zeiss FITC filter cassette and signals were analyzed using software Prog Res 2.3.3.

Statistical analysis

Descriptive statistics including mean values were analyzed for all measured parameters and variability in the data was expressed as the mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) was performed to detect significant differences ($p \leq 0.05$) in the mean (Rohlf 1998). Duncan's multiple range test (DMRT) was used for post hoc analyses using SPSS v 16.0 statistical package. To study the karyotypic relationships among the collected landraces of *S. indicum*, scatter diagram of A1 versus A2 was drawn following the descriptions of Paszko (2006). In order to further clarify the chromosomal relationship between each of the studied landrace, principal components analysis (PCA) was conducted according to McVean (2009). In this study, nine karyological variables (A1, A2, TF%, AsK%, CV_{CL}, CV_{CI}, CV_r, AI and TCL) were used to plot the principal components using the InfoStat version 2013d (free version).

RESULTS

In the present study, four landraces of *S. indicum* differing in seed coat colour viz., Black seeded til (BT), Dark brown seeded til (DBT), Light brown seeded til (LBT) and White seeded til (WT) were used for karyotype analysis (Fig. 1). Nearly 99% seeds of each sesame landrace germinated within 3- 6 days after imbibition. Distinct diversity in the floral morphology pertaining to four different landraces of *Sesamum* was noted. The length of the corolla was 15- 20 mm with characteristic pigmentations on the lower lip. The flowers in black seeded til (BT) showed intense purple pigmentation in lower lip of corolla while in other landraces (DBT, LBT and WT) the intensity of the pigmentation ranged from pale lavender/ purple to light pink to white respectively (Fig. 1).

Figure 1. Flower and seed morphology of four Indian landraces of *S. indicum*. a & e) Black seeded til; b & f) Dark Brown seeded til; c & g) Light Brown seeded til; d & h) White seeded til. Dotted arrows indicate pigmentation patterns in lower lip of the corolla.

Karyo-morphometric analysis

Standardization of enzymatic maceration of root tip cells at 37 °C is the most crucial step to obtain well scattered metaphase chromosomes. In the present study, enzymatic maceration of root tips of all the collected landraces was performed for 55- 90 min and finally the time was optimized to 85- 90 min to obtain cytoplasm free well scattered chromosomes. The giemsa staining was done for 20 min. For each landrace, at least 20 countable metaphase plates were studied to determine diploid chromosome number.

Somatic chromosome number of 2n= 26 was observed in all the studied landraces of *S. indicum* (Fig. 2; Table 1). All the landraces were characterized by small sized chromosomes ranging from 1.24 ± 0.02 to 2.87 ± 0.09 µm (Fig. 2; Table 1). A significant variation in the total chromatin length was observed among the studied accessions. Black seeded til (BT) was characterized by highest total chromatin length (52.75 ± 0.24 µm), while the lowest (44.85 ± 0.35 µm) being found in Dark brown seeded til (DBT). The detailed karyotype analysis revealed nine pairs of chromosomes with nearly median primary constriction, three pairs with sub median primary constrictions and a single satellite pair in each of the studied landraces (Fig. 2). The ordering of satellite (sat) bearing pair was found to be constant (5th pair) in all the landraces having identical haploid karyotype formula: 3Sm + 9m + 1Sm.Sat (Fig. 2; Table 1).

In the present study, several karyo-morphometric variations were also noted among the studied landraces. Low values of intra-chromosomal asymmetry index (A1) and inter-chromosomal asymmetry index (A2) were observed in all the studied landraces (Table 2). Asymmetric Index (AI), the product of coefficient of variation in chromosome length (CV_{CL}) and coefficient of variation in centromeric index (CV_{CI}) was found to be low (ranging from 1.464 to 1.964) in all studied accessions of *Sesame* (Table 2). Whereas, Ask% and TF% showed moderate values for all the four *Sesamum* landraces (Table 2). Analysis of the karyotype asymmetric indices also revealed that all studied landraces except Black seeded til (BT) belong to the group 2A of Stebbins classification while Black seeded til (BT) belongs to group 2B (Table 2).

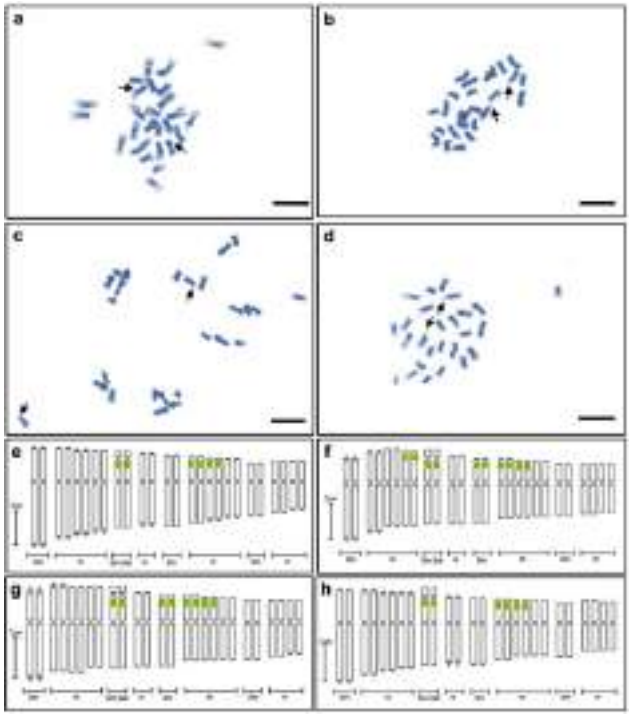

Figure 2. Panel A: EMA based giemsa stained mitotic metaphase plates of four Indian landraces of *S. indicum* showing 2n= 26 chromosomes. a) Black seeded til; b) Dark Brown seeded til; c) Light Brown seeded til; d) White seeded til. Arrows indicate secondary constricted chromosomes. Bar= 5 µm. Panel B: Comparative ideograms of the studied four landraces. Also showing positive CMA fluorescent band on respective chromosomes, Bar= 1 µm.

Fluorochrome banding analysis

In the present study, fluorochrome staining of *Sesamum* somatic chromosomes using DAPI and CMA was standardized for the first time. Chromosomes were stained properly with DAPI when incubated for 30 min, while for CMA, staining time was optimized at 60 min. The CMA staining of *Sesamum* chromosomes revealed the presence of distinct CMA positive (CMA^{+ve}) signals/zones in all the studied landraces However, the number of chromosomes showing CMA^{+ve} signals varied among them (Table 3). Based on the CMA signalling patterns, chromosomes were grouped into two basic types: type A [chromosomes with no CMA^{+ve} signals] and type B [chromosomes (including one pair of sat-bearing chromosomes) with distal CMA^{+ve} signal on short arm]. Both the type A and B chromosomes were present in all the four landraces of *Sesamum* while, the number of each type was found to be landrace specific (Table 3). The Black seeded til (BT) and White seeded til (WT) were characterized by six chromosomes with distal CMA^{+ve} signal on short arm (Fig. 3b and 3k), while the Dark brown seeded til (DBT) showed ten chromosomes with distal CMA^{+ve} signal on short arm (Fig. 3e). The Light brown seeded til (LBT) was characterized by eight chromosomes with distal CMA^{+ve} signal on short arm (Fig. 3h). However, we could not detect DAPI $^{+ve/-ve}$ signals on chromosomes of any of the landraces studied (Table 3).

Table 1. Chromosome morphometric analysis of four Indian landraces of *S. indicum**.

S. indicum landraces	Zygotic chromosome number (2n)	Length of longest chromosome (µm)		Length of shortest chromosome (µm)		Total chromatin length (µm) (Mean ± S.D.)	Ordering no. of SAT bearing pair	Karyotype formulae (n)
		Absolute (Mean ± S.D.)	Relative (Mean ± S.D.)	Absolute (Mean ± S.D.)	Relative (Mean ± S.D.)			
Black seeded Til	26	2.87 ± 0.09^{b}	5.44 ± 0.14^{b}	1.36 ± 0.02 ^{c}	2.58 ± 0.05^{a}	52.75 ± 0.24^{c}	5th	3Sm+9m+ 1Sm.Sat
Dark Brown seeded Til	26	2.20 ± 0.12^{a}	4.90 ± 0.24^{a,b}	1.24 ± 0.02^{a}	2.77 ± 0.06^{b}	44.85 ± 0.35^{a}	5th	3Sm+9m+ 1Sm.Sat
Light Brown seeded Til	26	2.15 ± 0.14^{a}	4.76 ± 0.28^{a}	1.28 ± 0.02^{a}	2.84 ± 0.07^{b}	45.20 ± 0.42^{a}	5th	3Sm+9m+ 1Sm.Sat
White seeded Til	26	2.62 ± 0.07^{b}	5.42± 0.06^{b}	1.33± 0.03^{b}	2.76 ± 0.03^{b}	48.29 ± 0.74^{b}	5th	3Sm+9m+ 1Sm.Sat

*Values followed by same letter are not significantly different according to Duncan's multiple range tests test (P=0.05).

Table 2. Comparative karyometric analysis of four Indian landraces of *S. indicum**.

S. indium landraces	A1	A2	TF%	AsK%	CV_{CL}	CV_{CI}	CV_{r}	AI	Stebbin's group
Black seeded Til	0.603	0.004	37.061	62.293	22.496	8.732	15.639	1.964	2B
Dark Brown seeded Til	0.619	0.007	37.747	61.360	19.056	10.218	17.877	1.947	2A
Light Brown seeded Til	0.628	0.009	38.228	60.886	17.369	8.433	14.562	1.464	2A
White seeded Til	0.636	0.015	38.163	61.007	21.370	7.523	12.311	1.607	2A

*Values followed by same letter are not significantly different according to Duncan's multiple range tests test (P=0.05). A1: Intra-chromosomal asymmetry index; A2: Inter-chromosomal asymmetry index; TF%: Total form percent; AsK%: Asymmetric karyotype percent; CVCL: Coefficient of variation of chromosome length; CVCI: Coefficient of variation of centromeric index; CVr: Coefficient of variation of arm ratio; AI: Asymmetry index.

Table 3. Fluorescent banding patterns in four Indian landraces of *S. indicum.*

S. indicum landraces	Maximum no. of chromosomes with CMA^{+ve} bands	Position of CMA^{+ve} bands in chromosome	CMA karyotypes (n)	Maximum no. of chromosomes with $DAPI^{+ve/ve-}$ bands
Black seeded Til	6	Distal part of short arm	20A+6B	Nil
Dark Brown seeded Til	10	Distal part of short arm	18A+8B	Nil
Light Brown seeded Til	8	Distal part of short arm	18A+8B	Nil
White seeded Til	6	Distal part of short arm	20A+6B	Nil

CMA^{+} signals in four studied landraces are incorporated in the Idiogram (Fig. 2)

Scatter plot and principal component (PCA) analyses

The scatter diagram of A1 versus A2 revealed that Dark brown seeded til (DBT) and Light brown seeded til (LBT) were placed close to each other, thereby forming a cluster, while the Black seeded til (BT) and White seeded til (WT) were positioned away from the cluster (Fig. 4). In the present study, PCA was further conducted to clarify the karyotypic relationship between the landraces using different karyo-morphometric parameters. In this eigenvector-based multivariate analysis, the component 1 (PC1) was found to be 59.6% of the total variation whereas component 2 (PC2) was 33.4% (Fig. 5). The obtained cophenetic correlation was 0.998, indicating a good fit between the eigenvalues and eigenvectors distance matrix. The PCA plot (Fig. 5) displayed the close positioning of Dark brown seeded til (DBT) with Light brown seeded til (LBT), which was similar to that of the scatter plot (Fig. 4). On the other hand, the Black seeded

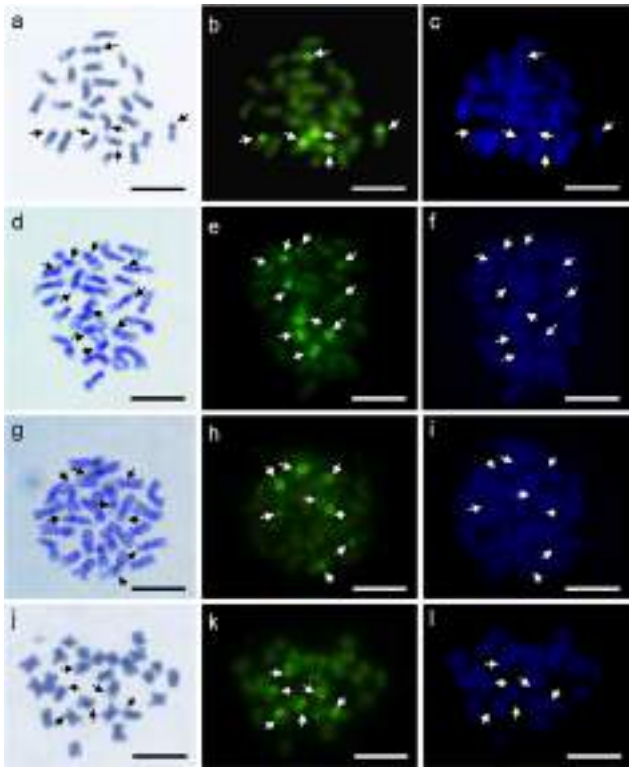

Figure 3. Somatic metaphase chromosomes (2n= 26) of four Indian landraces of *S. indicum* stained with Giemsa, CMA followed by DAPI. a- c) Black seeded til; d- f) Dark Brown seeded til; g- i) Light Brown seeded til; j- l) White seeded til. Bar= 5 µm. Arrows indicate the chromosomes showing CMA^{+ve} signals/ zones when stained with CMA fluorochrome.

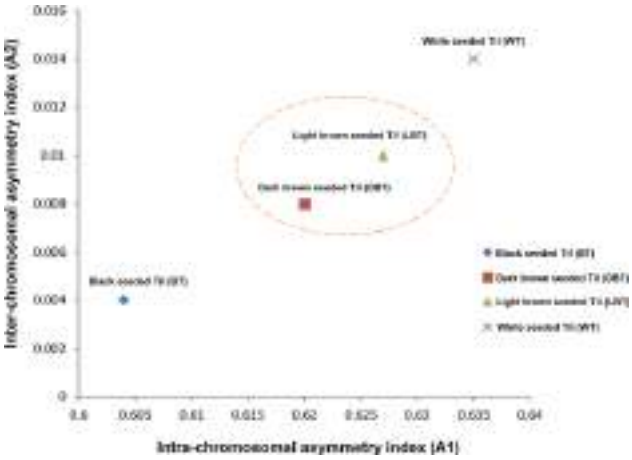

Figure 4. Scatter diagram of intra-chromosomal asymmetry index (A1) versus inter-chromosomal asymmetry index (A2) of four Indian landraces of *S. indicum*.

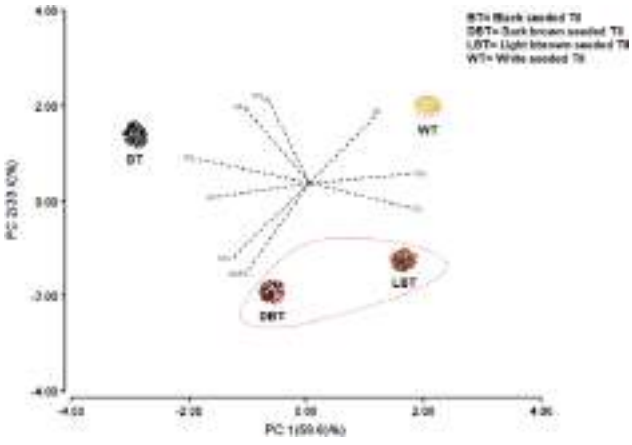

Figure 5. Principal component analysis (PCA) plot showing grouping of four Indian landraces of *S. indicum* based on nine karyomorphometric variables.

til (BT) and White seeded til (WT) were located at a significant distance from each other (Fig. 5).

DISCUSSION

The present study demonstrates a comprehensive karyo-morphometric analysis of four Indian landraces of *S. indicum* based on giemsa, CMA and DAPI banding analysis. Due to its characteristic life forms and immense nutritive value, *S. indicum* has attracted the attention of researchers and breeders to plan a successful conservation strategies and improvements in breeding programs. However, only a few reports are available on the cytogenetics of Indian varieties of *Sesamum* till date may be owing to very small size of the chromosomes (< 3 µm) and technical limitations (Raghavan and Krishnamurthy 1947; Mukherjee 1959). In the present study, we have adopted the EMA based chromosome analysis to obtain cytoplasm free well scattered metaphase chromosomes of the species. The combination of enzymatic maceration and air dying methods is a very useful technique to analyze chromosome morphology, constrictions and types of chromosomes in detail (Fukui 1996). This method was instrumental in obtaining uniformly spread chromosomes against a cytoplasm free background in several crop species with small and medium sized chromosomes (Kurata and Omura 1978; Moscone 1996; Yamamoto 2007; Jha 2014; Jha and Halder 2016; Jha *et al.* 2017; Jha and Saha 2017; Ghosh *et al.* 2018).

The results obtained from the present analysis offers several insights into the karyological characterization of different *S. indicum* landraces viz. Black seeded til (BT), Dark brown seeded til (DBT), Light brown seeded til (LBT) and White seeded til (WT). The diploid chromosome number (2n= 26) in all studied landraces

of *S. indicum* is in agreement with the earlier reports (Morinaga *et al.* 1929; Kobayashi 1949; Raghavan and Krishnamurthy 1947). Raghavan and Krishnamurthy (1947) identified 13 pairs of somatic chromosomes with terminal constrictions in this species, while Kobayashi (1991) classified five pairs of median and eight pairs of submedian chromosomes including one pair (10^{th} pair) of sat-bearing chromosomes in *S. indicum*. In the present study, a significant variation in chromosome size (ranging from 1.24 µm- 2.87 µm) has been scored among the *Sesamum* landraces. Mukherjee (1959) reported two pairs of chromosomes having secondary constrictions in *S. indicum*, while the present EMA based analysis clearly revealed the presence of nine pairs of chromosomes with nearly median primary constriction, three pairs were submedian and a single pair (5^{th} pair) of sat-bearing chromosomes in all the studied landraces and which can be considered as the modal karyotype for Indian Sesame.

In addition to the EMA based giemsa staining, the fluorochrome banding patterns are documented here for the first time in four studied Indian landraces of *Sesamum*. The application of nucleotide specific fluorochromes i.e. GC-specific CMA, AT-specific DAPI in chromosome analysis has been reported to be very expedient in proper karyological characterization of many plant species (Schweizer 1976; Moscone *et al.* 1996). In the present study, CMA banding analysis provides a comprehensive cytogenetic characterization of four Indian landraces of *S. indicum*. Based on both karyomorphology and CMA signalling patterns, distinct homologies could be established between the studied landraces. Presently, we could not locate $DAPI^{+ve}$ bands in any of the studied samples. However, the differences in distribution of CMA^{+ve} signals/ zones clearly delimit each of the studied landraces of *Sesamum*.

In the present study, all the collected landraces of *S. indicum* exhibited symmetrical karyotypes based on categories of Stebbins (1971). However, the analyses of scatter diagram of A1 versus A2 and PCA plot unambiguously delimit each of the studied landrace (Fig. 4 and 5). PCA is a true eigenvector-based multivariate analysis, which can be used to project samples onto a series of orthogonal axes and to statistically clarify the genetic relationship among the studied samples (McVean 2009). The results obtained from scatter plot of A1 vs A2 and PCA analysis provide a strong relationship with that of the fluorochrome (CMA) banding analysis. The Dark brown seeded til (DBT) and Light brown seeded til (LBT) exhibited maximum CMA^{+ve} signals/ zones and appeared close to each other, while both the Black seeded til (BT) and White seeded til (WT) characterized by minimum CMA^{+ve} signals (i.e. six chromosomes with distal CMA^{+ve} signal on short arm) positioned distantly in the scatter diagram of A1 versus A2 and PCA plot.

As a whole, the present study involving EMA based giemsa staining techniques demonstrates an explicit karyo-morphometric characterization of four Indian landraces of *S. indicum* for the first time. Distinct landrace-specific variation in the distribution of CMA^{+ve} signals/ zones in somatic chromosomes was also established in the species. The grouping of the studied landraces was also corroborated by the analysis of scatter diagram of A1 versus A2 and PCA plot which revealed a strong relationship with that of the fluorochrome banding analysis. However, further studies employing in situ hybridization techniques like FISH/ GISH and DNA barcode analysis are required for clarification of evolutionary processes within the particular species.

In conclusion, the present karyo-morphometric analysis explicitly characterizes four important Indian landraces of *S. indicum* for the first time. Application of EMA method of chromosome analysis followed by giemsa and fluorescent dye CMA which targets GC rich constitutive heterochromatin regions on chromosomes has clearly demonstrated that the method may be used as useful tool to characterize and differentiate *S. indicum* at the varietal level.

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Agapetes smithiana Sleumer var. *smithiana* -- a threatened plant on the verge of extinction

Populations of *Agapetes smithiana* Sleumer var. *smithiana* (Ericaceae: Vaccinioideae) are decreasing day by day in their natural habitats since its discovery by Gammie (1892) from Lachung Valley in Sikkim. The taxon is endemic to the eastern Himalaya of India (Sikkim & Darjeeling of West Bengal), Nepal, and Bhutan (Ghosh & Mallick 2014). Currently, the taxon is survived by a single to six individual plants in their natural habitats in Sikkim (Versay WS: Singh 2002–based on herbarium specimen by P. Singh 24981 at BSHC; Damthang: Sahu 2004 - based on herbarium specimen by A.K. Sahu 26669 at BSHC); West Bengal (Lower Tonglu in Darjeeling: Chamberlain 1975– based on herbarium specimen by D. Chamberlain 49 at DD and one reference (Panda & Reveal 2012); Nepal (an unknown locality under Eastern Nepal: H. Hara in (Hara et al. 1982) and Bhutan (Chhukha District: D.G. Long & S.J. Rae in A.J.C. Grierson & D.G. Long, 1991 and Trongsa District (Hara 1971). Based on herbarium consultations and field visits, present work provides its field description, distribution, current status and conservation aspects along with live images.

Agapetes smithiana Sleumer was first described by William Wright Smith (1911) based on specimens collected by George Alexander Gammie in 1892 as *Pentapterygium sikkimense* W.W.Sm. from Lachung Valley in the state of Sikkim. After Gammie, this species was collected by Charles Gilbert Rogers in 1899 from the lower Tonglu region of Darjeeling Himalaya in the state of West Bengal, India. Since Rogers collection (1899), no further collections were made for long time until Chamberlain (1975) who collected from Dilpa of lower Tonglu Valley. Following Chamberlain, Singh (2002: herbarium data), Sahu (2004: herbarium data), and Panda (2011: herbarium data) collected and reported this species from different localities of Sikkim and Darjeeling Himalaya, respectively. Hara (1982) reported from a locality under eastern Nepal based on his collection of two fruiting materials deposited at Tokyo University Herbarium (TI 6300562 & 6300563, fr). Hara (1971) also reported from Chendebi-Tashiling area in Trongsa district of Bhutan based on his collection in 1967 at an altitude of about 2300m. D.G. Long & S.J. Rae (1991) in A.J.C. Grierson & D.G. Long reported from Chukka District (north of Jumudag) of Bhutan based on their collections.

Agapetes smithiana Sleumer var. ***smithiana*** in *Bot. Jahrb. Syst*. 70: 106. 1939; Panda & Reveal, *Phytoneuron* 2012–8: 2. 2012. *Pentapterygium sikkimense* W.W.Sm. in *Rec. Bot. Surv. India* 4: 268. 1911. Type:

Image 1. *Agapetes smithiana* Sleumer var. *smithiana*: a–d—Chitrey-Manebhanjang-lower Tonglu population, Darjeeling | a—epiphytic Habit on *Quercus* tree trunk | b—twigs and branches | c—close-up of twigs showing flowers and fruits | d—putting awareness board in front of habit | e—distribution map.

India, Sikkim Himalaya, Lachung Valley, 7500 ft, 14.09.1892, *G.A. Gammie 1216* (lectotype: K!barcode no. K000729429).

Usually epiphytic dwarf shrub on tree trunks, 0.1–1m long. Stems rigid, terete, lenticellate, sparsely strigose-hispid; branches. Leaves compactly 2–3-stichous, 2–10 mm apart, coriaceous, subsessile; petioles 1–3 mm long, puberulous. Inflorescence cauline, 1–4-fascicled in a corymb. Flowers 12–16 mm long including pedicels with bract and bracteoles; pedicels greenish-pink, sparsely hirtellous, 4–5 mm long. Calyx campanulate, winged, light green with pinkish wings, persistent in fruits. Corolla greenish-yellow, tubular, 10–13 × 4 mm, 3.5–4.5 mm diam., glabrous. Stamens 10, encircling the pistil, distinct, 8–8.5 mm long; filaments slightly adnate to ovary disc. Pistil ca. 12 mm long. Fruit a berry, ovoid, 12–16 × 10–12 mm, light green (immature) to white (mature), glabrous, with an accrescent, winged calyx.

Distribution*:* Endemic to eastern Himalaya of India (Sikkim and Darjeeling), eastern Nepal, and Bhutan.

Flowering: April–early September; December.

Fruiting: July–August; December–January.

Habit: Epiphytic on tree trunks or rarely in rock crevices.

Habitat: Subtropical-temperate forests at altitudes ranging from 2,300–2,650 m.

Specimens examined: 24981 (BSHC), 18.v.2002, India, Sikkim Himalaya, Chitrey to Uttarey, coll. P. Singh; Damthang, 2,133–2,438m, ii.2004, coll. A.K. Sahu; 26669 (BSHC: fl.). West Bengal, Darjeeling, 3km

NW of Chitrey, along Sandakphu Trek route, 2,650m, 27.135 N & 88.167 E, 11.xii.2011, S. Panda 81 (CAL!); below Tonglu at Dilpa, 2,530m, 02.iv.1975, D. Chamberlain 49 (DD).
Local name: *Chara-ko-khorsanejato-pahelo* (Nepalese of Manebhanjang, Chitrey & Lamedura).
Notes: Author assumes Lower Tonglu population near Dilpa collected by Chamberlain (1975) may be similar to Chitrey population collected by Panda (2011) as both possessing nearly same altitude and 'Dilpa' basti is located about 1 km down of Chitrey toward Nepal side (Dilpa is located under Elam district in Nepal).

Conservation status: As a result of detailed herbarium consultations in different Indian herbaria as well as extensive field visits in Darjeeling (2011–2018) and Sikkim (2000–2004; 2007) Himalaya, currently four smaller populations were traced in Indian eastern Himalaya, viz., Versey (Chitrey-Uttarey route) in West Sikkim, Damthang in South Sikkim, Dilpa-Lower Tonglu and Chitrey in Darjeeling. Unfortunately, no further collections were made from the Lachung valley (type locality) in Sikkim after Gammie (1892). Populations of Chhukha and Trongsa districts in Bhutan showed a few individual plants epiphytic on tree trunks (D.G. Long & S.J. Rae 1991). Nepal populations collected by Hara (deposited in TI-Tokyo University Herbarium) also showed the smaller populations survived by only two individual plants epiphytic on tree trunks. The author put up a board in front of Chitrey population of Darjeeling in 2014 and 2019 to create awareness among the local Nepalese for conservation. The taxon is not assessed yet as per the IUCN Red List of Threatened Species (2019), but the taxon will qualify as Critically Endangered based on Criteria A [A4c], B [B2,b,c] and D [<50 in each population based on field visit and herbarium consultation].

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GAULTHERIA AKAENSIS PANDA & SANJAPPA (ERICACEAE: VACCINIOIDEAE): A CRITICALLY ENDANGERED, ENDEMIC ETHNOMEDICINAL PLANT FROM INDIAN EASTERN HIMALAYA ON THE VERGE OF EXTINCTION[1]

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Introduction

Gaultheria akaensis Panda & Sanjappa was first discovered in December, 2002 from Aka Hill in Arunachal Pradesh by S. Panda (Coll. no. 30824, CAL) and described by Panda and Sanjappa (2006). Since its discovery in 2002, till the present author's last field visit in November 2017, the Aka Hill population survives as a few plants with scanty flowering and irregular fruit formation without viable seeds (as observed during field visits in 2004, 2009, 2011, 2014, 2017). There is no trace of any other known population in Arunachal Pradesh since its discovery. Fortunately, four smaller discrete populations of *G. akaensis* were observed in Darjeeling Himalaya across extensive field visits (December 2011, June 2012, June 2014, October 2016, May 2017, and November 2018). The Darjeeling populations are comparatively better than the Aka Hill population with respect to the number of individuals, but less flower and fruit formation followed by less viable seeds was noticed in them. Very few individual plants were seen with flowers, and even flowering occurs irregularly.

Unfortunately, there is no increase in the number of individuals of *Gaultheria akaensis* observed since its discovery in 2011. Therefore, there is an urgent need to conserve this species in its natural habitats by increasing their population and protecting the natural habitats which are vulnerable, as they comprise rocky roadside slopes. The present work monitors these natural habitats regularly, putting up signboards to create awareness among the local people. The study attempts to observe the species' natural biology and reproduction. The present work also provides taxonomic details, field description, phenological observations, live images, distribution, uses and conservation aspects to enable field identification and other natural habitats if found, as well as to conserve it before its extinction in the wild.

Taxonomy

Gaultheria akaensis Panda & Sanjappa, *Edinburgh J. Bot*. 63(1): 15. 2006; Panda *et al.*, *Phytoneuron* 2012–35: 1. 2012; Panda, *J. Threatened Taxa* 5(7): 4118–4121. 2013; Panda & Sanjappa in Sanjappa & Sastry, Fasc. Fl. India Fasc. 25 Ericaceae: 205. 2014.

Type: India: Arunachal Pradesh, West Kameng district, Aka Hill, 3 km from Nechephu, 27 km toward Tenga, left bank of the bridge, 1,800 m, 25.xii.2002, *S. Panda* 30824 (holo. & iso. CAL). *Diplycosia indica* M.R. Debta & H.J. Chowdhery, *J. Bot. Res. Inst. Texas* 3: 147. 2009.

Type: India: West Bengal, Darjeeling, Singalila National Park, Kaniyakata to Kalapokhri, 2,950 m, 02.vi.2006, *M.R. Debta* 40813 (holo. CAL; iso. BSD). Fig. 1.

Decumbent, bushy, dwarf, stout shrub, 0.2–1.0 m high. Stem terete, profusely branched, rust red hirsute; branches terete, densely rust red hirsute. Leaves papery to subcoriaceous, lamina narrowly ovate, ovate-elliptic to elliptic, 2.5–4.2 × 1.6–2.8 cm, serrate at margin with long setose cilia, cilia *c*. 5 mm long, rounded to broadly cuneate at base, mucronate at apex, glabrous, dark green above, light green and setulose beneath; venation conspicuous brochidodromous with 3−4 pairs lateral veins; petioles stout, 2–3 mm long, setulose. Racemes 6–13 mm long, white puberulous, 3–5-flowered. Flowers *c*. 8 mm long; pedicels

Fig. 1: *Gaultheria akaensis* Panda & Sanjappa population at Chitrey-Lamedhura, Darjeeling

greyish-white to light green, *c*. 3 mm long, glabrous; bract 1, basal, pinkish, ovate, *c*. 2 × 1.5 mm, ciliate at margin, acute at apex, glabrous; bracteoles 2, opposite, median on pedicel. Calyx campanulate, persistent in fruits, light green, *c*. 3 mm long; lobes light green to greyish-white, ovate-triangular, *c*. 2 × 1 mm, ciliate at margin, acuminate at apex, glabrous. Corolla deciduous, urceolate, light pink to white, *c*. 5 mm long, glabrous, lobes ovate, *c*. 1 × 1 mm, entire at margin, rounded at apex. Stamens 5 (–7), *c*. 2 mm long, loosely epipetalous; filaments greyish-white, *c*. 1 mm long, slender, papillose, glabrous, dilated at middle; anthers dark brown, oblong, *c*. 1 mm long, each lobe with 2 minute apical awns. Pistil *c*. 3.5 mm long; ovary globose, light green, *c*. 1 × 1 mm, puberulous; ovules numerous on axile placenta in each locule; disc minutely 10-dentate; style impressed, light green, *c*. 2.5 mm long, slender, glabrous. Capsule globose, dark pink, *c*. 3 × 3 mm, enclosed in a dry accrescent calyx with *c*. 2 mm long pedicel, puberulous. Seeds numerous, minute, obconical, scariose.

Distribution: Endemic to Indian Eastern Himalaya (**Arunachal Pradesh**: West Kameng district, Aka Hill, 3 m from Nechephu (27° 16.434′ N & 92° 57.439′ E at 1,800 m); **West Bengal**: Darjeeling district, Chitrey-Lamedhura (26° 59.488′ N & 88° 06.690′ E at 2,377 m), Lamedhura (27° 00.731′ N & 88° 05.302′ E at 2,744 m), Gairibas-Kaiankata (27° 03.377′ N & 88° 01.844′ E at 2,872 m), Kaiankata-Kalipokhri (27° 04.327′ N & 88° 00.277′ E at 2,902 m).

Habitat: Grows in moist and humus-covered loose rocky soil, rarely in landslide areas, in association with *Gaultheria fragrantissima* Wall., *G. stapfiana* Airy Shaw, *G. nummularioides* D. Don, *Lyonia ovalifolia* (Wall.) Drude, *Rhododendron arboreum* Sm., *Polygonum barbatum* L., *Impatiens discolor* DC., and *Lycopodium clavatum* L. at an altitude of *c*. 1800 m.

Flowering: Usually during late May–June and December–January (both in Darjeeling and Aka Hill populations); uncommonly noticed in Darjeeling populations during April, September, and November. **Fruiting**: July–August; December–January.

Specimens examined:

Arunachal Pradesh: West Kameng district, Aka Hill, 3 km from Nechephu, 1,800 m, 25/12/2002, *S. Panda* 30824 (CAL); 15.v.2010, *S. Panda* 274 (Barasat Govt College Herb.); 21.xi.2014, *S. Panda* 334 (Darjeeling Govt College Herb.).

West Bengal: Darjeeling district, 3 km from Chitrey toward Lamedhura, 11.xii.2011, 2,300 m, *S. Panda* 78 (Darjeeling Govt College Herb.-DGC); near Lamedhura, 2,400 m, 11.xii.2011, *S. Panda 79* (DGC); Singalila National Park, Kaiankata-Kalapokhri road, near Kaiankata, 2,900 m, 12.xi.2011, *S. Panda 80* (DGC); Kaiankata to Kalapokhri, 2,950 m, 2.vi.2006, *M.R. Debta 40813* (BSD, CAL; type material of *Diplycosia indica*).

Ethnomedicinal uses: Tender leaf extract mixed with *Gaultheria fragrantissima* leaves (1:1) applied to cure acute rheumatic and sciatic pain by the Akas. Nepalese of Manebhanjang, Chitrey and Lamedhura use its tender leaves in a paste to relieve rheumatic pain.

Conservation Status: As a result of detailed herbarium consultations in different Indian herbaria as well as extensive field visits in different localities of Arunachal Pradesh (2002–2004, 2009–2011, 2014, 2017), Sikkim (2002–2004, 2007, 2009, 2013), Darjeeling Himalaya (2011–2018), and Northeastern states (2002, 2003, 2007), two different populations of *G. akaensis* were discovered and documented. (1) Aka Hill population in West Kameng district of Arunachal Pradesh (survived by a few individual plants – 0.5 sq. m area), and (2) four discrete populations in the Darjeeling Himalaya in West Bengal (i. Chitrey-Lamedhura – 2 sq. m area (Fig. 1), ii. Lamedhura – 5 sq. m area, iii. Gairibas-Kaiankata – 3 sq. m area, and iv. Kaiankata-Kalipokhri – 1 sq. m area). Unfortunately, both Arunachal and Darjeeling Himalayan populations do not produce viable seeds due to irregular fruit formation. Formation of flowers is also low, and flowering

Fig. 2: Informative signboard posted at the site of the Chitrey-Lamedhura population, Darjeeling

does not occur in all the plants. The Aka Hill population did not flower in 2004, 2007, 2011, 2014, and 2017.

Fortunately, all four discrete populations of Darjeeling produce flowers, but only in approximately 30% individuals. The author posted signboards in front of the Aka Hill and Darjeeling populations (Fig. 2) in 2011 and 2014, to create conservation awareness among the local people. *Gaultheria akaensis* is not assessed yet under IUCN (2017) Red List of Threatened Species (version 13, 2017), but would qualify as Critically Endangered based on Criteria A[A3c+A4c], B[B1ab(i+ii+iii) + B2ab(i+ii+iii)], C[C1a+C2a] and D.

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 Cytologia **84**(4): 313–317

Karyotype Analysis from Aerial Roots of *Piper nigrum* Based on Giemsa and Fluorochrome Banding

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Summary *Piper nigrum* is an important age-old herbal plant of the family Piperaceae. Chromosomal analysis a part of genomic research to conserve plant genetic resources has confronted difficulties in this particular species with conventional methods primarily due to its very small chromosome size and rich cytoplasmic contents. Different chromosome number from $2n=52$ to 128 have been reported in this species. A reassessment of chromosome analysis not attempted earlier with aerial roots and enzymatic maceration and air drying (EMA) method has been standardized in this species. Well scattered cytoplasm free metaphase plates stained with Giemsa revealed $2n=52$ very small chromosomes. Total chromosome length attains to $75.36\pm011\,\mu$m. Most chromosome pairs except one ranged from 0.99 to $1.81\,\mu$m. Karyotype formula is determined as 6M+26m+20sm and accordingly, idiogram is constructed. Fluorochrome banding with two contrasting nucleic acid dye DAPI and CMA provided positive signals for both of the stains on the very small chromosomes. The standardized EMA method and fluorochrome banding is repeatable and will help to evaluate other important species and genus within this family for the conservation of plant genetic resources.

Key words Aerial root, EMA method, Fluorochrome banding, *Piper nigrum*, Small chromosome, Karyotype.

Black pepper or *P. nigrum L.*, one of the three most important cultivated pipers, belongs to the family Piperaceae. The species ranked first as a spice in cookery for its flavor and aroma and is crowned as "Black Gold" and "King of Spices" (Gorgani *et al.* 2017). In traditional medicine 'Piper is an age-old herbal plant of Ayurveda' (Ahmad *et al.* 2012). Many reports on morphological, biochemical, molecular and biotechnological aspects of *P. nigram* are available (Narayanan 2000, Ravindran 2000, Nair and Gupta 2003, Nazeem *et al.* 2005, Parthasarathy *et al.* 2007, Shivashankar 2014) but the species has confronted extreme difficulties in the field of chromosomal studies. Chromosomal analysis in economically important crops has benefited the conservation of plant genetic resources for any crop improvement programs. Two basic chromosome numbers $x=12$ and 13 have been reported for the genus *Piper*. Since Johansen (1931) initiated chromosome analysis in the genus, a number of workers have attempted chromosome analysis in *P. nigrum* and reported different chromosome numbers. Janaki Ammal (1955) reported $2n=128$ chromosomes. Jose and Sharma (1984) reported $2n=104$, chromosomes. Sharma and Bhattacharyya (1959) reported $2n=48$, Dasgupta and Datta (1976) reported $2n=36$ and 60 chromosomes. Whereas $2n=52$ chromosomes were reported by Mathew (1958), Martin and Gregory (1962), Rahiman and Nair (1986). Even Nair *et al.* (1993) reported $2n=78$ chromosome. All authors have used conventional orcein staining for determination of chromosome number and reported that chromosomes of *P. nigrum* are very small in size and cells contain dense cytoplasm. No chromosome morphology was described in this species. However, it is agreed that chromosome analysis still provides foundational genomic information in a very cost-effective manner and "comparison of the molecular, genetic and cytological classification of chromosomes remains a highly relevant task" (Muravenko *et al.* 2009). It appears that the species did not receive adequate attention it deserves. A reassessment of chromosome analysis in this particular species with the application of the EMA method not attempted earlier was felt. EMA method was introduced for rice chromosomes by Kurata and Omura (1978) and has been applied in many plant species. The method is the basics of molecular cytogenetics and suitable for all types of plant chromosomes especially for cells having rich cellular contents and small chromosomes (Schweizer 1976, Kondo and Hizume 1982, Hizume 1991, Fukui 1996, Yamamoto 2012, Jha *et al.* 2015). EMA method followed by Giemsa staining not only helps in counting the actual chromosome number but also clears chromosome morphology through the removal of cell wall and cytoplasmic derbies. It was further revealed that earlier authors have used roots induced from vegetative cuttings due to problems of seed germination. Therefore, the present communication details the standardization of EMA based chromosome preparation method from aerial roots in *P. nigrum* along with the

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karyotype analysis through Giemsa and fluorochrome banding for the first time.

Materials and methods

P. nigrum aerial roots induced normally in nodal zones of a potted plant under moist and humid conditions were used for chromosome analysis (Fig. 1). A minimum of 20 healthy roots was collected in between 11.30 to 12 am and pre-treated separately with saturated paradichlorobenzene (PDB) and 0.5% colchicine solution for 4–5 h at 14–16°C. Acetic methanol (1 : 3) was used as a fixative and kept the roots in this solution for overnight and preserved in –20°C. Our earlier protocol (Jha *et al.* 2015) for EMA method with required standardization of enzyme maceration time from 55–100 min at 37°C was followed chromosome staining time with Giemsa, 4′-6-diamidino-2-phenylindole (DAPI) and chromomycin A_3 (CMA) was also optimized. Giemsa stained slides were de-stained in 70% methanol and air-dried prior to fluorescent staining. Basic steps of Kondo and Hizume (1982) were followed for fluorescent staining. However, 0.1 μg mL^{-1} and 0.2 μg mL^{-1} of DAPI solution for 15–30 min and 0.1 mg mL^{-1} of CMA solu-

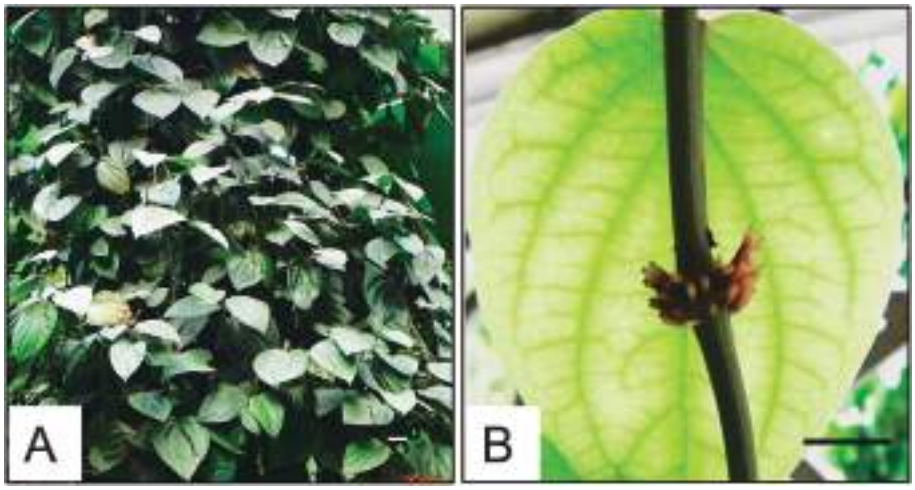

Fig. 1. *Piper nigrum* mother plant (A) and healthy aerial roots (B). Scale bar=1 cm.

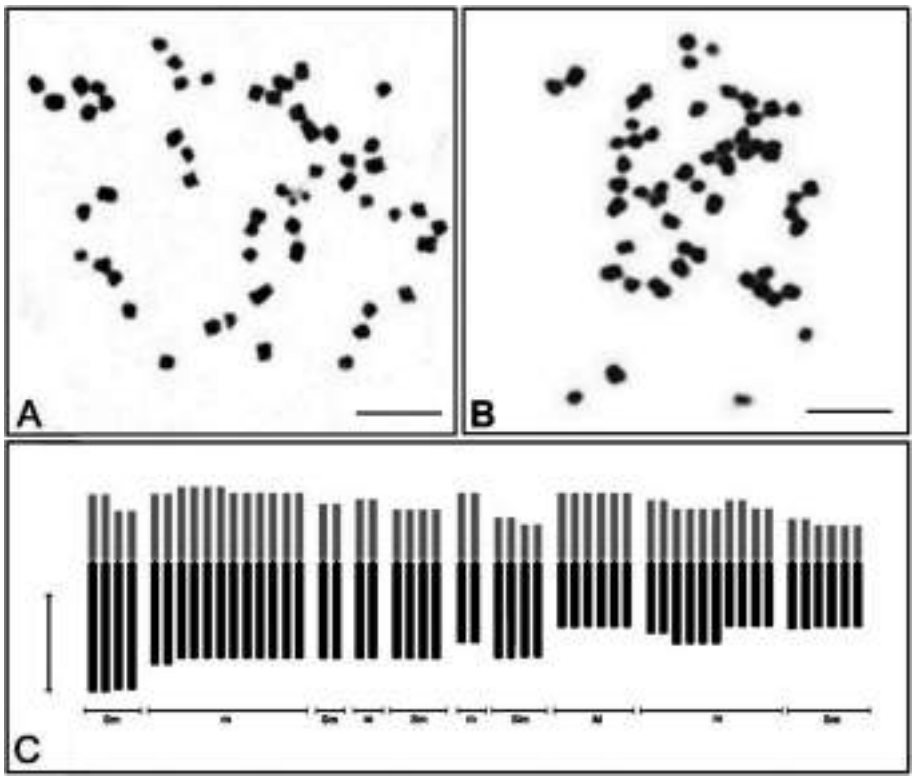

Fig. 2. EMA based chromosome preparation in *P. nigrum*. PDB treated Giemsa stained metaphase plate (A) and colchicine treated Giemsa stained metaphase plate (B) showing $2n=52$ chromosomes and ideogram (C). Scale bar in A and B=5 μm, Scale bar in C=1 μm.

tion for 60–100 min were tried for fluorescent staining. Slides were mounted in non-fluorescent glycerol and CMA slides were kept for maturation at 4°C for more than 48 h.

For counting of chromosome number and karyotype analysis a minimum of 50 well scattered Giemsa stained metaphase plates were examined. Similarly, 20–25 DAPI and CMA stained plates were also studied for verification of chromosome number and fluorescence response. A Carl Zeiss Axio Lab A1 microscope having a bright field, filter cassettes of DAPI and CMA, CCD camera fitted with a computer was used to capture all the photographs in the present studies. An Axiovision L. E4 software was used for the analytical part. For karyometric analysis standard karyological parameters were used (Jha *et al*. 2017).

Means and standard deviations were analyzed for all measured parameters. One-way analysis of variance (ANOVA) was performed to detect significant differences ($p \leq 0.05$) in the mean (Sokal and Rohlf 1995). Duncan's multiple range test (DMRT) was used for *post hoc* analyses using SPSS v 16.0 statistical package (SPSS Inc. IBM, Chicago).

Results and discussion

Processing of aerial roots (Fig. 1B) with PDB and colchicine yielded many metaphase (Fig. 2) and prometaphase plates. It revealed that both pretreating chemicals can be used for chromosome preparation. An important observation is that the aerial roots can be regularly used as an alternative tissue for chromosome preparation along with the induced roots from vegetative cuttings in *P. nigrum*. Enzymatic digestion time in EMA method and Giemsa staining time are two critical steps for the removal of the cell wall and rich cytoplasmic

Table 1. Chromosome morphometric data of *Piper nigrum*.

Chromosome number (2*n*)	Length of longest chromosome (μm)		Length of shortest chromosome (μm)		Total chromosome length (μm) (Mean±S.D.)	Total form percent (TF %) (Mean±S.D.)	Karyotype formulae (2*n*)
	Absolute (Mean±S.D.)	Relative (Mean±S.D.)	Absolute (Mean±S.D.)	Relative (Mean±S.D.)			
52	2.00±0.02	2.65±0.03	0.99±0.02	1.31±0.03	75.36±0.11	38.73±0.11	6M+26m+20Sm

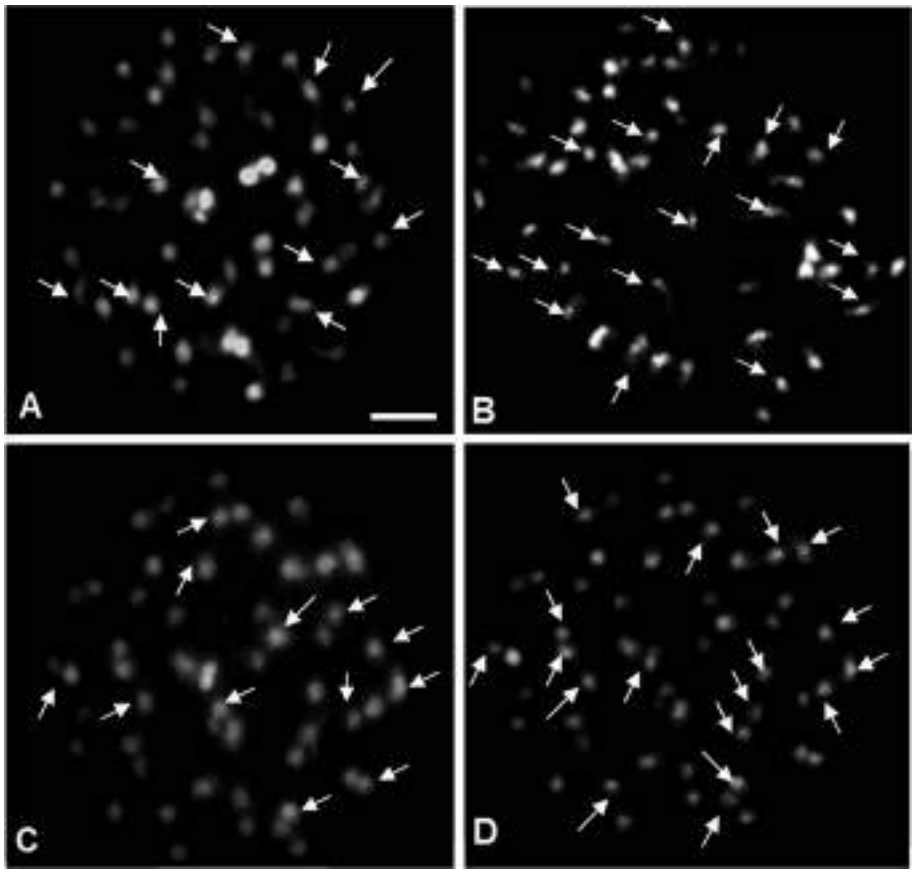

Fig. 3. A) Prometaphase (A) and metaphase plates (C) stained with CMA showing at least 12 positive signals (marked with an arrow). Prometaphase (B) and metaphase plates (D) stained with DAPI showing at least 18 positive signals (marked with arrow). Scale bar=5 μm.

contents and staining of such small chromosomes which is required for the correct determination of chromosome number and analysis of chromosome morphology. Both the steps were optimized and large number of cytoplasm free metaphase and pro-metaphase plates from aerial roots of *P. nigrum* were obtained when root tips were treated in enzyme solution for 95–100 min at 37°C and stained with 1.5% Giemsa solution in phosphate buffer for 30 min. Chromosomes of *P. nigrum* are very small in size and for confirming the diploid number more than 50 well scattered metaphase plates were studied. More than 95% cell counts revealed $2n=52$ chromosomes (Fig. 2A, B). For karyometric analysis five well-scattered metaphase plates with distinct chromosome morphology were used. Out of 26 pairs of small chromosomes, length of 25 pairs ranged from 0.99 to 1.81 μm and only one pair was 2 μm long. Detail chromosome morphometric data is presented in Table 1. Total chromosome length of 52 chromosomes appears from the table is 75.36 ±0.11 μm and karyotype formulae is 6M+26m+20sm. Idiogram of the species is presented in Fig. 2C. The present chromosome count $2n=52$ confirms the reports of Mathew (1958), Martin and Gregory (1962) and Rahiman and Nair (1986).

Fluorescent banding with CMA and DAPI which generally targets GC- and AT-rich constitutive heterochromatin regions on chromosomes and generate positive and negative signals have advanced cytogenetic research for many plant species (Schweizer 1976, Kondo and Hizume 1982, Hizume 1991, Moscone *et al.* 1996, Yamamoto 2012, Schwarzacher 2016). Not only they offer additional help in counting the chromosome number but also mark the chromosomes throughout the karyotype (Kondo and Hizume 1982, Hizume 1991, Guerra 2008). The concentration of fluorochrome dyes and staining time has been standardized in *P. nigrum*. DAPI staining was better with 0.2 μg mL^{-1} for 30 min while 0.1 mg mL^{-1} CMA took nearly 100 min time for differential staining in the same metaphase plates. It is reported that plants having small chromosomes face difficulties with fluorescent banding due to insufficient resolving power of light microscope and relatively low copy of repetitive sequences. However, for the first time *P. nigrum* revealed positive signals for two contrasting CMA and DAPI stains. The present chromosomal analysis was carried out on prometaphase and metaphase chromosomes of *P. nigrum* and has obtained at least 12 and 18 positive CMA and DAPI signals (Fig. 3). The standardized EMA method, Giemsa staining and fluorochrome banding are repeatable and well documented. The present observations not only conserve the genetic features of *P. nigrum* but also are useful in characterisation and conservation of intra and inter genetic diversity within the economically important genus *Piper*.

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Technique for the retention of leaf senescence of six ericoid wild plant taxa using ascorbic acid

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ABSTRACT

Efficacy of a growth promoter ascorbic acid (Vitamin C) on senescence deferral action was analysed using leaf discs of six wild ericoid plant taxa. Changes of some biochemical parameters like chlorophyll, protein, soluble and insoluble carbohydrates, RNA and DNA as well as activity of catalase enzyme were analysed as reliable senescence indices during detached leaf senescence of these six species under dark condition. With the progress of ageing duration from zero to 144 hours the levels of chlorophyll and proteins in leaf discs gradually declined in both control and ascorbic acid treated samples. However, in the chemical / Vitamin-C treated samples the rate of decline was found to be much slower. Concomitantly the levels of insoluble carbohydrate, RNA and DNA started declining right from 48, 96 and 144 hours of observation period both in treated and untreated samples. And here also ascorbic acid arrested the rapid rate of reduction of the levels of the biochemical parameters. On the other hand, soluble carbohydrate level started increasing irrespective of the treatments during the whole observation periods. However, the magnitude of increase was found to be low in case of the leaf samples which experienced treatment with ascorbic acid. The activity of the enzyme catalase was found to decrease progressively during the four observation periods (0, 48, 96 and 144 hours) regardless of the

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treatments. Ascorbic acid partially averted the rapid fall of the enzyme activity during the ageing periods. Ascorbic acid, a nonconventional senescence deferred thus seems to be a potent senescence deferral phytohormone /chemical / vitamin at least in case of six ericoid wild plant taxa.

***Keywords*:** Leaf senescence, Ericaceae, Ascorbic acid, India

1. INTRODUCTION

Senescence is a programmed deteriorative phenomenon occurring within cells, tissues, organs and organisms, which is culminated in the death of the concerned plant part or the organisms as a whole (Leopold and Kriedemann, 1975; Pati, 2007). As the process of senescence takes place at an exceedingly faster rate under detached condition of plant parts, the effect of any chemical having influence on the regulation of senescence can be quickly determined (Sabater, 1984).

Deferral of senescence by plant hormones like cytokinins is well established (Richmond and Lang, 1957; Vanstaden *et al*., 1988; Biswas and Ghosh, 1999). However, some gibberellins and auxins are reported to defer senescence in a number of plant species, but their efficiency is mostly not at par with cytokinins (Biswas and Ghosh, 1999). Again, many plant species do not respond positively for deferment of senescence in presence of auxin types.

In the present experiment, an attempt was made to ascertain whether ascorbic acid, a member of vitamin class (Vit. C), can regulate senescence of the experimental Ericoid plant species namely *Enkianthus deflexus* (Griff.) C. K. Schneid. var. *acuminatus* Panda & Sanjappa, *Gaultheria stapfiana* Airy Shaw, *G. hookeri* C. B. Clarke, *G. trichophylla* Royle var. *ovata* Panda & Sanjappa, *Vaccinium glauco-album* C. B. Clarke and *V. nummularia* C. B. Clarke under detached leaf condition.

Regulation of plant senescence by any chemical agent can be expeditiously and almost accurately determined under detached condition of plant parts. In fact, deteriorative processes during senescence of detached leaves simulate grossly with that of attached leaves under natural condition, the main difference being the speed at which the processes run. Thus, the principal aim of this work was to evaluate the efficiency of ascorbic acid on senescence retardation of six wild plant taxa, which are reported to be very sensitive towards any chemicals with regard to exhibiting senescence regulatory action (Biswas and Ghosh, 1999).

2. MATERIALS AND METHODS

In this investigation the experimental plants used were six highly elevated wild angiospermic plants of the family Ericaceae such as *Enkianthus deflexus* (Griff.) C. K. Schneid. var. *acuminatus* Panda & Sanjappa, *Gaultheria stapfiana* Airy Shaw, *G. hookeri* C. B. Clarke, *G. trichophylla* Royle var. *ovata* Panda & Sanjappa, *Vaccinium glauco-album* C. B. Clarke and *V. nummularia* C. B. Clarke.

The plant taxa were first carefully surface blotted using blotting paper. Uniformly sliced leaf discs, taken from mature leaves of the plants were treated with aqueous solution of ascorbic acid (100 μg/ml) or distilled water (control) in Petri dishes containing filter paper.

The experimental set-up was kept in dark condition and thus allowed the leaf discs to experimental treatment with ascorbic acid for 144 hours. At an interval of 48 hours the filter papers were remoistened with the test chemical or distilled water. At 48 hours intervals the biochemical data recorded include: chlorophyll, protein, soluble and insoluble carbohydrates, RNA and DNA contents as well as activity of the enzyme catalase.

Chlorophyll: To determine chlorophyll level, leaf tissues (100 mg) of each treatment were taken in 5 ml methanol in test tubes and kept in dark under laboratory conditions for 48 hours. The supernatant was decanted off and leaf samples were rinsed repeatedly with a little amount of methanol. The supernatant and washing materials were pooled together to make the final volume 10 ml. Subsequently, the absorbance of the chlorophyll extracts was measured at 650 nm and total chlorophyll content was estimated following Arnon's principle (1949).

Protein: The chlorophyll free leaf samples (100 mg) were solubilized by treating the leaf tissues with 0.5M NaOH at 80 ºC for one hour after making the samples free from phenol as per the method of Kar and Mishra (1976). A definite volume was made with the extraction medium. It was then estimated by allowing the protein solution to react with Folin phenol reagent and subsequent measuring of the OD values at 650 nm as per the method of Lowry *et al*. (1951).

Soluble carbohydrate: This was analysed from 100 mg leaf samples homogenised thoroughly using 80% boiling ethanol (5 ml). The homogenates in watch glasses were then evaporated to dryness and this was followed by removal of chlorophylls using solvent ether. The sugar was then pooled in test tubes by washing the watch glass using 80% ethanol. A definite volume was made with 80% ethanol and this was the source for soluble carbohydrate.
After necessary dilution, 1 ml sample was taken in test tube and 3 ml freshly prepared, precooled anthrone reagent (0.2% in conc. H_2SO_4) was added. The intensity of green colour was measured at 610 nm. This method was followed essentially as per Mc Cready *et al.* (1950).

Insoluble carbohydrate: Insoluble carbohydrate level was estimated from the residual material after extraction of leaf tissues (100 mg) with 80% boiling ethanol. The residue was dissolved in 25% H_2SO_4 and extraction was done at 80 ºC for 30 minutes. After necessary dilution, 1 ml of the acid extracted sample was taken in test tube and 3 ml freshly prepared, precooled anthrone reagant was added and the intensity of the green colour was measured in the same way as done in case of soluble carbohydrate.

Nucleic acids: Extraction of nucleic acids (RNA and DNA) was made from 100 mg fresh leaves following the method described by Cherry (1962). The levels of RNA and DNA were estimated from a common stock employing the method of Markham (1955) modified by Choudhuri and Chatterjee (1970).

RNA: For the estimation of RNA, 3 ml of diluted nucleic acid extract in a test tube was treated with an equal volume of freshy prepared orcinol reagent (1g orcinol powder in 100 ml of conc. HC1 containing 100 mg 0.1% $FeC1_3$, $6H_2O$) and boiled in a water bath for 20 min with glass marbles at the test tube tops. The mixture was then cooled, necessarily diluted and the intensity of green colour was measured at 700 nm.

DNA: 1 ml of the nucleic acid extract in a test tube was mixed with 5 ml freshly prepared diphenyl amine reagent (100 ml glacial acetic acid, BDH, AR + 2.7 ml H_2SO_4 + 1 g AR grade diphenyl amine). The mixture was boiled in a water bath for 30 min with glass marble at the top of the test tubes. After cooling, the intensity of the colour was measured at 610 nm.

Catalase: Leaf tissues (500 mg) of each treatment were homogenised with 8 ml of chilled 0.1M phosphate (Na_2HPO_4/NaH_2PO_4) buffer (pH 6.8). The homogenate was centrifuged at 3000 g for 15 min followed by 10,000 g for 20 min. The volume of the supernatant was made up to 10 ml with the same buffer, and this was assayed following the method of Snell and Snell (1971) modified by Biswas and Choudhuri (1978). The reaction mixture for catalase consisted of 1 ml of the above extract and 2 ml of H_2O_2 (0.05M), incubated together at 37 ºC for 30 min. The reaction was stopped by adding 1 ml 0.1% titanyl sulphate ($TiSO_4$) in 25% H_2SO_4 (v/v). After centrifugation for 6000 g for 10 min the intensity of yellow colour was measured at 420 nm.

3. RESULTS AND DISCUSSIONS

Chlorophyll and protein contents started declining rapidly with the advancement of leaf ageing irrespective of the treated and control samples. However, ascorbic acid arrested the rapid loss of both chlorophyll and protein levels (Table 1).

Table 1. Effect of ascorbic acid (A.A; 100 µg/ml) on the changes in chlorophyll (Chl, mg/g fresh weight) and protein (Pr, mg/g fresh weight) contents in leaf discs of six wild plant taxa during dark-induced ageing.
Leaf discs were treated with IAA or distilled water for 144 hours and the data were recorded at 48 hour intervals.

Plant taxa	Treatments (µg/ml)	Hours after leaf ageing							
		0		48		96		144	
		Chl.	Pr.	Chl.	Pr.	Chl.	Pr.	Chl.	Pr.
1. *Enkianthus Deflexus* var. *acuminatus*	Control	1.24	2.21	1.00	1.12	0.87	1.00	0.67	0.75
	A.A.	1.24	2.21	1.12	1.72	1.01	1.24	0.93	1.04
	LSD(P=0.05)	NC	NC	0.11	0.13	0.12	0.14	0.11	0.09
2. *Gaultheria hookeri*	Control	1.32	2.37	1.00	1.32	0.75	1.12	0.62	0.62
	A.A.	1.32	2.37	1.12	1.72	1.00	1.48	0.96	0.87
	LSD(P=0.05)	NC	NC	0.11	0.21	0.57	0.28	0.14	0.18
3. *G. stapfiana*	Control	1.18	2.15	0.82	1.70	0.58	1.02	0.35	0.56
	A.A.	1.18	2.15	1.05	1.97	0.85	1.54	0.72	0.98
	LSD(P=0.05)	NC	NC	0.07	0.16	0.06	0.10	0.05	0.07

4. *G. trichophylla* var. *ovata*	Control A.A. LSD(P=0.05)	1.12 1.12 NC	2.12 2.12 NC	0.92 1.12 0.11	1.02 1.11 0.08	0.68 1.00 0.07	0.82 0.92 0.16	0.48 1.12 0.17	0.62 0.78 0.11
5.*Vaccinium nummularia*	Control A.A. LSD(P=0.05)	1.45 1.45 NC	2.65 2.65 NC	1.16 1.32 0.10	1.85 2.32 0.15	0.80 1.05 0.07	1.32 1.85 0.10	0.66 0.80 0.05	0.78 1.60 0.07
6. *V. glauco-album*	Control A.A. LSD(P=0.05)	1.85 1.85 NC	2.54 2.54 NC	1.52 1.70 0.12	1.70 2.25 0.18	1.30 1.61 0.10	1.20 1.60 0.11	0.75 1.01 0.08	0.64 1.05 0.07

NC: Not calculated.

A differential result was observed when the changes of these two variables were recorded with stress-induced ageing duration. Soluble carbohydrate was found to increase while insoluble carbohydrate steadily declined with the progress of ageing duration (Table 2). Ascorbic acid ameliorated the ageing-induced rapid rise of the sugar levels as well as the progressive loss of the insoluble carbohydrate levels.

Table 2. Effect of ascorbic acid (A.A; 100 µg/ml) on the changes in soluble carbohydrate (Sol., mg/g fresh weight) and insoluble carbohydrate (Insol., mg/g fresh weight) contents in leaf discs of six wild plant taxa during dark-induced ageing.
Treatments and recording of data are the same as in Table 1.

Plant taxa	**Treatments (µg/ml)**	**Hours after leaf ageing**							
		0		**48**		**96**		**144**	
		Sol.	**Insol.**	**Sol.**	**Insol.**	**Sol.**	**Insol.**	**Sol.**	**Insol.**
1. *Enkianthus deflexus* var. *acuminatus*	Control A.A. LSD(P=0.05)	0.90 0.90 NC	7.98 7.98 NC	1.09 0.98 0.22	6.98 7.24 1.23	2.34 1.27 0.73	5.34 6.00 1.21	3.87 2.98 1.00	4.39 5.38 1.01
2. *Gaultheria hookeri*	Control A.A. LSD(P=0.05)	0.87 0.87 NC	6.25 6.25 NC	1.13 0.98 0.55	5.25 6.00 1.23	2.21 1.87 1.03	4.26 5.23 1.02	3.32 2.36 1.23	3.54 4.12 1.92
3. *G. stapfiana*	Control A.A. LSD(P=0.05)	0.85 0.85 NC	5.50 5.50 NC	1.05 0.92 0.08	4.05 4.68 0.38	1.95 1.25 0.11	3.52 4.05 0.35	2.30 1.52 0.15	2.15 2.75 0.21

4. *G. trichophylla* var. *ovata*	Control A.A. LSD(P=0.05)	0.98 0.98 NC	4.98 4.98 NC	1.11 1.00 0.11	3.98 4.18 0.35	1.98 1.28 0.25	2.28 3.08 1.01	2.21 1.98 1.00	1.20 2.98 1.12
5. *Vaccinium nummularia*	Control A.A. LSD(P=0.05)	0.65 0.65 NC	7.82 7.82 NC	0.85 0.78 0.07	6.75 7.09 0.38	1.68 1.02 0.10	4.68 5.51 0.28	1.85 1.15 0.11	3.60 4.01 0.20
6. *V. glauco-album*	Control A.A. LSD(P=0.05)	0.96 0.96 NC	6.75 6.75 NC	1.15 1.05 0.10	4.59 4.90 0.39	1.59 1.15 0.12	3.80 4.25 0.35	2.38 1.58 0.14	2.88 3.44 0.22

NC: Not calculated.

Both RNA and DNA (Table 3) levels gradually decreased with the stress-induced ageing of leaf samples. Ascorbic acid was found to significantly check the loss of both the nucleic acid levels and the effect was particularly significant when data were recorded after 48 hours of leaf ageing and subsequent observations.

Table 3. Effect of ascorbic acid (A.A.; 100 µg/ml) on the changes in RNA (µg/g fresh weight) and DNA (µg/g fresh weight) contents in leaf discs of six wild plant taxa during dark-induced ageing.
Treatments and recording of data are the same as in Table 1.

Plant taxa	**Treatments (µg/ml)**	**Hours after leaf ageing**							
		0		**48**		**96**		**144**	
		RNA	**DNA**	**RNA**	**DNA**	**RNA**	**DNA**	**RNA**	**DNA**
1. *Enkianthus deflexus* var. *acuminatus*	Control A.A. LSD(P=0.05)	407.0 407.0 NC	74.6 74.6 NC	367.2 397.2 12.1	61.3 70.0 0.91	258.4 302.3 14.5	51.4 67.0 11.3	201.6 249.6 12.6	40.4 51.3 14.6
2. *Gaultheria hookeri*	Control A.A. LSD(P=0.05)	401.5 401.5 NC	78.8 78.8 NC	378.8 399.3 12.8	67.6 75.9 10.7	298.4 300.1 10.7	52.6 67.1 11.3	199.5 209.4 13.0	42.4 57.3 13.4
3. *G. stapfiana*	Control A.A. LSD(P=0.05)	401.5 401.5 NC	55.8 55.8 NC	338.7 375.9 20.75	47.5 50.9 3.35	295.8 338.5 15.92	41.9 47.8 2.92	201.5 225.6 13.70	28.8 34.9 2.01

4. *G. trichophylla* var. *ovata*	Control A.A. LSD(P=0.05)	412.1 412.1 NC	65.3 65.3 NC	398.1 401.3 24.1	54.8 60.6 22.1	298.3 305.1 25.4	48.9 58.8 23.1	202.1 278.6 13.3	38.4 47.3 12.7
5. *Vaccinium nummularia*	Control A.A. LSD(P=0.05)	437.6 437.6 NC	60.5 60.5 NC	365.8 395.8 25.95	52.8 58.1 4.01	305.9 338.9 14.88	42.9 46.8 3.25	227.0 275.7 12.65	34.0 39.9 2.77
6. *V. glauco-album*	Control A.A. LSD(P=0.05)	444.9 444.9 NC	68.2 68.2 NC	379.8 425.0 22.75	57.0 62.1 4.01	336.6 378.8 17.90	50.1 54.9 3.81	225.6 275.9 14.54	32.5 40.5 3.05

NC: Not calculated.

The activity of enzyme catalase decreased with the progress of stress-induced ageing duration (Table 4). The chemical treatment of leaves with ascorbic acid alleviated the ageing-induced rapid loss of catalase.

Table 4. Effect of ascorbic acid (A.A.; 100 µg/ml) on the changes in catalase activity (ΔOD×Tv / t × v) in leaf discs of six wild plant taxa during dark-induced ageing.
Treatments and recording of data are the same as in Table 1.

Plant taxa	**Treatments (µg/ml)**	**Hours after leaf ageing**			
		0	**48**	**96**	**144**
1. *Enkianthus deflexus* var. *acuminatus*	Control A.A. LSD(P=0.05)	57.4 57.4 NC	49.4 51.0 1.4	39.5 46.8 2.0	31.21 40.1 2.1
2. *Gaultheria hookeri*	Control A.A. LSD(P=0.05)	51.6 51.6 NC	43.4 49.3 1.9	36.9 41.0 2.2	29.8 34.2 1.5
3. *G. stapfiana*	Control A.A. LSD(P=0.05)	50.5 50.5 NC	38.9 56.5 3.01	28.7 38.2 2.05	21.5 29.5 0.19
4. *G. trichophylla* var. *ovata*	Control A.A. LSD(P=0.05)	54.4 54.4 NC	42.1 50.0 2.10	32.0 46.2 2.01	26.7 31.8 2.21

5. *Vaccinium nummularia*	Control A.A. LSD(P=0.05)	48.8 48.8 NC	35.5 41.8 3.12	28.7 36.6 2.01	22.9 28.8 2.11
6. *V. glauco-album*	Control A.A. LSD(P=0.05)	70.8 70.8 NC	58.9 65.4 4.92	42.5 56.7 3.88	29.8 45.9 2.85

NC: Not calculated.

Senescence of detached leaves started immediately after the separation from other plants and occurs at a rapid rate with the progressive increase of catabolic activities and these ultimately result in death and decay of leaves. Literature on the senescence pattern and the effect of hormones on regulation of senescence in terrestrial plants are rather scanty (Biswas and Ghosh, 1999; Pati and Bhattacharjee, 2003; Pati, 2007).

Results of this investigation clearly reveal that during the dark induced ageing period of zero to 144 hours of the detached leaves of *Enkianthus deflexus* var. *acuminatus*, *Gaultheria stapfiana*, *G. hookeri*, *G. trichophylla* var. *ovata*, *Vaccinium glauco-album* and *V. nummularia*, the loss of chlorophyll, proteins, insoluble carbohydrates, RNA and DNA occur at a rapid rate. In IAA treated leaf samples the same trend of declining was recorded but the magnitude of loss was found to be much less than control samples. Numerous reports exist in the literature that during all types of senescence loss of some vital macromolecules like chlorophyll and proteins take place which is due to their degradation and /or subdued rate of biosynthesis (Woolhouse, 1967; Leopold and Kriedemann, 1975; Leopold, 1980; Sabatar 1984). Any chemical or external agents possessing the property to maintain the chlorophyll and protein levels during senescence are regarded as senescence retardants (Beevers, 1976; Biswas and Ghosh, 1999; Maity *et al*, 2000).

In this investigation ascorbic acid-induced partial arrestation of the rapid loss of chlorophyll, protein, insoluble carbohydrates, RNA and DNA is indicative of the senescence deferral action of ascorbic acid. Further corroboration regarding the significant the role of the experimental pretreating agent on senescence retardation in the six wild plant taxa can be made from some other biochemical studies like analysis on the enzyme activity catalase as well as the levels of soluble carbohydrate. Catalase is regarded as a scavenzer enzyme (Fridovich, 1976) and higher activity of this enzyme is the index of plant vigour. Rapid loss of catalase activity is indicative of the declining of plant potential (Bhattacharjee & Choudhury, 1986; Biswas and Ghosh, 1999 and Bhattacharjee, 2001; Pati, 2007).

In this investigation the chemical-induced retention of catalase activity during dark-induced detached leaf senescence is indicative of the retardation of senescence. Again, rapid increase of soluble carbohydrate during senescence is mostly due to the damage of cell membrane and subsequent leaching of sugar from the cells. In this study, the rapid increase of sugar in detached leaves of control samples and arrestation of higher leaching of sugar in ascorbic acid-treated samples is indicative of the efficacy of ascorbic acid on the maintenance of the membrane integrity at least to a certain extent. Possibly this chemical-induced maintenance of membrane integrity caused to check the rapid leaching of sugar from the cells. Hence, the sugar levels were found to be much less in the chemical treated leaf samples.

4. CONCLUSION

Considering all the biochemical parameters it can be concluded that ascorbic acid is a potent growth promoter for maintenance of membrane integrity as well as arrestation of overall senescence of the detached leaves of the plant taxa analysed. However, although the ongoing of senescence process during leaf ageing is inevitable, ascorbic acid treatment can retard faster rate of senescence efficiently in some plant species. Thus, ascorbic acid can be considered as a potent senescence deferral agent at least in case of six wild plant ericoid taxa analysed in this investigation.

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Original Article

Pharmacognosy and Phytochemical Screening of some Plant Derived Medicine to Treat Dysmenorrheal Pain by the Rajbanshi Community

Priyankar Roy[1], Palash Mandal[1*], Subhasis Panda[2], Sonia Mitra Roy [3], Arunika Subba[1]

ABSTRACT

Introduction: Dysmenorrhea is a sort of painful menstrual disorder. Several plants like *Allium sativum* L., *Areca catechu* L., *Zingiber officinale* Roscoe, *Crinum amoenum* Ker Gawl. Ex Roxb, *Cuscuta reflexa* Roxb. *Nymphaea rubra* Roxb. Ex Andrews, *Piper nigrum* L., *Citrus limon* (L.) Osbeck were used in appropriate ratio to make herbal formulation to cure dysmenorrheal pain by the traditional healers of Rajbanshi community. **Objective:** By semi-structured questionnaires in the course of scheduled interviews with the local herbal practitioners, four herbal formulations (coded as DYS1, DYS2, DYS3 and DYS4) were recorded. Pharmacognostic characterization was carried out for the authentication of the powder drugs which includes powder microscopy, fluorescence analysis and physicochemical evaluation. The presence of any therapeutic potential in DYS1, DYS2, DYS3 and DYS4 was determined by qualitative evaluation of phytochemicals of various successive solvent extracts based on their polarity. Thin layer chromatography was performed for screening various phyto-active compounds like arbutin, alkaloids, coumarins, cardiac glycoside, etc. **Results:** Powder microscopy revealed the presence of calcium oxalate crystal, stone cells etc. Various fluorescence colors were exhibited by these herbal drugs under UV after reacting with chemical reagents. Physical analyses values were also obtained in a satisfactory way. TLC and qualitative phytochemical analysis showed the presence of active phytoconstituents like arbutin, alkaloids, coumarins, cardiac glycoside etc. **Conclusion:** The results support the use of plants as a traditional medicine for the prevention disorders like Dysmenorrhea. Further evaluation is required for determining the efficacy of those herbal drugs and the active phytoconstituents involved in Dysmenorrheal therapy.

Key words: Dysmenorrhea, Traditional healer, Pharmacognostic evaluation, Antioxidant, Successive solvent extraction, Thin Layer Chromatography.

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INTRODUCTION

Plants have created the base of traditional medicine exercised that has been practiced since many years by the people in China, India, and many other countries. Some of the most primitive accounts of the use of the plant as drugs are originated in the Atharva-Veda, which is the origin of Ayurvedic medicine in India.[1] There is an ancient evidence of medicine discovery on Sumerian clay from Nagpur. Twelve very old medicinal formulation have been reported with plant element such as poppy and Mandrake.[2] Humans have produced medicine from plants and other organisms for centuries. A good number of the drugs used in modern medicines are formed indirectly from medicinal plants.[3] and about 90% of raw resources are derived from the wild sources. However traditional therapy is still restricted to certain areas or group of people because of the unavailability of effective scientific communication.[4] Therefore the requirement of the significance of herbal medicine and study of the knowledge of traditional healing practices by plants is quite essential. Several active principle constituents are found in those formulations, which could be explained by their function in animal and human system through pharmaceutical and pharmacological study.[5]

Rajbanshis are the fore runner of Koches and are living in Northern Region of West Bengal. Due to a distinctive climatic and ecological provision, a unique Biodiversity hotspot is situated in sub-Himalayan region of North Bengal. Rajbanshis are dominating this area for many years and they are mostly dependent on agriculture. But socio-cultural activities of the Rajbanshi community are directly and indirectly related to the plant resources. They are using various plant parts to make medicinal formulations for alleviating their diseases & disorders since time immemorial.[6] Previously we have found that the herbal practitioners (Kabiraj or Mahan) of Rajbanshi community exercise a total number of 31 types of plant species from 29 genera and

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24 families for preparation of 10 polyherbal formulations to treat various ailments and disorders like arthritis, lesions with pus formation, wound, dysentery, seasonal fever, liver problem, jaundice, nose bleeding along with frequent urination etc.[7] Our present study revealed that four polyherbal formulations (coded as DYS1, DYS2, DYS3 and DYS4) was made by mixing the various parts of several plants like *Allium sativum* L., *Areca catechu* L., *Zingiber officinale* Roscoe., *Crinum amoenum* Ker Gawl. ex Roxb., *Cuscuta reflexa* Roxb., *Nymphaea rubra* Roxb. ex andrews., *Piper nigrum* L., *Citrus limon* (L.) Osbeck. For the said treatment of Dysmenorrhea.

Literature survey did not show any information about pharmacognostic studies of the above polyherbs. For that reason, this study was concentrated on the standardization, by measuring various attributes such as powder microscopy, fluorescence analysis and physicochemical studies to explore the presence of active phytoconstituents and qualitative phytochemicals, extracted from different solvents according to their polarity.

MATERIALS AND METHODS

Study Area

Terai and Duars lie in the Northern half of the state of West Bengal and are surrounded by international boundaries of Bangladesh, Nepal and Bhutan. The area is a combination of the wide-ranging landscape - from the high mountainous region in the north to the vast Gangetic plains in the farthest south. The study area lies at 26.7072°N and 88.3558°E. The annual rainfall is recorded to be about 3900 mm and temperature varies between 7°C to 37°C. The uniqueness of the area has made it one of the treasures of West Bengal with diversified plant and animal populations. The region not only stands out for scenic beauty, along with flora and fauna but also in social atmosphere. The region is a blend of several exclusive tribal communities which makes it rich in culture as well. Some of the major tribes include Rajbanshi, Rabha, Santal, Munda, Oraon, Polia, Lepcha, Toto etc.[8,9]

Collection and authentication of plant material

Field survey was accompanied with detailed discussions in the form of questionnaires to the medicine men and local people of study areas, as some information was received from them as well. The information includes the mode of consumption, shelf life and ethnic values of the particular plant and its part (s) used. After these detailed discussions, the respective plants were collected properly both in its flowering and fruiting stage. The collected specimens were dried consequently and herbarium sheets were made. Voucher specimen was preserved in the NBU herbarium for further reference.

Sample preparation

Fresh plant parts are collected from the different part of the study area and dried and make the powder to preserve for future use. Polyhedral powder drugs DYS1, DYS2, DYS3 and DYS4 were prepared according to the herbal healers. The polyhedral powder sample was extracted through soxhlet apparatus in seven different solvents based on their polarity. The solvent extracts were heptane (HP), benzene (BZ), ethyl acetate (EA), acetone (AC), butanol (BT), methanol (MT) and water (AQ). The samples were then evaporated and reconstituted in methanol. These extracted samples were used for qualitative phytochemicals. For TLC and pharmacognostic studies, the samples were used in its powder form.

Pharmacognostic characterization

Organoleptic Characters

The colour, odour, taste and texture of the powder of DYS1, DYS2, DYS3 and DYS4 were observed and recorded.

Anatomy

Anatomical parameters have been taken into consideration for the study as they are usually the conserved characters for the specific species. The anatomical study revealed some unique and important characteristic features for the studied species. Transverse section of respective part of the studied plant species were done and treated with different reagents i.e. iodine, phloroglucinol and Toluidine blue stain. Iodine stain was used to check the presence of starch grains. Phloroglucinol stains the lignified tissues, whereas Toluidine blue helps to differentiate xylem and phloem cell wall.

Powder microscopy

The colour, odour, taste and texture of the powder of DYS1, DYS2, DYS3 and DYS4 were observed and recorded.[10] Microscopic examination was carried out in a standard method.[11] A small amount of powder of DYS1, DYS2, DYS3 and DYS4 were taken on glass slide, mounted on glycerin and observed under microscope. For the observation of lignified tissues, powder was stained with alcoholic solution of phloroglucinol followed by concentrated HCl. Similarly the powder was also stained with N/10 iodine solution to observe the starch granules and for the identification of fixed oil and fats, Sudan III were used.

Fluorescence analysis

Fluorescence study of the dried leaf powder was performed using standard procedure.[11,12,16] A small quantity of the sample was treated using fluorescence reagents (such as 1 N HCl, 50% H_2SO_4, $FeCl_3$, iodine solution, acetic acid glacial, 0.1 N NH_4OH, 1% CH_3COOH). The powder samples after treatment with various chemical reagents were subjected to fluorescence analysis. Observations were made under visible light and under UV light of short (254 nm) and long wave length (365 nm) separately.[12]

Physicochemical parameters

Coarse powder of the plant root was used to perform quality control parameters such as total ash, acid insoluble and water soluble ash, water and alcohol soluble extractive values and loss on drying.[13] three determinations were carried out for each parameter.

Preliminary phytochemical studies

The DYS1, DYS2, DYS3 and DYS4 powder extracted in various solvents were subjected for preliminary phytochemical screening to observe the presence or absence of phytoconstituents like tannin, triterpenoids, amino acid, steroids, cardiac glycosides and flavonoids by the standard methods.[14-17]

Thin layer chromatography

TLC was performed to analyze the variation in bioactive chemical constituents.[18] Readymade TLC plates (coated with silica gel 60 F254 on aluminum sheets) purchased from Merck Germany were used. The powdered sample was extracted with different procedures for the identification of each of the active constituents i.e. anthraglycosides, arbutin, cardiac glycosides, flavonoids, bitter principles, saponins, coumarins and alkaloids. The mobile phase solvent systems used were ethyl acetate: methanol: water (100:13.5:10) for the detection of anthraglycosides, arbutin, cardiac glycosides, bitter principles and alkaloids. The mobile phase ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26) was used for flavonoids identification and for the identification of saponin, the solvent system of chloroform: glacial acetic acid: methanol: water (64:32:12:8) was used while for the identification of coumarin, toluene : ethyl acetate (93:7) was used. The developed chromatograms were analyzed for the presence of drug constituents by spraying with suitable group reagent. The chromatograms were then

observed under UV-254 nm and UV-365 nm light. Photographs were taken with DSLR Nikon camera (D-3200) and the R_f values were calculated with the following formula:

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

RESULTS

Organoleptic evaluation

The powder of DYS1, DYS2, DYS3 and DYS4 were brown in colour and rough in texture. DYS1 and DYS4 were slightly bitter in taste, whereas DYS2 and DYS3 were partially sweet in nature. In DYS1, a characteristic pungent smell was observed, as garlic was used in that formulation in appropriate ratio according to the herbal healer's prescription.

Anatomy

Presence of starch grains were confirmed in species like *Citrus limon, Crinum amoenum, Zingiber officinale and Nymphea rubra* (Figure 1 A-D). Starch grains were stained with dark blusih-purple colour and were mostly present within the thick walled parenchymatous cells either near the cortex or pith. Starch grains were of various shape and sizes with prominent centered hilum in case of *Nymphea rubra*. Sclerenchymatous cells occur in patches in *Areca catechu*, confirmed through phloroglucinol staining (Figure 1 E). Presence of collenchymatous cells was seen in *Nymphea rubra* (Figure 1 F). Sclerenchymatous fiber cells were also found in *Piper nigrum* (Figure 1 K). In case of *Zingiber officinale*, prominent oil glands were observed (Figure 1 L). *Areca catechu,* when treated with toluidine blue stain, showed xylem and phloem wall differentiation along with sclerenchymatous fiber cells embedded within the phloem tissue. The cortical cells of *Allium sativum* were thick walled and showed prominent differentiation when stained with Toluidine blue (Figure 1 H). Within the cortical region of *Nymphea rubra*, some of the cells showed clear wall thickening and could be concluded as islet of sclerenchymatous cells (Figure 1 I). In case of *Piper nigrum* fruit coat, presence of thick walled osteosclereides was observed in rows above mesophyll cells (Figure 1 J)

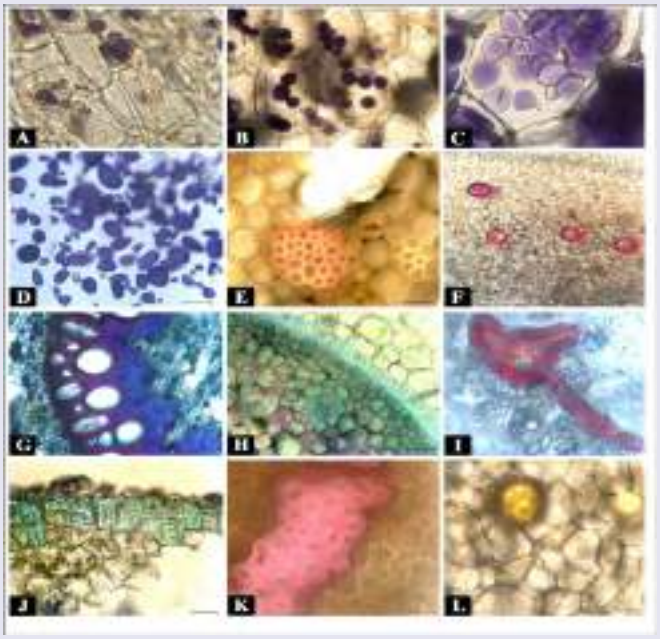

Figure 1. A-D: Starch grain: A. *Citrus limon*, **B.** *Crinum amoenum*; **C.** *Zingiber officinale*, **D.** *Nymphaea rubra*, **E.** Sclerenchymatous patch in *Areca catechu*; **F.** Collenchymatous cells in *Nymphaea rubra*; **G.** Ligning deposition in xylem and sclerenchymatous patches within phloem in *Areca catechu*; **H.** Lingnin deposition in cell wall of cortical cells in *Allium sativum*; **L.** Sclerenchymatous cells in *Nymphaea rubra*; **J.** Lignin deposition in Sclerenchymatous fiber cells in *Piper nigrum*; **K.** Sclerenchymatous patch in *Citrus limon*; **L.** Oil gland in *Zingiber officinale*: *Scale bar*: 330min (660x)

Powder microscopy

Powder microscopy has revealed some specific features and helps in authentication of the studied herbal drugs. In case of DYS1, presence of acicular type of calcium oxalate crystals and triangular starch grains with concentric striae were confirmed (Figure 2 A and B). In DSY2 starch grains of various shapes with central hilum and astrosclereids were observed. Literature showed the presence of astrosclereid in Nymphaeaceae family.[19] The astrosclereid is usually eight armed with somewhat prominent basal cells, arms are thick without any lumen and were broad-based to narrow tapering ends (Figure 2 C and D). DYS3 revealed the presence of pitted xylem vessels and round shaped uniform starch grains (Figure 2 E and F). In DYS4, elongated stone cells were visible but starch grains were absent (Figure 2 G and H). The four formulations have specifically shown the presence and absence of certain features which are significant tools for standardization of these powdered formulations.

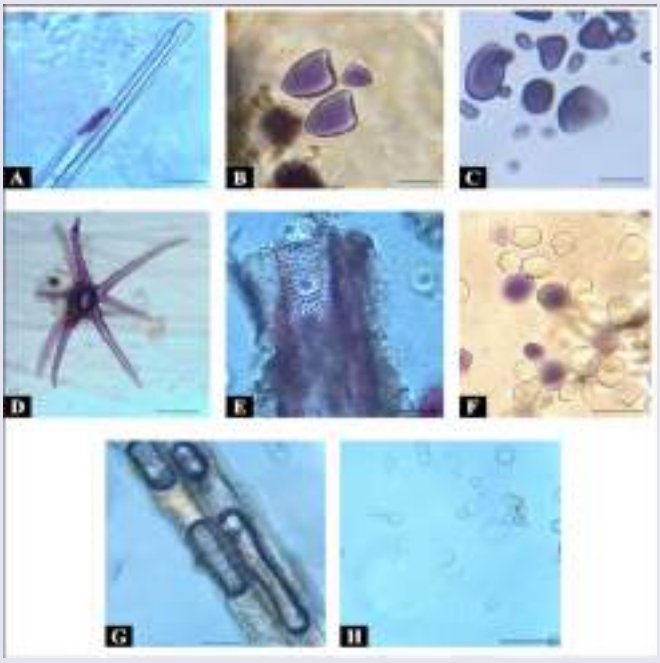

Figure 2. A & B: DYSI: **A.** Calcium oxalate crystal, **B.** Starch grains; **C & D.** DYS2: **C.** Starch grains; **D.** Abstrosclereid, **E & F.** DYS 3: **E.** Pitted vessels, **F.** Starch grains; **G & H.** DYS4: **G.** Stone cell, **H.** Starch grain absent; *Scale bar* = 330mm (660x)

Fluorescence analysis

Different fluorescent colours were exhibited after treating the powdered drugs with different chemical reagents (Figure 3). The colours were detected under visible and UV light and were identified by using the standard colour chart of German RAL Institute and noted down in Table 1. Characteristic blue and green colours were observed under UV light when these herbal drugs were interacted with acidic, alkaline or

Table 1: Fluorescence analysis of DYS1, DYS2, DYS3 and DYS4.

	(DYS1)			(DYS2)			(DYS3)			(DYS4)		
	VISIBLE LIGHT	UV-254	UV-365	VISIBLE LIGHT	UV-254	UV-365	VISIBLE LIGHT	UV-254	UV-365	VISIBLE LIGHT	UV-254	UV-365
50% KOH	Oyster white	Distant blue	Pearl night blue	Sand yellow	Signal blue	Black blue	Saffron yellow	Green blue	Pastel blue	Brown green	Sapphire blue	Green blue
10% FeCl3	Maize yellow	Ocean blue	Night blue	Daffodil yellow	Pastel turquoise	Sapphire blue	Daffodil yellow	Pastel turquoise	Pearl night blue	signal yellow	Black green	Grey blue
DISTILLED WATER	Light grey	Signal grey	Light blue	sand yellow	Graphite grey	Gentian blue	Oyster white	Black green	Sky blue	Beige red	Mint turquoise	Signal blue
HCL CONC.	Light ivory	Green blue	Emerald green	lemon yellow	Ocean blue	May green	Lemon yellow	Pearl gentian blue	Night blue	Traffic yellow	Mint turquoise	Grey blue
HCL 50%	Golden yellow	Pearl gentian blue	Leaf green	Ivory	Capri blue	Violet blue	Ivory	Pearl gentian blue	Water blue	Reed green	Pine green	Sapphire blue
H^2SO_4 CONC.	Traffic black	Dusty grey	Ultramarine blue	Fawn brown	Graphite grey	Ocean blue	Signal orange	Black green	Sky blue	Salmon pink	Mint turquoise	Azure blue
H^2SO_4 50%	Signal blue	Cement grey	Graphite black	Fawn brown	Blue green	Distant blue	Steel blue	Azure blue	Sky blue	Melon yellow	Mint turquoise	Gentian blue
HNO_3 CONC.	Dahlia yellow	Pearl gentian blue	Signal blue	Rape yellow	Blue green	Black blue	Daffodil yellow	Green blue	Ultramarine blue	Dahlia yellow	Signal black	Grey blue
HNO_3 50%	Sun yellow	Water blue	Traffic blue	Ivory	Blue green	Traffic blue	Sulfur yelllow	Green blue	Traffic blue	Rape yellow	Mint turquoise	Pigeon blue
CH3COOH CONC.	Signal white	Pearl black blue	Sky blue	Transparent	Pearl gentian blue	Pearl night blue	Green beige	Gentian blue	Traffic blue	Papyrus white	Mint turquoise	Signal blue
CH3COOH 50%	Pastel yellow	Pearl beige	Water blue	Sand yellow	Brilliant blue	Violet blue	Golden yellow	Gentian blue	Capri blue	Olive grey	Mint turquoise	Ultramarine blue
CHLOROFORM	Pure white	Sapphire blue	Cobalt blue	Transparent	Brillant blue	Signal blue	Green beige	Signal blue	Traffic blue	Light grey	Cobalt blue	Signal blue
BENZENE CONC.	Papyrus white	Traffic grey B	Signal blue	Transparent	Pearl gentian blue	Signal blue	Golden yellow	Blue green	Signal blue	Papyrus white	Mint turquoise	Cobalt blue
BENZENE 50%	Silk grey	Traffic grey B	Black blue	Transparent	Pigeon blue	Signal blue	Light ivory	Black green	Traffic blue	Pure white	Grey white	Ultramarine blue
METHANOL CONC.	Pure white	Traffic grey B	Gentian blue	Transparent	Pearl gentian blue	Signal blue	Ochre yellow	Ocean blue	Signal blue	Pure white	Mint turquoise	Signal blue
METHANOL 50%	Green beige	Chrome green	Light blue	Sand yellow	Blue green	Traffic blue	Light ivory	Pearl gentian blue	Light blue	Olive grey	Mint turquoise	Pearl night blue
ETHANOL CONC.	Pure white	Violet blue	Signal blue	Transparent	Sapphire blue	Pearl night blue	Oyster white	Traffic blue	Sky blue	Papyrus white	Night blue	Traffic blue
50% ETHANOL	Beige	Brilliant blue	Sapphire blue	Beige	Ocean blue	Capri blue	Light ivory	Sky blue	Light blue	Yellow grey	Cobalt blue	Traffic blue
ACETONE	Pure white	Sapphire blue	Ultramarine blue	Transparent	Night blue	Brillant blue	Green beige	Sapphire blue	Signal blue	Pebble grey	Gentian blue	Signal blue
PETROLEUM ETHER	Pure white	Sapphire blue	Ultramarine blue	Transparent	Night blue	Cobalt blue	Oyster white	Steel blue	Cobalt blue	Pure white	Cobalt blue	Violet blue
ALCOHOLIC NaOH	Oyster white	Saphire blue	Gentian blue	Light Ivory	Night blue	Traffic blue	Honey yellow	Gentian blue	Light blue	Olive grey	Violet blue	Signal blue
AMMONIA	Pastel yellow	Pegion blue	Azure blue	Pearl copper	Night blue	Night blue	Ivory	Traffic blue	Turquoise blue	Signal orange	Signal blue	Sapphire blue

Table 2: Physicochemical analysis of DYS1, DYS2, DYS3 and DYS4

	TOTAL ASH (%)	ACID INSOLUBLE ASH (%)	WATER SOLUBLE ASH (%)	ALCOHOL SOLUBLE EXTACTIVE (%)	WATER SOLUBLE EXTRACTIVE (%)	LOSS ON DRYING (%)
DYS1	8.18	1.12	4.88	6.17	13.81	11.63
DYS2	14.63	1.37	13.47	3.87	4.64	11.53
DYS3	20.82	8.38	19.41	4.9	4.13	8.22
DYS4	8.11	0.68	5.9	5.71	7.56	6.9

organic reagents. In DYS1 nad DYS2, Emerald to leafy green colours were fluoresced in presence of concentrated and 50% hydrochloric acid respectively. Mineral acids like nitric acid and sulphuric acid produced Traffic Blue and Gentian Blue respectively, when interacting with DYS1 and DYS4. Ammonia showed characteristic Turquoise and Sapphire Blue in response with drug fourmulation DYS3 and DYS4 respectively at UV 365 nm wavelength. DYS 3 also produced unique blue colours in presence of 50% acetic acid and methanol reagent.

Physicochemical characteristics

Total ash is mainly essential to check the purity of drugs showing the presence or absence of metallic salts or silica.[20,21] the results obtained from physicochemical studies were given in Table 2. Total ash of DYS3 showed the highest value among four herbal drugs, which was found to be 20.82% w/w and DYS4 confirmed the lowest value of 8.11% w/w. The percentage of ash was calculated with reference to the air-dried powder. The acid insoluble ash was noted and DYS3 showed the highest value of 8.38% and the lowest value of 0.68% was observed in DYS4. While the presence of material exhausted by water i.e. the water-soluble ash was recorded with high value of 19.41% in DYS3 and the low value of 4.88% in DYS1. Purpose of extractive values is a further way of inspection of the purity of the herbal formulation. The water-soluble extractive value was found to be 13.81% w/w which signifies that more amount of constituents in DYS1 was soluble in water than in alcohol (6.17% w/w). The percentage of loss on drying i.e. the moisture content in the powder was found to be 11.63% w/w, which is highest in DYS1. In case of DYS4 showed the lowest moisture content.

Qualitative phytochemical analysis

The biological activity of herbal formulation mainly depends on the phytoactive secondary metabolites present in it. Thus preliminary screening of phytochemicals which was performed to establish a chemical profile of a crude drug was a part of chemical evaluation.[22,23] these phytochemical tests (Table 3, 4, 5 and 6) revealed the presence of anthraquinones, cardiac glycosides, coumarine, flavonoids and steroids in different extracts of DYS1, DYS2, DYS3 and DYS4 obtained by using different solvents. Alkaloid and amino acid were found only at DYS1 and DYS3. Tannins were absent in all three formulation, except DYS4. In DYS4, soluble tannins were extracted with partial polar to polar solvents. Triterpenoids were found to be present in all formulations, while in DYS4, the same was found in benzene extract only.

Thin layer chromatography

Initial analysis of active constituents in DYS1, DYS2, DYS3 and DYS4 were carried out by thin layer chromatographic (TLC) method. Following particular spraying reagents for a specific active constituent, the results of TLC showed the presence of arbutin, bitter principle coumarins and saponin in all four formulations. It was found that except DYS2 all formulation showed the presence of anthrone. Anthrone and arbutin bands were most prominent in DYS4. Alkaloid was found only in DYS1 and a clear orange brown band was visible in case of DYS3 under visible light after Dragendorff reagent application. Five band of cardiac glycosides were found only at DYS4, which was clearly distinguishable under visible light, while flavonoids spots on TLC plate was found only in case of DYS2 and DYS4. R_f values were calculated for all the spot and the analysis were explained at the Table no 7.

DISCUSSION

There has been a remarkable utilization of herbal formulations in traditional folk medicine for the treatment of diseases and disorders, especially as anti-inflammatory and analgesic agent.[24] So scientific evaluation is required for each and every formulations mentioned in our traditional indigenous knowledge.[25] Thus in this study, the pharmacognostic features of these four herbal formulations for primary dysmenorrhea are being reported for the first time.

Therefore, the powder of DYS1, DYS2, DYS3, and DYS4 were subjected to certain standardization parameters. There are numerous modern tools available for the evaluation technique of plant drugs, but powder microscopy is considered to be the most fundamental, authentic, cheap and reproducible methods to recognize the source materials.[26] Presence of starch granules in DYS2 and DYS3 has been confirmed after staining with iodine solution, whereas phloroglucinol solution showed the presence lignified tissues. Oil globules have been found only in powder DYS3 after staining with Sudan III. Fluorescence study is also an important parameter for standardization of crude drug. The different chemical constituents present in plant extract showed characteristic fluorescence when suitably illuminated. Certain chemical substances that not naturally fluoresce themselves are actually treated with different reagents to attain fluorescence.[27] the presence of specific amounts of acid, sugar and inorganic matter in the powders are confirmed by water-soluble ash technique, whereas acid insoluble ash methods clearly specify the presence of silica, thus indicating the impurities with earthy materials.[28] The extractive values are useful to evaluate the chemical constituents present in crude drug and also help for estimation of specific constituents soluble in a particular solvent. For any drugs and herbal formulation, low moisture content is essential for higher stability of drugs. The general requirement for moisture in a crude drug should not be more than 14%.[29] Excess moisture content may support the growth of fungi and may cause contamination by other microorganisms resulting in the degradation of the drugs. But DYS1, DYS2, DYS3 and DYS4 powder showed the presence of moisture content less than the required standard (Table 2) which was not too high to promote the growth of microorganisms. The pharmacological importance of a drug is attributed to the various secondary metabolites present in it and a particular compound might possess a clinical significance. Therefore it is essential to separate the compounds present in the plants with an appropriate chromatographic method. TLC technique has proved its worth as a simple, inexpensive and reproducible method for the chemical and biological screening of plant extracts. It provides a basic idea of the polarity of a particular chemical constituent.[29] Development of TLC plates with appropriate group reagents indicate

Table 3 : Preliminary phytochemical analysis of different solvent extracts of DYS1

Phytochemical	HP	BZ	EA	AC	BT	MT	AQ
Amino acid	-	-	-	-	-	+	-
Alkaloids	-	-	-	-	-	-	+
Anthraquinones	-	-	-	-	-	-	-
Cardiac glycosides	+++	+++	++	++	+	+	+
Coumarin	-	-	+	+	+	+	++
Flavonoids	-	-	-	-	-	-	-
Saponins	-	+	++	+	+	-	-
Steroids	++	+	+	++	++	+++	++
Tannin	-	-	-	-	-	-	-
Tri-terpenoids	-	-	+	+	-	-	-

Table 4 : Preliminary phytochemical analysis of different solvent extracts of DYS2

Phytochemical	HP	BZ	EA	AC	BT	MT	AQ
Amino acid	-	-	-	-	-	-	-
Alkaloids	-	-	-	-	-	-	-
Anthraquinones	-	-	-	+++	++	+	-
Cardiac glycosides	+++	++	++	+	+	++	++
Coumarin	-	-	++	++	+	++	++
Flavonoids	-	-	++	++	+	+	+
Saponins	++	+	+	+	+	-	-
Steroids	+	+	+	++	++	+++	+
Tannin	-	-	-	-	-	-	-
Tri-terpenoids	++	+	+	+	+	-	-

Table 5 : Preliminary phytochemical analysis of different solvent extracts of DYS3

Phytochemical	HP	BZ	EA	AC	BT	MT	AQ
Amino acid	-	-	-	+	-	+++	++
Alkaloids	++	+	-	-	-	-	-
Anthraquinones	-	+++	++	+	-	-	-
Cardiac glycosides	+++	+++	+++	++	++	+	+
Coumarin	++	+++	++	+	+	++	++
Flavonoids	-	+	-	-	-	-	-
Saponins	-	+	+	+	-	-	-
Steroids	+++	+++	++	++	+	++	+++
Tannin	-	-	-	-	-	-	-
Tri-terpenoids	-	-	+	+	+	+	-

Table 6 : Preliminary phytochemical analysis of different solvent extracts of DYS4

Phytochemical	HP	BZ	EA	AC	BT	MT	AQ
Amino acid	-	-	-	-	-	-	-
Alkaloids	-	-	-	-	-	-	-
Anthraquinones	-	+	+	++	+	++	++
Cardiac glycosides	++	++	++	++	++	++	++
Coumarin	-	+	++	++	++	+++	+++
Flavonoids	-	-	++	++	++	++	++
Saponins	+	+	+	+	-	-	-
Steroids	+	+	++	+++	+++	+++	++
Tannin	-	-	+	+	+	+	+
Tri-terpenoids	+	+	-	-	-	-	-

Table 7: Detection of active phytoconstituents in powder formulation DYS1, DYS2, DYS3 and DYS4 by TLC method

Phytoconstituents	Solvent system	Detection reagents	(DYS1)		(DYS2)		(DYS3)		(DYS4)	
			No. Of Spot	Rf values	No. Of Spot	Rf values	No. Of Spot	Rf values	No. Of Spot	Rf values
ANTHRONE	Ethyl acetate: methanol: water (100:13.5:10)	KOH reagent	1	0.04	0	0	1	0.22	1	0.47
			2	0.29			2	0.33		
			3	0.55			3	0.4		
							4	0.66		
							5	0.77		
ALKALOIDS	Ethyl acetate: methanol: water (100:13.5:10)	Dragendorff reagent	1	0.69	0	0	1	0.71	0	0
ARBUTIN	Ethyl acetate: methanol: water (100:13.5:10)	Berlin blue	1	0.74	1	0.54	1	0.66	1	0.16
									2	0.24
									3	0.43
									4	0.68
									5	0.74
FLAVONOIDS	Ethyl acetate: Formic acid: glacial acetic acid: water (100:11:11:26)	NP/PEG reagent	0	0	1	0.97	0	0	1	0.93
BITTER PRINCIPLES	Ethyl acetate: methanol: water (100:13.5:10)	Vanillin-sulphuric acid reagent	1	0.04	1	0.06	1	0.12	1	0.16
			2	0.72	2	0.57	2	0.71	2	0.24
					3	0.67			3	0.31
									4	0.68
									5	0.76
CARDIAC GLYCOSIDES	Ethyl acetate: methanol: water (100:13.5:10)	Antimony-III chloride reagent	0	0	0	0	0	0	1	0.43
									2	0.56
									3	0.6
									4	0.66
									5	0.72

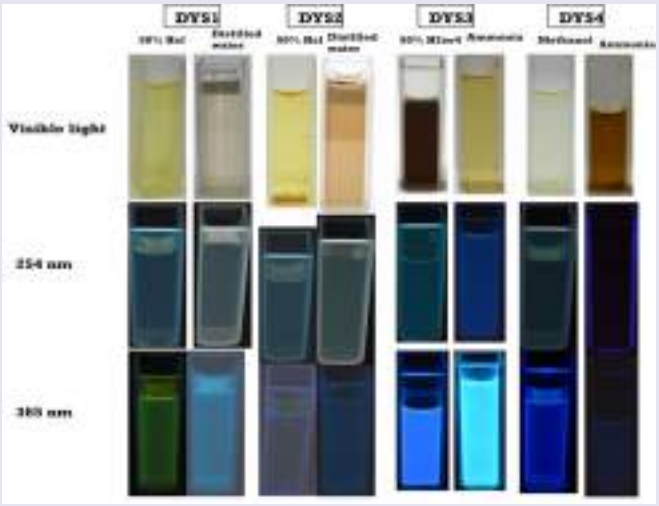

Figure 3: Different colours exhibited after treating the powder of DYS1, DYS2, DYS3 and DYS4 with different chemical reagents.

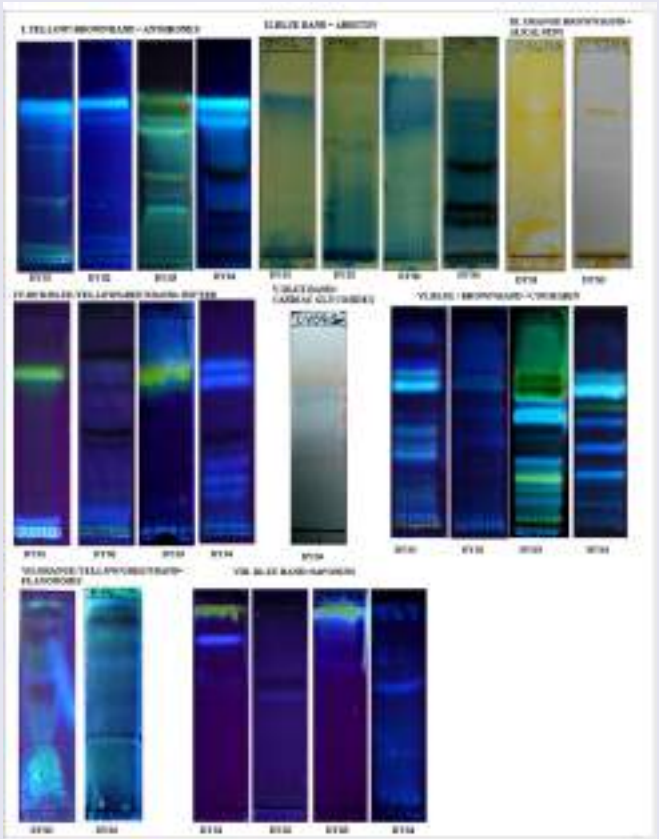

Figure 4: Preliminary investigation by Thin layer chromatography I. Anthrone II. Arbutin III. Alkaloid IV. Bitter Principle V. Cardiac Glycosides VI. Coumarin VII. Flavonoids VIII. Saponins.

the presence of anthrone, arbutin, bitter principle, cardiac glycosides, coumarins, flavonoids and saponins in DYS1, DYS2, DYS3 and DYS4 powder. The pattern of bands on TLC plates provides primary data and is used to show the uniformity and stability of herbal components. It is an effective and speedy way to differentiate between chemical classes which may not be fulfilled by macroscopic and microscopic analysis.[30] TLC is commonly used for generating fingerprints of herbal medicines as it is simple, versatile, sensitive to specific compounds.[31] Thus, TLC is a convenient method of determining the quality and possible adulteration of herbal drugs.

Phytochemicals are important for the establishment of pharmaceutical companies; the constituents are playing a significant role in the identification of crude drugs. The medicinal value of these plants lies in some chemical substances that produces a definite physiological action on the human body.[33] Qualitative phytochemical studies on the different solvent extracts of four herbal formulations revealed that some solvents are good for extraction of phytoconstituents while some others are not suitable. Table no. 3, 4, 5 and 6 showed that the alkaloids and amino acids were found in DYS1 and 3 only. Presence of alkaloid is evidenced by the presence of Dragendorff's band in TLC Plate (Figure 3). All the extracts indicated the presence of cardiac glycoside and steroids in large amount (Table no. 3, 4, 5 and 6). TLC fingerprinting and preliminary phytochemical screening, both proved the presence of coumarin. Coumarins act as competitive inhibitors of vitamin K, which is a blood clotting factor. Coumarins are potent anti-inflammatory drugs and it directs against cell-adhesion molecules, thus highly significant in inflammatory responses.[34]

CONCLUSION

All the scientific investigations support the traditional use of those powdered polyherbal formulation for the treatment of primary dysmenorrhea. The present study focused on establishing pharmacognostic standards for the identification and authentication of DYS1, 2, 3 and 4, as well as detection of adulterants. The important diagnostic features of all four polyhedral formulations established in this study may be useful for the further studies.

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GRAPHICAL ABSTRACT

SUMMARY

Dysmenorrhea is a sort of painful menstrual disorder. By semi-structured questionnaires in the course of scheduled interviews with the local herbal practitioners, four herbal formulations were recorded Several plants like *Allium sativum* L., *Areca catechu* L., *Zingiber officinale* Roscoe, *Crinum amoenum* Ker Gawl. ex Roxb, *Cuscuta reflexa* Roxb., *Nymphaea rubra* Roxb. ex Andrews, Piper nigrum L., Citrus limon (L.) Osbeck were used in appropriate ratio to make herbal formulation to cure dysmenorrheal pain by the traditional healers of Rajbanshi community. The present research article focuses on the evaluation of pharmacognostic identity and quality parameters to be used as herbal drug and thus validating its ethno-medicinal use to treat Dysmenorrhea.

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SHORT COMMUNICATION

Chemical-induced seed germination and enhancement of seed potential of seven wild plant taxa of Ericaceae in India

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ABSTRACT

Pretreatment of seeds of seven wild plant taxa (*viz*., *Gaultheria hookeri* C. B. Clarke, *G. stapfiana* Airy Shaw, *G. semi-infera* (C. B. Clarke) Airy Shaw, *G. trichophylla* Royle var. *ovata* Panda & Sanjappa, *Lyonia ovalifolia* (Wall.) Drude var. *ovalifolia*, *Pieris formosa* (Wall.) D. Don and *Vaccinium glauco-album* C. B. Clarke) in the family Ericaceae using Na-dikegulac (Na-DK) for 8 hours (4 + 4 h in two installments) before keeping in ambient storage condition (32±2 ºC) for different durations (0 and

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20 days) slowed down the rapid loss of germination and reduced the time (h) required for 50% seed germination (T_{50}). Concomitantly, the reduction of protein level as well as the activity of catalase of seed kernels during storage period was ameliorated to a significant extent in the chemical pretreated seed lots.

***Keywords*:** Seed germination, seed metabolism, T_{50} of germination, protein, catalase

1. INTRODUCTION

Deterioration of seeds is a natural catabolic process which results in serious impairment of seed viability and consequent termination of life span. This process may be accelerated by some pathogenic attack or by adverse environmental conditions. Maintenance of vigour and viability of seeds in tropical countries like India is a matter of serious concern to the crop growers because of high temperature and high relative humidity (RH) prevailing in major parts of the country almost throughout the year. These two environmental factors strongly impair seed and seedling health and cause to reduce percent seed germinability and seedling performance at a rapid rate (Copeland and McDonald, 1995; Desai *et al.,* 1997; Maity *et al*., 2000; Bhattacharjee, 2001).

Thus, Indian cultivators are very often compelled to use low vigour seeds in agriculture. To get rid of this problem, strategies are now being undertaken to improve the storage potential of seeds for enhancing their life span (Pathak and Basu, 1980; Chhetri *et al.*, 1993; Basu, 1994; Aditya et al. 2014). Being the experimental seeds are grown in temperate climate and in higher altitudes, these are often unable to germinate in the plane land agroclimatic condition. Keeping this problem in mind, an attempt is made in this investigation to enhance the seed germination and metabolism of seven wild plant taxa in the family Ericaceae using Na-dikegulac (Na-DK). Experiments of this investigation were carried out under ambient storage condition (32±2 ºC) condition to obtain more or less uniform and expeditious results. Thus, the objective of this investigation is to explore the efficacy of Na-Dk and metabolism on enhancing percentage of seed germination of a few wild plant taxa under ambient storage condition by analysing germination behaviour and metabolic status of seeds.

2. MATERIALS AND METHODS

Experiments of the present investigation were carried out with freshly collected seeds of seven wild plant taxa (*viz*., *Gaultheria hookeri* C. B. Clarke, *G. stapfiana* Airy shaw, *G. semi-infera* (C. B. Clarke) Airy Shaw, *G. trichophylla* Royle var. *ovata* Panda & Sanjappa, *Lyonia ovalifolia* (Wall.) Drude var. *ovalifolia*, *Pieris formosa* (Wall.) D. Don and *Vaccinium glauco-album* C. B. Clarke) in the family Ericaceae from Sikkim Himalaya at altitudes ranging from 2500 – 4000 m. Seven taxa are correctly identified at Central National Herbarium (CAL).

After surface sterilization (0.1% $HgCl_2$ for 90 seconds) the seed samples were separately pre-soaked in the aqueous solution of Na-dikegulac (Na-Dk, 100 µg/ml) for 4 hours and then dried back to the original dry weight of the seeds. This was repeated twice allowing maximum penetration of the chemicals present in the aqueous solution.

The pretreated seed lots were taken in separate cloth bags and kept under alternating freezing (4 hours) followed by thawing (32±2 ºC for 4 hours) in two installments. This experimental set up was kept at 32±2 ºC for 20 days allowing the seeds to experience natural ageing treatment. Data were recorded after zero (0) and 20 days. To analyse the percentage germination, four groups of 100 seeds *i.e.* 400 seeds of each treatment were transferred to separate Petri dishes containing filter paper moistened with 10 ml distilled water. Germination data were recorded after 96 hours of seed soaking following the International Rules for Seed Testing (ISTA, 1976). The time for 50% germination of seeds (T_{50}) was determined following the method described by Coolbear *et al*. (1984).

Protein contents as well as the activity of catalase enzyme was analysed from seed kernels of each sample. Protein level was estimated as per the methods of Lowry *et al.* (1951). Extraction and estimation of the enzyme catalase was made following the method of Snell and Snell (1971) as modified by Biswas and Choudhuri (1978). For assaying these enzymes, the blank was taken as zero time control and the activity was expressed as $(\Delta OD \times T_V)/(t \times v)$, where ΔOD is the difference of OD of the blank and sample. T_V is the total volume of filtrate, t is the time (min) of incubation with the substrate and v is the volume of filtrate taken for incubation (Fick and Qualset, 1975). Data were statistically analysed at the treatment and replication levels and least significant difference (LSD) values were calculated at 95% confidence limits (Panse and Sukhatme, 1967).

3. RESULTS

Data clearly revealed that under accelerated ageing condition percentage seed germination was significantly decreased both in control and treated seed lots. But the magnitude of decrease was found to be much less when seeds were pretreated with aqueous solution of Na-dikegulac. The seed pretreating agent also significantly reduced the time required for 50% germination (T_{50}) of seeds (Table 1). This effect was found remarkable at later observation periods. The level of protein (Table 2) experimental seeds were remarkably reduced in control samples than the treated ones. The activities of the enzyme catalase (Table 2) was found to decline with ageing process and the declining trend was arrested by the seed pretreateating agent.

4. DISCUSSION

The results of the present study show that during ambient storage the ageing and deterioration of experimental seeds as would be evident from the progressive fall of germination percentage and higher T_{50} hours (Table 1). Pretreatment of the seeds with Na-DK significantly alleviated the loss of germination and reduced T_{50} hours (Table 1), alleviated the loss of protein (Table 2) as well as catalase (Table 2) enzyme.

The proposal that a decrease in membrane lesions might play a significant role in deterioration of seeds has been supported by the work on solute leakage accompanying a loss in germinability and viability (Ching and Schoolcraft, 1968, Harmann and Granett, 1972, Powell and Matthews, 1977). The ability of seeds to reorganize its membrane rapidly as the desiccated tissue rehydrates is a crucial factor for successful germination and this is clearly

documented in the literature (Simon, 1974). Much evidence has been put forward to suggest that membrane status within the germinating embryo is an important factor in deterioration (Harmann and Mattick, 1976; Ponnachan *et al.*, 1993; Desai *et al.*, 1997; Kamalakkannan and Stanely, 2003; Mishra *et al.*, 2004a; Pati & Bhattacharjee 2015). Thus, in the present study, the concomitant reduction of seed germinability is the indicative of damage of seed membrane and consequent loss of seed vigour and viability. The chemical-induced substantial amelioration of all these deleterious effects are indicative of seed potentiation under adverse storage environment. Efficacy of the Na-DK on the maintenance of seed can also be supported from the data on biochemical analysis of seeds kept at ambient storage for 20 days. In the assayed seeds experimental chemical helped to check the decline of protein along with catalase.

The results therefore point out that although deterioration is a common phenomenon in treated and control sample of the seed species, the catabolic processes within the treated seed samples remained somewhat subdued, thereby rendering them tolerant against unfavourable storage environment of plane lands. Available reports show that during seed storage a loss of some vital cellular components including protein, carbohydrates, nucleic acids were also occurred (Kole and Gupta, 1982; Bhattacharjee and Gupta, 1985). Catalase is regarded as a scavenger enzyme (Fridovich, 1976) and higher activity of this enzyme is indicative of higher plant vigour (Sarkar and Choudhuri, 1980; Pati and Bhattacharjee, 2003; Pati & Bhattacharjee 2011 & 2012).

Table 1. Effect of seed pretreatment with Na-dikegulac (Na-DK;100 µg/ml) on percentage seed germination and T50 of seed germination of seven wild plant taxa in Ericaceae from Sikkim Himalaya.

Seeds were presoaked with the chemical or distilled water for 4h and then dried back to original seed weight. This was repeated twice. Pretreated seed samples were kept under ambient storage condition (32 ± 2 ºC) and data were recorded after zero (0) and 30 days.

Different seed samples	Percentage seed generation				T50 of seed germination			
	Days after analyses							
	0		30		0		30	
	Control	Na-DK	Control	Na-DK	Control	Na-DK	Control	Na-DK
Gaultheria hookeri	15.00	28.00	14.0	20.00	NA	NA	NA	NA
G. stapfiana	20.00	30.00	11.00	20.00	NA	NA	NA	NA
G. semi-infera	40.00	56.00	30.00	50.00	NA	78.00	NA	84.00
G. trichophylla var. *ovata*	16.00	26.00	09.00	15.00	NA	NA	NA	NA
Lyonia ovalifolia var. *ovalifolia*	41.00	54.00	30.00	51.00	NA	72.00	NA	78.00
Pieris formosa	43.00	55.00	32.00	50.00	NA	66.00	NA	72.00
Vaccinium glauco-album	40.00	50.00	26.00	48.00	NA	78.00	NA	NA
LSD (p = 0.05)	0.92	1.12	0.32	1.09	NC	5.17	NC	5.08

NC: Not calculated; NA: Non attainment of 50 % germination of seeds

Table 2. Effect of seed pretreatment with Na-dikegulac (Na-DK;100 µg/ml) on protein (mg/g fr. wt.) and catalase (ΔODxTv/txv) of seven wild plant taxa in Ericaceae from Sikkim Himalaya.

Different seed samples	Protein				Catalase			
	Days after analyses							
	0		30		0		30	
	Control	Na-DK	Control	Na-DK	Control	Na-DK	Control	Na-DK
Gaultheria hookeri	184.00	196.24	100.01	140.01	65.04	74.01	40.01	58.07
G. stapfiana	127.00	138.00	98.90	102.00	50.91	60.13	29.03	40.97
G. semi-infera	178.00	190.00	101.97	132.03	61.00	70.20	30.37	44.98
G. trichophylla var. *ovata*	168.04	180.01	98.88	194.00	58.88	68.28	29.00	42.03
Lyonia ovalifolia var. *ovalifolia*	100.28	112.39	79.07	98.00	44.13	50.01	23.07	38.88
Pieris formosa	163.89	172.92	84.04	102.98	51.07	60.18	28.98	40.01
Vaccinium glauco-album	174.00	188.01	93.06	113.09	52.78	62.25	29.18	41.05
LSD (p = 0.05)	10.04	11.08	8.27	8.38	4.04	4.78	1.93	2.24

Treatments and recording of data as in Table 1.

5. CONCLUSIONS

In this investigation, the chemical-induced arrestation of rapid loss of the enzyme activity was indicative of strengthening the defence mechanism by the chemical under adverse storage condition. It can be concluded from the results of this investigation that Na-DK is effective in enhancing storage potential of experimental seeds. Thus, invigouration property of the present seed pretreating agent seems to be apparent from our experimental results.

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RECOLLECTION OF *CRAIBIODENDRON HENRYI* W.W. SMITH (ERICACEAE: VACCINIOIDEAE), A POTENTIAL ETHNOMEDICINAL PLANT FROM ARUNACHAL PRADESH AFTER SEVEN DECADES[1]

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Introduction

Craibiodendron henryi W.W. Sm. was first described by William Wright Smith (1912) based on collections by Augustine Henry from Szemao forest in China. Surprisingly, in the same year and in the same journal, Smith (1912) first described *C. mannii* based on incomplete collections by Gustav Mann from Meghalaya in India, which were actually an Indian population of *C. henryi* W.W. Sm. The taxon *Craibiodendron mannii* was an imperfectly known species to Smith, as the specimen had only immature flower buds and leaves. The German botanist Gustav Mann, who was a gardener at the Royal Botanic Gardens, Kew, collected specimens of *C. henryi* (syn. *C. mannii*) from Juudmaka Pahar near Jowai, in the present Jaintia Hills district of Meghalaya in August, 1891. Subsequently, this species was collected by F. Kingdon-Ward from Dalai Valley ('Delei valley') of Arunachal Pradesh in 1928; by N.L. Bor from Naga Hills in Nagaland during 1935–1936; by A.A. Mao from Lower Subansiri district, Arunachal Pradesh, in 2010 (flower buds); and by S. Panda from Lower Subansiri district, Arunachal Pradesh in 2014 and 2019 (fruiting specimens). Therefore, the specimens collected by Mao and Panda from Arunachal Pradesh are considered as recollection after Mann in 1891 from Meghalaya, Kingdon-Ward in 1928 from Arunachal Pradesh, and Bor in 1936 from Nagaland.

Fig. 1: Immature flowering twig of *Craibiodendron henryi* collected from Pange Forest in May, 2010 (A.A. Mao 23282)

Fig. 2: Fruiting twig of *Craibiodendron henryi* at Pange forest in November 2014 (S. Panda 132)

Judd (1986) revised the genus *Craibiodendron* W.W. Sm. and included a single species, *C. henryi* (syn. *C. mannii*) from India. He mentioned that the 'amplified description of *C. mannii*' by Rao and Chakraborty (1982) is clearly conspecific to *C. henryi* in respect of the long acuminate leaves and deeply lobed campanulate corolla.

Panda and Sanjappa (2014) revised the genus in the Indian context and mentioned only one species, *C. henryi* (syn. *C. mannii*). Although Deb (1961) reported *C. stellatum* (Pierre) W.W. Sm. from Shugnu in Manipur, India (Deb 2633), the specimen is untraceable in CAL and ASSAM.

Recently, Mir *et al*. (2019) in their 'Checklist of Endemic Flora of Meghalaya' included *C. henryi* W.W. Sm. based on earlier collections by Mann and King's collector from Meghalaya in 1891. Rao and Chakraborty (1982) in their amplified description of *C. mannii* W.W. Sm. (synonym of *C. henryi*), mentioned that 'there is no recent collection of this plant so far [in the] herbarium consulted'.

Taxonomy: ***Craibiodendron henryi*** W.W. Sm., Notes Roy. Bot. Gard. Edinburgh 5: 158. 1912; Judd, J. Arnold Arbor. 67: 459. 1986; F. Ruizheng & P.F. Stevens in Wu *et al*., Fl. China 14: 460. 2005; Panda & Sanjappa in Sanjappa & Sastry, Fasc. Fl. India 25: 228. 2014. Type: China, Yunnan, Szemao forest, 6000 ft, *A. Henry* 13137 (lecto. E, *n.v*.; isolecto. K, image !).

C. mannii W.W. Sm., Notes Roy. Bot. Gard. Edinburgh 5: 159. 1912; T.A. Rao & S. Chakraborty, J. Bombay Nat. Hist. Soc. 79: 223. 1982; Judd, J. Arnold Arbor. 67: 459. 1986; Panda & Sanjappa in Sanjappa & Sastry, Fasc. Fl. India 25: 228. 2014.

Type: INDIA: Meghalaya, Jaintia Hill district, Juudmaka Pahar near Jowai, 1,828.8 m, August 1891, *G. Mann s.n*. (CAL!). Figs 1 and 2.

Vernacular Name: *Patohamang* (Apatanese of Manipolyang).

Description: Erect, robust treelet to medium-sized tree, up to 15 m tall (usually 5–10 m). Stems profusely branched, glabrous; twigs beset with minute deciduous hairs (seen in live specimens). Leaves alternate to subalternate, coriaceous; lamina lanceolate, oblong-lanceolate to elliptic, 6.5–12 x 2–3.7 cm, entire, slightly revolute at margin, cuneate at base, long acuminate to caudate-acuminate at apex, acumen up to 14 mm long, glabrous; venation brochidodromous with 8–12 pairs of secondary veins, obscure above, conspicuous beneath; petioles stout, 7–12 mm long, pubescent beneath. Inflorescence usually axillary, rarely terminal, panicle-like cymes, perulate; primary rachis 10–24 cm long, 14–45-flowered (*A.A. Mao* 23282, ARUN), sparsely pubescent, producing secondary and tertiary branches. Immature flower buds 4–5 mm long, greenish-white; pedicels 2–3 mm long, puberulous. Bract 1, basal, ovate-triangular, 1.5–2 mm long, caducous. Bracteoles 2, subbasal on pedicel, *c*. 1.5 mm long, caducous. Calyx lobes 5, basally connate, persistent in immature fruits, broadly ovate-triangular, *c*. 1.5 x 2 mm, puberulous inside, glabrous outside. Immature corolla greenish-white (*A.A. Mao* 23282, ARUN) globose-campanulate in bud, 2–4 mm long, glabrous. Stamens immature, 10, *c*. 1.5 mm long; filaments *c*. 1 mm long, slender, geniculate, basally dilated, anther-filament junction spurless; anther lobes minute. Ovary ovoid-globose, *c*. 1 x 1 mm, glabrous; style *c*. 1 mm long; stigma truncate. Fruits (*S. Panda* 132 & 516 DGC & MAC) loculicidal 5-valved capsule with persistent style and withered calyx, ovoid to globose-ovoid, 9–14 x 9–16 mm, fruiting pedicel sparsely pubescent, *c*. 9 mm long. Seeds obconical, ovoid, winged, *c*. 5 x 4 mm, scariose.

Distribution: INDIA: Arunachal Pradesh, Meghalaya and Nagaland. EXTRALIMITAL: China (Yunnan); northern Myanmar (Htawpaw); northern Thailand (Chiang Mai).

Habitat: Along rocky slopes in discontinuous patches in association with *Lyonia ovalifolia* and *Rhododendron* spp. at *c*. 1,600–2,000 m altitude.

Flowering: June to August (peak in July). **Fruiting**: Late September to late December (peak in mid-October to mid-November).

Specimens examined: **Arunachal Pradesh**: Lower Subansiri district, Forest in vicinity of Pange Inspection Bungalow 27° 32.896′ N & 93° 53.790′ E, 1,800 m, 31.v.2010, *A.A. Mao* 23282 (fl. buds: ARUN-Arunachal Pradesh Regional Centre Herbarium, Itanagar, Botanical Survey of India); Lower Subansiri district, Forest in vicinity of Pange Inspection Bungalow 27° 32.896′ N & 93° 53.790′ E, 1,800 m, 17.xi.2014, *S. Panda* 132 (fr: Darjeeling Govt College Herbarium-DGC & Maulana Azad College Herbarium-MAC); 1 km from Pange toward Talle Valley, 27° 32.565′ N & 93° 53.395′ E, 1,874 m, 12.xii.2019, *S. Panda* 516 (MAC); 1 km from Pange toward Manipolyang, 27° 32.155′ N & 93° 53.195′ E, 12.xii.2019, 1,846 m, *S. Panda* 524 (MAC); Dalai Valley ('Delei valley'), 1928, *F. Kingdon-Ward* 7974 (K, Cibachrome image!). Meghalaya: Juudmaka Pahar, Jowai, Jaintia Hill district, 1891, *G. Mann s.n*.; Jowai, 1891, *King's Collector* [G. Mann] *s.n*., acc. no. 38805 (ASSAM). Nagaland: Naga Hills, Kekrima, 1935, *N.L. Bor* 2828 (DD); Naga Hills, Pedi, May, 1936, *N.L. Bor* 6271 (ASSAM).

Ethnomedicinal uses: Extract of tender leaves is used to cure old wounds, skin rashes, and to clear skin spots, by the Apatanese of Manipolyang, Soro, and Hapoli. Extract of

seeds is boiled with coconut oil, allowed to cool and applied on the skin to remove skin spots.

IUCN Status: According to the IUCN Red List of Threatened Species (version 14, 2019), the species is not assessed as yet, but all three populations of *C. henryi* W.W. Sm. of Lower Subansiri, Arunachal Pradesh, are well protected under Talle Wildlife Sanctuary. Although these populations are restricted to three sites within an area of 2 sq. km, fortunately they are not reduced yet, as observed in December, 2019 by the first author, nearly 5 years after the last visit (November, 2014). The three sites are: Site I – Four trees and two shrubs near Pange Inspection Bungalow *c*. 10 sq. m; Site II – Two trees and two shrubs at intervals of *c*. 1 km towards Pange-Talle Valley trek route, *c*. 5 sq. m; Site III – Two trees just 1 km before Pange from Manipolyang, in an area *c*. 4 sq. m.

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