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Karyotype analysis on *Gloriosa superba* using enzymatic maceration and air-drying-based Giemsa, DAPI, and CMA staining techniques

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Summary *Gloriosa superba* is a highly valued vegetatively propagating rhizomatous medicinal plant. Unfortunately, unrestricted overexploitation from its natural habitat has made the species endangered. Intraspecific variations have been reported but, detailed genetic diversity analysis at the chromosomal level is unavailable. This study has standardized an enzymatic maceration and air drying (EMA) protocol followed by sequential staining with Giemsa, 4′-6-diamidino-2-phenylindole (DAPI), and chromomycin A_3 (CMA) in three Indian germplasm of *G. superba*. Chromosomal analysis on cytoplasm-free metaphase plates with distinct chromosomal configurations has revealed that two wild populations (I and II) are diploid with $2n=22$ chromosomes, and the horticulture population (III) has $2n=22–80$ plus chromosomes. Two pairs of interstitial secondary constrictions in two wild diploid populations are confirmed through Giemsa, DAPI, and CMA bandings. However, the horticulture population shows terminal secondary constrictions. The DAPI-positive banding pattern in two wild populations generates four and 10 DAPI-positive bands resulting in a differential karyotype formula for population I (2A+4B+16D) and population II (10A+4C+8D). Meiotic investigation of populations I and II has confirmed their diploid nature with 11 bivalents. This repeatable protocol may be useful for applying to any species/population of *Gloriosa* for conservation, phylogenetic analysis, and future crop improvement programs.

Keywords *Gloriosa superba* L., Enzymatic maceration and air-drying method, Giemsa staining, CMA staining, DAPI staining, Karyotype analysis.

Gloriosa superba L. is an economically valuable medicinal plant that belongs to the family Colchicaceae. It is the only Indigenous species of this genus found in various parts of India, including the foothills of the Himalayas. This plant contains many bioactive alkaloids, such as colchicine, lumicolichicine, colichicoside, thiocolchicoside, and gloriosine (Malayandi *et al.* 2019; Misra *et al.* 2020), which are present in different parts of the plant and are beneficial for treating various ailments, such as cancer, arthritis, gout, rheumatism, inflammation, ulcers, bleeding piles, and snakebites (Chatterjee and Ghosh 2015; Ionkova *et al.* 2022; Tirkey *et al.* 2023). Additionally, colchicine is used in traditional breeding for induced artificial polyploidy and as a mitotic blocker in plant cytogenetics (Niazian and Nalousi 2020; Cui *et al.* 2023; Wang *et al.* 2023). The genus *Gloriosa* has around 10 to 15 species with notable morphological variations within and among them (Vinnersten and Reeves 2003; Vinnersten and Manning 2007; Maroyi 2012; Chatterjee and Ghosh 2015; Umavathi *et al.* 2020). *G. superba* is in high demand resulting in its overexploitation in the wild. As a result, the International Union for Conservation of Nature (IUCN) has classified it as an endangered species (Mahajan *et al.* 2018). Therefore, it deserves conservation efforts to ensure sustainable use.

Various studies have been conducted on the morphology, biochemistry, and molecular genetics of this plant (Selvarasu and Kandhasamy 2017; Sahana *et al.* 2019; Misra *et al.* 2021; Mahajan *et al.* 2022), however, cytological studies are inadequate. Conventional methods have revealed a polyploid series with $2n=22$, 44, 66, 84, and 88 chromosomes within the genus along with $x=11$ basic chromosome number (Sharma and Sharma 1961; Vijayavalli and Mathew 1990, 1992; Ghosh *et al.* 2009). Limited chromosomal analyses through conventional methods have been carried out in some south and eastern Indian populations of *Gloriosa*, revealing intraspecific polyploid chromosome numbers with $2n=22$, 44, 88, and 90 (Vijayavalli and Mathew 1990, 1992; Ghosh

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et al. 2009). However, karyotype analysis remains incomplete as the authors could not mark the number and position of the secondary constrictions in this species.

Banding techniques have helped in proper chromosome identification with more clarity in many plant species (Fukui 1996; Moscone *et al.* 1996; Guerra *et al.* 2000; Hizume 2015; Yamamoto *et al.* 2019; Jha and Bhowmick 2021; Jha and Halder 2023). The primary objective of molecular cytogenetics is to prepare cytoplasm-free metaphase plates with distinct chromosome morphology. However, except for Sultana *et al.* (2019), no one has attempted fluorescent-based chromosome analysis in this species so far.

To address this gap, we collected three populations of *G. superba*, grew them in pots, documented floral morphology, and successfully developed a repeatable EMA protocol for carrying out detailed chromosomal analyses following sequential staining with Giemsa, 4′-6-diamidino-2-phenylindole (DAPI), and chromomycin A_3 (CMA). Detailed morphometric analysis in two wild populations with Giemsa, DAPI, and CMA stainings helped to generate many cytoplasm-free metaphase plates with distinct chromosome morphology that confirms the number and locations of secondary constrictions along with population-specific DAPI bands. Karyotype analysis is also carried out in a horticultural population. We also carried out gametic chromosome analysis in two diploid populations. The repeatable protocol may be useful for applying to any species/populations of *Gloriosa* for conservation, phylogenetic analysis, and crop improvement programs in the future.

Materials and methods

Plant material collection

Three populations of *Gloriosa superba* L. were collected from different regions of India. Two wild populations (I and II) were obtained from the natural habitats of the districts of Hooghly and Jhargram, West Bengal, respectively, while population III was collected from a nursery in New Delhi, and designated as a horticultural population. The plants were grown in pots and eventually flowered.

EMA protocol for chromosome preparation

At least ten root tips of 0.7–1 cm were collected for each population between 10–11 a.m. and they were pretreated with 2 mM hydroxyquinoline for 4 h at 20°C, fixed in 3 : 1 methanol : acetic acid overnight, and kept at –20°C. The EMA chromosome processing was performed using our previous protocol (Jha 2021) with necessary modifications. Fixed root tips were incubated in an enzyme mixture containing 1% cellulase Onozuka RS, 0.75% macerozyme R-10, 0.15% pectolyase Y-23, and 1 mM EDTA for 75–85 min at 37°C. After enzymatic digestion, the root tips were transferred using a Pasteur pipette to a Petri dish filled with water and incubated for 15–20 min. Each root tip was placed individually on a one-end frosted glass slide and macerated with a fine-tip metallic tweezer and freshly prepared fixative (1 : 3, acetic acid : methanol). The slides were then air-dried for at least 2 h before being used for sequential staining of chromosomes.

Sequential staining of chromosomes

Air-dried chromosome preparations were then stained with 1.5% Giemsa solution for 10–15 min before being studied under a microscope. To study fluorescent bandings in these germplasms, sequential staining of chromosomes with two fluorochromes of DAPI and CMA was carried out as described by Jha and Bhowmick (2021), with the required modifications. The slides were then de-stained in 70% methanol and sequentially stained with DAPI and CMA. The treatments were carried out at room temperature under minimal light. For DAPI staining, the slides were incubated in McIlvaine buffer for 30 min, followed by staining with 0.2 µg mL^{-1} DAPI for 10–15 min. They were then rinsed with the buffer, counterstained with 0.25 mg mL^{-1} actinomycin D for 6–10 min, rinsed again with the buffer, and mounted with glycerol. For CMA staining, the slides were de-stained in the fixative for at least 1 h, air-dried, and then incubated with McIlvaine buffer for 30 min, followed by McIlvaine buffer containing 5 mM $MgCl_2$ for 15 min. Slides were stained with 0.2–0.5 mg mL^{-1} CMA for 100–120 min, mounted with glycerol, and stored at 4°C

Table 1. The salient karyomorphometric features of two diploid populations of *Gloriosa superba* deciphered by EMA-based Giemsa, DAPI, and CMA staining.

Population of *G. superba*	Somatic and gametic chromosome number	Diploid TLC in µm (mean±SD)	CSR in µm (mean±SD)	ACL in µm (mean±SD)	No. of SAT chromosomes (pair number)	Diploid Karyotype formula	Number of detected fluorescent bands/signals			Diploid fluorescent karyotype formula*
							DAPI positive	CMA positive	Total	
Population I (Hooghly)	$2n=22$; $n=11$	163.30^{b}±8.86	5.3±0.55–11.96±1.55	7.42^{q}±0.40	4 (5th and 7th)	18m+2sm.sm+2m.sm	2	4	6	2A+4B+16D
Population II (Jhargram)		134.74^{a}±10.66	4.10±0.17–10.37±1.22	6.12^{p}±0.48		16m+4sm+2sm.sm+2m.sm	14	4	18	10A+4C+8D

CSR: Chromosome size range, ACL: average length of chromosomes, TCL: Total chromosome length, SAT: satellite bearing chromosomes; Values followed by same letters are not significantly different at $p \leq 0.05$ according to ANOVA and DMRT for each data point; *Fluorescent banding types- A: Centromeric DAPI-positive, B: Nucleolar CMA-positive, C: Centromeric DAPI-positive+Nucleolar CMA-positive, D: DAPI-CMA neutral.

for 72 h. All the microphotographs were taken using a Carl Zeiss Axio Lab A1 microscope equipped with DAPI and CMA filter cassettes and attached to a CCD camera. For each population of *G. superba*, a minimum of ten Giemsa-stained metaphase plates were evaluated to determine and confirm the somatic chromosome numbers. Different chromosome morphometric data such as the length of the long arm and short arm, absolute chromosome length, relative chromosome length, average chromosome length (ACL), and total chromosome length (TCL) were measured and calculated for each population from the captured photographs using an Axiovision L. E4 software. Chromosome types, such as median point (M), median region (m), submedian region (sm), subterminal region (st), terminal region (t), and terminal point (T), were determined following the classification of Levan *et al.* (1964). Idiograms were prepared using the mean values obtained from at least five metaphase plates of populations I and II. Chromosomes were numbered from 1st to 11th according to their total length in descending order. More than ten metaphase plates of each population were used to analyze for the determination of DAPI and CMA fluorescent banding patterns, and chromosomes were classified into four types based on their fluorescent banding patterns. Centromeric DAPI-positive band-bearing chromosomes were designated as type A, while those with nucleolar CMA-positive bands were designated as type B. The chromosomes with both centromeric DAPI-positive and nucleolar CMA-positive bands were designated as type C, and the chromosomes showing neither DAPI nor CMA bands were designated as type D.

Gametic chromosome analysis

For gametic chromosome analysis, *Gloriosa* flower buds from populations I and II were fixed in 1 : 3 acetic alcohol at 11 a.m. for 24 h. Slides were then prepared with 2% acetocarmine following the standard protocol (Sharma and Sharma 1980).

Statistics analysis

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 an SPSS v 16.0 statistical package (SPSS Inc. IBM, Chicago).

Results

The study reveals significant differences in the floral features of population III, collected from New Delhi, as compared to populations I and II. These differences include flower size, shape, and color, as shown in Fig. 1A–C. The study also found differences in tuber sprouting and flowering time. Population III started sprouting and flowering in February and April, respectively, while populations I and II sprouted in June and flowered in August.

The study successfully standardized the cost-effective EMA method in two Indian wild populations of *G. superba*. It is also the first report of DAPI and CMA bandings in this species. Enzyme digestion for 80–85 min followed by a 10 min Giemsa staining protocol produced well-scattered metaphase plates with distinct chromosome morphology, as shown in Fig. 2A–F. The study confirmed $2n=22$ chromosomes in populations I and II by analyzing more than ten cytoplasm-free metaphase plates produced by the EMA method and sequential staining with Giemsa, DAPI, and CMA. The TCL is significantly higher in population I (163.30±8.86 µm) than in population II (134.74±10.66 µm). The size of the chromosome varies in a range of 11.96±1.55 to 5.3±0.55 µm in population I and 10.37±1.22 to 4.10±0.17 µm in population II. Both populations have two pairs of satellite-bearing sm chromosomes (5th and 7th pairs). The karyotype formula in population I is 18m+2sm.sm+2m.sm, and in population II, it is 16m+4sm+2sm.sm+2m.sm. The idiograms of populations I and II are presented in Fig. 2G and 2H, respectively. A few polysomatic cells are also documented in both populations.

Four chromosomes revealed DAPI-negative and CMA-positive bands in both populations I and II, representing the location of secondary constriction in the

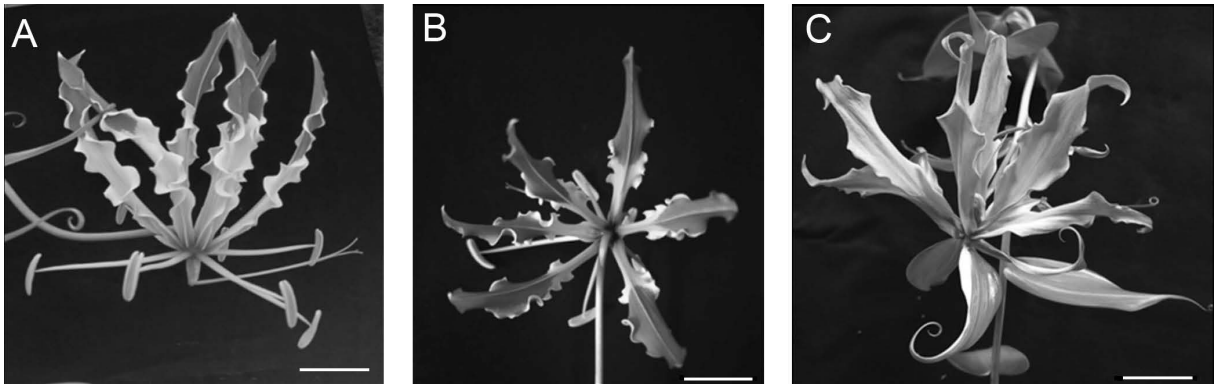

Fig. 1. Flower morphology of *Gloriosa superba*. (**A**) Population I, (**B**) Population II, and (**C**) Population III. Scale bars=2 cm.

interstitial position. No other chromosomes showed a CMA-positive band in the two populations. A differential DAPI-positive band pattern was noted in both populations. Two DAPI-positive bands are detected within the karyotype (Fig. 2B) of population I, whereas 14 interstitial DAPI-positive bands have been recorded in population II (Fig. 2E) for the first time in the metaphase plate. The Giemsa, DAPI, and CMA staining study also found a completely different chromosomal pattern in population III, comprising a polyploid nature with diverse chromosome numbers ranging from $2n$ = more than 22 to 80 plus (Fig. 3A–G). Although detailed karyotype analysis was not performed, terminal secondary constrictions are detected in several chromosomes (Fig. 3A–G). Interestingly, this particular population shows many DAPI-positive bands in terminal and interstitial

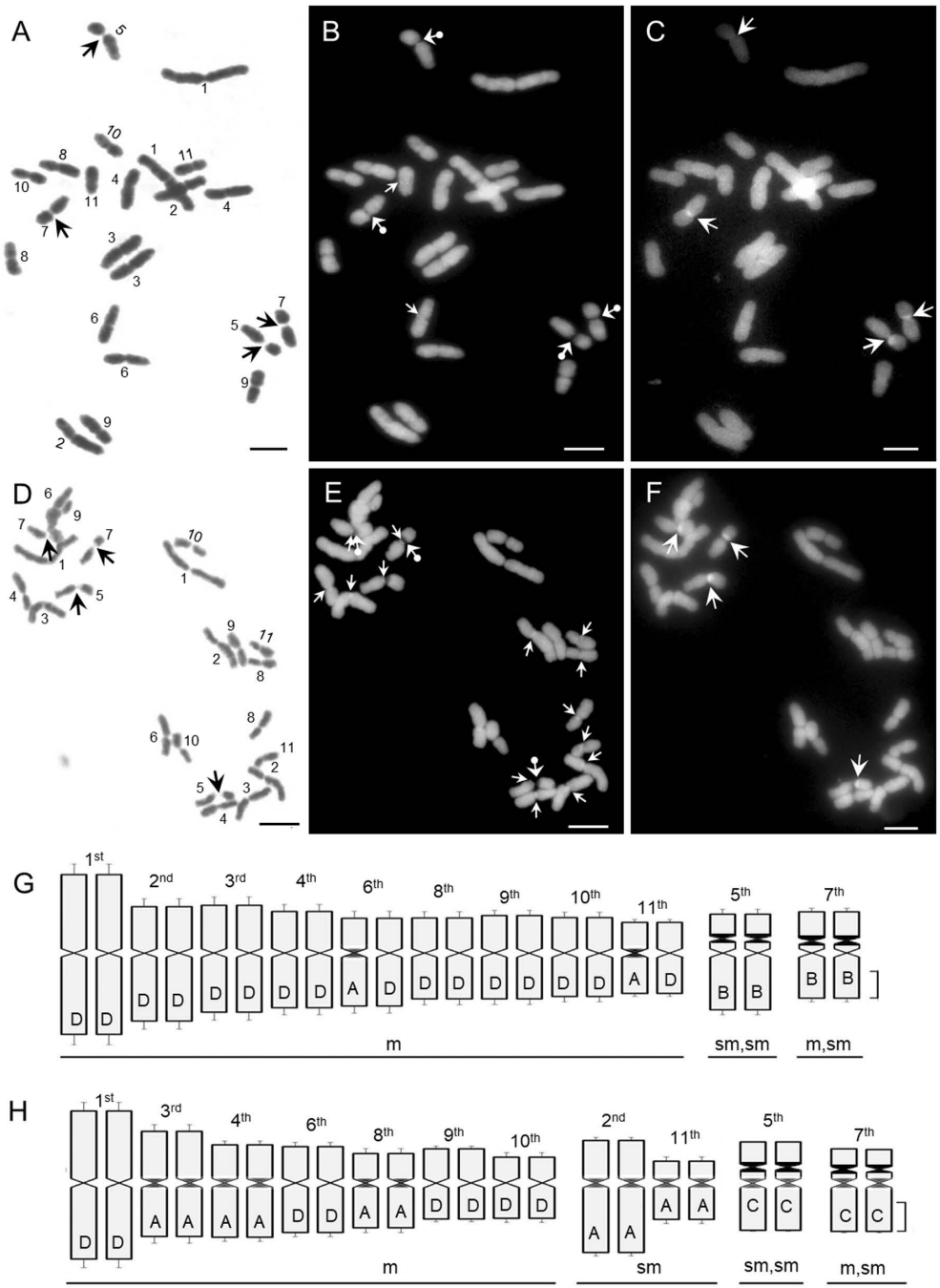

Fig. 2. Somatic metaphase chromosomes ($2n$=22) of *Gloriosa superba*. Population I (Hooghly) was stained with Giemsa (**A**), DAPI (**B**), and CMA (**C**); population II (Jhargram) was stained with Giemsa (**D**), DAPI (**E**), and CMA (**F**); Scale bars=5 µm. Positions of secondary constrictions in two pairs of satellite-bearing chromosomes are indicated with black arrows in Giemsa-stained metaphase plates (A, D). DAPI-positive bands (B, E), and CMA-positive bands (C, F) are marked with white arrows while DAPI-negative bands (B, E) are marked with round-ended white arrows. Somatic idiograms of *G. superba* population I (**G**) and population II (**H**). Scale bars=2 µm. The positions of DAPI-positive and CMA-positive bands are indicated with dark black and gray colored regions in ideograms (G, H), respectively.

regions on their chromosomes (Fig. 3E) compared to diploid populations.

Meiotic investigation in populations I and II revealed n=11 bivalents (Fig. 4A–D). Populations I and II had normal fruiting and seed sets, but population III failed to bear fruits and seeds.

Discussion

Flowers play a vital role in the morphology of plants. Our study has found significant differences in the floral morphology of population III compared to populations I and II (Fig. 1A–C). The morphological variations in this species are reported to be influenced by agroclimatic conditions (Vijayavalli and Mathew 1990; Ghosh *et al.*

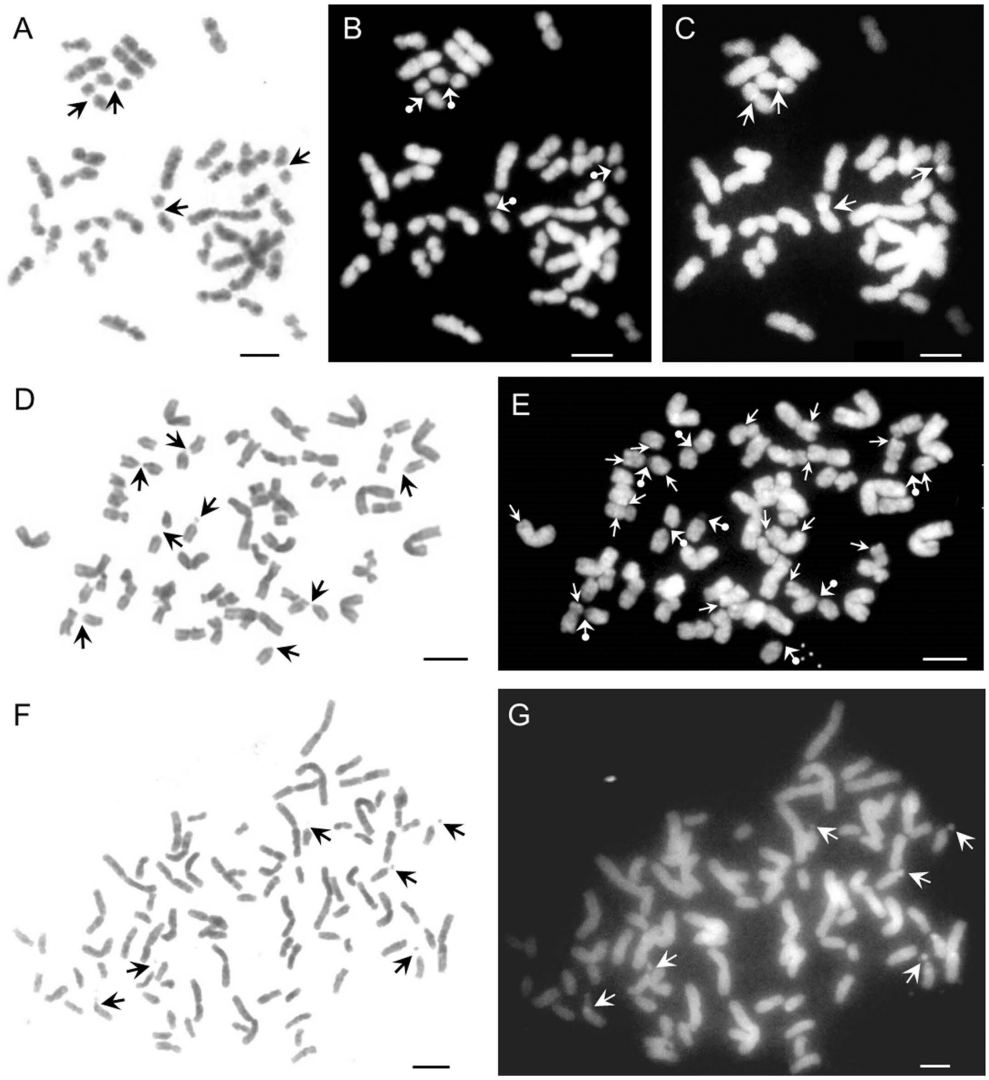

Fig. 3. Somatic metaphase chromosomes of *Gloriosa superba* population III (New Delhi) stained with Giemsa (A, D, F), DAPI (B, E), and CMA (C, G). Positions of secondary constrictions are indicated with black arrows in Giemsa-stained metaphase plates (A, D, F). DAPI-positive bands (E), and CMA-positive bands (C, G) are marked with white arrows, while DAPI-negative bands (B, E) are marked with round-ended white arrows. Scale bars=5 µm.

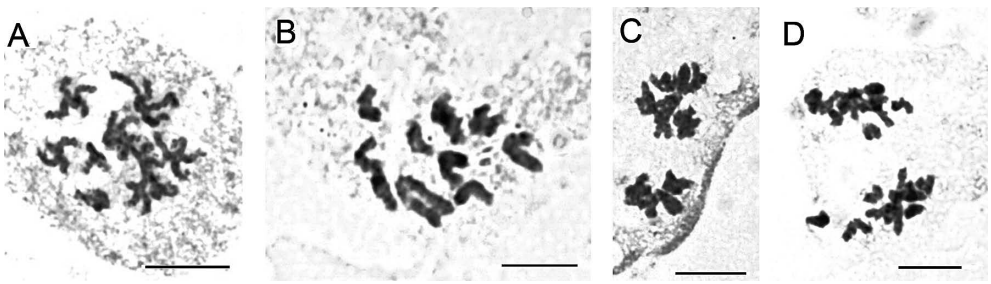

Fig. 4. Meiotic chromosomes of *G. superba*. (**A, B**) metaphase I with 11 bivalents, (**C, D**) anaphase I. Scale bars=5 µm.

2009), whereas chromosomal features are not affected by environmental influences. Population III displays diverse chromosome numbers ranging from $2n =$ more than 22 to 80 plus (Fig. 3A–G), which could be attributed to the population's anomalous floral morphology and sterility. This underscores the potential utility of morphological and chromosomal correlations in future studies and conservation efforts for *Gloriosa*.

Meiotic investigation in both populations I and II revealed n=11 bivalents with a tendency toward secondary association (Fig. 4A–D) without affecting the seed setting. Secondary association of bivalents resulting from a high degree of clumping of chromosomes at metaphase I, have been reported in different *G. superba* morphotypes (Vijayavalli and Mathew 1992).

The literature review presents inadequate cytogenetic information about *G. superba*. However, authors who worked on some Indian populations have reported different chromosomal counts ($2n$=22, 44), intraspecific karyotype diversity, deletion of a chromosome arm, heteromorphic pairing, and polysomaty. Diversity in the karyotype formula has also been presented as 8m+14sm by Narain (1981), 11m and 10m+1sm by Vijayavalli and Mathew (1990), and 4M+16m+2sm, 8M+10m+4sm, and 8M+14m by Ghosh *et al.* (2009). There was no karyotype formula presented for the polyploid population. All the earlier studies have failed to identify the number and position of secondary constrictions in any *Gloriosa* populations.

The present chromosomal analysis for the first time presented EMA-based Giemsa staining and fluorescent banding with DAPI and CMA in any Indian *G. superba* populations. This EMA-based chromosome preparation method can overcome the technical limitations of classical chromosome preparations (Jha and Halder 2023). We have successfully standardized the method to obtain large numbers of morphologically distinct metaphase plates for karyotype analysis of *G. superba* following Giemsa and DNA base-specific fluorochromes (DAPI and CMA) for the first time. Applications of Giemsa, DAPI, and CMA stainings on the same metaphase plates have helped us to confirm the numbers and patterns of secondary constrictions in diploid populations as well as to unravel different patterns of secondary constrictions in the polyploid population. Karyotypes of both diploid populations carry two pairs of interstitial secondary constrictions on the 5th and 7th pairs of chromosomes. With DAPI and CMA stainings, these pairs show DAPI-negative and CMA-positive banding patterns (Fig. 2B, C, E, F). Secondary constrictions are part of the nucleolar organizing region (NOR) and are generally comprised of GC-rich sequences (Guerra *et al.* 2000). Accordingly, DAPI and CMA stains produced contrasting banding patterns on the 5th and 7th pairs of chromosomes. These fluorescent banding patterns also confirm the occurrence of secondary constrictions at interstitial positions (Fig. 2B, C, E, F). Interstitial secondary constrictions have been confirmed through conventional and fluorescence banding in cultivated lentils (Ladizinsky 1979; Jha 2021). The present study is another confirmation that fluorescent banding can be used for the identification of secondary constrictions and to unravel hidden molecular features directly on the chromosomes.

In population I, fluorescent banding with DAPI has generated DAPI-positive bands in two chromosomes within the karyotype (Fig. 2B), whereas 14 interstitial DAPI-positive bands were detected in population II for the first time (Fig. 2E). These chromosomal banding patterns have unraveled differences between the two morphologically alike karyotype populations. Besides the secondary constricted chromosomes, no other chromosomes within the karyotypes of both populations show CMA-positive bands.

On the other hand, Giemsa, DAPI, and CMA stainings confirm the detection of terminal secondary constrictions on several chromosomes in population III (Fig. 3A–G) instead of interstitial observed in populations I and II (Fig. 2A–F). This particular population shows many DAPI-positive bands in terminal and interstitial regions on their chromosomes (Fig. 3E) compared to diploid populations. Further study is needed to verify the taxonomic status of population III. All these facts highlight the necessity of the application of basic molecular cytogenetics investigation in the genus *Gloriosa*, particularly in the context of chromosomal diversity, conservation, and potential taxonomic implications.

Author contributions

TBJ designed and experimented. TBJ, MH, and PC were involved in experimental data acquisition, analysis, and interpretation of the data obtained by the experiment. TBJ and MH have finalized the manuscript. All the authors read and approved the final manuscript.

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