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Polymorphism in *Gloriosa superba*

Seemanti Ghosh[1], Biswajit Ghosh[2] and Sumita Jha[1]*

[1]*Centre of Advanced Study in Cell and Chromosome Research, Department of Botany, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, India and*
[2]*Department of Botany RKMVC College, 24 Parganas (North), Rahara, West Bengal, India*

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Abstract

Gloriosa superba L. is a highly valued indigenous medicinal plant. It is available throughout India with noticeable variations in morphology and colchicine content. In the present study, morphological, phytochemical, cytological and molecular data are employed to screen the polymorphism, if present, in five different populations of *G. superba* L. Based on gross morphological traits, the plants can be grouped into two clusters: Amtala (AM) and Baruipur (BR) plants belonging to one cluster, and Siliguri (SG), Darjeeling (DJ) and Sikkim (SK) plants belonging to the other. Colchicine content of the tubers ranged from 0.06% in AM plants to 0.37% in BR plants. Cytological analysis revealed that the SK plants showed 44 chromosomes, i.e. tetraploid status, whereas the other four were diploid. Assessment of the karyomorphology of the four diploid populations indicated a close similarity between AM and BR plants, whereas the SG plants exhibited slightly different karyomorphology and lacked *sm*-type chromosomes. Random Amplified Polymorphic DNA (RAPD) analysis revealed 76% polymorphism among the populations. Cluster analysis using RAPD resulted in three main cluster groups; BR and SG plants clustered together indicating that they are most closely related when compared with other populations.

Keywords: colchicine; diversity; karyomorphology; markers; RAPD

Introduction

Plant populations vary in space and time (Linhart *et al*., 1981). The phenotypic expressions are pertinent to habitats and differentiation is the reflection of variable environmental conditions. Consequently, populations from dissimilar habitats might show variable morphological expressions. Various chemical, physiological and genetic changes have also been reported in response to environmental instability (Brown, 1979; Hamrick, 1983). Morphological features alone are inadequate for studying genetic polymorphism, owing to their limited representation of the total genome and the high degree of environmental modification to which they are susceptible (Forrest *et al*., 2000). Molecular markers are of high value for assessing general levels of genotypic variation within and between populations (Forrest *et al*., 2000). In spite of the accumulating molecular data, chromosome information continues to be important in assessing phylogenetic relationships (Carr *et al*., 1999).

Gloriosa superba (Liliaceae) is an indigenous medicinal plant (Anonymous, 1956), distributed throughout India from 68.7°E to 97.25°E and 8.4°N to 37.6°N and 0 to 2043 m.a.s.l. The plants of this species have proved highly adaptable to extreme conditions, including temperatures ranging from 22 to 32°C. Tubers of *G. superba* are sold in Indian herbal market as an important source of the alkaloid colchicine. Apart from its action on mitosis, colchicine reduces the inflammation and relieves the pain associated with acute gout (Insel, 1996), and it is primarily used for this purpose. Colchicine therapy diminishes the metabolic activity of leukocytes, resulting in reduced phagocytosis of urate microcrystals, thereby interrupting the cycle of new crystal deposition (Seegmiller *et al*., 1962). Identification of this medicinal

* Corresponding author. E-mail: sjha_123@yahoo.co.in

herb is essential because these are usually sold as tubers that cannot be identified according to morphological and histological characteristics. It was observed that an extreme degree of variability existed in *G. superba* with respect to habitat, colchicine content and morphological characteristics of plants in different parts of India, and there is no report that addresses the variation among the plants of various habitats.

Random Amplified Polymorphic DNA (RAPD) employs single short primers with an arbitrary sequence to generate genome-specific 'fingerprints' of multiple amplification products. Polymorphisms found between RAPD profiles can serve as genetic markers (Williams *et al.*, 1990), and thus this technique has been widely used to produce DNA polymorphisms in many medicinal plant species (Garcia-Mas *et al.*, 2000; Cabrita *et al.*, 2001; Ran *et al.*, 2001; Ho *et al.*, 2002; Aros *et al.*, 2006).

The purpose of the present investigation was to analyse the extent of diversity, if any, among different populations of *G. superba*.

Materials and methods

Collection of plant material

Tubers of *G. superba* (~1 year old) were collected from five different locations of the eastern part of India, Sikkim (SK), Baruipur (BR), Amtala (AM), Darjeeling (DJ) and Siliguri (SG), during the harvest season (October). The altitude ranged from 0 to 2043 m.a.s.l. and the temperatures during harvest ranged from 24 to 32°C. The details of the collections from the five locations are summarized in Supplementary Table 1 (available online only at http://journals.cambridge.org). These tubers were planted and maintained in the Experimental Garden of the Department of Botany, University of Calcutta, and tubers or other plant parts were procured from these stocks.

Morphological study of different populations

The morphological characteristics measured for a sample of 15 plants per collection location were plant height, leaf size, internodal length, tuber size, phyllotaxy and flower colour. Measurement of 1-year-old tubers were taken; only mature leaves were used for the determination of size, and internodal length was measured from the lower and mature parts of each plant.

Extraction and quantification of colchicine from tubers

Colchicine was extracted and analyzed following the method of Hayashi *et al.* (1988) from 15 tubers per population. The tubers were thoroughly washed, dried at 50°C, crushed into powder with mortar and pestle and extracted with methanol for 6 h at 40°C. The extract was evaporated, and the residue redissolved in distilled water, partitioned against *n*-hexane and finally the aqueous phase containing colchicine was extracted with chloroform. The chloroform was evaporated and the residue dissolved in high-pressure liquid chromatography (HPLC)-grade methanol, passed through 0.22 μm filter and subjected to HPLC following Hayashi *et al.* (1988). A quantitative analysis was performed on Shimadzu liquid chromatograph (LC-10 AD) employing a reverse-phase ODS-Hypersil C-18 column (150 mm × 4.6 mm id), a Supelco guard column (Pelliguard TM LC-18 kit with 2 cm × 4.6 mm cartridge) and isocratic elution with acetonitrile:methanol:water (35:5:60). The flow rate was 1 ml/min and all chromatographs were plotted at 243 nm, using a UV detector. Colchicine was identified by comparing the retention time of the sample with that of the authentic colchicine (Sigma®; Ghosh *et al.*, 2002) and spiking with standard.

Cytological analysis

Root tips were pretreated in *p*-dichlorobenzene for 4 h at 16°C, fixed overnight in acetic acid and ethanol mixture (1:3) and stained with 9:1 2% aceto-orcein:HCl (w/v). Tissues were then squashed with 45% acetic acid and observed under a light microscope. The mitotic metaphase plates were photographed to prepare karyotypes from 1500× magnification, following the nomenclature of Levan *et al.* (1964). The chromosomes were classified into four size groups: A, 12.0–9.0 μm; B, 8.99–6.0 μm; C, 5.99–4.0 μm; D, 3.99–3.0 μm. At least 15 individuals per population were analysed cytologically, and for each individual at least 15 metaphases were scored.

PCR amplification of DNA

DNA was isolated from fresh and young leaf (3–4 weeks old) tissues from 15 plants of each population, using the CTAB method of Doyle and Doyle (1987). Twenty-four arbitrary sequence decamers (Operon Technologies Inc., Alameda, CA) were used as primers for PCRs. Each 25 μl PCR contained 400 μM of each dNTP, 1.5 mM $MgCl_2$, 25 pmol primer, 0.25 U *Taq* polymerase, 2.5 μl *Taq* buffer (Promega Corporation, Madison, WI) and 70 ng of template DNA, and was amplified for 45 cycles of 1 min at 92°C, 1 min at 35°C, 2 min at 72°C and one final cycle of 10 min at 72°C. RAPD fragments were separated electrophoretically on 1.5% Tris-Borate-EDTA (TBE) agarose gels and stained with ethidium bromide. Each PCR was repeated at least three times and only

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reproducible fragments were counted. Populations were identified on the basis of presence (1) or absence (0) of polymorphic bands. For quantification of similarity/diversity, calculating distances using Jaccard's similarity coefficient made pairwise comparisons of banding patterns. The resulting distance matrix was used in Unweighted Pair Group Method with Arithmatic mean (UPGMA) cluster analysis to generate the dendrogram for estimating the relationship among populations using the NTSYSpc ver. 2.11v software.

Statistical analysis

Results presented in the tables are mean ± standard error (SE). Data presented are from 15 replicates and each experiment was performed three times. The results were subjected to analysis of variance (ANOVA) to detect the significance of differences among the treatment means (Sokal and Rohlf, 1987). The treatment means were then compared using Duncan's multiple range test (DMRT) at a 5% probability level and different letters (Sokal and Rohlf, 1987) indicate differences.

Results

Variation in morphology and colchicine content

Variations in morphology and colchicine content were noted among the plants of the five populations (Tables 1 and 2). One-year-old SK plants were the smallest (107 ± 1.88 cm) and those of DJ population the largest (173.2 ± 2.32 cm). BR tubers were small (7.7 cm × 1.34 cm long) when compared with those of DJ (12.36 cm × 2.12 cm long). SK plants bore small leaves (5.9 cm × 2.3 cm) with a maximum internodal length of 8.76 cm. BR leaves were large (10.02 cm × 3.64 cm) with an internodal length of 5 ± 0.15 cm. AM and BR plants showed opposite phyllotaxy, whereas those from the other three populations showed alternate phyllotaxy. BR and SG plants had a high colchicine content (0.37 and 0.22%), whereas SK plants contained little colchicine (0.06%; Table 2).

Cytological variation

Cytological analysis revealed that four populations were diploid ($2n = 22$), while the SK plants were tetraploid ($2n = 44$; Fig. 1). However, the root tips of all plants in all the populations showed polysomaty. The frequency of metaphase plates showing <22 chromosomes varied from 17.5 to 20.2% and those showing >22 varied from 9.8 to 22.4% (in AM, BR, DJ and SG). In SK, ~18.3% mitoses showed between 22 and 44 chromosomes. An assessment of the karyomorphology of the four populations (Fig. 2) indicated close similarity between AM, BR and DJ, whereas the SG plants lacked *sm*-type chromosomes (Table 2).

RAPD variation

Out of 24 primers, 9 amplified weakly or not at all, whereas 15 produced clear, intense amplification profiles. Primers with high GC content (≥60%) were the most informative (Supplementary Table 2, available online only at http://journals.cambridge.org). Within-population variation was absent; so a representative plant of each population was used to compare the populations.

A total of 241 RAPD products were scored (average of 16 bands per primer), varying from 7 (OPA-09) to 25 bands (OPA-08) per primer, of which 183 were polymorphic. An average level of 76% of polymorphism was observed.

Out of the five populations studied, BR produced the maximum number of DNA amplified fragments (150) while SK produced 126 bands, which is the minimum number. SG and BR plants showed maximum common bands (130), while the minimum common bands (73)

Table 1. Details of morphological characters and measurements of the five populations of *Gloriosa superba*

Characters	SK	DJ	AM	BR	SG
Leaf length (cm)	5.94 ± 0.2b	8.78 ± 0.2a	9.26 ± 0.1a	10.02 ± 0.1a	8.54 ± 0.15a
Leaf width (cm)	2.3 ± 0.1c	3.02 ± 0.05b	3.42 ± 0.03a	3.64 ± 0.04a	2.52 ± 0.06c
Internodal length (cm)	8.76 ± 0.2a	5.88 ± 0.1c	5.08 ± 0.07c	5 ± 0.15c	6.32 ± 0.19b
Height of plant (cm)	107.0 ± 1.9c	173.2 ± 2.3a	135.0 ± 1.7b	149.8 ± 1.9b	163.8 ± 2.3a
Tuber size (cm)	8.82 ± 0.23c	12.36 ± 0.2a	7.7 ± 0.16c	7.6 ± 0.15c	10.34 ± 0.14b
Diameter of tuber (cm)	1.7 ± 0.05b	2.12 ± 0.05a	1.34 ± 0.06c	1.4 ± 0.04c	1.62 ± 0.03b
Phyllotaxy	Alternate	Alternate	Opposite	Opposite	Alternate
Flower colour	Red	Red	Pinkish red	Pinkish red	Red

Values represent means ± standard error of three experiments with 15 replicates; means followed by different letters differ at $P \leq 0.05$, according to ANOVA and DMRT within a row ($n = 30$).

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Table 2. Colchicine content of the tubers (1 year old) and cytological analysis of the plants of the five collected populations of *Gloriosa superba*

Population	Colchicine content (% dw)	Chromosome number	Karyotype formula
AM	0.37 ± 0.01a	22	2A*M* + 4B*m* + 2C*M* + 8C*m* + 4D*m* + 2D*sm*
BR	0.22 ± 0.02bc	22	2A*M* + 4B*M* + 2C*M* + 8C*m* + 4D*m* + 2D*sm*
DJ	0.13 ± 0.02c	22	2A*M* + 4B*m* + 6C*M* + 4C*m* + 2D*m* + 4D*sm*
SK	0.11 ± 0.01c	44	–
SG	0.06 ± 0.01c	22	4A*M* + 6B*m* + 2C*M* + 6C*m* + 2D*M* + 2D*m*

Values represent means ± standard error of three experiments with 15 replicates; means followed by different letters differ at $P \leq 0.05$, according to ANOVA and DMRT within a column ($n = 30$).
M, median chromosome; *m*, median region chromosome; *sm*, sub-median region chromosome (according to Levan *et al.*, 1964).

were produced by AM and SK plants. The average of bands per population is 8.4, 9.3, 9.8, 10 and 9.6 in the SK, DJ, AM, BR and SG, respectively.

Among the primers used, the highest percentage (100%) of polymorphic fragments was obtained with OPA-09. Primer OPA-08 produced 25 RAPD loci, whereas OPA-09 generated 7 RAPD loci (Fig. 3).

All of the five populations could be identified individually using one population-specific RAPD marker. Primer OPA-05 yielded a 300 bp fragment only in AM, OPA-09 a 900 bp fragment only in DJ and OPA-09 a 500 bp fragment in only SK.

The phylogenetic analysis identified three clusters (Fig. 4): BR and SG plants clustered together, AM comprised a separate cluster while DJ and SK plants formed another cluster.

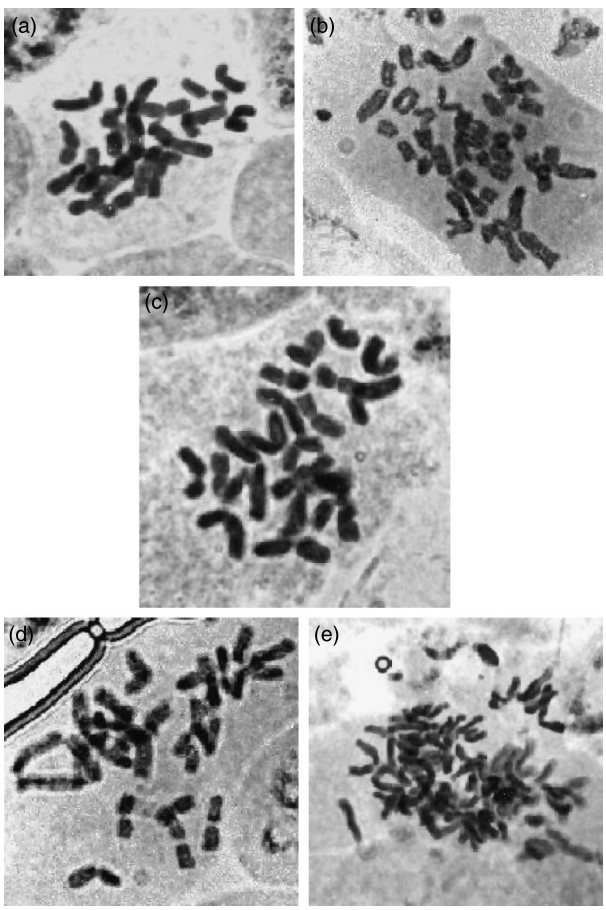

Fig. 1. Metaphase plates from the root tips of the plants of five different populations of *Gloriosa superba*. (a) Amtala, bar 10 μm; (b) Baruipur, bar 10 μm; (c) Darjeeling, bar 10 μm; (d) Siliguri, bar 10 μm and (e) Sikkim, bar 15 μm.

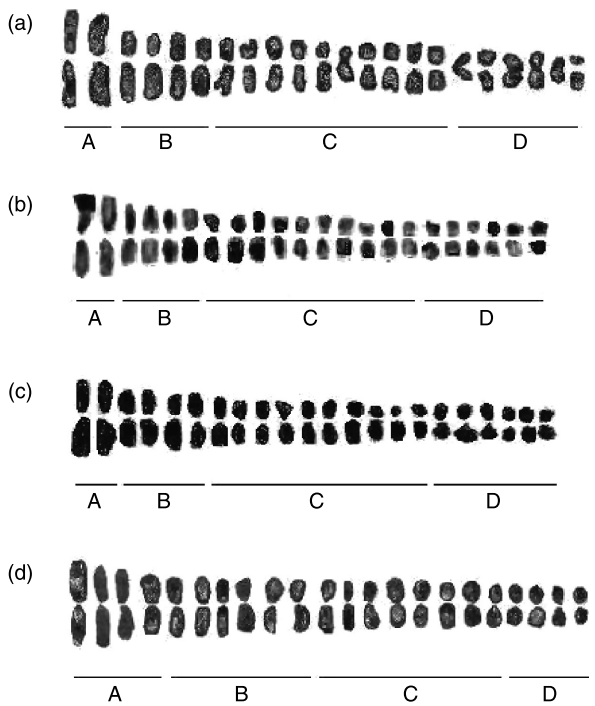

Fig. 2. Comparative karyotypes of the different populations of *Gloriosa superba*. (a) Amtala; (b) Baruipur; (c) Darjeeling; (d) Siliguri.

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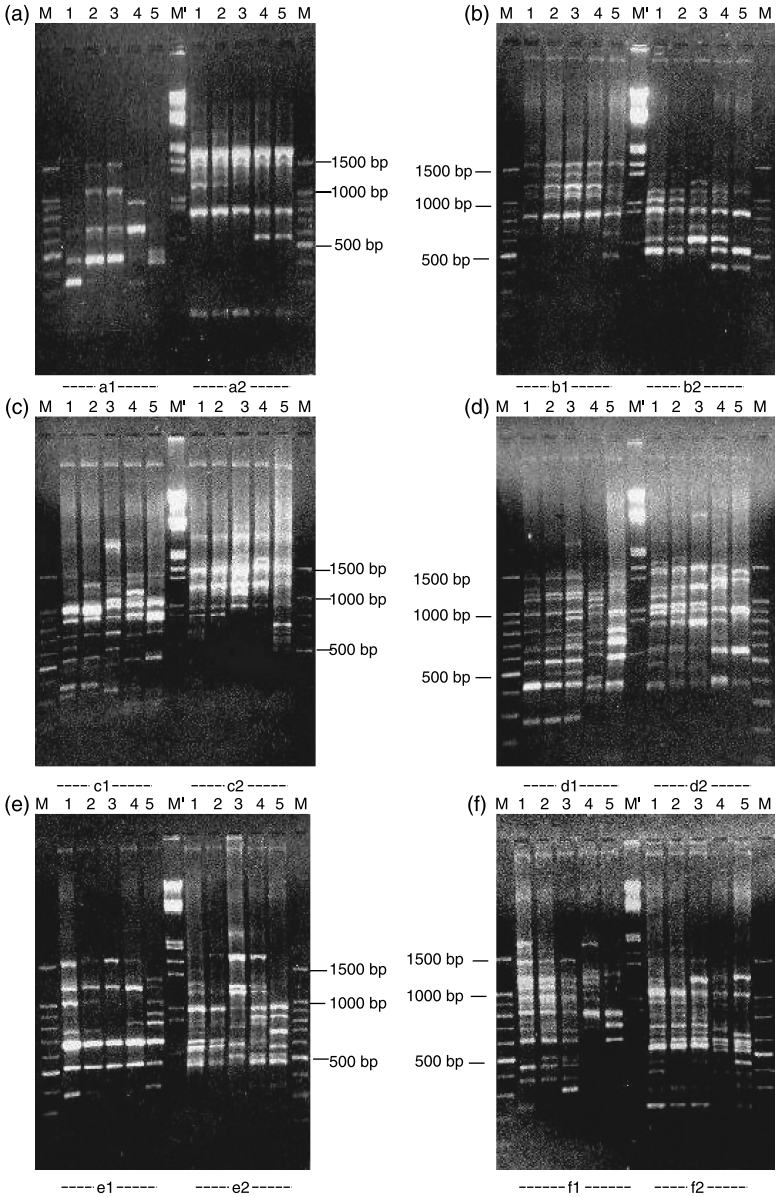

Fig. 3. Amplification profiles of *Gloriosa superba* obtained with 12 primers. Lane M, 100 bp ladder; lane M′, molecular size marker (*Eco*RI + *Hin*dII double digest); lanes 1–5, Sikkim, Siliguri, Baruipur, Amtala, and Darjeeling, respectively.

Discussion

Using markers that reflect the variation present within the genome, genetic polymorphism can be assessed. In the present study, based on gross morphological traits, five populations of *G. superba* sampled from different environments can be grouped into two clusters: AM and BR plants belonging to one cluster, and SG, DJ and SK plants belonging to the other. It is known that environmental effects and growth practices may affect morphological markers. Consequently, populations from dissimilar habitats might show variable morphological expressions. Populations of *Trifolium alexandrinum* from disturbed environments have been reported to have distinctly shorter leaves (length and width) than undisturbed populations (Mahmood and Abbas, 2003). However, morphological features alone are not sufficient to assess genetic polymorphism and secondary compounds, commonly used as markers, are in many cases believed to have some adaptive significance to the plant (Forrest *et al.*, 2000). In the present study, significant variation in colchicine content was observed in

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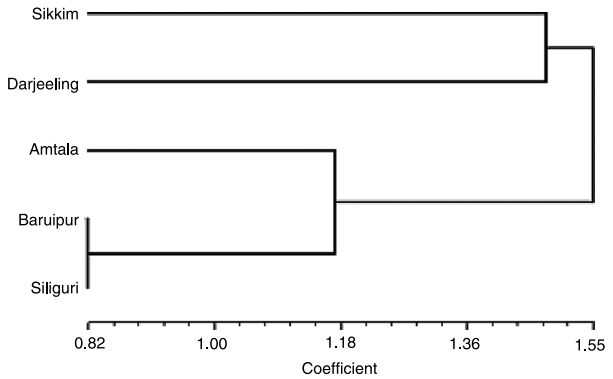

Fig. 4. Dendrogram of the five populations of *Gloriosa superba*.

tubers from different populations of *G. superba*. RAPD analysis of the five populations revealed a close similarity between BR and SG plants, which was also evident from phytochemical investigations. However, the morphological and cytological data in this study did not match with this finding.

Identification of genetic polymorphism is essential for *G. superba* since this plant is extensively used as a herbal medicine all over India and a wide range of variation exists in terms of alkaloid content. As flowering is rare and seed set does not occur, the only method open for its improvement is selection of desirable genotypes, based on higher colchicine content, better adaptability, etc. and molecular markers can provide important insights for selection of such desirable genotypes of *G. superba*.

The genetic diversity of the germplasm has been assessed in various plant collections and in spite of a narrow genetic base, high level of polymorphism has been obtained (Iqbal *et al.*, 1997). In these cases, the unique bands/profiles have been pointed out for one or a few accessions (Martin *et al.*, 1997), but in general, even when using a large number of primers, it was not possible to identify a single primer that could distinguish between all the accessions (Lashermes *et al.*, 1993; Iqbal *et al.*, 1997). In our investigation, while the genetic base of these randomly selected populations of *G. superba* was found to be quite narrow, the extent of polymorphism was high (76%), possibly because the polymorphism was estimated within plants of the same species growing in different locations under different environmental conditions. As our objective was to identify plants with high colchicine content, character related to alkaloid content and morphology of plant were considered for variation studies. High degree of variability among selected populations in variation analysis suggests applicability of clonal propagation for multiplication of high colchicine yielding plants.

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