Karyotype, RAPD and ISSR Analysis of Four Specimens in *Gynura nepalensis* DC.

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Summary Four specimens of *Gynura nepalensis* DC. were investigated for authentic characterization by differential cytogenetical and PCR based molecular analysis. Three specimens out of four were collected from different localities of Bangladesh viz. i) from Netrokona district (specimen A), ii) from Dhaka district (specimen B) and iii) from Bogra district (specimen D). On the other hand, specimen C was collected from Canada. The diploid chromosome number of four specimens were 2n=20 with different centromeric formulae such as 14m+6sm in specimens A, B and D while 20m in specimen C. Total length of 2n chromosome complement was 211.61 µm in specimen A, 217.58 µm in specimen B, 168.82 µm in specimen C and 197.80 µm in specimen D. The maximum range of chromosome length was found in specimen B (6.67–14.30 µm) and minimum in specimen C (6.44–10.12 µm). The four specimens have distinct CMA- and DAPI-banding pattern which confined to the terminal regions of respective chromosomes. Karyotype analysis revealed that the number, location and distributions of GC- and AT-rich repeats are different in these four specimens. Heteromorphicity in respect of occurrence of CMA- and DAPI-banding pattern in the homologue members were observed in specimen A, C and D. The heteromorphicity might be a result of deletion of banded region from the respective homologue members. One interstitial CMA band was found on the short arm in a member of pair V in specimen A and in pair IV of specimen C. Two terminal DAPI bands were found in two chromosomes of specimens A and B. These CMA- and DAPI-banded chromosomes were unique and thus could be used as marker chromosomes for the respective specimens. Only specimen C showed RAPD bands with three primers. However, the four specimens had distinguishable ISSR-banding patterns with seven primer combinations. Specimen C showed maximum ISSR bands. The combined RAPD and ISSR analysis placed specimen C in a separate cluster with the highest genetic distance. In spite of very distant location specimens A and D were placed in a sub-cluster with narrow genetic distance.

Key words Karyotype, RAPD, ISSR, *Gynura*.

The genus *Gynura* Cass. belongs to Asteraceae comprising of 40 herbaceous species distributed in tropical Asia and Africa (Airy-Shaw 1897). It is probably one of the easiest tropical vegetables that can be grown. These herbs have been using as traditional medicine for household remedy against various human ailments such as eruptive fevers, rash, kidney disease, migraines, constipation, hypertension, diabetes and cancer (Perry 1980). From Indian subcontinent, *G. nepalensis* was reported earlier by Hooker (1882) from the temperate Himalaya and later Hajra *et al.* (1995) from Himalayas and the North-East regions of India. However, the genus and any species belonging to the genus were not reported earlier in any of the relevant floristic literature covering Bangladesh territory (Prain 1903, Ahmed *et al.* 2008). Recently a perennial and terrestrial herb with yellow flowers in this genus was collected from Kendua of Netrokona district, Bangladesh. Moreover, two samples were also collected from Dhaka and Bogra districts (Rahman and Asad 2013). A specimen with similar phenotype was collected from Canada Airport by Md. Shahabuddin (personal communication). After critical taxonomic studies, the specimens have been identified as *Gynura nepalensis* DC., referring to the descriptions of Hooker (1882), Davis (1979) and Hajra *et al.* (1995). These specimens were planted in the Botanic Garden, University of Dhaka and the voucher specimen preserved at the Salar Khan Herbarium (DUSH), Department of Botany, University of Dhaka. Therefore, this might be a new report of this genus and as well as species from Bangladesh (Afroz *et al.* 2014).

The literature and internet sources revealed that except 2n chromosome number, there is no genomic information or report available about this herbal plant in home and abroad. Genomic information is essential for characterizing any specimen. Genome analysis using cytogenetical and molecular marker technologies provide information about the organization and distribution of genetic materials useful for authentic identification of a specimen.

Since *G. nepalensis* is an important herbal medicinal...
plant, the genomic information of this species is essential for further research work. Unfortunately such information is not available in Bangladesh. Therefore, a detail karyotype and molecular analysis of the species is necessary. In this study, a combination of cytogenetic and molecular approaches have been undertaken for the first time to characterize the four species of *G. nepalensis* collected from different localities.

Materials and methods

Four specimens of *G. nepalensis* DC. were studied in this investigation. These specimens were initially collected from different localities of home and abroad. Among four specimens three were collected from different parts of Bangladesh such as i) from Netrokona district (specimen A), ii) from Dhaka district (specimen B) and iii) from Bogra district (specimen D). Another one (specimen C) was collected from Canada Airport. The plants were grown and maintained in the Botanic garden, Department of Botany, University of Dhaka, Bangladesh.

Chromosome preparation

Healthy roots were collected and pretreated with 0.002 M 8-hydroxyquinoline for 6 h at 18°C followed by 15 min fixation in 45% acetic acid at 4°C. These were then hydrolyzed in a mixture of 1 NHCl and 45% acetic acid (2:1) at 60°C for 2 min. The root tips were stained and squashed in 1% aceto-orcein. For CMA- and DAPI banding, Alam and Kondo’s (1995) method was used with slight modification. After hydrolyzing and dissecting, the materials were squashed with 45% acetic acid and kept on dry ice. The cover glasses were removed quickly from dry ice and allowed to air dry for at least 24 h before study. For CMA-staining, the air-dried slides were first pre-incubated in McIlvaine’s buffer (pH 7.0) for 30 min followed by Distamycin A (0.1 mg mL⁻¹) treatment for 10 min. The slides were rinsed mildly in McIlvaine’s buffer supplemented with MgSO₄ (5 mM) for 15 min. One drop of CMA (0.1 mg mL⁻¹) was added to the materials for 15 min in a humid chamber and then rinsed with McIlvaine’s buffer with MgSO₄ for 10 min. Slides were mounted in 50% glycerol and kept at 4°C for overnight before observation. These were observed under Nikon (Eclipse 50i) fluorescent microscope with blue violet (BV) filter cassette. For DAPI-staining, after 24 h of air drying, the slides were first pre-incubated in McIlvaine’s buffer (pH 7.0) for 30 min and treated in Actinomycin D (0.25 mg mL⁻¹) for 10 min in a humid chamber. The slides were immersed in DAPI solution (0.01 mg mL⁻¹) for 20 min and mounted with 50% glycerol. These were observed under a Nikon (Eclipse 50i) fluorescent microscope with an ultra violet (UV) filter cassette.

DNA isolation

Leaves were harvested and total genomic DNA was extracted by using modified CTAB method (Doyle and Doyle 1987). DNA concentration was quantified through spectrophotometer (Analylykjen, Specord 50, Germany). The A 260/280 readings for DNA samples were 1.8–2.0.

PCR amplification and primer survey

The PCR reaction mixture for 25 µL containing template DNA (25 ng) 2 µL, de-ionized distilled water 18.8 µL, Taq buffer A 10× (Tris with 15 mM MgCl₂) 2.5 µL, primer (10 µM) 1.0 µL, dNTPs (2.5 mM) 0.5 µL and Taq DNA polymerase (5 U µL⁻¹) 0.2 µL. The PCR amplification was done in an oil-free thermal cycler (Biometra UNOII, Germany) for 46 cycles after initial denature 94°C for 5 min, denature at 94°C for 1 min, annealing at 34–36°C for 30 s, extension at 72°C for 3 min and final extension at 72°C for 5 min. In the present study, three oligonucleotide random primers and seven microsatellite primers were used for screening for RAPD and ISSR assay, respectively.

Gel electrophoresis

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing 10 µL ethidium bromide (10 mg mL⁻¹) and 100 mL 1× TAE buffer at 50 V and 100 mA for 1.0 h. Ladder DNA of 1 kb was electrophoresed alongside the RAPD and ISSR product as marker, respectively. DNA bands were observed on UV-trans-illuminator and photographed by a gel documentation system (BioSciTec, Gelscan 6.0 Professional, Germany) for both the markers.

Scoring and data analysis

After gel electrophoresis, the photographs were critically discussed on the basis of presence (1) or absence (0), size of bands and overall polymorphism of bands. These were carried out for further investigation. The scores obtained using all parameters such as, RAPD- and ISSR-analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, Nei’s (1972) gene diversity, genetic distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among the specimen using the computer program "POPGENE32" (Version 1.32).

Results and discussion

Conventional karyotype

The four specimens of *G. nepalensis* DC. were found to possess 2n=20 chromosomes (Figs. 1–4). Similar chromosome number of this species was reported earlier (Mehra *et al.* 1965, Shetty 1964) thus the present find-
Karyotype, RAPD and ISSR in *Gynura nepalensis* DC.

Findings confirmed the previous reports on 2n chromosome number. Moreover, different chromosome number for various *Gynura* species such as 2n=18 (in *G. angulosa*), 2n=22 (in *G. japonica*) and 2n=40 (in *G. angulosa, G. crepidiodes*) were reported by different authors (Mehra and Sidhu 1960, Suzuka 1953, Mehra *et al.* 1965, Darlington and Wylie 1955, Jose and Mathew 1990, Tzanoudakis 2017).
In the present aneuploid nature of the genus *Gynura* and Kypriotakis 1987). The above records indicated the chromosome number of the four specimens of *G. nepalensis*. The present result revealed the constant 2n = 20 chromosomes.

Total length of 2n chromosome complement and the range of chromosome length of three specimens (A, B, D) were almost similar while it was much less in specimen C (Table 1). The centromeric formula of 14m+6sm was observed in specimens A, B and D, whereas specimen C was found to possess 20 metacentric chromosomes which revealing strictly symmetric karyotype. According to Stebbins (1971), asymmetric karyotypes are advanced and symmetric karyotypes are primitive in nature. In this regard, specimen C seems to be comparatively primitive than the rest three specimens (Table 1, Figs. 13–16). This indicated that the karyotype of the rest three specimens might be originated from such a symmetric karyotype by deletion of heterochromatic parts of six chromosomes resulting the formation of sub-metacentric chromosomes.

Fluorescent banding pattern

Chromomycin A3 (CMA) and 4’-6-diamidino-2-phenylindole (DAPI) were two fluorochromes specific to GC- and AT-rich repetitive segments on chromosomes, respectively (Schweizer 1976). Fluorescent banding gives decisive analysis of karyotype, even chromosome having similar morphology and other conventional karyotypic features. In this study, these two fluorochromes were used for karyotype analysis of four *G. nepalensis* specimens.

The four specimens possessed distinct CMA-banding patterns in the metaphase chromosomes. The number, location, distributions, intensities and percentage of CMA-bands varied in different specimens. Most of the CMA-bands were present at the terminal regions of respective chromosomes in four specimens (Figs. 17–20, Table 1). CMA-bands represented the GC-rich repeats (Schweizer 1976). The presence of maximum terminal CMA-bands revealed that the GC-rich repetitive sequences were preferentially accumulated at the terminal ends rather than the other parts of the respective chromosomes.

Six CMA bands were found in specimens A and B followed by three in specimen D and two in specimen C. The specimen B possessed the highest GC-rich repeats (1.22%), while the lowest was in specimen C (0.41%) (Table 1). On the other hand the highest eight DAPI bands were found in specimen B followed by four DAPI bands in specimen C and two DAPI bands in specimens A and D (Figs. 21–24, Table 1). The highest AT-rich repeats were present in specimen B (1.78%) and the lowest in specimen D (0.59%) (Table 1).

Like CMA, most of the DAPI-bands (AT-rich regions) were present at the terminal regions of respective chromosomes in four specimens. Therefore, both GC- and AT-rich repeats have tendency to accumulate at chromosome ends (Figs. 21–24). Sumner (1990) explained equilocal distribution of heterochromatin where the heterochromatins have the tendency to localize a particular location of different chromosomes. The present finding regarding the distribution of GC- and AT-rich repeats suggesting the presence of heterochromatin at the chromosomal ends.

Heteromorphicity

Heteromorphicity in respect of CMA-banding pattern was observed in a member of chromosome pairs III and V in specimen A, pair IV in specimen C and pair III, VII in specimen D. In these pairs, a chromosome had CMA-positive band at the terminal end whereas no band was found in their respective homologue members (Figs. 17, 19, 20). Moreover, a member of pairs I and VIII in specimen A, pairs VI and VII in specimen C and pairs I and IX in specimen D had a terminal DAPI band which was absent in their homologue members (Figs. 21, 23, 24). The probable reason for the absence of bands in the homologous members due to deletion of GC-rich and AT-rich repetitive sequences from the respective chromosomes.

Marker chromosomes

Most of the CMA-bands were present at the terminal regions of respective chromosomes in four specimens. In contrast, only one interstitial CMA-band was found in the short arm of a member of pairs V and IV in specimens A and C, respectively. This type of banding pattern was not observed in any other chromosomes of the rest two specimens (Figs. 17, 19, arrow). Therefore, these two chromosomes could be used as marker chro-

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**Table 1.** Comparative orcein-, CMA- and DAPI-karyotype analysis of four specimens of *Gynura nepalensis* DC.

<table>
<thead>
<tr>
<th>Specimens</th>
<th>2n</th>
<th>Range of chromosomal length (µm)</th>
<th>Total length of 2n chromosome complements (µm)</th>
<th>Centromeric formulae</th>
<th>No. of CMA-bands</th>
<th>% of GC-rich repeats</th>
<th>No. of DAPI-bands</th>
<th>% of AT-rich repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20</td>
<td>6.90–13.11</td>
<td>211.61</td>
<td>14m+6sm</td>
<td>6</td>
<td>1.09</td>
<td>2</td>
<td>0.76</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>6.67–14.03</td>
<td>217.58</td>
<td>14m+6sm</td>
<td>6</td>
<td>1.22</td>
<td>8</td>
<td>1.76</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>6.44–10.12</td>
<td>168.82</td>
<td>20m</td>
<td>2</td>
<td>0.41</td>
<td>4</td>
<td>1.78</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>7.36–11.96</td>
<td>197.80</td>
<td>14m+6sm</td>
<td>3</td>
<td>0.64</td>
<td>2</td>
<td>0.59</td>
</tr>
</tbody>
</table>

m=metacentric chromosome, sm=sub-metacentric chromosome
mosomes of specimen A and specimen C, respectively.

Four specimens had different DAPI banding pattern. A member of pairs I and VIII of specimen A and both the members of pair I of specimen B had two terminal DAPI-bands (Figs. 21, 22). Except these chromosomes, two terminal DAPI bands were not observed in other two specimens (Figs. 23, 24). Therefore, these chromosomes could also be used as DAPI marker chromosomes for the identification of respective specimens.

From the ongoing discussion, it revealed that the chromosomes of four specimens of *G. nepalensis* possessed different structural organization in respect of deletion and distributions of GC- and AT-rich repetitive sequences. These variations of chromosomes might be the probable major reasons for karyotype diversification of the four specimens.

**RAPD analysis**

Initially, 10 different primers were used, of which only three primer combinations showed clearer and reproducible bands. A total of thirteen RAPD bands were found only in specimen C with the three primers. Three bands were found with OPG-6 and five with both OPG-3 and OPG-9 (Figs. 25, 26, 27, Table 2). However, no band was observed in other three specimens suggesting the lack of those sequences (loci) in their genomes.

**ISSR analysis**

A total of seven ISSR primers were used in this study. The seven primers generated 35 distinct bands of which 27 were considered as polymorphic and thus showed 22.86% polymorphisms (Figs. 28–34, Table 2). Two common bands of different sizes were observed with primer UBC 826 revealing that the four specimens sharing similar genomic sequences. On the other hand, no common band was found with other six primers suggesting the unique nature of banding pattern. A unique band is a band found in a specimen with a certain primer but absent in other specimen with the same primer. The unique bands were specimen specific and thus could be used as molecular markers. Specimen B had three unique bands with primer UBC 807, two in specimen C with primer UBC 810, one in specimen A with primer UBC811, two in specimen C with primer UBC 819, four in specimen C with UBC 822 and one in specimen B with primer UBC 827 (Figs. 28, 29, 30, 31, 32, 34). ISSR markers provide basic genetic knowledge and the polymorphic bands could differentiate the genotypes of four specimens. Thus the four specimens of *Gynura nepalensis* could be characterized on the basis of ISSR markers.

**Features of specimen C**

Morphologically the four specimens of *G. nepalensis* DC. look similar. Unlike morphological similarity, the
four specimens differed in respect of cytogenetical and molecular features. Among the four specimens, specimen C (collected from Canada) differed sharply from the rest three specimens. It had only metacentric chromosomes whereas both metacentric and submetacentric types of chromosomes were found in the other three specimens.

Three to five RAPD bands were observed only in specimen C with three different primers. In contrast, different patterns of ISSR bands were found in this specimen with seven primer combinations. The combined RAPD and ISSR analysis placed specimen C alone in a complete separate cluster with the highest genetic distance (1.6094) and other three specimens in a different cluster with minimum genetic distance (0.2973). Therefore, the specimen C is different from the rest three specimens.

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