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COMPARATIVE KARYOTYPE AND RAPD ANALYSIS OF FOUR VARIETIES OF *ALLIUM CEPA* L. AND A SPECIES OF *ALLIUM FISTULOSUM* L.

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Abstract

Four varieties of *Allium cepa* L. *viz*., BARI Piaz-1, BARI Piaz-3, BARI Piaz-4 and BARI Piaz-5 and a species of *Allium*, namely *A. fistulosum* L. were cytogenetically studied. In addition, DNA fingerprinting by using RAPD was also carried out to make phylogenetic dendrogram. The chromatins were homogeneously distributed in the interphase nuclei and prophase chromosomes of five specimens and they were found to possess 2n = 16 chromosomes. The highest number of acrocentric chromosomes was found in *Allium fistulosum* while no acrocentric chromosome found in BARI Piaz-4. This result indicated advanced karyotype in *A. fistulosum.* Each specimen showed characteristic RAPD fingerprinting. The unique RAPD fragments could be used as marker for these respective specimens. The cytogenetical and RAPD data support that *A. fistulosum* is totally different from rest four specimens.

Introduction

Allium species are important herbaceous plants used as spices as well as vegetables in the world. In early classifications of angiosperms, *Allium* species were placed in the Liliaceae, later they were more often included in the Amaryllidaceae on the basis of their fluorescence structure (Paknia and Karimzadeh 2010). The genus *Allium* comprises of some 750 species (Friesen *et al.* 2006), including both wild and cultivated plants (Negi 2006).

Allium includes many economically important species which are extensively used as a condiment in the preparation of many kinds of foods and it is an integral part of the world diet. In case of onion (*Allium* sp.), the main usable part is the fleshy scaly-leaf bases, usually known as bulbs. Moreover, the whole immature plant, the leaves and also the peduncle are used as vegetables. Onion contains 89% water, 4% sugar, 1% protein, 2% fibre and 0.1% fat. It contains vitamin B and a trace of vitamin C and also trace of iron, calcium and volatile oil known as allylpropyl-disulphide (Yawalkar 1985). It has anti-rheumatic, diuretic and antibacterial properties and reduces the insulin requirement of diabetic patients and decreases serum cholesterol. It is regarded as a stimulant and aphrodisiac (Ghani 2003).

In Bangladesh, onion ranks first and second among all spices and condiment crops grown in respect of production and area, respectively (BBS 2004). Bangladesh Agriculture Research Institute (BARI) has successfully released five varieties of onion, namely *Allium cepa* (BARI Piaz-1, BARI Piaz-2, BARI Piaz-3, BARI Piaz-4 and BARI Piaz-5) and a species of *Allium fistulosum*. They were characterized on the basis of phenotypic and agronomic features without any basic knowledge of their genome. It is well-known that, for authentic identification of a specimen, genomic information is essential. The karyotype analysis is a dependable method in the identification and design of the chromosomes of animals and plants (Okumus and Hasan 2000). A complete idea about the karyotype of a germplasm is an important prerequisite for effective plant

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genetic and breeding studies (Martinez-Gomez *et al.* 2003) and also provides valuable information related to the mechanism of genome evolution (Wilkinson 1994) since it is very much species specific.

The diploid chromosome number of *Allium cepa* is well established i.e., 2n = 2x = 16 (Okumus and Hassan 2000, Kim *et al.* 2002, Mukherjee and Roy 2012, Alam *et al.* 1999). Like *Allium cepa*, in *Allium fistulosum* the diploid chromosome number was also reported as 2n = 2x = 16 (Joachimiak *et al*. 1993, Barthes and Ricroch 2001, Jenkins and Okumus 1992, Shigyo *et al*. 1998, McCallum *et al*. 2012). Except Alam *et al.* (1999), most of the previous researchers were confined to 2n chromosome count. Alam *et al*. (1999) applied differential staining *viz*., orcein, geimsa, CMA, DAPI on local and Indian onion. They were able to characterize the two *A. cepa* specimens.

In addition, molecular characterization is more reliable and authentic. In recent years, RAPD analysis through the PCR has been widely used in molecular characterization and traces the phylogeny of diverse plant and animal species. This technique has contributed widely in the studies of genetic diversity and phylogenetic analysis (Savolainen and Chase 2003, Nybom 2004). These markers are used as versatile tools to provide the correct estimate of genetic diversity. The main advantages of RAPD analysis over other molecular methods are low sample DNA requirements, the high frequency of detectable polymorphic bands (Williams *et al.* 1990) and independent from the effects of environmental factors (Powell *et al.* 1995, Garcia *et al.* 1998, Kuras *et al.* 2004).

A combination of karyotype and RAPD analysis gives multidimensional genomic information. Several authors tried this combination for authentic characterization of specimens in a species and to solve different taxonomic problems (Alam and Kondo 1995, Fawzia and Alam 2011, Hiron *et al*. 2006). There is so far no reports and combined analysis by karyotype and RAPD on onion. In this research, a combined karyotype and RAPD analysis have been undertaken to characterize five specimens of *Allium* released by BARI.

Materials and Methods

The four varieties of *Allium cepa* L. and a species named *Allium fistulosum* L. were investigated. The specimens were collected from the Regional Spices Research Center (RSRC) of Bangladesh Agricultural Research Institute (BARI). These were maintained in the Botanical garden, Department of Botany, Jagannath University, Dhaka.

The young healthy roots from bulbs of *Allium cepa* and seedlings of *Allium fistulosum* were collected and pretreated with para dichloro benzene (PDB) for 2 hrs at 20 - 25°C. Then the roots were fixed in 45% acetic acid for 15 min at 4°C. The pretreated RTs were hydrolyzed for 10 s at 60°C in a mixture of 1N HCl and 45% acetic-acid (2 : 1). The root tips were stained and squashed in 1% aceto-orcein. These were observed under a binocular microscope (XSZ-107BN).

Tender leaves were harvested and total genomic DNA was extracted by using modified CTAB method (Doyle and Doyle 1987). DNA concentration was quantified through spectrophotometer (Analylikjena, Specord 50, Germany). The PCR reaction mixture for 25 µl contained template DNA (25 ng) 2 µl, de-ionized distilled water 18.8 µl, Taq buffer A 10× (Tris with 15 mM $MgCl_2$) 2.5 µl, primer (10 µM) 1.0 µl, dNTPs (2.5 mM) 0.5 µl and Taq DNA polymerase (5U/µl) 0.2 µl. PCR amplification was done in an oilfree thermal cycler (Biometra UNOII, Germany) for 46 cycles after initial denature at 94°C for 5m, denature at 94°C for 1 min, annealing at 36°C for 30 sec, extension at 72°C for 3 min and final extension at 72°C for 5 min. Five random primers, *viz.*, primer-4 (5'-GGG TAA CGC C-3'), primer-5 (5'-TCA CGT CCA C-3'), Primer-7 (5'-CCC GCC TTC C-3'), primer-8 (5'-GTC CTC GTA G-3') and primer-11 (5'-

CAC GGC TGC G-3') were used in the present study for screening. The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing ethidium bromide (10 mg/ml) 8 µl and 100 ml 1× TAE buffer. Agarose gel electrophoresis was conducted in 1× TAE buffer at 50 volts and 100 mA for 1.5 hrs. DNA ladder (1 kb) was electrophoresed alongside the RAPD reactions as marker. DNA bands were observed on UV-transilluminator and photographed by a gel documentation system. The PCR products were analyzed after gel electrophoresis. The photographs were critically examined on the basis of presence (1) or absence (0), size of bands and overall polymorphism of bands. These were carried out for further investigation. RAPD analysis was then combined to create a single data matrix. This was used for estimating linkage distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among the varieties using computer program Popgene (version 1.31). Linkage distances were computed from frequencies of polymorphic markers to estimate genetic relationship between the studied five *Allium* specimens using UPGMA.

Results and Discussion

The four varieties of *Allium cepa* L. and a species named *Allium fistulosum* L. were found to possess 2n = 16 chromosomes. No satellite or secondary constriction was found in these specimens. A similar diploid chromosome number for four specimens of *A. cepa* was reported by different scientists (Joachimiak *et al*. 1993, Barthes and Ricroch 2001, Jenkins and Okumus 1992, Shigyo *et al*. 1998, McCallum *et al*. 2012). Thus, the present report on the 2*n* chromosome number of the four specimens correlates with the earlier reports. Moreover, 2n = 32 was also reported for *Allium fistulosum* (Warasy and Begum 2013), which did not correlate with the present findings.

The five specimens showed different centromeric formulae i.e. 10m + 4sm + 2ac in both BARI Piaz-1 and BARI Piaz-3. BARI Piaz-4 had 8m + 8sm and 8m + 6sm + 2ac were found in BARI Piaz-5 while 8m+4sm+4ac in *A. fistulosum* (Table 1). Among the specimen except BARI Piaz-4 each had acrocentric chromosomes. Presence of acrocentric chromosomes revealed more heterogenous karyotype. According to Stebbins (1971), heterogenous karyotype is an advanced character. Therefore, BARI Piaz-4 possessed relatively primitive karyotype.

The smallest and largest total length of 2n chromosome complement was found in BARI Piaz-4 and BARI Piaz-3, respectively (Table 1). On the basis of total length, the five specimens could be categorized into two groups: (i) bigger length- length was around 80 µm long found in BARI Piaz-1, BARI Piaz-3 and BARI Piaz-5 and (ii) smaller length- length was around 55 µm in BARI Paiz-4 and *A. fistulosum* (Table 1).

Table 1. Comparative karyotype analysis of five *Allium* specimens.

Specimens	2n	Range of chromosome length (µm)	Total length of 2n chromosome complements (µm)	Centromeric formulae
A. cepa (BARI Piaz-1)	16	3.69-5.72	76.49	10m + 4sm + 2ac
A. cepa (BARI Piaz-3)	16	3.97-6.65	84.80	10m + 4sm + 2ac
A. cepa (BARI Piaz-4)	16	2.66-4.20	53.75	8m + 8sm
A. cepa (BARI Piaz-5)	16	3.80-5.97	77.16	8m + 6sm + 2ac
A. fistulosum	16	2.64-4.33	55.65	8m + 4sm + 4ac

m = Metacentric, sm = Sub-metacentric, ac = Acrocentric.

BARI Piaz-4 and *A. fistulosum* showed karyomorphological similarities upto certain extent. For example: (i) the total length of diploid complements were almost similar, (ii) in both the specimen, the largest chromosome were almost 1.5 time bigger than the smallest one and (iii) the range of chromosomal length was same in both the specimens.

The pair wise comparison of chromosome in BARI Piaz-4 and *A. fistulosum* indicated probable occurrence of inversion. In BARI piaz-4, the IV and VIII pair was sub metacentric (Fig. 13, arrow). The same pairs in *A. fistulosum* were acrocentric (Fig. 15, arrow). The total length of pair IV in BARI Piaz-4 and *A. fistulosum* was almost same, while the total length of pair VIII in these two specimens was exactly same. Moreover, the length of short arm in pair IV and VIII in BARI Piaz-4 and corresponding short arms of these pairs in *A. fistulosum* indicating the occurrence of inversion on short arms including centromere, i.e. a pericentric inversion.

These two specimens have great karyomorphological similarities except the presence of four acrocentric chromosomes in *A. fistulosum*. The pericentric inversion in pair IV and VIII in BARI Piaz-4 might be the cause of evolution of acrocentric chromosomes in *A. fistulosum.*

Five different primer combinations were used for RAPD fingerprinting analysis of five *Allium* specimens. In primer-4 and primer-7, one common band and in primer-11, two common bands of different sizes were observed which revealed the presence of similar genomic sequences among the five specimens (Figs 16, 18, 20). On the other hand, no common band was found with other two primers and the results indicated that these specimens were not sharing much DNA fragments amplified with these primers (Figs 17, 19).

In case of primer-5, no band was found in BARI Piaz-1 and BARI Piaz-3 (Fig. 17). This indicated the lack of those alleles in their genomes. Here both BARI Piaz-5 and *Allium fistulosum* showed two unique bands (Fig. 17, Table 2). On the other hand, a unique band was found in BARI Piaz-3 with primer-7 (Fig. 18), BARI Piaz-1 with primer-8 and two unique bands in *Allium fistulosum* with primer-8 (Fig. 19). These bands were considered as unique since they were absent in other specimens with those primers. The unique RAPD fragments could be used as marker for these respective specimens.

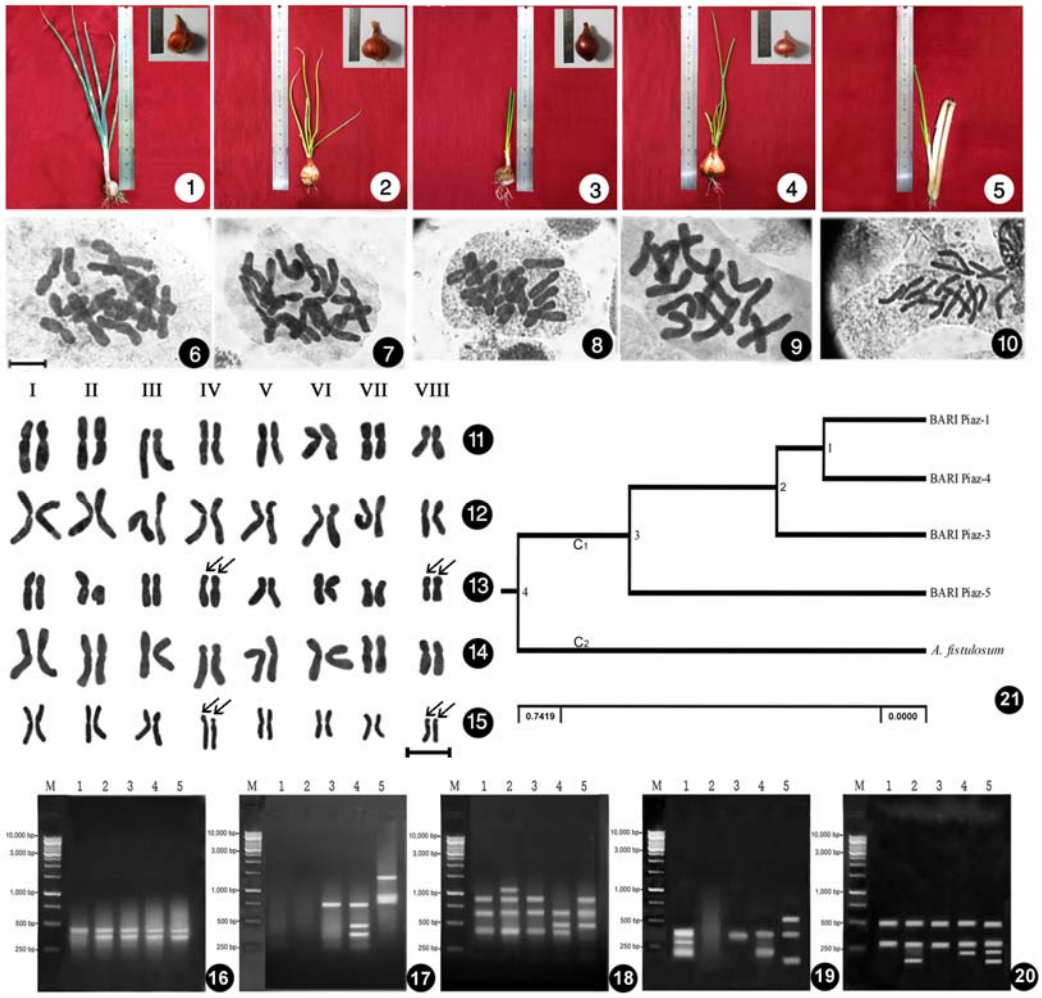
Table 2. Compilation of RAPD analysis of five *Allium* specimens.

Primer codes	Total bands	Size ranges (bp)	No. of poly-morphic bands	No. and size (bp) of variety specific unique bands	Polymor-phism %
Primer-4	2	400 - 350	1	-	50.00
Primer-5	5	1300 - 375	5	480 bp and 375 bp in BARI Piaz-5, 1300 bp and 800 bp in *A. fistulosum*	100.00
Primer-7	5	1100 - 400	4	1100 bp in BARI Piaz-3	80.00
Primer-8	5	500 - 150	5	270 bp in BARI Piaz-1, 500 bp and 150 bp in *A. fistulosum*	100.00
Primer-11	4	500 - 150	2	-	50.00

On the basis of RAPD banding pattern, the five specimens were initially grouped into two different clusters (C_1 and C_2) (Fig. 21). Cluster 1 comprises of the four specimens of *Allium cepa,* whereas cluster 2 formed with *Allium fistulosum.* It indicated that *Allium fistulosum* is totally different from rest four specimens. This result correlated with their karyological and morphological features.

Allium fistulosum L. was found to possess 2n = 16 chromosomes (Fig. 15). Similar diploid chromosome number was reported earlier (Joachimiak *et al*. 1993, Barthes and Ricroch 2001,

Jenkins and Okumus 1992, Shigyo *et al*. 1998, McCallum *et al*. 2012). Moreover, 2n = 32 was also reported for *Allium fistulosum* (Warasy and Begum 2013), which did not correlate with the present findings.



Figs 1-21. Plant morphology, orcein-stained mitotic metaphase and RAPD analysis of five *Allium* specimens. 1. Plant morphology of BARI Piaz-1, 2. Plant morphology of BARI Piaz-3, 3. Plant morphology of BARI Piaz-4, 4. Plant morphology of BARI Piaz-5, 5. Plant morphology of *Allium fistulosum* (Bulb inset), 6. Orcein-stained mitotic metaphase chromosomes of BARI Piaz-1, 7. BARI Piaz-3, 8. BARI Piaz-4, 9. BARI Piaz-5, 10. *Allium fistulosum*, 11. Karyotype prepared from orcein-stained mitotic metaphase chromosomes of BARI Piaz-1, 12. BARI Piaz-3, 13. BARI Piaz-4, 14. BARI Piaz-5, 15. *Allium fistulosum*, (Bar = 5 µm for Figs 6-15), 16. RAPD analysis with primer-4, 17. primer-5, 18. primer-7, 19. primer-8, 20. primer-11, M = 1 kb DNA ladder (1 = BARI Piaz-1, 2 = BARI Piaz-3, 3 = BARI Piaz-4, 4 = BARI Piaz-5, 6 = *Allium fistulosum*), 21. Cluster analysis by UPGMA among five *Allium* specimens based on different RAPD markers.

The probable reason for the disagreement regarding 2n chromosome number is that the earlier workers might work on a tetraploid specimen of *A. fistulosum.* If the previous specimen was a tetraploid, must show some gigas features. From the description of the material by Warasy and Begum (2013), no significant morphological difference was found between the previous and

present materials. Further there will be two probable reasons: (i) either the earlier specimen was different cytotype of *A. fistulosum* or (ii) chromosome doubling did not carry any morphological change.

A difference between karyotypes of formerly reported *Allium fistulosum* and the karyotype of the present specimen was found. Therefore, further genomic investigation is necessary to elucidate the taxonomic status of the specimen used in this study.

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