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Molecular and embryological analyses of rare sexual plant in Buffelgrass (*Cenchrus ciliaris* L.)

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Abstract

Buffelgrass (*Cenchrus ciliaris* L.) is an important forage grass, which reproduces predominantly through apomixis. Apomixis in *C. ciliaris* limits possibilities of genetic improvement of this species by hybridization and studying phylogenetic relationship. Obligate apomictic and sexual plants are required for the development of mapping population for genetic studies in apomixis. We identified an obligate sexual plant of *C. ciliaris* using pistil-clearing technique and DNA profile of this rare sexual plant was developed using RAPD technique. A 10-mer primer OPJ-13 produced a plant-specific band of about 0.22 kb, while OPP-14 primer produced specific band of about 1.2 kb. The DNA profile can be used for identification of this plant and may be useful in the genetic studies involving this plant as a parent.

Key words : Apomixis, Buffelgrass, *Cenchrus ciliaris* L., DNA profile, RAPD marker, Sexual plant.

Introduction

Buffelgrass (*Cenchrus ciliaris* L.) is an important perennial forage grass which grows throughout the semi-arid tropics. It reproduces predominantly by aposporous apomixis (Dwivedi *et al*., 2007). Apomixis provides a means of clonal propagation through seeds because the progenies produced through apomixis are genetically identical to the mother plant. Absence of sexual reproduction in *C. ciliaris* has severely limited the possibilities of genetic improvement of this species by hybridization and studying phylogenetic relationship among different species of this agamic complex. Clonal mode of reproduction and polyploidy nature has further complicated overall genetic analysis and molecular studies for apomixis (Pessino *et al*., 1999). In recent years molecular markers linked to apospory have been reported in several grasses including *C. ciliaris* (Gustine *et al*., 1997; Pessino *et al*., 1998; Ozias-Akins *et al*., 1998; Martinez *et al*., 2003; Dwivedi *et al*., 2007). Such molecular markers are proving to be of great help in genetic, cytogenetic and molecular analysis of apomixis (Gustine *et al*., 1997; Ortiz *et al*., 1997; Ozias-Akins *et al*., 1998).

Although apomixis is prevalent in several plant species, it is predominant in the Rosaceae, Compositae and Poaceae families (Richard, 1986). In most of the species exhibiting apomictic mode of reproduction, apomixis has been found dominant over sexuality. Under such condition occurrence of obligate sexual plants in natural population becomes rare and over a period of time apomictic individuals outnumber sexual ones. Since *C. ciliaris* is protogynous, open pollination leads to fertilization by neighbouring apomictic plants that gives rise to either facultative or obligate apomictic types. Offtype plants have been occasionally isolated in this species (Bray, 1978; Gupta *et al*. 2001). Obligate sexual and apomictic plants are needed for genetic and molecular studies in apomixis. Female parent is required for the development of mapping population(s) for genetic analysis of apomixis and genetic improvement of this species. Sexual plant has been reported to show characteristically different morphology as compared to the normal (apomictic) plants. We identified a sexual plant in buffelgrass germplasm by embryosac analysis using pistil-clearing technique. DNA profile of the sexual plant was developed using RAPD technique. The DNA profile of this obligate sexual plant will be very useful for distinguishing it from other plants of buffelgrass as well as in the genetic studies involving this plant as a parent.

Materials and Methods

Existing germplasm collections (183) and 133 plants from F_2 mapping population (Dwivedi *et al*., 2007) of *C. ciliaris* maintained at IGFRI were used in the present study. More than 300 plants of *C. ciliaris*, primarily selected on the basis of their morphology and growth were screened through embryosac analysis using the pistil-clearing

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technique (Young *et al*., 1979). Inflorescence were collected at 75% stigma exertion stage and fixed in a mixture of 95% ethanol (40 ml), 40% formaldehyde (3 ml), glacial acetic acid (3 ml) and distilled water (14 ml). The inflorescence were kept submerged in the solution for 24 hrs, then transferred to 70% ethanol and stored at 4°C until used for pistil-clearing technique within a week. Pistils were excised out and passed through an ascending series (85% and 100%) of ethanol followed by passing the pistils through mixture of ethanol and methyl salisylate in 1:1 ratio for 2 hrs, then through the mixture in 1:3 ratio for 4 hrs and finally incubated overnight in 100% methyl salisylate. Slides were prepared by mounting the processed embryo sacs with methyl salisylate and examined under DIC microscope. More than 25 embryo sacs per plant were analyzed to ascertain mode of reproduction.

Genomic DNA of selected *C. ciliaris* plants (obligate sexual and obligate apomicts) from germplasm collections and F_2 mapping population was isolated using CTAB method (Dellaporta *et al*., 1983) as well as using genomic DNA isolation kit (DNeasy Plant Mini Kit, QIAGEN Gmbh, Germany). For genomic DNA isolation using CTAB method, young leaves (1g) from the selected plants were ground into a fine powder using liquid Nitrogen and transferred to 10 ml of freshly prepared extraction buffer (100 mM Tris-HCl, (pH 8.0), 20 mM EDTA, (pH 8.0), 1.4 M NaCl, 2% CTAB) and mixed thoroughly. The homogenate was incubated at 65° C for one hour in a water bath. Then equal volume of chloroform : isoamyl alcohol (24:1) was added and mixed by gently inverting the tubes several times. After centrifugation at 12,000 rpm for 10 min at room temperature, the supernatant was collected in a fresh tube and above step was repeated. To the supernatant 0.6 volume of isopropanol was added, mixed gently by inverting the tubes and incubated at -20°C for one hour, then centrifuged at 12,000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, air-dried the pellet and dissolved in 500 ml of TE (10 mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). RNase treatment was given by adding RNase (@ 10 mg/g leaf tissues) in the tubes and incubating at 37°C for one hour. The residual proteins were removed by extracting once with phenol, chloroform & isoamyl alcohol (25:24:1 ratio) and twice with chloroform & isoamyl alcohol (24:1). DNA was precipitated by adding equal volume of absolute ethanol, incubating at -20°C for one hour and centrifuging at 4° C. The DNA pellet was washed with 70% ethanol, air dried and finally dissolved in 500 ml of TE.

About 100 mg young leaf tissue from the selected plants was used for genomic DNA isolation using the DNA isolation kit. Leaf material was ground into a fine powder using liquid Nitrogen and the kit protocol was used for isolation and purification of DNA. Isolated genomic DNA was run on 0.8% agarose gel to check quality and quantity of DNA. Two bulks were made by pooling genomic DNA from 10 obligate sexual progeny plants (Spp) and 10 obligate apomictic progenies (App) for RAPD analysis along with genomic DNA from the sexual (Sx) plant.

Polymerase chain reaction (PCR) for RAPD analysis was carried out in 20 ml of reaction volume consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 10 mM of dNTP mix, 10 pm of 10-mer primer (Operon Technologies, USA), 3 units of Taq DNA polymerase (Bangalore Genei, Bangalore) and 5 ng of template genomic DNA. PCR was performed in a PTC-100 Thermalcycler (MJ Research, UK). The PCR conditions were 94°C for 4 min, followed by 40 cycles of amplification (94°C for 1 min, 37°C for 1 min and 72°C for 2 min) and 72°C for 10 min. PCR products were separated by agarose gel (1.6%) electrophoresis at a constant voltage (2 V/cm) along with DNA size markers. The gel was stained with ethidium bromide by adding it in the gel @ 0.5 mg ml^{-1} and analyzed using gel documentation system (GelDoc-It Imaging System, UVP, USA). RAPD results were analyzed based on the banding patterns observed in the three consecutive independent PCR under similar conditions.

Results and discussion

One hundred eighty three (183) plants from germplasm collections and F_2 mapping population were examined for their mode of reproduction using the pistil-clearing technique. In *C. ciliaris*, apomictic embryo sac is produced through apospory (Dwivedi *et al*., 2007) and contains four-nucleated embryo without antipodal cells. In case of sexual mode of reproduction normal meiosis occurs and eight-nucleated embryosac (Fig. 1A) is formed with one egg cell, two synergids, two polar nuclei and three antipodal cells at chalazal end. Thus, a four-nucleate apomictic embryosac (Fig. 1B) can be clearly distinguished from the eight-nucleated sexual embryosac. At least 25 embryos from obligate apomictic plants and about 30 embryos from obligate sexual plants were analyzed. When apomictic and sexual embryos were observed in the same plant, it was designated as facultative apomict. Majority of the plants in F_2 mapping population were facultative apomict, while obligate sexual (12) and obligate apomictic plants (17) were also

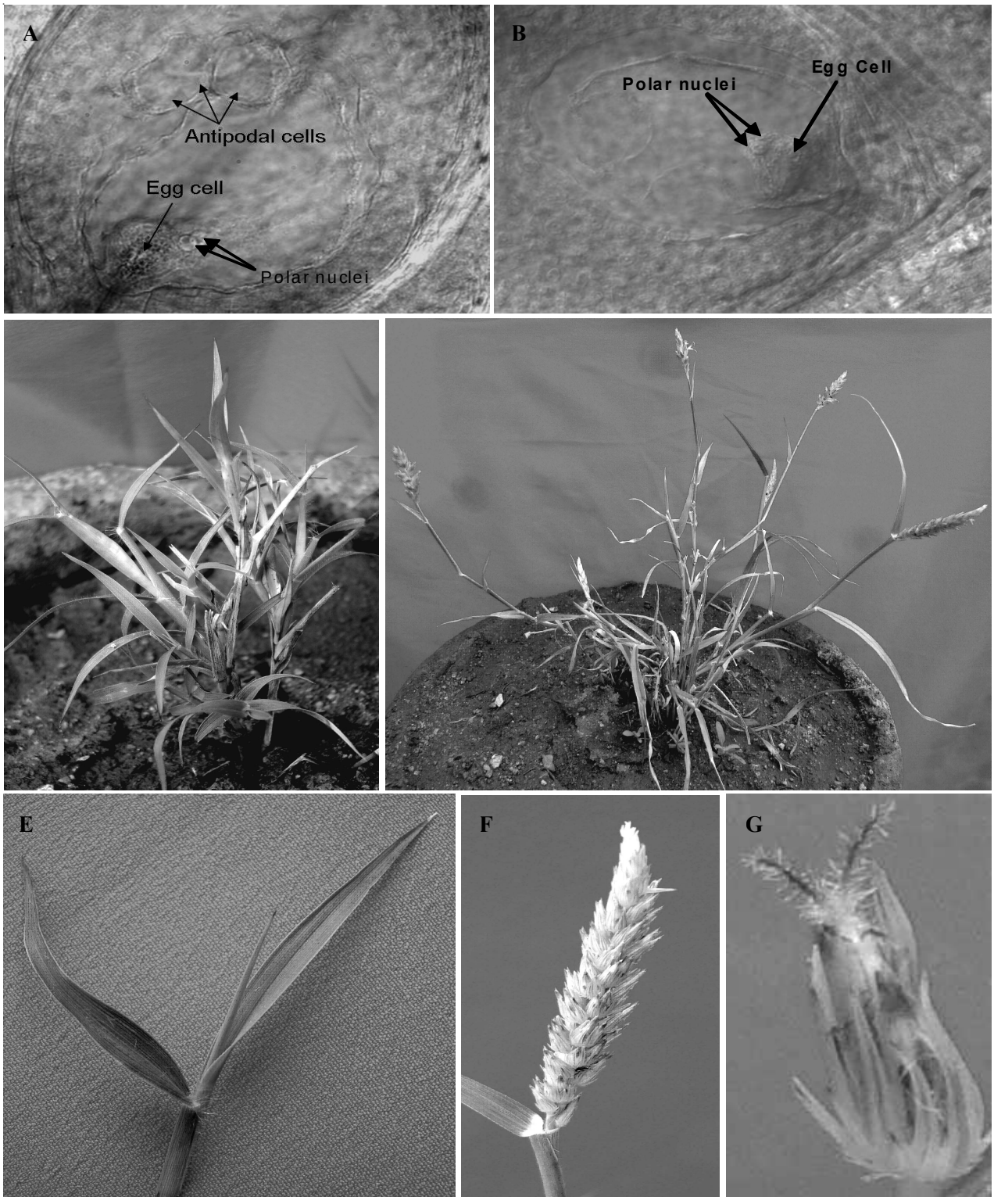

Fig. 1 : (A) eight-nucleated sexual embryosac with prominent antipodals, (B) four-nucleated apomictic embryosac without antipodal cell, (C) sexual plant (IGFRI-CcSx-08/1) of buffelgrass in vegetative stage, (D) the plant in reproductive phase, (E) leaf morphology of the sexual plant, (F) inflorescence and (G) floret of the sexual plant.

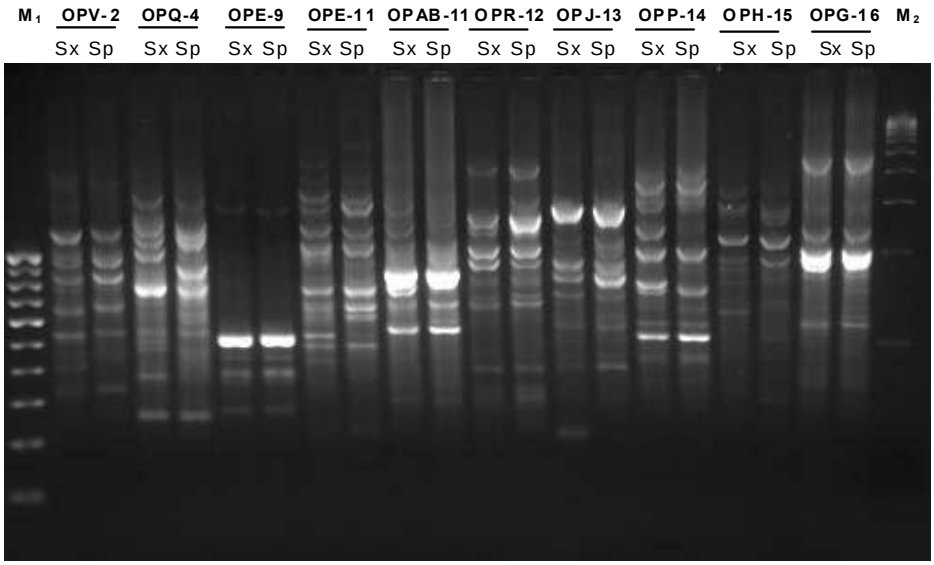

Fig. 2 : RAPD pattern with 10 random primers for sexual *C. ciliaris* plants. Sx = the sexual plant; Sp = sexual progeny from F_2 mapping population; M_1 = 100 bp DNA ladder M_2 = 500 bp DNA ladder.

observed. In a few cases, development of multiple apomictic embryosacs was also observed. The germplasm collections were either facultative or obligate apomicts. An off-type plant was observed, wherein more than 50 embryosacs (in two flowering seasons) were analyzed and all of them were eight-nucleated sexual embryos. Occurrence of sexual forms in natural population of *C. ciliaris* has been occasionally reported (Bray, 1978; Gupta *et al*. 2001). The sexual *C. ciliaris* plant (named as IGFRI-CcSx-08/1) was observed to be very short in stature with distinct morphology (Fig. 1C). The plant bears awnless panicles of smaller size with a few florets (Fig. 1D & 1F). Leaves are comparatively thick, smaller in size (Fig. 1E) and originate on nodes having shorter internodes. The plant was characterized as protogynous (Fig. 1F), self-incompatible with poor seed setting on open pollination. Being perennial in nature, it is maintained by vegetative propagation.

Fifty random primers were screened for polymorphic response in sexual plants using RAPD (Fig. 2). Polymorphic primers (OPJ-13 and OPP-14), showing clear-cut distinguishing bands in the sexual plant (IGFRI-CcSx-08/1), obligate apomictic and sexual progenies were used for fingerprinting of the sexual plant. This resulted into identification of two bands specific to the sexual plant. Random primer OPJ-13 produced a sexual plant-specific band of about 0.22 kb, while OPP-14 produced another specific band of about 1.2 kb (Fig. 3). The bands were neither present in other obligate sexual plants in F_2

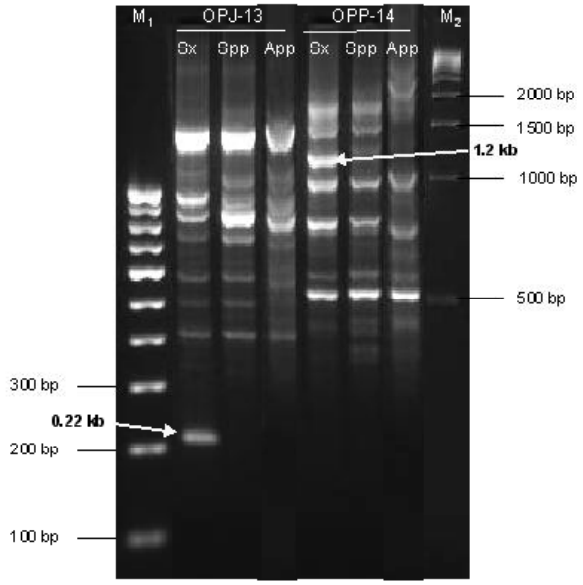

Fig. 3 : DNA fingerprint of the sexual *C. ciliaris* plant (IGFRI-CcSx-08/1). M_1 = 100 bp DNA ladder; Sx = the sexual plant; Spp = pool of (DNA from) 10 sexual plants from F_2 mapping population; App = pool of 10 apomictic plants from F_2 mapping population; M_2 = 500 bp DNA ladder.

mapping population nor in the apomictic plants. Thus, the bands were very specific to the sexual plant. Prominence of the bands observed every time in the repetitive PCR added conformity about the linkage of the DNA marker with the sexual plant. This can be used as RAPD marker for identification of the rare sexual plant

(IGFRI-CcSx-08/1). Using the fingerprint the plant can be easily distinguished from any other *C. ciliaris* plant. The sexual plant is considered as an elite and precious genetic material for genetic studies in apomixis, improvement of *C. ciliaris* by hybridization and studying phylogenetic relationships. The plant-specific RAPD markers may be useful in the genetic studies involving this plant as one of the parents in hybridization.

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