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The utility of ISSRs for the identification of interspecific hybrids between pearl millet (*Pennisetum glaucum* [L.] R.Br.) × napier grass (*Pennisetum purpureum* Schumach)

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Abstract

Interspecific hybrids between pearl millet (*Pennisetum glaucum*) and napier grass (*Pennisetum purpureum*) give rise to perennial fodder crops characterized by high biomass, broad clumps and good palatability. These hybrids are triploid and developed by hand pollination of napier grass pollen on pearl millet panicles. The progeny shows a high percentage of pearl millet genotype due to self-pollination in the female parent. Identification of hybrids at a young stage based on morphological characters is difficult. DNA-based molecular markers have high discriminating power and were used to assess genetic differences between hybrids and their parents. Genetic diversity was studied in 18 pearl millet × napier grass hybrids along with their parents and two released national checks using inter simple sequence repeat (ISSR) markers. Eight ISSR primers gave rise to 125 bands, of which 120 bands were polymorphic. Polymorphic information content and ISSR primer index ranged from 0.40 to 0.49 and 8.88 to 11.14, respectively. The hybrids showed the presence of unique bands, besides those shared with male and female parents. Female (pearl millet) parents formed a separate group in the dendrogram constructed based on ISSR polymorphism. The male (napier grass) parents formed a separate group along with hybrids, indicating a higher similarity of hybrids with the male parents. Principal component analysis and STRUCTURE analyses showed a similar grouping. The close resemblance of hybrids to the male parents confirmed their interspecific origin. The study revealed that ISSR marker analysis could be a quick and reliable method to identify interspecific hybrids at an early stage of growth.

Keywords: genetic diversity, inter-simple sequence repeat, interspecific hybrids, molecular markers

Introduction

India has a livestock population of approximately 512 million and there is a net deficit of around 61% in green fodder availability (Ahmed *et al.*, 2017). Cultivation of perennial grasses has emerged as an economically feasible alternative to meet the fodder demand (Gate *et al.*, 2018). Perennial grasses show low infestation by natural pests and it prevents soil erosion and increases soil carbon content (Lewandowski *et al.*, 2003).

Napier grass or elephant grass (*Pennisetum purpureum* Schumach) is one of the promising perennial fodder grasses which is a native of tropical Africa and was introduced in India during the early part of the 20th century (Babu *et al.*, 2009). It produces high green biomass yield, ease of propagation and resistance to diseases and pests (Kandel *et al.*, 2015). However, due to the pubescence on leaves and stems, it has low palatability. Pearl millet (*Pennisetum glaucum* [L.] R.Br.), also known as bajra in

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India, is an important dual-purpose crop used for human and animal food. It is soft, succulent and nutrient-rich, and has high value as a fodder crop. However, it is a seasonal crop and shows lower biomass yield in comparison to napier grass.

In order to improve the fodder quality traits of napier grass, interspecific crosses between napier grass and pearl millet have been attempted and the resultant hybrids showed high biomass and desirable quality traits (Burton, 1944). The interspecific hybrids (2n = 3x = 21AA'B) are sterile allotriploids (Techio *et al.*, 2005). Pearl millet × napier grass hybrids (B × N hybrids) supply quality green fodder continuously for 3–5 years from the same field (Gate *et al.*, 2018). Their inability to set seed necessitates fresh interspecific crossing. B × N hybrids are usually identified based on morphological characteristics such as perennial growth, high tillering and palatability. Scoring for these traits requires screening of large F1 populations and the traits are not visible at the early stages of growth (Khajudparn *et al.*, 2012). Molecular tools such as EST-SSRs (simple sequence repeats present in expressed sequence tags), which require sequence information of the two plant species, have been used for confirmation of interspecific hybrids between pearl millet and napier grass (Dowling *et al.*, 2014).

Inter simple sequence repeats (ISSRs) are single primer-based markers that amplify genomic regions lying between two repeats. Their use does not require prior sequence information and is cost-effective. The ISSR markers are less laborious to score than other molecular markers such as amplified fragment length polymorphism or SSRs and yield more reliable bands than random amplified polymorphic DNA (Costa *et al.*, 2016). ISSRs have been successfully used to determine interspecific hybrids of *Bromus* species (Sutkowska *et al.*, 2015), bamboo species (Lin *et al.*, 2010), *Coffea* species (Ruas *et al.*, 2003), loquat species (Wang *et al.*, 2017) and mung bean species (Khajudparn *et al.*, 2012). In the present study, ISSR markers have been utilized to facilitate the identification of B × N hybrids. These markers could be used for the detection of B × N hybrids at an early stage of growth, hence reducing the population size to be screened for favourable morphological traits.

Materials and methods

Plant materials and hybridization

The crossing programme between pearl millet and napier grass was carried out at the time of flowering during September–October 2011 at BAIF Development Research Foundation, Central Research Station, Urulikanchan, Pune 412 202 (M. S.) India. Three types of crosses involving pearl millet genotypes BAIF Bajra-1, BAIF-INC-Bajra-3 and BAIF-INC Bajra-14 as the female parents and napier grass genotypes BRN-01 and FD-444 as male parents were carried out as shown in Table 1. Pearl millet inflorescences were covered with butter paper bags to avoid cross-pollination. The emasculation of the florets was not carried out since pearl millet is protogynous. The receptiveness of stigma on the protruding styles towards the upper part of the inflorescence was visually confirmed. Napier grass (BRN-01 and FD-444) pollen was collected by gently tapping inflorescences in a petri dish in the early morning. The pollen was applied to the stigma of pearl millet with a paintbrush and the inflorescences were bagged immediately. The same procedure was repeated over 4–5 days. Panicles of pearl millet were harvested and the grains were collected at maturity. The F1 grains were sown in the summer of 2012 and used for scoring B × N hybrids.

Table 1. List of the pearl millet × napier grass hybrids and their parents involved in crosses

Parents involved in crosses	Hybrids of the pearl millet × napier grass
BAIF Bajra-1 × BRN-01	BNH-2, BNH-11, BNH-12, BNH-13, BNH-14, BNH-15, BNH-16, BNH-17, BNH-18, BNH-19, BNH-20,
BAIF-INC-Bajra-3 × BRN-01	BNH-21, BNH-22
BAIF Bajra-1 × FD-444	BNH-23, BNH-24, BNH-25
BAIF-INC Bajra-14 × BRN-01	BNH-26, BNH-27
National checks	BNH-10, CO-3

DNA extraction and ISSR amplification

Fresh leaves of 18 B × N hybrids, two national checks and four parent plants were ground to a fine powder in liquid nitrogen. DNA was extracted from 100 mg of leaf powder using a modified CTAB method (Murray and Thompson, 1980). The quality and quantity of the genomic DNA were checked by 1% agarose gel electrophoresis and spectrophotometrically (Nanodrop^{TM} 2000, Thermo Fischer Scientific, India) Mumbai, Maharashtra. Genetic fingerprinting was carried out using eight ISSR primers (Table 2) which were selected based on distinct and reproducible banding patterns. The PCR reactions were carried out in 20 µl volume, containing 2× Go Taq green master mix (Promega, India), 10 mM ISSR primer, 20 ng genomic DNA and water. PCR amplification was carried out in a thermo-cycler (MJ research PTC-200) with an initial

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Table 2. Comparison of primers, total number of loci, polymorphic loci, percentage polymorphism, polymorphic information content, primer index for amplification profiles of pearl millet × napier grass hybrids and parents generated using eight ISSR primers

Sr. no.	Primer sequence[a] (5′-3′)	No. of amplified loci	Total no. of polymorphic loci	Percentage polymorphism	Polymorphic information content (PIC)	ISSR primer index (SPI)
1	$(AG)_8T$	19	19	100	0.44	10.11
2	$(CA)_8A$	14	13	92.85	0.43	9.74
3	$(ATG)_6$	11	11	100	0.44	10.35
4	$(AG)_8YT$	12	12	100	0.40	8.88
5	$(AG)_8YA$	12	10	83.33	0.49	11.14
6	$(AC)_8G$	19	19	100	0.46	10.52
7	$(AC)_8YG$	17	16	92.85	0.48	11.09
8	$(GA)_8T$	21	21	100	0.40	8.99
	Total	125	121	96.8		

[a]Y = C or T (pyrimidine) and R = A or G (purine).

denaturation at 94°C for 5 min followed by 45 cycles at 94°C for 30 s, annealing at 50°C for 45 s and extension at 72°C for 60 s, and then a final extension step at 72°C for 5 min (Nimbalkar *et al.*, 2018; Takawale *et al.*, 2019). The amplified products were separated on a 2.0% agarose gel in 1.0× TAE buffer and bands were detected by ethidium-bromide staining. Stained gels were documented using a gel documentation system (BIO-RAD, India).

Data analysis

The presence (1) or absence (0) of bands in all the genotypes were manually scored. The number of polymorphic bands specific to B × N hybrids, male and female parents, respectively, as well as the shared bands between both parents was documented as alleles using Microsoft Excel 2016. Polymorphism information content (PIC), which detects polymorphism within a population by considering the number of alleles that are expressed and the relative frequencies of those alleles, was calculated (Nagy *et al.*, 2012). The ISSR primer index (SPI) was calculated by adding the PIC values of all loci amplified by a given primer. The binary data were used for the Unweighted Pair Group Method with Arithmetic Means (UPGMA) cluster analysis and a dendrogram was constructed using PAST 3.12 software (Hammer *et al.*, 2001). Principal component analysis (PCA) was performed for all samples using matrix variance–co-variance and between-groups approach employing PAST 3.12 software and plotted using the convex hull option.

The possible admixture of the 18 hybrids along with parents and national checks was analysed using STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000) that is based on the Bayesian model. The software parameters were set as Burn-in period (10,000), Markov Chain Monte Carlo (MCMC = 100,000) where the admixture model was used assuming correlated-allele frequencies. Simulations were run setting $K = 1$–5 with five iterations. The best fit number was selected by submitting results to STRUCTURE HARVESTER that is based on the maximum ΔK value (Earl and vonHoldt, 2012).

Results

The F1 progeny of four crosses were evaluated for phenotypic characters such as perenniality, presence/absence of leaf pubescence and total green biomass. After 5–6 cuttings carried out over years, 18 promising heterotic clumps were identified and named as BNH lines (Table 1).

Out of 15 primers tested, eight ISSR primers that showed distinct banding patterns were selected for final analysis (Table 2). A total of 125 bands showing 96.8% polymorphism were recorded for these eight ISSR primers. The polymorphic bands ranged from 10 to 21 for the primer $(AG)_8YA$, and $(GA)_8T$, respectively, with an average of 15 bands per primer. PIC per primer ranged from 0.40 to 0.49 (Table 2). The SPI, which is indicative of the efficiency of the maker system, ranged from 8.88 to 11.14.

To verify the genetic relationship of hybrids with their parents, the percentage of ISSR bands shared by hybrids and their parents and those unique to hybrids was calculated. B × N hybrids (BNH-14, BNH-21 BNH-15, BNH-23, BNH-2 and BNH-18) shared a higher percentage of bands with the male parents (32.56, 28.57, 27.66, 25.64, 24.39 and 24.32%, respectively) than with the female parents (Table 3). The highest number of unique bands was recorded in BNH-13 (17 bands) and lowest (10 bands) in BNH-25 (Table 3).

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Table 3. Genetic relationship based on sharing of ISSR bands by pearl millet × napier grass hybrids with their parents as well as unique bands observed in the hybrids

Sr. no.	Hybrids	Total no. of bands	Bands shared with male	Bands shared with female	Bands shared with both parents	Bands for hybrid only
1	BNH-11	48	12	11	10	15
2	BNH-12	48	10	8	14	16
3	BNH-13	47	10	9	14	17
4	BNH-14	43	14	8	10	11
5	BNH-15	47	13	9	10	15
6	BNH-16	46	11	10	11	14
7	BNH-17	46	11	9	11	15
8	BNH-18	37	9	8	9	11
9	BNH-19	45	9	11	12	13
10	BNH-20	41	10	9	8	14
11	BNH-21	42	12	5	12	13
12	BNH-22	39	9	6	13	11
13	BNH-23	39	10	7	11	11
14	BNH-24	37	8	8	10	11
15	BNH-25	38	7	9	12	10
16	BNH-02	39	8	9	9	13
17	BNH-26[a]	36	17	-	-	19
18	BNH-27[a]	36	19	-	-	17

[a]Maternal parent not involved in the present study.

The UPGMA dendrogram constructed from the binary data (presence or absence of band) for each genotype revealed that female parents formed a distinct cluster from the B × N hybrids as well as male parents, with a bootstrap value of 61% (Fig. 1). CO-3, which represented a nationally released check for B × N hybrid, was used as a positive control since it did not involve any of the parents used in the present study. This genotype was separated from the group formed by hybrids and male parents, with a bootstrap value of 75%. The two male parents grouped with one of the hybrids (BNH-21) (Table 3; Fig. 1). All other hybrids formed a cluster with bootstrap values of <50%. PCA complemented the inferences drawn from the dendrogram and the first two components of PCA contribute to 20.6% variation. The convex hull revealed separation of hybrids from the parents and checks BNH-10 and CO-3 (Fig. 2).

The results of STRUCTURE analysis processed in STRUCTURE HARVESTER revealed the highest ΔK value (29.76) of $K = 3$ suggesting a division of all genotypes in three populations where 25 independent runs were used (Fig. 3). The structuring patterns indicated distinctness of female parents, hybrids origin confirmed based on admixture pattern. As demonstrated by the dendrogram and PCA, STRUCTURE also showed the genetic closeness of all hybrids to the male parents.

Discussion

Pearl millet is a protogynous plant. The styles protrude from the spike from the top to bottom over 2 days, during which stigma receptivity is known to last only for 12–24 h. Anthesis starts when the styles dry from the centre of the spike and proceeds in both directions. Since cross-pollination between napier grass pollen and pearl millet stigmas was carried out over 4–5 days, there were chances of self-pollination in pearl millet. The F1 hybrids were difficult to distinguish based on morphology and it was only observing regrowth after 3–4 cuttings, or lack of panicle emergence, it was possible to confirm their identity. Identification of interspecific hybrids of *P. purpureum* Schumach. × *P. glaucum* [L.] R. Br. based on flow cytometry also failed, due to highly similar nuclear genomic DNA content in the two parents (Dowling *et al.*, 2013). Simple sequence repeat (SSR) markers have been developed for napier grass, but these markers showed about 50% cross-amplification in pearl millet, hence limiting their use to distinguish the two species (Sousa Azevedo *et al.*, 2012). Though EST-SSRs have been used for identifying pearl millet × napier grass hybrids, the methods used for scoring SSRs are tedious (Dowling *et al.*, 2014). In comparison, ISSR markers are neutral types of markers, cheaper, easier to score and reproducible. Based on the previous reports

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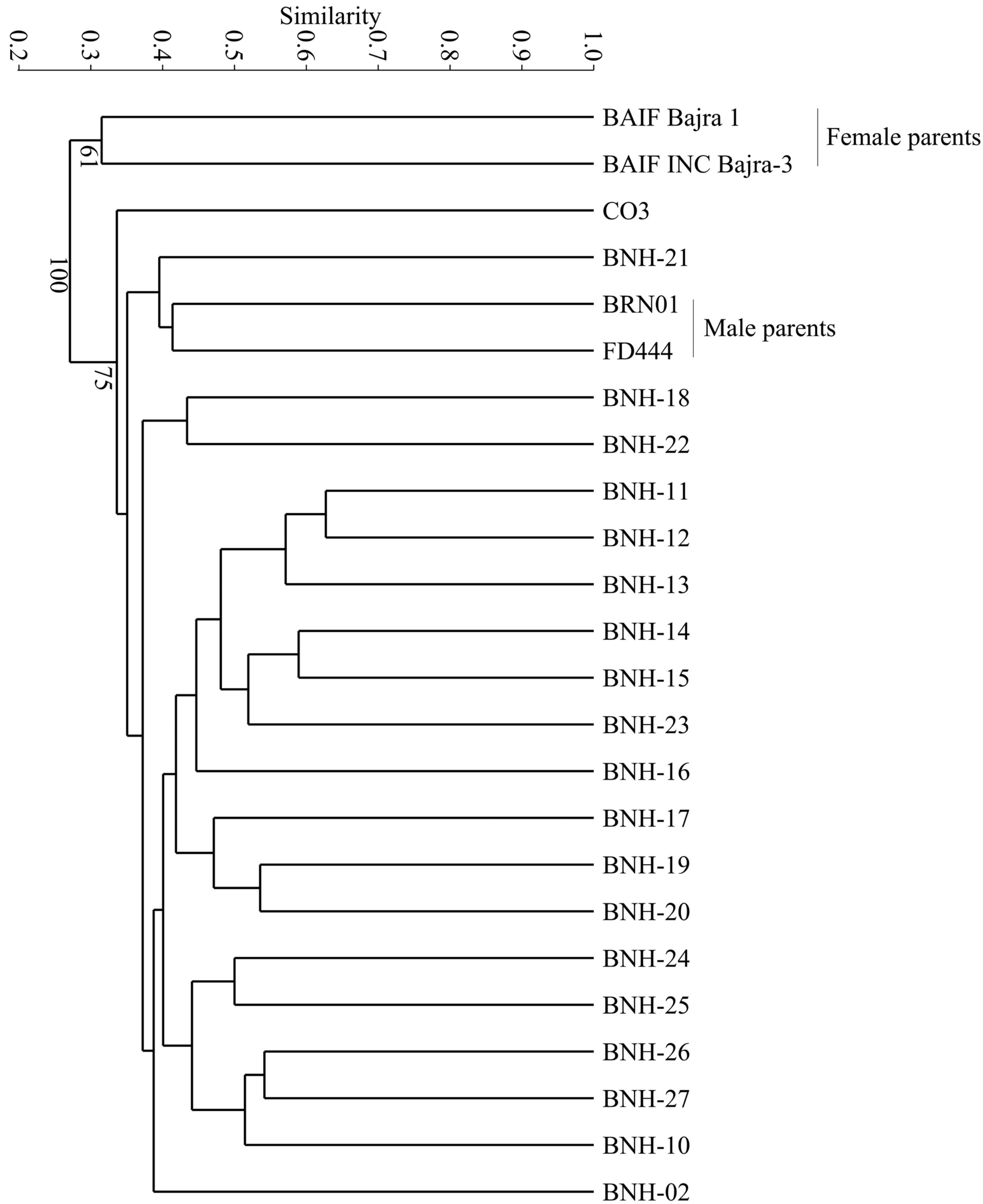

Fig. 1. UPGMA cluster analysis showing the relationship among 18 pearl millet × napier grass hybrids, their parents and the pearl millet × napier grass hybrid national check CO-3 and BNH-10 using ISSR markers. The dendrogram was calculated using Jaccard's coefficient and bootstrap values (%) at nodes were generated by 1000 replications.

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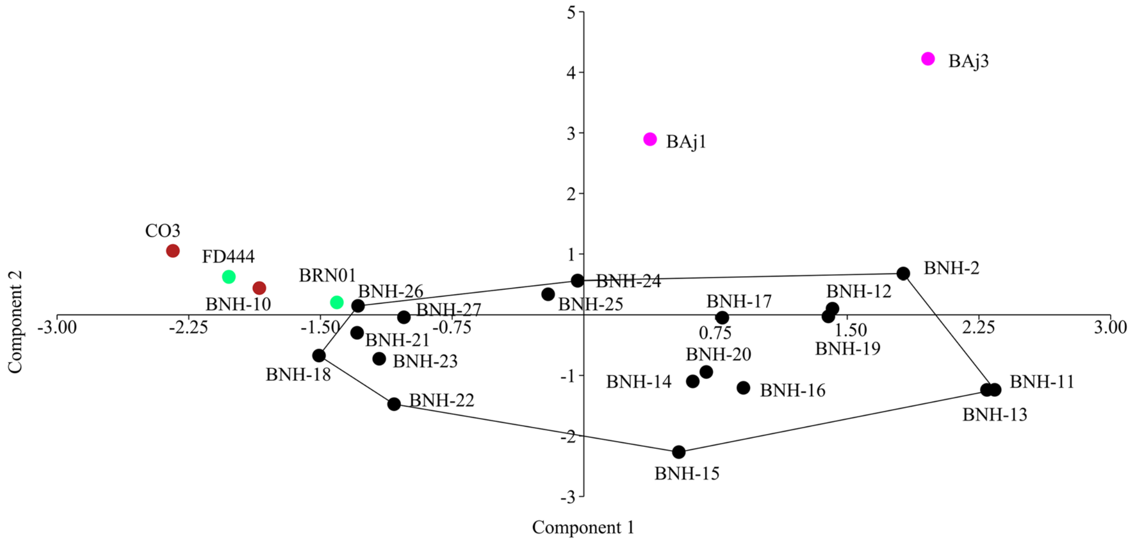

Fig. 2. Principal component analysis of 18 pearl millet × napier grass hybrids, their parents and the pearl millet × napier grass hybrid national checks CO-3 and BNH-10. The convex hull option was used to delimit hybrids. Component 1 (10.8) and component 2 (9.8) contribute to 20.6% of the total genetic variance. Blue dots: female parents (pearl millet); green dots: male parents (napier); black dots: pearl millet × napier grass hybrids, and brown dots: national checks.

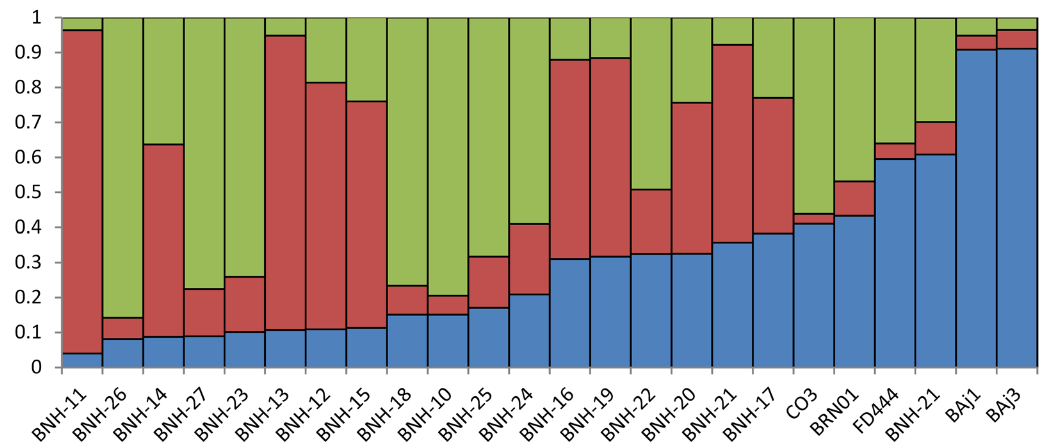

Fig. 3. ISSRs revealed the Bayesian genetic structure of pearl millet × napier grass hybrids and their parents in each vertical line using STRUCTURE software version 2.3.4 and STRUCTURE HARVESTER program. Based on $\triangle K$ value (29.76) bar plot for $K = 3$ was selected and genotypes were sorted by Q value.

from this laboratory (Nimbalkar *et al.*, 2018; Takawale *et al.*, 2019), di nucleotide-based ISSR primers were found to be most informative and yielded higher polymorphism. These have therefore been used for identifying the hybrids.

The PIC value of a marker is the probability that the marker genotype of the progeny differs from either parent, and ISSRs show a maximum PIC value of 0.5 since they are dominant markers (Nagy *et al.*, 2012). All markers used in this study showed PIC values >0.4, suggesting their usefulness in distinguishing the hybrids from the parents.

The relation between parents and hybrids can be studied based on the number of shared loci between them. Bands shared between one of the parents and the hybrid is indicative of its genetic relatedness with that parent, while bands shared with both parents indicate the true hybrid nature of the progeny (Wang *et al.*, 2017). On the other hand, the presence of private/unique bands in hybrids is indicative of a potential rearrangement in their genomes (Lin *et al.*, 2010; Sutkowska *et al.*, 2015). In the present study, the percentage of bands shared with the male parent was higher than the female parent, which could be attributed to the higher genetic content in napier grass (allotetraploid) as compared to pearl millet (diploid). This was also observed in another similar hybridization

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experiment of napier grass and pearl millet (Dowling *et al.*, 2014). The bands shared by hybrids with the male parent could arise from the homologous genome A' or the distinct genome B. Development of napier grass-specific sequence characterized amplified region markers from some of these bands shared with the male parents requiring a single PCR amplification for the identification of the B × N hybrids would be undertaken in future work.

Cluster or grouping analysis, either dendrogram, PCA or STRUCTURE, demonstrated clear separation of the female parents suggesting genetic distinctness than hybrids and male parents. The male parents were grouped along with hybrids with lower bootstrap values (>50). Mort *et al.* (2000) reported genotypes with lower bootstrap values (>50) have lower genetic differences; therefore, the position of the hybrids was found to be changed in all three grouping methods and no strict patterns were observed. Further, the association of hybrids with male parents was also reported earlier (Gonzalez and Hanna, 1984; Dowling *et al.*, 2014). For CO-3, no parents were involved in the present study. In all three grouping analyses, CO-3 was grouped inside pearl millet but formed an outlier to the rest of the genotypes confirming its distinct genetic makeup.

In summary, the study revealed that the ISSR technique is quick and reliable for marker-assisted verification in interspecific hybrids. The identification of informative ISSRs and their utilization in identifying true hybrids in this study provides a novel molecular tool in the napier grass and pearl millet breeding programme.

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Author contributions

PST and SSJ did the crossing, selection and propagation of hybrids. SSJ and RAB did the molecular and statistical analysis. All authors wrote and approved the manuscript.

Conflict of interest

None.

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