Development of EST Derived Microsatellite Markers in Buffel Grass and Their Cross Transferability to Other Forage Grasses

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Development of EST derived microsatellite markers in Buffel grass and their cross transferability to other forage grasses

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Introduction

Buffel grass (*Cenchrus ciliaris* L.) is an important perennial grass grown widely in arid and semiarid regions of the world. It is one of the prominent species of the *Dichanthium-Cenchrus-Lasiurus* grass cover spread over the Peninsular India (Dabadghao and Shankarnarayan, 1973). *C. ciliaris* is a warm season, C_4 grass well adapted to survive harsh conditions, elevated CO_2 levels and wide range of climates and soil regimes. It is considered an excellent pasture grass as it provides highly nutritious and palatable forage during drought periods. Despite its excellent forage characteristics genetic improvement through conventional breeding methods has been difficult due to its apomictic mode of reproduction, and is mostly confined to the selection of elite lines from natural variants. (Bhat *et al*., 2001). Knowledge of genetic diversity and phenetic relationships among accessions is prerequisite for breeding programmes. The study of morphological variability is the only approach for assessing genetic diversity especially in minor crops. An assessment based only on agro-morphological traits might be biased because distinct morphotypes can result from spontaneous mutations. However, with molecular markers, genetic resources can accurately be assessed and characterized (Capo-chichi *et al*., 2004). Buffel grass has been extensively studied to understand the genetic regulation of apomixis targeting the genomic regions shared and conserved across grass family, while limited studies have been done so far to assess the genetic variation and utilization in breeding for agronomically important traits and absence of adequate genomic resources impeded further improvement. Cenchrus cultivars and accessions have previously been characterized using random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR) and Sequence tagged site (STS) markers. While microsatellites are the most preferred markers due to their locus specificity, co-dominant nature, high polymorphism and reproducibility, their development and application is highly time consuming and expensive and has been limited to a few agriculturally important crops (Powell *et al*., 1996). An alternative way is to search the abundant sequence data already available in public databases to identify SSR's. Nevertheless, markers developed in this way present a valuable resource for subsequent comparison between the model species and the related species. Therefore, the objectives of the present study were to develop EST based SSR markers and test their transferability potential in other related forage grasses.

Materials and Methods

Perfect and compound microsatellites >10 bp with motifs ranging in length from 1 to 6 only were identified using a PERL script misa.pl from 21,729 EST's of *C. ciliaris*. A minimum unit size of twelve for mono-nucleotide repeats, six for a di-nucleotide repeat, four for tri and a minimum of three for tetra, penta and hexa-nucleotide repeats were set to detect the SSR loci. Primers from the flanking sequences of SSRs were designed in a batch mode by using PRIMER3 program and PERL5 module. A threshold criterion of 20–23 nucleotide length, an optimum annealing temperature of 55–65 °C and GC content of 45–55% were set to design primers. 346 SSR primers were successfully designed of which a subset of 117 primers representing mono, di tri, tetra and compound repeats were custom synthesized by Integrated DNA Technologies (San Diego, USA). The putative functions of sequences containing SSRs were annotated from the non-redundant protein database of NCBI using BLASTX. QBLAST based homology search in BLAST2GO V2 program was used to retrieve the associated GO terms and to assign plausible associations of biological processes, cellular components and molecular functions. Twenty three genotypes representing six species of Cenchrus and five varieties of four other grasses were used to validate the developed EST-SSR markers. Genomic DNA was extracted from 200mg of freshly frozen leaf tissue using the CTAB method. The DNA concentrations were quantified using a PICODROP and the quality was checked on 0.8% agarose gels. 25 ng of DNA was used as template for each PCR reaction.

Amplification and cross transferability of SSRs: The PCR reactions were carried out in a 20 ul reaction volume consisting of 1.0 ul of 10X PCR reaction buffer, 1.0 U of TaqDNA polymerase (Merck), 0.2 mM of dNTPs, 0.5uM of each primer and 25 ng of genomic DNA using a BIOXPER thermocycler. The following program was set; an initial

denaturation of 3 min at 94^0C followed by 35 cycles of 30 s at 94^0 C, 45 s at optimal annealing temperature, 90 s at 72^0 C and a final extension step of 10 min at 72^0C. The PCR products were resolved on 2% agarose gels stained with ethidium bromide and visualized using Geldoc (UV-Vis) for SSR band detection. A 100-bp DNA ladder was used to estimate the amplicon sizes.

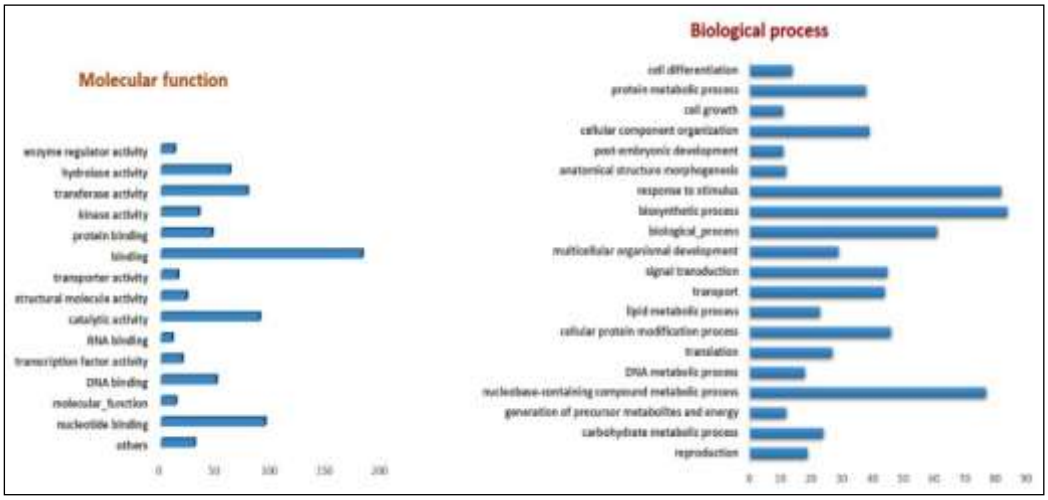
Results and Discussion

Cenchrus ciliaris is a drought tolerant pasture grass occupying the arid and semiarid tracts of the world. Since tropical grasses have been less intensively studied limited molecular markers are available for germplasm characterization. In the present study we have developed EST- derived SSR markers from 21,729 publicly available EST sequences. 10 % of the ESTs have found to have one or more SSR motifs (Table 1), generally a frequency of 2-12% SSRs in ESTs were reported in many flowering plants (Morgante 2002), differences in the SSR frequencies may be due to different search parameters employed (Asp *et al*., 2007). Majority of repeats motifs were shorter than 15 bp in contrast to previous reports of 12-19 bp in Setaria and Lolium. The average density of SSR was 2.8 KB which is much higher than Setaria and Citrus. Trinucleotide repeat motifs constituted the majority of the identified SSRs followed by mono, di, tetra, penta and hexa nucleotide repeats (Table 2). The most abundant repeat motifs found in the present study were CCG/CGG, AG/CT, AGGG/CCCT for trinucleotide, dinucleotide and tetranucleotide repeats, respectively, which was quite similar to earlier reports in Lolium, Brachypodium, Sorghum and Rice. In this study, 117 primer pairs were developed and evaluated in a set of Cenchrus germplasm and further their transferability was tested among six species of Cenchrus and four other important forage grasses. Majority of the primers produced clear amplicons suggesting the utility of EST based SSRs as they are developed from the coding regions of the genome. Gene ontology (GO) annotations based on BLASTX analysis assigned putative functions to ESTs broadly into genes regulating the biological functions related to response to stimulus, signal transduction and transport, genes showing similarity based on molecular function like DNA binding, transferase (Figure1).

Table 1: Summary of the EST data mining in Cenchrus

Summary of ESTs	
Total number of sequences examined	11086
Total size of examined sequences (bp)	8335083
Total number of identified SSRs	2939
Number of SSR containing sequences	2291 (20.66%)
Number of sequences containing more than 1 SSR	478
Number of SSRs present in compound formation	214
Frequency of SSR	1/ 2.8 Kb

Fig. 1: Functional annotation of transcripts containing SSRs



Conclusion

Cenchrus ciliaris is an excellent pasture sown grass grown throughout the arid and semiarid regions of the world. Due to apomictic mode of reproduction, genetic improvement has been limited to selection of elite genotypes. For successful forage breeding programme availability of molecular markers is prerequisite not only to characterize the germplasm but also to introgress novel alleles. In the present study ESTs were exploited for development of microsatellite (SSR) markers and their cross transferability potential in other forage grasses was tested

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