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Short Communication

Development of microsatellite markers in finger millet (*Eleusine coracana* Geartn.)

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Abstract

In the present study, for the first time an attempt was made to isolate microsatellites in finger millet using pre-cloning enrichment (selective hybridization) strategy which resulted in isolation of very good number of micro satellites (323).While developing the microsatellite-enriched library for finger millet, more than 2200 colonies were screened, and 1021 recombinant clones ranging from 500-1000bp were selected for further sequencing. Sequence analysis revealed that only 645 clones (63%) contained microsatellite motif regions. Monorepeats C and T were abundant, whereas, AG and CT class of dinucleotide repeats accounted about 21% and 30% respectively. Apart from the abundant, mono, di-nucleotide and tri repeats, about 58 tetranucleotide and 62 penta repeats were also isolated.

Key words: SSR, finger millet, enrichment, microsatellites, ragi

Finger millet (*Eleusine coracana* Geartn.) commonly called as Ragi, a subsistence crop mainly grown on dry lands. The grain has high nutritional value and excellent storage qualities, which make it an important famine food. Nutritionally the grain rich in fiber, calcium and iron content. It is a recommended diet for the diabetics. The crop productivity in this crop is affected due to drought. In general, plants have developed several mechanisms to cope with water-limited situations. The combination of relevant physiological traits must be pyramided in order to achieve an overall improvement in drought tolerance as well as enhanced productivity. However, breeding for these physiological traits is complex as these traits have polygenic inheritance and are also difficult to quantify. Thus, molecular breeding strategy where tightly linked DNA based molecular markers are used to identify the desirable genotype is expected to significantly complement the breeding processes to introgress relevant traits.

QTL mapping allows one to statistically identify chromosomal regions containing genetic factors contributing to variation in a polygenic trait (Meng et al. 2015). Once the tightly linked markers have been identified, they can be used to develop marker assisted selection strategy for breeding application. Molecular markers are pre-requisite for assessing the polymorphism which further helps in the development of genetic map. The co-dominant markers such as SSR (Simple Sequence Repeats) are essential for detecting useful traits in segregating population but finger millet is a less investigated crop in terms of molecular breeding study and also that markers specific to the crop have not yet been developed.With this background, a study was taken up with the major objective to develop SSR markers in finger millet and also to characterize the isolated markers.

Development/Isolation of SSR markers

The pre-cloning enrichment (selective hybridization) strategy was followed to fish out microsatellites from fingermillet genome. This strategy employs the whole pool of digested DNA by subjecting them to repeat oligomer hybridization, thereby leading to selective

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enrichment. The steps followed in the microsatellite isolation by pre-cloning enrichment (selective hybridization) strategy are:

DNA extraction by CTAB method, Restrict digestion of extracted DNA using RsaI or Hae III, Linker ligation to DNA fragments with linkers, *viz*., Super SNX24 forward- 5' GTTTAAGGCCTAGCTAG CAGAATC and Super SNX24+4p reverse-5' pGATTCTGCTAGCTAGGCCTTAAACAAAA.

Further enrichment of microsatellite repeats using biotinylated oligos $(CA)_{17}$, $(AG)_{16}$, $(AGC)_8$, $(AGG)_8$, $(AAC)_{14}$, $(ATC)_{14}$, $(AAG)_{14}$, $(ACGC)_5$, and $(ACCT)_8$., Capturing of repeat regions from enrichment PCR product by streptavidin beads, Cloning into pTZ57 R/ T vector and sequencing of repeat containing DNA fragments with nested M13 forward and reverse primers using ABI prism sequencer (Fig. 1) and Sequence analysis for the presence of microsatellite regions using the web-based software –"Tandem Repeat Finder" and primer designing to specific microsatellite repeats using the Fast PCR software programme and Optimization of annealing temperature for microsatellite primers (Fig. 2) were carried out.

The work on development of SSR markers and their characterisation was undertaken in the present study. An attempt was made to isolate microsatellites in fingermillet using pre-cloning enrichment strategy which resulted in isolation of 323 micro satellites. While developing the microsatellite-enriched library for finger millet, more than 2200 colonies were screened 1021 recombinant clones ranging from 500-1000bp (Fig. 1) (Table 1) were selected for further sequencing. Sequence analysis revealed that only 645 clones (63%) contained microsatellite motif regions, while many traditional microsatellite isolation studies have reported an average of 50-60% positive clones (Zane et al. 2002) after sequencing and the average percent of false positives in such studies was estimated to be 48.7% (Squirrel et al. 2003). The short length of

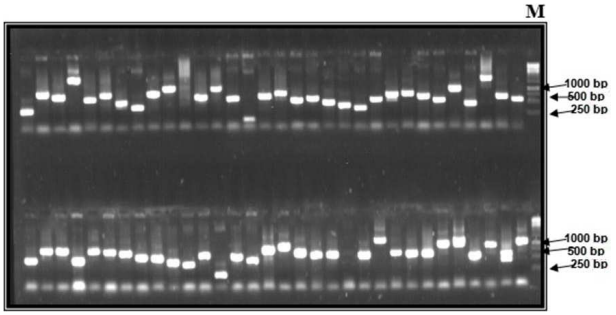

Fig. 1. Screening of the microsatellite enriched colonies by colony PCR. The amplified, enriched, recovered microsatellite repeat fragments (pure gold DNA) were cloned into pTZ57R/T (T/A cloning vector) and transferred into *E.coli*. Based on blue white selection, the recombinant clones were confirmed by colony PCR reactions and product was resolved on 1.0% agarose gel.; M-Marker (1kb)

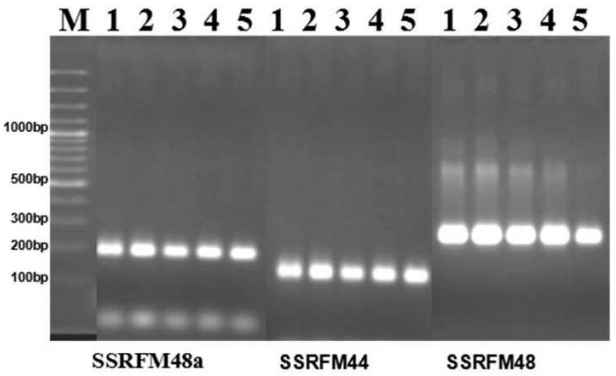

Fig. 2. Standardization of annealing temperature of SSR primers for locus specific amplification. The in-house developed microsatellite primers were initially standardized for optimum annealing temperature at different gradient temperatures. The gel depicts standardization of annealing temperature of few SSR primers Lane 1-59.9^{0}C, 2-61.6^{0}C, 3-64.0^{0}C, 4-66.1^{0}C, 5-69.6^{0}C; M-Marker (100bp)

Table 1. Summary of sequences generated from the enrichment library

No. of colonies screened	No. of clones sequenced	Clones with SSR motif	Clones without SSR motif	Clones with repeats > 15 bp	No. of primers designed
2200 1021	645	376	323	323	

More than 2000 clones were screened to get the bigger fragment size, however one thousand and twenty one bigger fragment clones ranging between 500-1000bp were sequenced with nested M13 forward and reverse primers using ABI prism sequencer. The sequences were analyzed for the repeat motifs regions using the web-based Tandem Repeats finder programme. The results revealed that three hundred and twenty thee repeat motifs were identified with >15bp, for which primers have been designed.

Table 2. Different types of nucleotide repeats and their frequency in enrichment library

Type of nucleotide repeats	Number of nucleotide repeat motifs in clones
Monorepeats	165
Direpeats	1047
Trirepeats	207
Tetrarepeats	58
Pentarepeats	62
Total	**1539**

The generated sequence information from the library designated more of di-nucleotide repeats (67%) than mono (11%), tri (14%) tetra (4%) and penta (4%) nucleotide repeats

oligonucleotides used as probes could be a possible reason for the resulting high proportion of false positives. The length of the probe applied for hybridization can influence the type and length of microsatellites captured. Michael Seringhaus et al. (2008) found that longer oligonucleotide probes not only favored the isolation of relatively long arrays, but also eliminated the mismatches. The protocol in this study made use of (28-30 bp long) oligonucleotides, other whereas successful studies (Stajner et al. 2005) used much longer probes (200-550 bp). It has been suggested that short probes for hybridization tend to select fragments with shorter interrupted repeats. In other investigations (Lopes et al. 2002; Senda et al. 2004), 2 rounds of hybridization have been performed to eliminate false positives. Santana et al. (2018) suggested the enrichment of genomic libraries to enhance identification of microsatellites. Enrichment ratios published in the literature vary from 20-95% depending mainly on the procedure and method used (Stajner et al. 2005). In fact, the enriched library prepared in this study resulted in a higher percentage (63%) of microsatellites.

Characteristics of isolated SSR markers

Out of the 1021 sequences, 645 sequences contained the microsatellite repeats. The generated sequence information from the library consisted more of di-nucleotide repeats (67%) than mono (11%), tri (14%) tetra (4%) and penta (4%) nucleotide repeats (Table 2). In the present study, monorepeats C and T were abundant. AG and CT class of dinucleotide repeats accounted about 21% and 30% respectively and this is consistent with the previous studies in finger millet (Dida et al. 2007), *Coffea* genus (Poncet et al. 2006), hop (*Humulus lupulus* L.) *Lolium temulentum* (Senda et al. 2004) and *Mangifera indica* L. (Srivastav et al. 2021), for all of which enrichment strategy was employed further supporting the conclusion that these repeats are abundant in plants and mango (Srivastav et al. 2021). GC repeats were not found, which is consistent with the reports suggesting that these repeats are extremely rare in most genomes (Cui et al. 2005). Lower frequencies of GC repeats have been attributed to methylation of cytosine, which in turn increases the chance of mutation to thymine, by deamination (Simmen 2008). Apart from the abundant, mono, di-nucleotide and tri repeats, about 58 tetranucleotide and 62 penta repeats were also isolated, with less success. This failure could be due to selective enrichment of specific microsatellite sequences. However, the high success in enrichment of similar motifs in a separate study using similar enrichment (Métais et al. 2002) strategy invalidates this point. Although di-nucleotide repeats are the most commonly used class of microsatellite markers in plants, there has been concern over the difficulty experienced in genotyping them due to a high frequency of strand slippage artifacts. In contrast, tri- and tetra-nucleotide repeat based markers have been shown to produce a higher proportion of discrete PCR products due to reduced level of stuttering (Gastier et al. 1995).

The sequencing was done in both the directions in order to increase the quality of sequences for primer designing after alignment. About 323 primers were designed out of which, 175 primers showed locus amplification. Few primers could not be optimized and they either produced faint bands or did not show reproducible banding patterns. Most of primers yielded sharp bands at a annealing of 55 ± 5^0C and, the bands were not sharp at 60^0C and often characterized with a smear or spurious bands presumably due to the high stringent annealing temperature. Although Squirrel et al. (2003) have indicated that optimization in itself could be a source of attrition but other investigations have pointed out the fact that PCR optimization may lead to insignificant improvement in band interpretability (Ashworth et al. 2004). Out of 175 primers which showed locus specificity in the present study, very less number (10) of SSR markers revealed polymorphism between parents and also in mapping population. The low level of polymorphism can be attributed to genetic divergence of species. One of the contributions of this study is generation of SSR marker systems, both genomic and genic which can

be used by the diverse groups for molecular characterization of finger millet.

Authors contribution

Conceptualization of research (SMS, T.GP, MU), Designing of the experiments (SMK, JNM, MSS, TGP, MU), Contribution of experimental materials (SH), Execution of field/lab experiments and data collection (SMK, JNM), Analysis of data and interpretation (SMK, MSS), Preparation of the manuscript (SMK).

Declaration

The authors declare no conflict of interest.

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