HYBRIDITY AND GENETIC PURITY TESTING OF PEARL MILLET [*Pennisetum glaucum* (L.) R. BR.] HYBRID SHRADDHA

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Abstract— The hybridity and genetic purity of pearl millet hybrid Shraddha was analyzed by PCR-based SSR markers in present research. Amongst the 20 primers screened to identify the specific marker associated with hybrid and parental lines, only six primers *viz.*, PSMP-2084, PSMP-2013, PSMP-2202, PSMP-2089, PSMP-2270 and PSMP-2272 were able to produce both female and male parent specific alleles and helped to assess hybridity and genetic purity of the hybrid. SSR marker PSMP-2084 amplified allele size 232 and 455 bp and PSMP-2272 amplified alleles of size 270 and 370 bp which were specific to female parent (RHRB 1A) whereas, alleles of size 343 and 370 bp amplified by PSMP-2084 and PSMP-2272 respectively, were specific to male parent (RHRBI 138). Also, PSMP-2013 amplifying allele at 134 and 180 bp, PSMP-2089 amplifying allele at 142 and 331 bp and PSMP-2202 amplifying allele at 148 and 162 bp were distinguished the hybrid by expression of parent specific alleles. Another marker, PSMP-2270 amplified alleles at 154, 420 and 512 bp in hybrid and both of its parents. Study leads to conclude that, PSMP-2084, PSMP-2013, PSMP-2202, PSMP-2089, PSMP-2270 and PSMP-2272 markers were hybrid specific and can be utilized for hybridity and genetic purity testing of hybrid Shraddha.

Index Terms— Pearl Millet, Genetic Purity, Hybridity, SSR, Molecular Markers.

I. INTRODUCTION

Conventional genetic purity analysis methods in the field are time consuming, laborious, resource intensive and drastically affected by environmental factors, therefore, a technique that is rapid and not affected by environment is needed for assessment of genetic purity and selection of parental lines for use in true to type hybrid development programmes. In contrast to morphological traits, which can be influenced by temperature, soil type, nutrients, insects, etc., the use of molecular markers can provide new insights to better understand the genetic variation within the germplasm collection. Genetic purity assessment prior to developing hybrids can aid in better exploitation of diversity.

Genetic variation in pearl millet has been studied mainly by morphological descriptors and isozyme markers, which are not only limited in number but also get affected by environmental conditions and stages of development (Tostain and Marchais ,1989). Besides genetic variation, molecular makers were also been used for evaluating the genetic stability of crops (Koshy *et al*. 2013). Pallavi *et al*. (2011) in sunflower, Liu *et al.* (2008) in tomato, Dunja *et al*. (2014) in cabbage identified SSR markers associated with hybridity and genetic purity testing of hybrids. Hipi et al. (2013) showed that SSR markers were more reliable for assessing genetic purity as compared to morphological marker.

Farmers can exploit the full potential of any hybrid only when they get genetically pure seeds of the hybrid. Hence, ensuring the genetic purity of certified seeds of hybrid is mandatory, which is done through field grow out test (GOT) based on the morphological characters of plants grown up to maturity. GOT based morphological markers are being land and labour intensive, time consuming, cumbersome and influenced by the environment, hence there is a need to identify rapid and reliable alternatives like DNA based assays. In India, the seed crop of pearl millet is primarily grown during December to April in the southern states and the grain crop is sown between June and July in the northern and central India. Thus, the time available for ensuring genetic purity of produced seed lots through GOT is rather short. Apart from this, plant morphological characters have limitations like small variability observed between cultivars and the environmental effect on the expression of character. To overcome these limitations rapid and reliable techniques, *viz.* electrophoresis of seed esterases was developed for characterizing pearl millet cultivars (Rao *et al.*, 2001).

Therefore, the present study was undertaken with the objective to identify efficiency and accuracy of SSR markers as could be found more rapid, practical and efficient tool to test the hybridity and genetic purity of hybrid Shraddha.

II. MATERIALS AND METHOD

The present investigation was conducted at State Level Biotechnology Center, M.P.K.V., Rahuri during 2013. The experimental material was developed and provided by the AICPMIP, Pearl millet Research Scheme, Dhule, (MS) India during 2012-2013. All recommended agronomical practices and plant protection measures were followed as and when required for raising good crop.

Plant materials

The true-to-type breeder seed, obtained from Pearl millet Breeder, Pearl millet Research Scheme (Dhule) affiliated Mahatma Phule Agriculture University, India. The seed material for the present investigation comprise of pearl millet F_1 hybrid 'Shraddha' and its parents 'RHRB 1A' (female), 'RHRBI 138' (male).

Genomic DNA isolation

Young (15-20 days old) and healthy leaves were harvested for genomic DNA extraction collected from individual plants. Total genomic DNA was isolated and purified according a modified Cetyl trimethyl ammonium bromide (CTAB) protocol and the DNA quality for each sample assessed on 1.2% agarose gel, then stored at 4 ◦C or −20 ◦C for further use (Saghai-Maroof *et al.*, 1990).

SSR analysis and PCR amplification

The genomic DNA was amplified using twenty SSR primer pairs. The SSR primers were synthesized by Bangalore Genei (India) Biotech Co. according to the reported sequences of microsatellite markers. The PCR reactions for SSR were carried out according to method given by Sarla *et al*. (2003) with some modifications. The volume of the reaction mixture was 20 µl, consisting of template DNA (2.0 µl), 1 × PCR buffer (2.0 µl), with 1.5 mM of $MgCl_2$, 1.6 µl of dNTPs, 1 µl each of forward and reverse primers and 0.3 µl of Taq DNA polymerase sterile distilled water (12.1 µl). SSR-PCR was performed in a thermal cycler (Eppendorf master cycler) which was programmed for 40 cycles of 94 ^{0}C (5 min.), 50-55 ^{0}C (40 sec.), 72 ^{O}C (30 sec.), then followed by final extension at 72 OC for 10 min. Following the amplification, the PCR products of SSR were loaded on 2.5 % agarose gel, which was prepared in 90 ml of sterile distilled water containing 10 x TBE buffer (10 ml), ethidium bromide (4 µl). Electrophoresis of the amplified products were performed at 100 V (1.5- 2 hrs). After separation the gel was viewed under gel documentation system (Kodak Molecular Imaging Software (v. 5.0.1.2.7) and photographed.

Genetic Purity Data Analysis

Clear and distinct bands amplified by SSR primers were scored for the presence and absence of the corresponding band among the genotypes. The scores 1 and 0 indicate the presence or absence of bands, respectively. A dendrogram was constructed by using the unweighted pair group method with arithmetic average (UPGMA) with the XLSTAT to show a genetic relationship by the similarity coefficient. The software used for the analysis of the scored data was NTSYS-pc version 2.02 (Rohlf 1994).

III. RESULTS AND DISCUSSION

All twenty SSR primers produced polymorphic bands and revealed a high DNA polymorphism among the genotypes. High level of polymorphism revealed that hybrid included in this study were considerably genetically diverse. This was obvious as different parental lines have been used in development of this hybrid. Being allogamous in nature, pearl millet genotypes are highly heterogeneous reflecting high variability within and among the genotypes. Protogyny and time lag between stigma emergence and anther dehiscence favor complete cross pollination leading to greatest diversity. A high level of polymorphism found during research was in accordance with the findings of Autunes *et al*. (1997) in pearl millet, Kale and Munjal (2005) in pearl millet, Jeya Prakash *et al.* (2006) in sorghum and Govindaraj *et al.* (2009) in pearl millet.

Based on the complementary banding patterns between the hybrid and its parents, the SSR markers PSMP 2013, PSMP 2202, PSMP 2084, PSMP 2089, PSMP 2270 and PSMP 2272 were identified as the specific markers which enable to distinguish and identify Pearl millet hybrid Shraddha form their parental lines. The expression of alleles in hybrids confirmed that they have their origin either of the parents and absence of any other allele in hybrid was the result of genuineness of hybrid and the banding pattern of hybrids confirmed the hybridity. Similar type of work done and reported by Pallavi *et al.* (2011) in sunflower and Arun Kumar *et al.* (2014) in pearl millet.

In hybrid Shraddha, the SSR marker PSMP 2084 (Plate 1) amplified three alleles of size 332, 343 and 455 bp. The alleles of 232 and 455 bp were expressed in its female parent (RHRB 1A) and allele of size 343 bp was expressed in its male parent (RHRBI 138). The expression of alleles in hybrid Shraddha confirmed that they have their origin either of the parents and absence of any other allele in hybrid was the result of genuineness of hybrid.

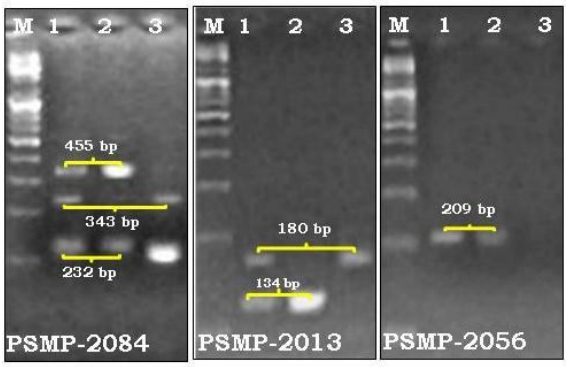

Fig. 1: SSR profile of PSMP-2084, PSMP-2013 & PSMP-2056 obtained on Ethidium bromide stained PAGE (2.5 %)
Lane M=100 bp ladder; 1= Hy. Shraddha; 2=RHRB 1A; 3= RHRBI 138

Another SSR marker PSMP 2013 (Plate 1) shown two amplicons at 134 and 180 bp in hybrid; out of which, amplicon of size 134 bp was also seen in its

female parent (RHRB 1A) and amplicon of size 180 bp was found in its male parent (RHRBI 138) and no other band found in hybrid. Hence, the hybrid and its parents showed necessary matching banding pattern for recognition and authenticity of hybrid Shraddha. The similar types of findings were reported by Pallavi *et al.* (2011) in sunflower and Arun Kumar *et al.* (2014) in pearl millet. The SSR marker PSMP 2056 (Plate 1) amplified single allele of size 209 bp in hybrid Shraddha and its female parent (RHRB 1A).

Likewise, SSR marker PSMP 2089 (Plate 2) amplified two alleles of size 142 and 331 bp in hybrid Shraddha. The allele of size 142 bp was also amplified in its female parent (RHRB 1A) and other allele of 331 bp was amplified in hybrid and both of its parents. Another allele of 137 bp was found only in its male parent. The identified SSR marker PSMP 2089 had both female and male specific bands in hybrids showing complementary banding pattern to both the parents and found vital for hybridity test. These findings are in agreement with results reported by Bhosale *at el.* (2015) in sunflower.

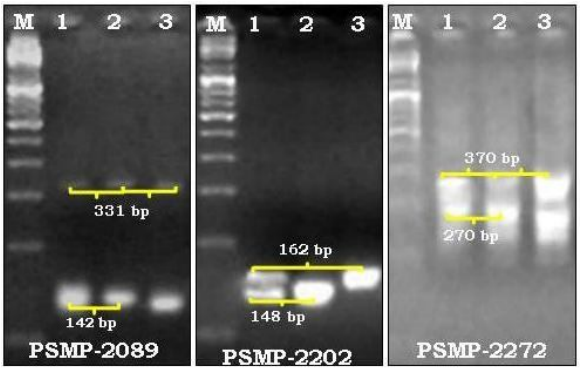

Fig. 2: SSR profile of PSMP-2089, PSMP-2202 & PSMP-2272 obtained on Ethidium bromide stained PAGE (2.5 %)
Lane M=100 bp ladder; 1= Hy. Shraddha; 2=RHRB 1A; 3= RHRBI 138

Similarly, the SSR marker PSMP 2202 (Plate 2) amplified allele of size 148 bp in hybrid and its female parent (RHRB 1A). On the other hand the pollen parent had an amplicon at 162 bp which was also present in hybrid. Also, the SSR profile of PSMP 2272 (Plate 2) in hybrid Shraddha amplified two alleles of size 270 and 370 bp. The amplicon of 370 bp is present in its both parents (male parent RHRBI 138 and female parent RHRB 1A), while other amplicon at 270 bp is present in hybrid and its female parent RHRB 1A. But the amplicon of 266 bp is present in its male parent RHRBI 138 only. However, hybrid exhibited the alleles of both parents confirming the heterozygosity and genuineness of the hybrid. (Liu *et al.*, 2007 and Naresh *et al.*, 2009).

The profile of PSMP 2270 (Plate 3) amplified total of three alleles among all genotypes. Hybrid Shraddha and both of its parents (RHRB 1A and RHRBI 138) expressed alleles at 154, 420 and 512 bp in common. SSR marker PSMP 2206 (Plate 2) amplified allele of size 197 bp in hybrid Shraddha and its female parent (RHRB 1A) and other amplicon at 194 bp was present only in its male parent (RHRBI 138). SSR marker PSMP 2237 (Plate 3) amplified allele of size 233 bp in hybrid Shraddha and its male parent and other amplicon at 228 bp was present only in its female parent.

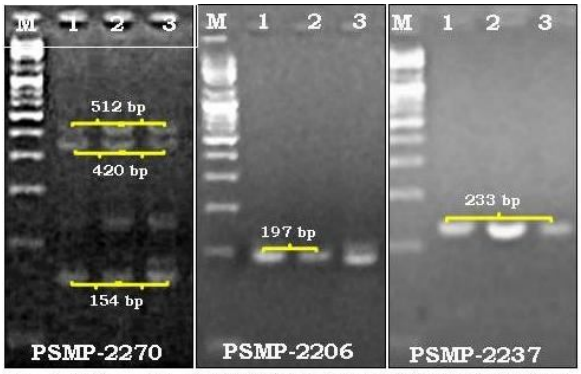

Fig. 3: SSR profile of PSMP-2270, PSMP-2206 & PSMP-2237 obtained on Ethidium bromide stained PAGE (2.5 %)
Lane M=100 bp ladder; 1= Hy. Shraddha; 2=RHRB 1A; 3= RHRBI 138

Similarly, SSR marker PSMP 2224 (Plate 4) amplified allele of size 162 bp in hybrid Shraddha and its male parent. Other amplicon of 156 bp was amplified only in female parent. The SSR marker PSMP 2263 and PSMP 2086 (Plate 4) amplified amplicon at 270 and 122 bp respectively in hybrid Shraddha and its both parents. Similar results were reported by Pallavi *et al.* (2011) in sunflower and Arun Kumar *et al.* (2014) in pearl millet.

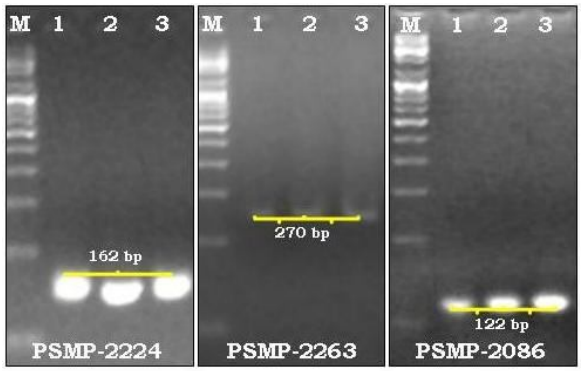

Fig. 4: SSR profile of PSMP-2224, PSMP-2263 & PSMP-2086 obtained on Ethidium bromide stained PAGE (2.5 %)
Lane M=100 bp ladder; 1= Hy. Shraddha; 2=RHRB 1A; 3= RHRBI 138

The SSR PSMP 2248 profile (Plate 5) had shown two amplicons at 169 and 175 bp. The amplicon at 169 bp was expressed in hybrid and its female parent (RBRB 1A), but the other of 175 bp was present only in male parent (RHRBI 138).

The SSR marker PSMP 2090 PSMP 2246 and PSMP 2001 amplified allele of size 176 bp, 272 bp and 200

bp respectively, in hybrid Shraddha and both of its parents. Marker PSMP 2273 (Plate 6) amplified allele at 170 bp in hybrid Shraddha and its female parent (RHRB 1A) while, other amplicon at 165 bp is expressed in male parent only (RHRBI 138).

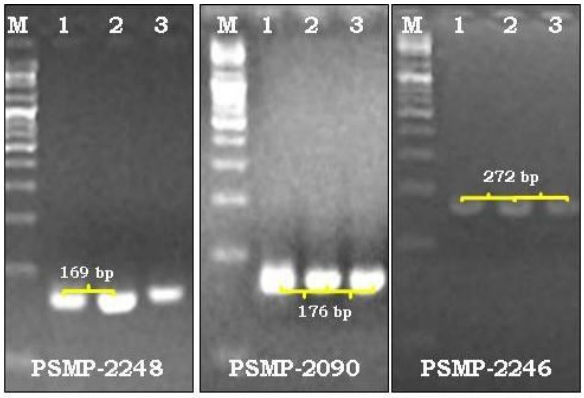

Fig. 5: SSR profile of PSMP-2248, PSMP-2090 & PSMP-2246 obtained on Ethidium bromide stained PAGE (2.5 %)
Lane M=100 bp ladder; 1= Hy. Shraddha; 2=RHRB 1A; 3= RHRBI 138

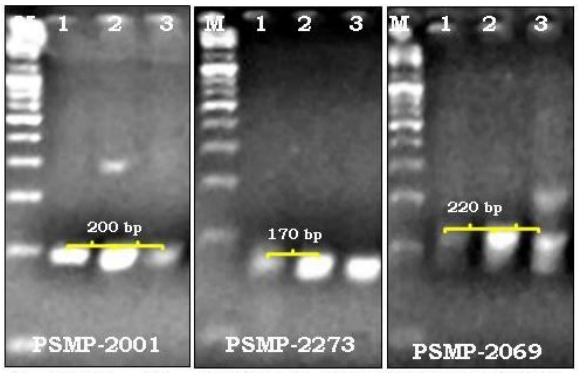

Fig. 6: SSR profile of PSMP-2001, PSMP-2273 & PSMP-2069 obtained on Ethidium bromide stained PAGE (2.5 %)
Lane M=100 bp ladder; 1= Hy. Shraddha; 2=RHRB 1A; 3= RHRBI 138

Overall, the hybrid Shraddha either expressed the bands from both the parents i.e., A line and R line, or no extra band indicating it's a true to type hybrid. Hence, the SSR markers are useful tools for genotype identification and for the assessment of genetic relationships as well are highly efficient and reproducible for genetic purity testing in pearl millet. These results are in conformity with those reported by Chowdari *et al.* (1998) in pearl millet, Nandakumar *et al.* (2004) in rice, Prasanna *et al.* (2007) in pearl millet, Liu *et al.* (2008) in pearl millet.

Based on the SSR profile, the hybrid can be identified and differentiated from parents by using seven markers *viz.*, PSMP 2084, PSMP 2013, PSMP 2089, PSMP 2263, PSMP 2272, PSMP 2056 and PSMP 2202. The discriminating power of these specific markers will be helpful for variety identification by comparing SSR markers and morphological traits in tests of distinctiveness, uniformity, and stability (DUS). It will be helpful in testing purity of F_1 hybrids and for registering elite germplasm. Thus, the SSR markers were more reliable, accurate and efficient than the commonly used GOT. It is in agreement with the results found to Zhao *et al.* (2012) in cauliflower, Dhaliwal *et al.* (2013) in chilli and Pereira *et al.* (2013) in pearl millet.

This study showed that SSR markers were more reliable for assessing genetic purity as compared to morphological marker. The results of study are expected to be useful in the verification of genetic purity of hybrid seeds accurately. This is in agreement with the conclusions reported by Hipi *et al*. (2013) in maize. These markers have an advantage of co-dominance inheritance, easy scoring of the alleles, reproducibility and accessibility to laboratories (Paniego *et al.*, 2002). The use of SSR markers for genetic purity testing has also been demonstrated in maize (Wang *et al*., 2002) and in rice (Nandakumar *et al*., 2004).

The study suggested that molecular marker analysis can be utilized as its reliable and less time consuming for purity testing of hybrids. It has been clearly brought out by the present study that identification and use of such hybrid specific markers can effectively reduce the cost and simplify the procedure of hybridity and purity testing.

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REFERENCES

[1] S. Tostain and L. Marchais, "Enzyme diversity in pearl millet (*Pennisetum glaucum* L.) Africa and India", *Theor. Appl. Genet.*, vol. 77, pp. 634-640, 1989.

[2] E. P. Koshy, B. K. Alex and P. John, "Clonal fidelity studies on regenerants of *Psophocarpus tetragonolobus* (L.) Dc. using RAPD Markers", *The Bioscan*, vol. 8, no. 3, pp. 763-766, 2013.

[3] H. M. Pallavi, R. Gowda, K. Vishwanath, Y. G. Shadakshari and K. Bhanuprakash, "Identification of SSR markers for hybridity and seed genetic purity testing in sunflower (*Helianthus annuus* L.)", *Seed Science & Technology*, vol. 39, no. 1, pp. 259-264, 2011.

[4] L. Liu, Y. Wang, Y. Gong, X. Zhai, F. Yu and H. Shen, "Genetic Purity Test of F1 Hybrid Tomato Using Molecular Marker Analysis", *Acta Horti*., vol. 771, 2008.

[5] Šamac. Dunja, M. Bogović, D. Vincek, J. Martinčić, and B. Salopek-Sondi, "Assessing the authenticity of the white cabbage (*Brassica oleracea* var. capitata f. alba) cv. 'Varaždinski' by molecular and phytochemical markers", *Food Research International*, vol, 60, pp. 266-272, 2014.

[6] A. Hipi, M. Surahman, S. Ilyas, Giyanto, "Seed Genetic Purity Assessment of Maize Hybrid Using Microsatellite Markers", *International Journal of Applied Science and Technology*, vol. 3, no. 5, pp. 66-71, 2013.

[7] K. S. S. V. P. Rao, A. Varier, T. K. Mohapatra, and S. P. Sharma, "Electrophoresis of seed esterases and RAPD analysis for identification of hybrids and parental lines of

pearl millet [*Pennisetum glaucum* (L.) R.Br.]", *J. Plant Varieties and Seeds.*, vol. 14, no. 1, pp. 41-52, 2001.

[8] M. A. Saghai-Maroof, K. M. Soliman, R. A. Jorgensen, and R. W. Allard, "Ribosomal DNA spacer-length polymorphisms in barley-mendelian inheritance, chromosomal location, and population-dynamics", *Proceedings of the National Academy of Sciences of the United States of America*., vol. 81, pp. 8014-8018, 1984.

[9] N. Sarla, S. Bobba, and E. A. Siddiq, "ISSR and SSR markers based on AG and GA repeats delineate geographically diverse Oryza nivara accessions and reveal rare alleles", *Cur. Sci.*, vol. 84, no. 5, pp. 683-690, 2003.

[10] F. J. Rholf, "NTSYS-pc. Numerical Taxonomy and Multivariate Analysis System", version 1.80. Exeter Software, Setauket, New York: Applied Biostatistics Inc., 1994.

[11] M. S. Autunes, M. J. V. Vasconcelos and D. A. M. Netto, "RAPD analysis of pearl millet cultivars". *Int. Sorghum Millet News*, vol. 38, pp. 146-150, 1997.

[12] A. A. Kale and S. V. Munjal, "Molecular analysis of mitochondrial DNA of lines representation a specific CMS-fertility–restorer system of pearl millet (*Pennisetum glaucum* (L.) R. Br.) by RAPD Markers", *Indian J. Genet*., vol. 65, no. 1, pp. 1-4, 2005.

[13] S. P. Jeya Prakash, K. R. Biji, G. S. Michael, G. K. Murthy and R. Chandra Babu, "Genetic diversity analysis of sorghum (*Sorghum bicolor* L. Moench) accessions using RAPD markers", *Indian J. Crop Sci.*, vol. 1, no. 1-2, pp. 109-112, 2006.

[14] M. Govindaraj, B. Selvi, D. Arun Prabhu and S. Rajarathinam, "Genetic diversity analysis of pearl millet (*Pennisetum glauccum* [L.] R. Br.) accessions using molecular markers", *African Journal of Biotechnology*, vol. 8, no. 22, pp. 6046-6052, 2009.

[15] M. B. ArunKumar, M. Dadlani, R. Kumar and S. R. Jacob, "Identification and validation of informative SSR markers suitable for ensuring the genetic purity of brinjal (*Solanum melongena* L.) hybrid seeds", *Scientia Horticulturae*, vol. 171, pp. 95-100, 2014.

[16] S. M. Bhosle, R. L. Chavhan, V. R. Hinge, and M. K. Ghodke, "Molecular fingerprinting and assessment of genetic purity of sunflower (*Helianthus annuus* L.) hybrids by using RAPD and SSR markers", *The Bioscan*, vol. 10, no. 2, pp. 773-779, 2015.

[17] L. W. Liu, Y. Wanga, Y. Q. Gonga, T. M. Zhaob, G. Liua, X. Y. Lia and F. M. YUC, "Assessment of genetic purity of tomato (*Lycopersicon esculentum* L.) hybrid using molecular markers", *Sci. Hortic*., (Amsterdam). Vol. 115, pp. 7-12, 2007.

[18] V. Naresh, K. N. Yamini, P. Rajendrakumar and V. Dinesh Kumar, "EST-SSR marker-based assay for the genetic purity assessment of safflower hybrids", *Euphytica*, vol. 170, pp. 347-353, 2009.

[19] K. V. Chowdari, A. P. Davierwala, V. S. Gupta, P. K. Ranjekar and O. P. Govila, "Genotype identification and assessment of genetic relationships in pearl millet [Pennisetum glaucum (L.) R. Br] using microsatellites and RAPDs", *Theor. Appl. Genet.*, vol. 97, pp. 154-162, 1998.

[20] N. Nandakumar, A. K. Singh, R. K. Sharma, T. Mohapatra, K. V. Prabhu and F. U. Zaman, "Molecular fingerprinting of hybrids and assessment of genetic purity of hybrid seeds in rice using microsatellite markers", *Euphytica*, vol. 136, pp. 257–264, 2004.

[21] B. M. Prasanna, A. C. C. Chandra Shekara, S. R. Bhat and B. B. Singh, "Genetic Diversity Analysis of Elite Pearl Millet Inbred Lines Using RAPD and SSR Markers", *J. Pl. Biochem. & Biotech*., vol. 16, no. 1, 2007.

[22] L. Zhao yan, C. Fang, Y.Yibin, and R. Xiuqin, "Identification of rice seed varieties using neural network", *Journal of Zhejiang University Science*, 2005.

[23] M. S. Dhaliwal, S. K. Jindal, A. K. Gaikwad, and K. Singh, "Genetic Diversity Analysis and DNA Fingerprinting of Elite Chilli Pepper Lines Using SSR Markers", *International J. Veg. Sci*., vol. 19, no. 3, pp. 207-216, 2013.

[24] G. S. Pereira, R. P. Mendonça Neto, E. V. R. Von Pinho and B. L. Carvalho, "Identification of pearl millet cultivars using both microsatellites and enzymatic markers", *Genet. Mol. Res*., vol. 12, no. 1, pp. 1-14, 2013.

[25] N. Paniego, M. Echaide, M. Muñoz, L. Fernandez, S. Torales, P. Faccio, I. Fuxan, M. Carrera, R. Zandomeni, E. Y. Suárez, and H. E. Hopp, "Microsatellite isolation and characterization in sunflower (*Helianthus annuus* L.)", *Genome*, vol. 45, pp. 34-43, 2002..

[26] J. Wang, G. Y. Zhong, E. C. L. Chin, J. C. Register, R. D. Riley, W. S. Nieber and J. S. C. Smith, "Identification of parents of F1 hybrids through SSR profiling of maternal and hybrid tissue". *Euphytica*, vol. 124, pp. 29-34, 2002.

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