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Fingerprinting of Bottle Gourd (*Lagenaria siceraria*) Hybrids and their Parental Lines Using Microsatellite Markers and their Utilization in Genetic Purity Assessment

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Abbreviation:
CTAB-Hexadecyl trimethyl ammonium Bromide; **GOT**-Grow out test; **SSR**-simple sequence repeat

A B S T R A C T

Use of morphological differences, between true hybrids and off types in grow out test (GOT) for genetic purity analysis, are not always apparent and cannot be recognised easily. Further, morphological traits are costly, tedious to score and environment sensitive. Alternatively, it is suggested that recent breakthrough in molecular markers can be employed in genetic purity analysis. Microsatellite markers are used for fingerprinting of bottle gourd hybrids in order to assess variation within parental lines. Fifty two microsatellite markers were employed to fingerprint to three hybrids and their parental lines. Three Simple Sequence Repeat (SSR) markers were found to be polymorphic across the hybrids and to produce unique fingerprints for each of the three hybrids. These highly informative primers not only differentiated the parent genotypes, but also confirmed the parentage of the true hybrids. The microsatellite marker, LSR 47 amplified alleles specific to different parental lines of AS lattu; likewise, LSR 77 primer for AS 3417 and LSR 15 primer for AS 606. Results indicate that microsatellite procedures are excellent genomic tools for parentage confirmation and hybridity determination, and may also enhance efficiency of breeding programmes. This technique is simple to use, more accurate and not affected by environment when compared with GOT.

Introduction

The bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] is a diploid (2n = 2x = 22), self compatible, annual monoecious, cucurbitaceous vegetable. Two subspecies of bottle gourd, viz. *L. siceraria* subsp. *siceraria* (domesticated 4000 years ago in Africa) and *L. s.* subsp. *Asiatica* (domesticated at least 10,000 years ago in Asia), have been identified (Heiser,1979). India is the secondary centre of the diversity of bottle gourd, and bottle gourd is grown year-round, extensively throughout the country, except in very cool regions during winter. The development of varieties/hybrids for earliness, yield and resistance to insect pests and diseases is the main breeding objective. Mass selection has been recommended as one of the breeding methods for the improvement of fruit shape for bottle gourd (Bisogin and Storck,2000).

The breeding of bottle gourd for high yield and downy mildew resistance through marker-assisted selection is being carried out at the Indian Institute of Horticultural Research. A few varieties in South India have been found to be resistant to zucchini yellow mosaic virus and powdery mildew. Telangana region of the state of Andhra Pradesh in India is endowed with a rich variability of bottle gourd, especially with regard to fruit characteristics (Sivaraj and Pandravada, 2005).

A lot of information is known on the medicinal aspects of bottle gourd (Milind and Satbir, 2011); however its potential as a possible food security crop has been lowly documented. In nature, bottle gourd exhibits great morphological and genetic variability (Branwell *et al*., 1987) This alone could indicate its wide environmental adaptation (Koffi *et al*.,2009). The plant also demonstrates an indeterminate growth habit when there is enough supply of water. This allows farmers to have a constant supply of fresh green leaves for consumption and animal fodder. Young immature fruits are consumed in the same manner as pumpkin fruits, while the seeds are a rich source of essential amino and fatty acids (Loukou *et al*.,2007; Koffi *et al*.,2009). Bottle gourd does not require complex field management practices. It grows well with small amounts of nitrogen fertilizer and it is a natural weed smother (Koffi *et al*.,2009). It is often intercropped with cereal crops and can act as a live mulch (Ouma and Jeruto, 2010).

The genetic purity of a variety/hybrid refers to the absence of seeds of other genotypes than the specific one. Therefore, seed testing or genotype identification

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is of prime importance for assuring good quality seed. It is estimated that for every 1% impurity in the hybrid seed, the yield reduction is 100 kg per ha². Thus, it is of critical importance to evaluate the genetic purity in seed production and trade (Ashok *et al*.,2011).Genetic purity of hybrid seeds is assayed conventionally by the 'grow-out test' (GOT), which involves growing plants to maturity and assessing several morphological characteristics that distinguish the hybrids. The environmental influences on morphological characters and time factor make it difficult to collect the morphological data, besides other limitations in unambiguous differentiation of genotypes. Molecular markers can be used because they offer a faster and reliable technique for precisely assessing the genotype of a plant. Among the different molecular markers such as RAPD, ISSRs, Microsatellites or SSRs, RFLP, AFLP and isozymes markers. Microsatellites have been successfully employed in many genetic diversity studies and are useful for a variety of applications in plant genetics and breeding (Liu *et al*.,2000; Gutierrez *et al*.,2002; Powell *et al*.,1996; Tejaswini and Pulak, 2016)

Evaluation of genetic resources is crucial for breeders to develop new cultivars or for further improvement of the existing ones in response to changes in consumer demand. Molecular markers have enormous potential to explore genetic diversity by detecting polymorphisms, are useful tools for breeding, genotype identification, determination of genome organization and evolution in plants (Adeniji *et al.,*2012). Microsatellites (SSR) markers are a popular source of genetic markers owing to their high reproducibility, multi-allelic nature, co-dominant inheritance, abundance, and wide genome coverage. High level of polymorphism makes SSR an ideal marker for mapping and diversity studies, fingerprinting and population genetics (Nunome *et al*., 2003).

The objective of the present study to identify SSR based molecular markers for genetic purity analysis of commercial bottle gourd hybrids AS lattu, AS 3417, AS 606 and their parental lines using agarose gel electrophoresis and to develop a more efficient method for genetic purity analysis of Bottle gourd hybrids than conventional GOT method.

Materials and Methods

Plant material

The studies pertaining to DNA fingerprinting for the identification of bottle gourd hybrids using SSR marker system were conducted at the Adithya Biotech Lab and Research, Raipur, Chhattisgarh. The plant material for this study comprised of three hybrids and their female and male parents: AS lattu (AS 14×AS 16), AS 3417 (AS 34×AS 17), AS 606 (AS 60×AS 6) obtained from Aditya seeds Pvt Ltd, Raipur (Table 1). Fresh young leaves from all plants were collected for DNA extraction.

DNA extraction

Genomic DNA was isolated according to a modified CTAB method (Zhu *et al*., 2010). Quantification of DNA was accomplished by analyzing the DNA on 0.8% agarose gel using diluted uncut lambda DNA as a standard. Finally, all the genomic DNA samples were diluted to a final concentration of 40ng/µl with 1X TE buffer (10mM Tris-HCL; pH 8.0; 1mM EDTA). DNA samples were stored at -20ºC for further use.

SSR-PCR amplification and agarose gel electrophoresis analysis

A total of 52 SSR markers were used to identify polymorphic marker among the parents. The forward and reverse primers of these SSR markers were synthesized from Eurofins Genomics India Pvt Ltd., Banglore. From the nine markers that were found to be polymorphic (Table 2), three markers were selected to test the genetic purity of hybrid seeds since they have produced clear, scorable and unambiguous polymorphic bands among the parents.

PCR amplification was conducted in a 20µl volume containing 40 ng of genomic DNA, 10X buffer,1 U *Taq* DNA polymerase, 1.5mM MgCl2, 2.5mM dNTPs, 6 pmol forward and reverse primer. The PCR protocol consisted of an initial denaturation at 94ºC for 5 min, followed by 35 cycles of 94ºC for 30 sec, primer annealing was conducted at 53-54ºC for 40 sec, 72ºC for 1 min and final extension step of 72ºC for 10 min. The amplification reaction was carried out in thermo cycler (Applied Biosystems). The PCR products were analysed on 2% agarose gel along with 100bp molecular weight marker and photographed under UV light using Bio-Rad gel documentation system.

Results

The three bottle gourd hybrids and their parental lines were analyzed for microsatellite polymorphisms. All of the primer pairs had good amplification. Out of 52 microsatellite primers, nine pairs i.e. LSR11, LSR15, LSR20, LSR40, LSR47, LSR56, LSR61, LSR74,LSR77 were scorable on agarose gel and showed polymorphism in parents.The parental polymorphism survey identified three informative markers (LSR 47, LSR 77, LSR 15), which were used to fingerprint the hybrids. The three markers collectively amplified a unique fingerprint for all hybrids and therefore, were effective in distinguishing them from one another . The parents and the hybrid plants were carefully observed on the basis of morphology to determine if they were true hybrids. The polymorphisms observed between the parents aware used as markers for hybrid identification. Comparing the SSR banding pattern of parents with respective hybrids, genuine hybrids were confirmed.

The LSR 47 primer demonstrated an amplified allele of size 150 bp in the male parent (AS16) and the hybrid (AS lattu). Alternatively, the female parent (AS 14) had an amplicon of 140 bp. However, hybrid (AS lattu) exhibited the alleles of both parents, confirming the heterozygosity of the hybrid with the presence of two bands at 140 and 150 bp (Fig. 1).

Similarly, hybrid AS 3417 was identified and distinguished by the LSR 77 marker, as shown in (Fig. 2). The hybrid demonstrated the complementary banding pattern of both parents. The marker had an amplicon of 280 bp in its female parent (AS 34); the same marker had a different amplicon of size 290 bp in male parent (AS 17); the hybrid showed both the amplicons at 280 and 290 bp. Thus, it confirmed the genuine nature of the hybrid.

The primer LSR 15 amplified a specific allele size of 295 bp in the hybrid (AS 606) and male parent (AS 6), but not in its female parent (AS 60). However, the LSR 15 amplified a different allele of size 278 bp in the female parent. The same allele size of 278 bp was expressed in the hybrid, but not in its male parent (AS 6). Thus,the presence of

Table 1. Name of bottle gourd hybrid and their parental lines used in this study

Sl. No.	Hybrid	Female	Male	Source
1.	AS lattu	AS 14	AS 16	Aditya seeds, Raipur.
2.	AS 3417	AS 34	AS 17	
3.	AS 606	AS 60	AS 6	

Table 2. Sequences of SSR primer pairs used in this study which shows polymorphism in parental lines.

Sl. No.	Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
1.	LSR 11	TTCGCCTCAGTCCATCTAGTTT	ATGTCGTACCTTTTCCCCTTTT
2.	LSR 15	CTTACCTTCACAAAACCCCATC	ACTCTGTTTCGACTCTGCTTCC
3.	LSR 20	AACTGAAACCATTAACGAAGGC	AATAAGCAGCAACCATGTCAAC
4.	LSR 40	TTCCATCCAGACCAAACCTATC	CAAAGGCCATAGACAAACACAA
5.	LSR 47	CAATAGAGTAGGGTGGGGCATA	TAAAATAGTGGGAGAGCAAGGG
6.	LSR 56	TAATAATGCCACTGCACATGGT	AGATGAATCCCAATATCCCAGA
7.	LSR 61	TGTTTATATTGGCAGGAGGAGG	GGCAGGGAATTTTCATAGAGTG
8.	LSR 74	TGGGGTAGAAATTGAAGAGGAG	CTCAAAATAGCCCAACCCTAAA
9.	LSR 77	GACAGATCCTTCTGGGACTTTT	TTCTGCAATAGAGTACGTTGGC

Figure -1: Amplification results of primer LSR 47 from bottle gourd hybrid AS lattu and their parental lines. Lanes F represent female line AS 14 (amplification size 140bp), Lanes M represent male line AS 16 (amplification size 150bp), Lanes H represent hybrid AS lattu (amplification sizes 140:150 bp), Lane L represent 100bp ladder (100bp-1000bp).

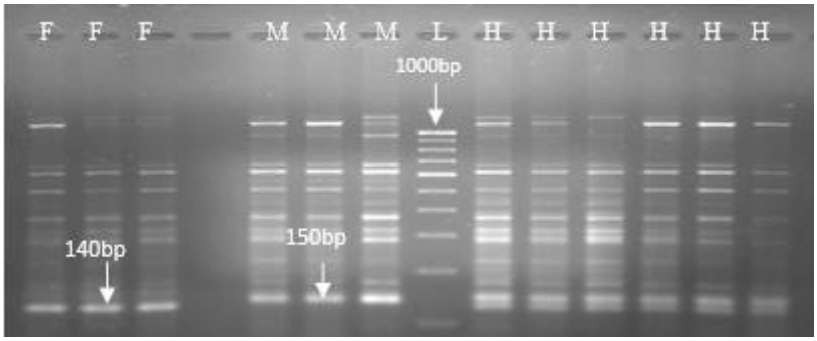


Figure – 2: Amplification results of primer LSR 77 from bottle gourd hybrid AS 3417 and their parental lines. Lane L represent 100bp ladder (100bp-1000bp). Lanes F represent female line AS 34 (amplification size 280bp), Lanes M represent male line AS 17 (amplification size 290bp), Lanes H represent hybrid AS 3417 (amplification sizes 280:290 bp)

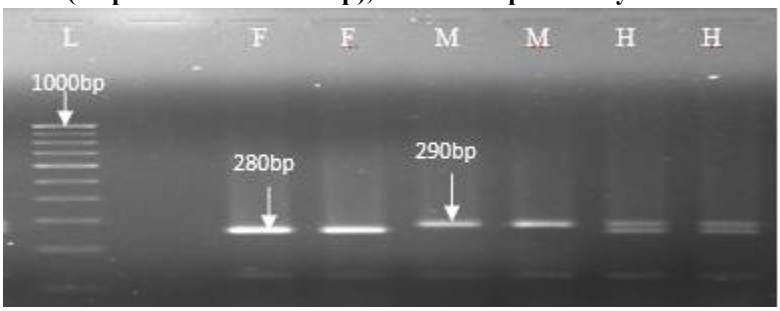
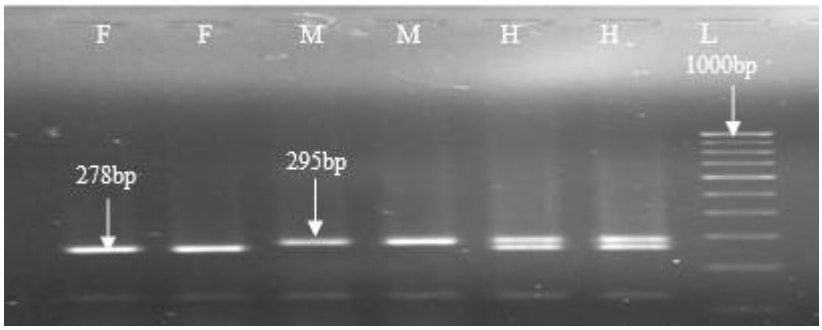


Figure -3: Amplification results of primer LSR 15 from bottle gourd hybrid AS 606 and their parental lines. Lanes F represent female line AS 60 (amplification size 278bp), Lanes M represent male line AS 6 (amplification size 295bp), Lanes H represent hybrid AS 606 (amplification sizes 278:295 bp), Lane L represent 100bp ladder (100bp-1000bp).



both female and male parent alleles indicated the result of crossing between two parents (hybrid). The observed banding pattern, as shown in (Fig. 3), was highly specific to hybrid AS 606 and was not observed in any other hybrids included in this study.

Discussion

Assessment of seed purity is one of the most important quality control components in hybrid seed production. Traditionally, GOT has been employed to assess the purity of hybrid seeds using morphological traits. Although GOT is used to determine the genetic purity of hybrids, it is tedious, space-demanding and time-consuming, and often does not allow the unequivocal identification of genotypes. Hence, a recent development in molecular markers has been suggested for genetic purity testing, since they are used to precisely assess the genotype, and not the phenotype (Sundaram *et al*., 2007) . Among different types of molecular markers, SSR markers have been reported as useful for testing genetic purity, since they are co-dominant. Additionally, using SSR, the heterozygous state can be easily discerned from the homozygous state.

Agarose gel electrophoresis was used to analyze SSR fragments. In this study agarose gel electrophoresis was selected for analysis of the amplifications because of it's simple and affordable technology. The agarose gel electrophoresis results showed that SSR molecular markers were valuable for genetic analysis of bottle gourd because of it's high polymorphism. Based on the agarose gel electrophoresis primers, LSR 47, LSR 77, LSR 15 produce stable, distinct and polymorphic amplification.

Conclusion

In summary, it is concluded from this study that it is possible to differentiate the bottle gourd hybrids more accurately and efficiently from its parental lines using molecular markers. These molecular markers would be more efficient than GOT, since DNA markers would be more accurate for determining hybrid seed purity. Further, marker analysis will also result in considerable savings for the seed industry as this technique may avoid the cost of storage for a whole season and cost of acquiring land and cultivation.

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