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Utilization of SSR markers for hybridity assessment in F_1's chickpea plants

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Abstract
Simple sequence repeat markers were used for fingerprinting of hybrids, assessing variation within parental lines, and testing the genetic purity of hybrid seed lot in chickpea as a self-pollinating crop. In this study, 10 simple sequence repeats (SSR) markers were employed for parental polymorphism and F_1 hybridity assessments. One TA-14 SSR marker was found polymorphic between the used parents and produced a specific allele for each parent. This TA-14 SSR marker differentiated the 45 F_1 hybrids from 70 F_1's plants for their true hybrid nature. This marker can be used as referral markers for the unambiguous identification and protection of these hybrids. The analysis of plant-to-plant variation within the parental lines of the hybrid, using informative markers indicated residual heterozygosity at marker loci. This highlights the importance of SSR markers in maintaining the genetic purity of the parental lines. To utilize this SSR marker effectively for the detection of impurities in hybrids. Hence, The SSR marker information developed through this study will be of immense help for the hybrid seed industry to select the genetic purity of the crop.

Keywords: Outcrossing, purity, hybridity, kabuli, fingerprinting

Introduction
Chickpea (*Cicer arietinum*) is the second-largest cultivated food legume crop in the world [2]. Chickpea has an important role in human nutrition as a very wise and good source of protein, carbohydrates, fats, vitamins, fibres, and minerals. Its use both as human food and animal feed, coupled with its ability to fix atmospheric nitrogen makes it a very important crop.
There are two distinct types of chickpea, "Kabuli" and "Desi" differing in their geographic distribution and different plant type habitat. Kabuli types are usually taller and white flowers. Desi types are generally shorter, possessing small leaflets, pods, seeds, and predominantly pinked flowers [9].
Chickpea is predominantly a self-pollinating species and due to its small flower, the crossing is difficult and tedious [14].
For a self-pollinated crop like chickpea, one of the challenges is the production and supply of adequate quantities of pure seeds to the farmers. And maintenance of high-level genetic purity of hybrid seeds and is to exploit the moderate level of heterosis in this crop. It is estimated that for every 1 per cent impurity in the hybrid seed, the yield reduction is 100 kg per hectare. Thus, there is a need for an assay to assess the genetic purity of seeds that is both accurate and faster, so seed produced in the chickpea season can be released for commercial cultivation in the ensuing true seeds for higher production to the farmers.
The authenticity of the variety is one of the most important characteristics of a good quality seed. A genetic purity test is done to verify any deviation from the genuineness of the variety during its multiplications. A genetic purity test is compulsory for seed certification and hybrid seeds productions. Higher genetic purity is a necessity for the commercialization of any hybrid seeds. Besides, the success of any hybrid technology depends on the availability of quality seed supplied in time at a reasonable cost. The genetic purity during production stages is prone to contamination due to the presence of pollen shedders, outcrossing with foreign pollens etc., besides physical admixtures. Thus, the use of seeds with less genetic purity results in segregation of the traits, lower yields and genetic deterioration of varieties.
Traditional Grow out Test (GOT) based on morphological markers are time-consuming and are environmental dependent. To overcome this disadvantage, molecular markers are being used in many of the crops. The use of molecular markers particularly the co-dominant markers like SSR markers are of great importance for rapid assessment of hybrid and parental line

identification [6]. The use of these markers is well known and is a routine nowadays in a selection of desirable plants in several crop species. Therefore, the present study aimed to use SSR markers to assure the hybridity of F1 plants derived from crosses made for developing significant hybrid lines in chickpea.

Materials and Methodology

Genotypes used for crossing program

Hybridization programs were executed during *Rabi* 2018-2019 between PKV Kabuli 4 as the female parent (moderately susceptible) to Fusarium wilt and WR-315 (resistant parent) used as the male parent to obtain the F1 plants.

During the second season, *Rabi* 2019-20 the 75 F_0 seeds were planted on a field of Pulses Research Unit, Dr. PDKV., Akola. Total 75 F_0 seeds were obtained, and out of which 70 seeds were germinated and tested for hybridity using polymorphic markers. This low seed setting is usual in chickpea as earlier reported by [14].

The total genomic DNA was isolated from the fresh leaf tissues of 10- to 15-day-old seedlings of the parental genotypes as well as the F1s using modified cetyltrimethylammonium ammonium bromide (CTAB) extraction method [8] followed by purification of extracted DNA by removing RNA and protein impurities. Purified DNA was then analyzed for quality and quantity using 0.8 % agarose gel and finally normalized to a concentration of ~50 ng/µl. The list of the ten markers used for the parental polymorphism study given in Table:1.

The PCR temperature regime comprised an initial denaturation for 4 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 57 -60 °C and elongation at 72 °C for 50 s and final elongation at 72 °C for 8 min.

Table 1: The list of the markers used in the present study

S. N.	Marker	Position	Nucleotide sequence	Annealing temperature (°C)
1	TA-1	Forward	TGAAATATGGAATGATTACTGAGTGAC	58.9
		Reverse	TATTGAAATAGGTCAGGCTTATAAAAA	55.9
2	TA-2	Forward	AAATGGAAGAAGAATAAAAACGAAAC	55.3
		Reverse	TTCCATTCTTTATTATCCATATCACTACA	58.2
3	TA-3	Forward	AATCTCAAAATTCCCCAAAT	50.1
		Reverse	ATCGAGGAGAGA AGA ACCAT	55.3
4	TA-14	Forward	TGACTTGCTATTTAGGGAACA	54.0
		Reverse	TGGCTAAAGACAATTAAAGTT	50.1
5	TA-18	Forward	AAAATAATCTCCACTTCACAAATTTTC	55.9
		Reverse	ATAAGTGCGTTATTAGTTTGGTCTTGT	58.9
6	TA-27	Forward	ACAATTCCACTTAATCTTTGC	52.0
		Reverse	AATTTAGCCTACAGACACACACA	57.1
7	TA-28	Forward	TAATTGATCATACTCTCACTATCTGCC	60.4
		Reverse	TGGGAATGAATATATTTTTGAAGTAAA	54.3
8	TA-37	Forward	ACTTACATGAATTATCTTTCTTGGTCC	58.9
		Reverse	CGTATTCAAATAATCTTTCATCAGTCA	57.4
9	TA-59	Forward	ATCTAAAGAGAAATCAAAATTGTCGAA	55.9
		Reverse	GCAAATGTGAAGCATGTATAGATAAAG	58.9
10	TA-64	Forward	ATATATCGTAACTCATTAATCATCCGC	58.9
		Reverse	AAATTGTTGTCATCAAATGGA AAATA	53.8

Recording and analysis of molecular data

For analyzing the genetic purity of hybrids, the banding patterns of SSR markers were compared and markers showing polymorphism between parents were identified. The size of a polymorphic marker was measured using the online fragment size calculator (http://home.sandiego.edu/cloer/bio182s04/frag_calculator.html), which was further used to test respective hybrids [13]. The purity of the F1 s was confirmed when they showed the presence of male and female parent alleles or only from male parent allele at a marker locus.

Result and Discussion

Characterization and identification of cultivars are crucial to varietal improvement, release and in the nuclear seed production program. It is mandatory to maintain the genetic purity of hybrid seeds for successful crop production. The unambiguous characteristic pattern of hybrids can be obtained using DNA markers and had been termed as DNA fingerprinting. The use of DNA markers to obtain genotype-specific profiles had distinct advantages over morphological and biochemical methods. The morphological markers are influenced by the environmental conditions, labour intensive and time-consuming. However, the biochemical markers such as isozyme and protein patterns are least influenced by the environment but exhibit limited polymorphism and often do not allow discrimination between closely related inbred lines [3]. Molecular markers overcome most of these drawbacks of morphological and biochemical markers that can be useful to distinguish varieties and off-types.

Parental polymorphism

Ten SSR primers were used to screen polymorphism among the two parental genotypes (PKV Kabuli 4 and WR-315) involving the development of the F1 generation. Among these one marker TA-14 showed the clear polymorphic loci, between the used parents.

The SSR primer pairs, TA-14_F, TGACTTGCTATTTAGGGAACA and TA-14_R, TGGCTAAAGACAATTAAAGTT clearly showed the variability between two parents and were reproducible when used to characterize the F_1 plants. The amplified PCR product was separated using 10 % polyacrylamide gel electrophoresis and finally stained by silver staining protocol to visualize intact and clear bands [16].

Hybridity assessment

In the present investigation, F1 plants were screened with one polymorphic SSR marker to identify the true F1 plants in a cross for developing the true hybrids lines for its commercial purpose. In this context, the hybridity of 70 F1 plants derived from the cross (PKV Kabuli 4 X WR-315) was tested by TA-14 SSR marker. This SSR marker was selected from the polymorphic markers, which amplified unique loci to each parent. Out of 70 F_1 plants, only 45 F_1 plants (60 %) were confirmed as true hybrid plants based on one SSR amplified marker. The representative gel image shown in Figure:1. These found rates of confirmed hybrids agree with the earlier finding with 65 % [16] in chickpea crops. These 45 plants were used to obtain F2 seeds for developing the hybrid lines. The TA-14 SSR marker amplified both female (160 bp) and the male parent (150 bp) specific loci in F_1 plants (Figure 1).

The banding pattern of this hybrid showed both the amplicon at 160 bp and 150 bp. Thus, it is confirmed the genuine crossing and heterozygotic condition of the hybrid. The SSR marker identified had both female and male-specific bands and are useful in genetic purity testing. These markers have the advantage of co-dominance inheritance, easy scoring of the alleles, reproducibility, and ease of access to laboratories [10]. The use of SSR markers for genetic purity testing has been demonstrated in maize [7] in rice [13]; in sunflower [5].

Other F1 plants of this cross amplified either allele of female parents or new alleles (i.e., does not present in either parent) at SSR marker locus. This genetic impurity might be resulted due to selfing or crossing of the female parent with male plants of the different genotypic constitution or the admixture of seeds at the time of sowing.

The huge potentiality of molecular markers for measuring genetic purity as compared to morphological or biochemical traits has previously been reported in several crops [7]. Target locus-based markers have been utilized successfully for parentage verification, hybrid identification, and purity testing [5, 1]. More recently, molecular markers have been used to confirm the parentage of hybrids in cotton (Muhammad *et al*. 2009), and in chickpea [17].

Thus, determining hybrid purity is important to develop hybrids lines and to select the pure genetic material from the segregant population also for studying the genetics of desirable traits. False F_1 plants mislead target traits having economical values. In chickpea, selfing and admixing commonly occur. Therefore, the identification of true F_1 using molecular markers in the present study will certainly reduce the chance of errors. Moreover, it will help to save time, effort and money required to handle an incorrect segregating population and defiantly reduce the time in the varietal developmental program.

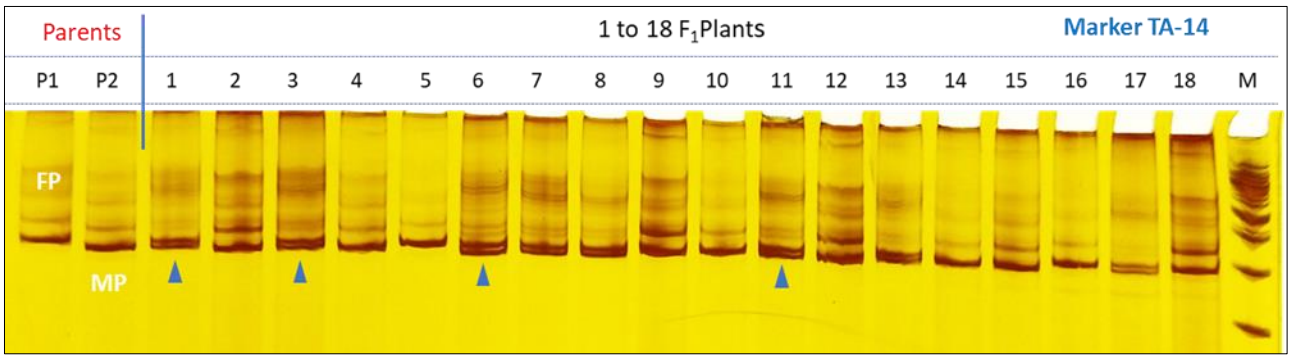

Fig 1: FP-Female Parent – PKV Kabuli 4 MP – Male Parent – WR-315▲ True F_1 Hybrids Plants

Conclusions

The present study showed that SSR markers are quick, effective and results are generally reliable with morphological analysis in the field study. TA-14 SSR marker identified in the study could be employed for routine hybridity testing between parents of PKV Kabuli 4 and WR-315. The SSR marker information developed through this study will be of immense help for the hybrid seed industry to select the genetic purity of the crop.

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