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Sanchit Thakur
Department of Crop Improvement, CSKHPKV, Palampur, Himachal Pradesh, India

Jai Dev Sharma
Department of Tea Husbandry & Technology, CSKHPKV, Palampur, Himachal Pradesh, India

Kamal Dev Sharma
Department of Agricultural Biotechnology, CSKHPKV, Palampur, Himachal Pradesh, India

Corresponding Author:
Sanchit Thakur
Department of Crop Improvement, CSKHPKV, Palampur, Himachal Pradesh, India

Confirmation of hybridity using DNA-based markers is essential in chickpea (*Cicer arietinum* L.)

Sanchit Thakur, Jai Dev Sharma and Kamal Dev Sharma

Abstract
Confirmation of hybridity is essential to exclude selfed plants in plant breeding programmes. The morphological markers for confirmation of hybridity in chickpea are unavailable in closely related germplasm and this results in ambiguity in identification of true hybrids. In present study, cold tolerant parent ICC-16349 (donor) and cold susceptible parent GPF-2 (recipient) were crossed to generate 80 putative hybrids. The parents were screened using 51 simple sequence repeat (SSR) markers, of which only one i.e TA 180 exhibited polymorphism. Screening of 80 putative hybrids using TA 180 revealed that only 34 (42.5%) of the putative hybrids were true hybrids. The study indicated that in chickpea breeding programmes, hybridity must be confirmed using DNA based markers to avoid inclusion of selfed plants as hybrids.

Keywords: Chickpea, hybridization, SSR and true hybrids

Introduction
Chickpea (*Cicer arietinum* L.) (2n=2x=16) is one of the important pulse crop that was first grown in Turkey about 7000 BC (Philologos, 2005) [10]. It is a self pollinated crop and belongs to kingdom Plantae, order Fabales, family Fabacae, genus *Cicer* and species *arietinum*. The crop is grown traditionally in semi-arid zones of Middle-East, Pakistan and India. According to Vavilov (1926) [15], there are two primary centres of diversity of chickpea, namely, South-West Asia and Mediterranean while Ethiopia is designated as the secondary centre. The centre of origin of the crop is considered to be in South-Eastern Turkey and Northern Syria where it is believed to be evolved from its progenitor *Cicer reticulatum* (Maesen, 1987) [6]. Chickpea is also known as Bengal gram, Garbanzo bean, Chana and Shonagalu in different chickpea producing areas of the world. India which contributes to about 60% of the total world's production, is the largest producer and consumer of Chickpea (Varshney *et al*. 2014) [14]. In India, chickpea is mainly produced in Madhya Pradesh, Maharashtra, Rajasthan, Uttar Pradesh, Andhra Pradesh, Karnataka, Chhattisgarh, Bihar and Jharkhand and these states contribute more than 95% to the total production. India still imports it from other nations besides being the largest producer of chickpea due to low productivity which is due to the abiotic and biotic stresses and use of low yielding varieties.

Lack of diagnostic morphological markers for the confirmation of hybidity owing to insufficient genetic variability in cultivated chickpea species along with shortage of polymorphic markers is a major constraint for identification of true hybrids in chickpea (Atalay & Babazogles, 2012) [1] are the major hindrances to confirm hybridity in this crop. Consequently, there are chances of categorizing false hybrids as true hybrids leading to errors in the breeding programmes resulting in wastage of resources and time. At present, no information is available for the extent of inclusion of false hybrids in chickpea breeding programmes. For hybridity confirmation in chickpea, DNA-based markers may be the markers of choice to ascertain the hybrid nature. Among vast categories of DNA markers available for chickpea, simple sequence repeats (SSRs) are preferred due to codominant nature, locus specificity, high reproducibility and ease to use (Tautz & Renz, 1984) [13]. SSRs, also known as sequence tagged microsatellite site or microsatellite markers (Beckmann & Soller, 1990) [2] are widely used in genetic diversity analysis, population genetics, marker assisted selection and genetic mapping. Most of the important legumes in India including chickpea are accompanied by lack of genomic resources as limited SSR markers have been reported so far. SSR markers being codominant, detect alleles of both male and female parents and hence, are ideal for differentiation of true hybrids from the selfed individuals. The present study was formulated to estimate the extent of false positive hybrids in chickpea and to confirm the hybridity of F_1 plants by using SSR markers.

Materials and Methods

Hybridization was conducted between two parents having contrasting traits *viz*., cold tolerance (ICC-16349) and cold susceptibility (GPF-2) where 'ICC-16349' was selected as a donor and 'GPF-2' as a recipient. The seeds of hybrids and parents was sown in 10" diameter pots using a standard potting mixture (Soil: Sand: FYM::1: 1: 1). At 3-4 leaf stage, a small amount of leaf tissue from each putative hybrid was harvested. The leaves were transported immediately in ice to lab for DNA extraction. The leaves were crushed in liquid nitrogen and DNA was isolated by using CTAB method (Murray and Thompson, 1980) [8]. The DNA of parents was amplified by using a set of 51 SSR markers developed by Winter *et al*. (1999) [17] and Gaur *et al*. (2011) [3] (Table 1). Off the 51 SSR primers, only one i.e TA 180 generated polymorphism between the parents. For polymerase chain reaction (PCR) assay, 10 µl PCR reaction mixture was prepared which constituted 6.7 µl sterile double distilled water, 1 µl 10X Taq buffer, 0.3 µl DNTPs (2 mM), 1.2 µl DNA (25-50 ng/µl), 0.2 µl DNA polymerase (1U/µl) and 0.3 µl each forward and reverse primer. PCR profile with initial denaturation of 5 min at 95^0C; 35 cycles with denaturation at 94^0C for 30 seconds, annealing temperature as per melting temperature of primer used, followed by extension at 72^0C for 1 min; and a final extension at 72^0C for 8 mins. The amplification products were stored at 4^0C and were resolved by gel electrophoresis in horizontal agarose system at 100 V for 2 hrs staining using ethidium bromide (0.5 µg/ml) in 3% agarose gel (0.5X TAE Buffer). Gel documentation system was used to visualize the amplified products and size of the amplicon was estimated by using 100 bp ladder. Genetic polymorphism was estimated by comparing size of the bands. The F_1s that showed alleles of both the parents were termed as hybrids usually referred to as true hybrids in present study to differentiate those from putative hybrids.

Results and Discussion

Crosses between chickpea genotypes 'ICC-16349' and 'GPF-2' generated a total of 80 putative hybrid seeds, out of which 25 were from reciprocal crosses and 55 were from direct crosses. Of the 51 primer pairs of SSRs used to amplify DNA of both the parents, only one i.e. TA 180 (1.2% of total SSRs) showed polymorphism between the parents. Hence, TA 180 was used for confirmation of hybridity of putative hybrids (Figure 1). Out of 80 putative hybrids screened, only 34 amplified both the alleles corresponding to ICC-16349 and GPF-2 suggesting that those 34 (42.5%) were true hybrids and 57.5% putative hybrids were false hybrids.

The study suggested that in chickpea breeding programmes, hybridity must be confirmed using DNA based markers to avoid inclusion of selfed plants in breeding programmes. The study further indicated that chickpea breeders usually include significant number (>50%) of selfed plants as hybrids in breeding programmes, thereby jeopardizing the objective of chickpea improvement. Similar studies regarding hybridity confirmation were conducted by various workers. SSR markers have already been used for confirmation of hybridity in chickpea **(**Smitha and Katageri, 2019) [12]. They identified 13 markers which were polymorphic for both the parents i.e. Super Annigeri-1 × BS 100B and Super Annigeri-1 × BS 72C2. However, only one marker ICCM0299 was able to detect the presence of both the parental alleles in F_1s and thus, was used for confirmation of hybridity. Morais *et al*. (2016) [7] genotyped common bean with 24 microsatellite markers. Out of 342 F_1s obtained from 21 different parental crosses, 325 (82.91%) were confirmed as true hybrids. Johnson *et al*. (2019) [4] conducted hybrid testing and studied heterosis in relation to genetic divergence in chickpea under rice based cropping system. A total of 25 SSR markers with known sequences were used out of which only SSR21 and SSR22 were polymorphic between the parents. Reena & Jaiwal (2014) [11] confirmed intra-specific and inter-specific F_1 hybrids for salt tolerance in mungbean using trait specific SSRs. Sixteen different intra-specific and inter-specific hybrid populations obtained by three type of crosses among salt susceptible and salt tolerant lines were tested for hybridity by using 15 gene specific SSRs. Only two primers i.e. SSR3435 & SSR4041 produced polymorphism between the parents. The SSR markers in addition to hybridity confirmation have also been used for testing genetic purity in maize (Wang *et al*., 2002) [16] and rice (Nandakumar *et al*., 2004) [9].

Identification and characterization of hybrid cultivars is important for varietal improvement, seed production and release. For successful crop production, genetic production of hybrid seed must be maintained. Use of DNA markers offer distinct advantages over biochemical and morphological markers. Morphological markers are highly influenced by environmental factors and are time and labour consuming. Also, biochemical markers e.g. protein and isozyme markers are least affected by environment but they fail to differentiate closely related genotypes due to limited polymorphism (Luchhese *et al*., 1999) [5]. DNA markers overcome most drawbacks of biochemical and morphological markers and are useful for identification of hybrids.

The present study revealed that SSRs were robust and reliable markers for confirmation of hybridity in chickpea. The study also revealed that chickpea crossing might generate high proportion of selfed plants that may be designated falsely as hybrids. The study further demonstrated that putative hybrids must be confirmed by the use of SSR markers to identify true hybrids and to omit any errors of inclusion of false hybrids in breeding programme.

Table 1: Description of SSR markers used in the present study

Sr. No.	Primer name	Forward (5'-3')	Reverse	T_m (^{0}C)
1	TA8	AAAATTTGCACCCACAAAATATG	CTGAAAATTATGGCAGGGAAAC	55.00
2	TA203	ATAAAGGTTTGATCCCCATT	TGTGCATTCAGATACATGCT	55.00
3	TR43	AGGACGAAACTATTCAAGGTAAGTAGA	AATTGAGATGGTATTAAATGGATAACG	55.00
4	TA30	TCATTAAAATTCTATTGTCCTGTCCTT	ATCGTTTTTCTAAACTAAATTGTGCAT	55.00
5	TA113	TCTGCAAAAACTATTACGTTAATACCA	TTGTGTGTAATGGATTGAGTATCTCTT	55.00
6	TA59	ATCTAAAGAGAAATCAAAATTGTCGAA	GCAAATGTGAAGCATGTATAGATAAAG	55.00
7	TA28	TAATTGATCATACTCTCACTATCTGCC	TGGGAATGAATATATTTTTGAAGTAAA	55.00
8	TA2	AAATGGAAGAAGAATAAAAACGAAAC	TTCCATTCTTTATTATCCATATCACTACA	55.00
9	TA146	CTAAGTTTAATATGTTAGTCCTTAAATTAT	ACGAACGCAACATTAATTTTATATT	55.00
10	TA72	GAAAGATTTAAAAGATTTTCCACGTTA	TTAGAAGCATATTGTTGGGATAAGAGT	55.00

11	TA116	AATTCAATGACGAATTTTTATAAGGG	AAAAAGAAAAGGGAAAAGTAGGTTTTA	55.00
12	TA130	TCTTTCTTTGCTTCCAATGT	GTAAATCCCACGAGAAATCAA	55.00
13	TR20	ACCTGCTTGTTTAGCACAAT	CCGCATAGCAATTTATCTTC	55.50
14	NCPGR209	ATTGTTTGTTGGAGTGATGG	CACGGTTTCATTGTCTTGTT	55.00
15	TA22	TCTCCAACCCTTTAGATTGA	TCGTGTTTACTGAATGTGGA	55.00
16	TA80	CGAATTTTTACATCCGTAATG	AATCAATCCATTTTGCATTC	55.00
17	TA176	ATTTGGCTTAAACCCTCTTC	TTTATGCTTCCTCTTCTTCG	55.00
18	TR44	TTAATATTCAAAAACTCTCTTGTGCAAT	TTTACAACAGCGCTTGTATTTAGTAAG	55.00
19	TR35	ACTTTGGTTTAACATTTTCGGTAGTTA	AGTATCAACGTCATGTGTAACTCGTAT	55.00
20	TR1	CGTATGATTTTGCCGTCTAT	ACCTCAAGTTCTCCGAAAGT	55.00
21	TA180*	CATCGTGAATATTGAAGGGT	CGGTAAATAAGTTTCCCTCC	55.00
22	TA14	TGACTTGCTATTTAGGGAACA	TGGCTAAAGACAATTAAAGTT	55.00
23	TA78	CGGTAAATAAGTTTCCCTCC	CATCGTGAATATTGAAGGGT	55.00
24	TA64	ATATATCGTAACTCATTAATCATCCGC	AAATTGTTGTCATCAAATGGAAAATA	55.00
25	NCPGR264	TGGGAATCTTGTTGGTTCTT	TGAAAGGAGATGGAAAAAGC	57.10
26	TS43	AAGTTTGGTCATAACACACATTCAATA	TAAATTCACAAACTCAATTTATTGGC	55.00
27	TA5	ATCATTTCAATTTCCTCAACTATGAAT	TCGTTAACACGTAATTTCAAGTAAAGAT	55.00
28	NCPGR263	CAAGGATGAATGTGTGTGTG	CATAGTATCCTCGGTTTCCC	55.50
29	NCPGR136	GGACTGAGTGAGTTCGTCTT	GTATCCTCGGTTTCCCTATC	54.00
30	NCPGR117	GAACTTCTTCAATCTCACGG	CTAGCACGATGAAAGGATTC	54.50
31	NCPGR247	CAATGATTGGTTCTCTCCTC	GGTTTGACTAAAATATGGCG	54.50
32	NCPGR281	GCAATGATTGGTTCTCTCCT	GTGGAATTCTTTAGGGTTTGAC	56.50
33	NCPGR231	AACCTCCGTCCACACATTTC	GGTCGAAGCCATTGTTTTGT	59.40
34	NCPGR224	TGGAATTAGTTGATGTGACAA	ATTTCCCGTGTCTTTGAGAT	59.20
35	NCPGR214	ATTTCCCGTGTCTTTGAGAT	GGAATTAGTTGATGTGACAATG	54.50
36	NCPGR127	CATAATGCAAGGGCAATTAG	CTCTTATCTTCATGTTGCCG	55.50
37	NCPGR111	AATAACTCCATTTGGCTTGA	GCGGTAATTACACAATACAGG	54.50
38	NCPGR142	TAACTCCATTTGGCTTGAGA	TAACCTTATATGGTAGGCGG	54.50
39	NCPGR252	TTGCCCTGAGGAATACATTA	GGTTGTTGAAGGCATAACTG	54.30
40	NCPGR255	TCAGTGGTATTGAGACATCG	CCATCTTCAAAAGTGAACCT	54.00
41	TA25	AGTTTAATTGGCTGGTTCTAAGATAAC	AGGATGATCTTTAATAAATCAGAATGA	55.00
42	TA42	ATATCGAAATAAATAACAACAGGATGG	TAGTTGATACTTGGATGATAACCAAAA	55.00
43	GA 11	GTTGAGCAACAAAGCCACAA	TTCTTGTCTGGTTGTGTGAGC	55.00
44	TS83	AAAAATCAGAGCCAACCAAAAA	AAGTAGGAGGCTAAATTATGGAAAAGT	55.00
45	TA96	TGTTTTGGAGAAGAGTGATTC	TGTGCATGCAAATTCTTACT	55.00
46	TA37	ACTTACATGAATTATCTTTCTTGGTCC	CGTATTCAAATAATCTTTCATCAGTCA	55.00
47	TA27	GATAAAATCATTATTGGGTGTCCTTT	TTCAAATAATCTTTCATCAGTCAAATG	55.00
48	NCPGR254	GCCTTTTTCAATTTCTCTCA	CCCAAAGAAGACAAAACAAC	54.50
49	NCPGR261	GATTGTGTGGCAAAATCCAT	ACTCTCAGGTTGCTGTTCTGA	58.90
50	NCPGR146	AACGTGAAATTCCACCACTA	GAGTCGATTTCGTGTTGATT	55.40
51	TA96	TGTTTTGGAGAAGAGTGATTC	TGTGCATGCAAATTCTTACT	55.00

*polymorphic primer

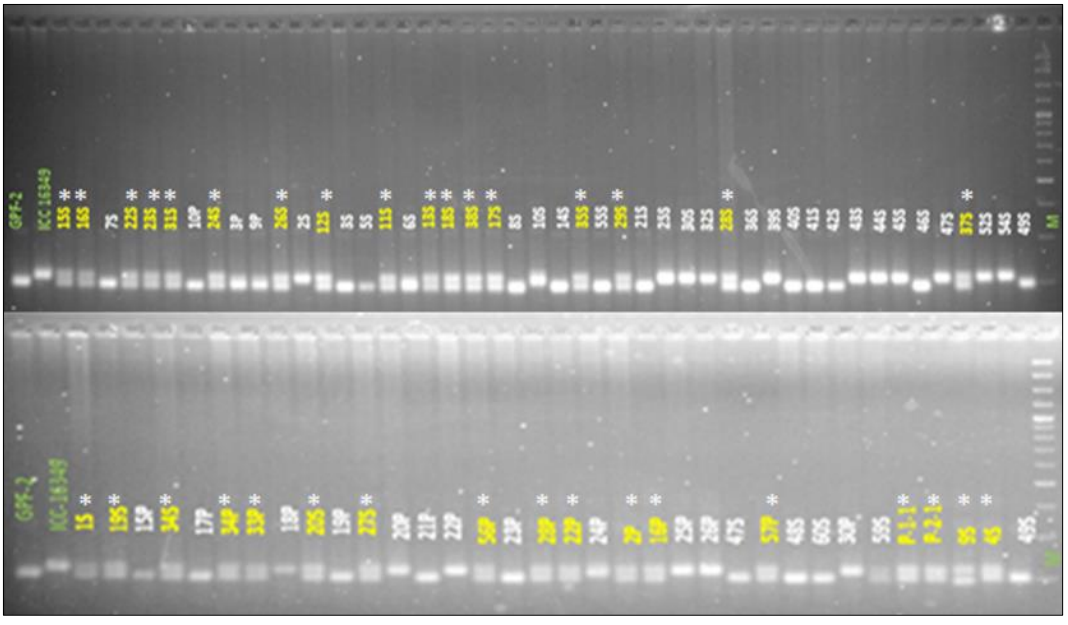

Fig 1: Amplification pattern of parents (ICC-16349 and GPF-2) and putative hybrids as revealed by SSR marker TA 180. Names of parents and hybrids are given at the termini of lanes. M=100 bp DNA ladder, * = true hybrids

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