Pak. J. Bot., 52(6): 2157-2163, 2020. DOI: http://dx.doi.org/10.30848/PJB2020-6(24)

AUTHENTICATION OF INDIGENOUSLY DEVELOPED CHILLI (*CAPSICUM ANNUUM*L.) HYBRIDS THROUGH ISSR MARKERS

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Abstract

Hybrid technology promises higher yields. For *Capsicum annuum* L., hybrid production is preferred because the vigorous yield uniformity, tolerance against biotic and abiotic stresses and other excellent horticultural traits. From the breeder's perspective, development of hybrids permits better control of intellectual property rights including control and protection of parental lines. Nowadays elite chilli hybrids have become popular due to their best performance. At present indigenously developed 10 elite chilli hybrids (Hybrid-1 to Hybrid-10) along with their 6 elite parents/inbred lines were used for molecular study to authenticate the F_1 progeny. The DNA based hybrid purity testing was carried out by using 16 inter simple sequence repeat polymerase chain reaction (ISSR-PCR) markers. Out of 16;8 ISSR primers, which included six di-and two tri-nucleotide primers amplified clear and polymorphic bands in 1-10 hybrids with their parents. The number of amplicons per primer ranged from 4 (CA1, CA2) to 16 (CA9) with an average 9.125 amplicons per primers. The average numbers of polymorphic bands per primer were 2.12. F_1 purity was verified by the presence of parental specific band in each hybrid. The number of polymorphic bands ranged (2 to 4) but CA1, CA2, CA3 showed no polymorphic and parent specific bands. ISSR markers are beneficial and efficient for assessing the genetic purity of F_1 hybrids and provide better opportunity for selection of superior genotypes for breeding purposes. This is the first report on the use of ISSR markers which provides the basis for authentication and identification of each of the progenitors of F_1 hybrids of *Capsicum annuum* Land could be cited as ready reference for hybrid development and authentication in chillies.

Key words: Genetic purity, ISSR markers, Hybrid, Chilli, *Capsicum annuum* L.

Introduction

Chilli (*Capsicum annum* L.) is an important member of *Solanaceae* family. Chilli was originated from the Central and Southern America and most likely from Mexico (Salvador, 2002). Chilli is a requisite spice essentially used in every cuisine due to its pungency, flavour, colour and aroma. It is the richest source of vitamin A, C and E (Simonne *et al*., 1997). Amongst twenty-five, there are five major cultivated species recognized as domesticated in the genus *Capsicum* i.e., *Capsicum annuum*, *C. frutescens*, *C. chinense*, *C. baccatum* and *C. pubescens* (Bosland & Votava, 2000). In Pakistan, only two species (*Capsicum annum* and *Capsicum frutescence*) are found to be cultivated. However, most of the chilli varieties cultivated on large scale as a cash crop belong to *Capsicum annuum* (Iqbal *et al*., 2012).

Generally, India is the largest producer of chilli and is presently considered as universal spice grown throughout the country. The total production of chilies all over the world was estimated be 2.098 billion tonnes that constituted about 85% of production in 2007 year. In this production, India contributed 36%, China (11%), Bangladesh (8%), Peru (8%) and Pakistan (6%) (Karvy, 2008). The total cultivated area under chillies in Pakistan was estimated as 64.82 thousand hectares with the annual production of 143.15 thousand tonnes (Anon., 2015-16). Due to the utilization of chillies in culinary purpose, more than 90% of the production is consumed within the country leaving only a small portion for export (Amjad & Anjum, 2007).

Very few commercial hybrids are available in chilli locally. The greater extent of the out crossing and large number of viable seeds produced by crossed chilli facilitate in development of commercial hybrids (Sreelathakumary and Rajamony, 2004). In order to pool the best traits, hybrid technology offers the promising outcome. Such as for *Capsicum annuum*, hybrid production is thought preferable due to its uniformity, vigour and tolerance to both biotic/abiotic stresses and other outstanding horticultural traits. By breeder's point of view, the development of hybrid cultivars is better for control of intellectual property rights which include parental lines control and protection. Nowadays, elite chillies hybrids have become popular due to their best performance.

All companies need quality controls to find out the hybrid seed purity. Methods used to verify hybrid seed purity require growing a representative sample of the F_1 seed and later classifying it by using descriptors of differences as true hybrid seed or off-types. This method is time consuming, space demanding and often does not allow the unambiguous recognition of genotypes. Various morpho-biochemical and molecular markers are used to characterize different plant species (Shinwari *et al.,* 2013; Jan *et al.,* 2016; Shinwari *et al.,* 2018; Jan *et al.,* 2018). However, estimation of genetic variability at molecular level is very precise and steadfast than variability at phenotypic level as the former is less dependent and influenced by environmental factors (Williams *et al.,* 1990; Turi *et al.,* 2012; Shah *et al.,* 2015; Jan *et al.,* 2017). Genetic analysis in *Capsicum* species has been

carried out using morphological and molecular markers. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeats-PCR (ISSR-PCR) markers assessments proved fruitful in cultivars genetic fingerprinting (Collins & Symons, 1993; Graham *et al.*, 1994), The RAPD approach has to be used with care, since inconsistent reproducibility might be encountered. Only few crop and animal species have information on ISSR loci. They leave no room for another choice to get highly reproducible markers without any prerequisite for earlier sequence information for different genetic analysis. The generated PCR products give information of multi-locus profiles, which could be expressed on agarose gel. The ISSR method is thought fit for F_1 hybrid purity because of reproducible results. It has been successfully used in *Capsicum* species by Kochieva *et al*., (2004).

National Agricultural Research Centre (NARC), Islamabad Pakistan has started an extensive hybrid chilli programme. Local chilli hybrids development is the major step taken for self-sufficiency on the available resources; as imported hybrid seed which is being sold at higher prices is out of reach for farmers. The present study was therefore designed with a view to assess the genetic purity of F_1 hybrids produced in NARC in comparison with their parents at molecular level.

Materials and Methods

The study was conducted in two phases. In the first phase, the purity of the parental lines was assessed. In the second phase, the hybrid combinations developed from the parental source were authenticated at the National Institute for Genomics and Advanced Biotechnology (NIGAB), NARC Islamabad, Pakistan.

Plant material: Plant material comprised of 10 chilli hybrids and their parental sources as: NARC-14/9, NARC-15/6, NARC-16/4, NARC-16/5, NARC-16/8 and NARC-16/9. For the purpose of the hybrid security, these parental lines were coded. However, the salient features of the parental lines have been presented in Table 1.

Experimental design and layout: The seeds of the crosses along with their parents/inbreds were harvested and were raised thereafter on the nursery beds under the greenhouse conditions. The material was transplanted to the plots sized 3.0 m × 1.0 m with plant to plant and row to row distances of 40 cm and 75 cm, respectively. The experiment was conducted with three replications in randomized complete block design (RCBD). Each entry was transplanted on a single bed accommodating 16 plants in total on both sides of the bed.

DNA extraction: Sampling of fresh leaves from each of the hybrids and their parents was made and stored at -80°C till further processing. Total genomic DNA was extracted from leaves by using the protocol of Shahzad *et al*., (2012) method. One % agarose gel was used for quantification of extracted DNA. For DNA quantification same quantity of DNA sample and lambda DNA standards of 25, 50, 100, 200, 300ng/µl was loaded in gel. DNA sample were observed under ultraviolet light with the help of Gel Documentation System. Quantification of DNA samples was assessed by measuring the intensity of the bands with those of Lambda DNA standard. Samples of DNA were diluted to 25 ng/µl for ISSR-PCR analysis.

Dissolution and dilution of primers: Sixteen sequenced ISSR-PCR markers were used in PCR reactions as described by Kumar *et al*., (2001) to detect the hybrids (F_1) purity of chillies (Table 2). The primers were dissolved in Tris-EDTA (TE) buffer to make100pmol/µl primers stock which was further diluted with TE buffer to a working concentration of 20 pmol/µl.

Polymerase chain reaction: The master mixture for polymerase chain reaction (PCR) was prepared in a volume of 40µl. The PCR master mixture contained 1X Taq buffer having 3mM $MgCl_2$, 0.2mM dNTPs mix, 20 pmol of the primers, one unit of Taq DNA polymerase (Fermentas, life sciences) and 25ng DNA template. PCR amplification was performed in an Applied Biosystems Thermal Cycler (Veriti 96 well) at 94ºC for 10 minutes, followed by 35 cycles each comprising of three steps; denaturation at 94ºC for 1 minute, primer annealing for 1 minute (optimized for each primer given in Table 1) and initial extension for 2 minutes at 72ºC. Final extension was carried out at 72ºC for 10 minutes.

Gel electrophoresis: The scoring of amplified products of PCR were carried out initially on 2% agarose gel with added ethidium bromide and unresolved bands, if any were further resolved on 5% polyacrylamide gel at voltage of 3000 kV for 10 hours. Bands were visualized using a UV Trans illuminator.

Data analysis

The absence and presence of bands were scored by comparing hybrids with their parents. First of all the differences in terms of bands (presence or absence) among parents were noted. The presences of both of the parental bands in F_1s were termed as hybrids. Absence of male parent band in F_1 denoted the selfed cross.

Data for number of fruits per plant and fresh green weight of fruits per plant was recorded for each plant in each hybrid population. The data for all authenticated hybrids in a population were averaged and the mean was subjected to standard error of mean analysis for comparisons (Table 4).

Table 1. Salient features of the chillies parental/inbred lines.

S. No.	Inbred lines	Salient features
1.	NARC-14/9	Long and thin fruit with light green in colour
2.	NARC-15/6	Long fruit size with medium green fruit colour, moderately resistant to chilli mosaic virus and Phytophthora
3.	NARC-16/4	Long fruit in size with light green in colour
4.	NARC-16/5	Long, thick fruit in size with medium green in colour, moderately resistant to chilli mosaic virus
5.	NARC-16/8	Long fruit with light green in colour
6.	NARC-16/9	Long fruit and light green in colour

Table 2. List of ISSR primers used.

S. No.	Marker name	Repeat motif	Primer sequences (5 ′ – 3 ′)	Annealing temperature (°C)
1.	CA1	(TG)7	5'ACACGTACAGTGTGTGTGTGTGTG3'	50°C
2.	CA2	(TG)7T	5'CATGCACACTGTGTGTGTGTGTGT3'	55°C
3.	CA3	C (GA)7	5'GATGATACGAGAGAGAGAGAGA3'	50°C
4.	CA4	T (GA)8	5'CGTAATGAGAGAGAGAGAGAGA3'	50°C
5.	CA5	(GA)8C	5' GAGAGAGAGA GAGAGA GGT 3'	55°C
6.	CA6	(CA)7	5'GATACTGATACACACACACACACA3'	62°C
7.	CA7	T (GT)9	5' CGTATGTGTGTGTGTGTGTGT3'	60°C
8.	CA8	GC (GCC)4	5'CGAGC GCCGCCGCCGCC 3'	55°C
9.	CA9	TA (CAG)4	5' AAA TC CAGCAGCAGCAG 3'	60°C
10.	CA10	CT (AAT)4	5' GTG CT AATAATAATAAT 3'	60°C
11.	CA11	T3 (ATT)4	5' GA TTT ATTATTATTATT 3'	55°C
12.	CA12	TC (ATT)4	5' GAA TC ATTATTATTATT 3'	50°C
13.	CA13	CGA(ATT)4	5' AGC AA ATTATTATTATT 3'	60°C
14.	CA14	AA (AAT)4	5'TG AAA AATAATAATAAT 3'	58°C
15.	CA15	A3 (AAT)4	5' ACG AAA AATAATAATAAT 3'	50°C
16.	CA16	GA (ATT)4	5'AGT GA ATT ATTATTATT 3'	55°C

Table 3. ISSR primers showing total number of amplified bands and polymorphic band per primer.

S. No	DNA marker name	Total genotypes	Number of genotypes shown amplification	No. of alleles/ genotype	Polymorphic bands
1	CA1	183	175	4	0
2	CA2	183	174	4	0
3	CA3	183	171	6	0
4	CA4	183	169	8	3
5	CA6	183	170	9	3
6	CA7	183	173	8	2
7	CA8	183	178	14	4
8	CA9	183	175	16	4
Mean			**173.125**	**9.125**	**2.25**

Results

ISSR amplification: Sixteen ISSR primers (Table 1) were initially used to screen the chilies germplasm. Eight of these sixteen primers gave reproducible and scorable amplified bands. The number of amplicons per primer ranged from 4 (CA1 and CA2) to 16 (CA9) with an average 9.125 amplicons per primer. The average number of polymorphic bands per primer was 2.125 (Table 3). In our results ISSR primers showed very good DNA polymorphism.

DNA based hybrid authentication: The hybrid purity of F_1 hybrid can be easily estimated by comparison of polymorphic and parents specific bands between F_1 population and their parents. In the present study, ISSR markers were used for authentication of hybrids because DNA profiling proved useful to assess the genetic purity of hybrids and parental verification in crosses in short time. Eight out of sixteen ISSR primers, which included six di- and two tri-nucleotide primers expressed clear amplified and polymorphic bands in 1-10 hybrids with their parents. The hybrid purity of 97% in Hybrid 1 (P4 × P2) and 100% in reciprocal Hybrid 9 (P2 × P4) and their respective populations (32 plants and 5 plants) was verified by the comparison of polymorphic bands between F_1 and their parent specific band respectively. The data is presented in Table 4. The primer CA 9 produce 4 polymorphic bands followed by primers CA8 (3), CA7 (2), CA6 (2), CA4 (2) polymorphic bands (Table 3). On the basis of these parent specific bands the genetic purity of F_1 Hybrid 1 and Hybrid 9 recognized with their parents. The primers CA1,CA2 and CA3 showed no polymorphism and parent specific band, one plant out of 32 in Hybrid 1 progeny did not give amplified band with any ISSR primers (Figs. 1 and 2).

The F_1 purity of Hybrid 2 (P1 × P2) that is reciprocal to Hybrid 3 (P2 × P1) their individual plants (28 and 14) gave 96.60% and 100% purity, respectively (Table 4) confirmed by discriminative ISSR loci expressed by primer CA9 (4) and CA 8 (3) followed by primers CA7 (2), CA6 (2) and CA4 (2) (Fig. 2).

The hybrid purity of progeny (6) and (8) of F_1 Hybrid 4 (P7 × P6) and Hybrid 8 (P7 × P5) 83.30% and 95% respectively (Table 4) recognized by variable amplified band shown by primer CA 9 produce 4 polymorphic band followed by primers CA8 (3), CA7 (2), CA6 (2) and CA4 (2) polymorphic bands (Fig. 3). Only 1 plant of Hybrid 4 recognized as out cross by primer CA9 and 1 plant of Hybrid 8 as non-hybrid due to no DNA amplification of desired band. The yield of these two plants is lower than other pure F_1 hybrid plants (Table 4).The DNA based F_1 purity 100% of Hybrid 5 (P1 × P4), Hybrid 6 (P4 × P3) Hybrid 7 (P5 × P4) and Hybrid 10 (P4 × P6) in their progenies (21) (21) (23) and (3) respectively scored through more reliable reproducible ISSR primers.

Table 4. Parentage, number of plants tested and authenticated, hybrid purity, number of fruits per plant, fresh green yield per plant in ten chilli hybrids tested.

Hybrid No.	Parentage	Number of plants tested	Number of hybrid plants authenticated	Number of non-hybrid plants	% Hybrid purity	Number of fruits / plant (Hybrid plants)	Number of fruits/ plant (Non-hybrid plants)	Fresh green yield/ plant (kg) (Hybrid plants)	Fresh green yield/ plant (kg) (Non-hybrid plants)
1.	P4 × P2	32	31	1	97	445.94 ± 77.48	125	3.63 ± 0.63	1.01
2.	P1 × P2	28	27	1	96.60	343.04 ± 80.55	113	4.37 ± 1.03	1.44
3.	P2 × P1	14	14	0	100	410.14 ± 64.15	320*	4.22 ± 0.66	2.94*
4.	P7 × P6	6	5	1	83.30	905.8 ± 82.9	681	6.69 ± 0.61	5.03
5.	P1 × P4	21	21	0	100	698.2 ± 141.7	488*	6.59 ± 1.33	3.53
6.	P4 × P3	21	21	0	100	599.1 ± 85.56	399*	4.4 ± 0.63	2.11*
7.	P5 × P4	23	23	0	100	585.79 ± 109.6	488*	5.15 ± 0.96	3.53*
8.	P7 × P5	21	20	1	95	484.26 ± 899.9	154	4.26 ± 0.79	1.35
9.	P2 × P4	5	5	0	100	645.4 ± 52.63	768*	2.32 ± 0.19	3.78*
10.	P4 × P6	3	3	0	100	968.66 ± 109.86	768*	5.85 ± 0.66	3.78*

* The number of fruits per plant and fresh green yield of female parent given for comparison

In addition to DNA based authentication of F_1 hybrids; the plant wise yield related data (number of fruit per plant and yield per plant) of hybrids population also supported the inference drawn from molecular data. The Table 4 depicted significant differences for fruits number per plant between 10 hybrids and their respective parents. The maximum fruits number per plant was accounted for the Hybrid 10 (968.8 ± 109.86) followed by Hybrid 4 (905.8 ± 82.9). Statistically, the difference between the above two mentioned hybrids were considered to be significant. However, there existed a significant difference with respect to the minimum fruits per plant value recorded in the non-hybrid plant of hybrid 2 (113). The number of fruits per plant between the two sets of reciprocal hybrids i.e; Hybrid 1 (P4 × P2) to Hybrid 9 (P2 × P4) and Hybrid 2 (P1 × P2) to Hybrid 3 (P2 × P1) was 445.94 ± 77.48, 645.4 ± 52.63, 343.04 ± 80.55 and 410.14 ± 64.15 respectively was found statistically significant when compared to their respective parents P1 (488), P2 (320) and P4 (399) (Table 4). The Hybrid 5 (P1 × P4), Hybrid 6 (P4 × P3), Hybrid 7 (P5 × P4) and Hybrid 8 (P7 × P5) gave number of fruits per plant as 698.2 ± 141.7, 599.1 ± 85.56, 585.79 ± 109.6, and 484.26 ± 899.9 respectively which had highly significant differences from their respective parents as evident from the Table 4.

Our findings showed that all the hybrids differed potentially for yield per plant (Table 4). The highest yield per plant was recorded in hybrid 4 (6.69 ± 0.61 kg) followed by Hybrid 5 (6.59 ± 1.33 kg) the difference of which was considered to be as highly significant. Minimum yield per plant was noticed in the non-hybrid plant of hybrid 1 (1.01 Kg). The fresh fruit yield per plant (kg) of each hybrid was compared to its parents so as to assess the performance of F_1 over its parents. Fresh fruit yield per plant (3.63 ± 0.63 kg) of Hybrid 1 (P4 × P2) significantly l differ from parents P2 (2.94 kg), P4 (2.11 kg) but showed high difference from its reciprocal Hybrid 9 (P2 × P4) (2.32 ± 0.19 kg). Hybrid 2 (P1 × P2) and Hybrid 3 (P2 × P1) are hybrid of mutual parental combination. The observed fresh fruit yield per plant in Hybrid 2 (4.37 ± 1.03 kg), Hybrid 3 (4.22 ± 0.66 kg) were significantly differed from P1 (3.53kg) and P2 (2.94 kg). The fresh fruit yield per plant (4.4 ± 0.63 kg, 5.15 ± 0.96 kg, 4.26 ± 0.79 kg and 5.85 ± 0.66 kg) of Hybrid 6 (P4 × P3), Hybrid 7 (P5 × P4), Hybrid 8 (P7 × P5) and Hybrid 10 (P4 × P6) respectively predicted significant difference from their respective parents P4 (2.11kg), P5 (3.53 kg) and P7 (3.53 kg) (Table 4).

The primer CA9 gave 16 amplified bands per individual sample followed by CA8 15 bands per sample; out of which 4 and 3 respectively were polymorphic bands and specific for male and female parents.CA 4, CA6 and CA7 also recognized these hybrids as true F_1 hybrid with 2 polymorphic bands of each as shown in Fig. 1. The number of fruits per and yield per plant of all hybrids is higher than their respective parents (Table 4).

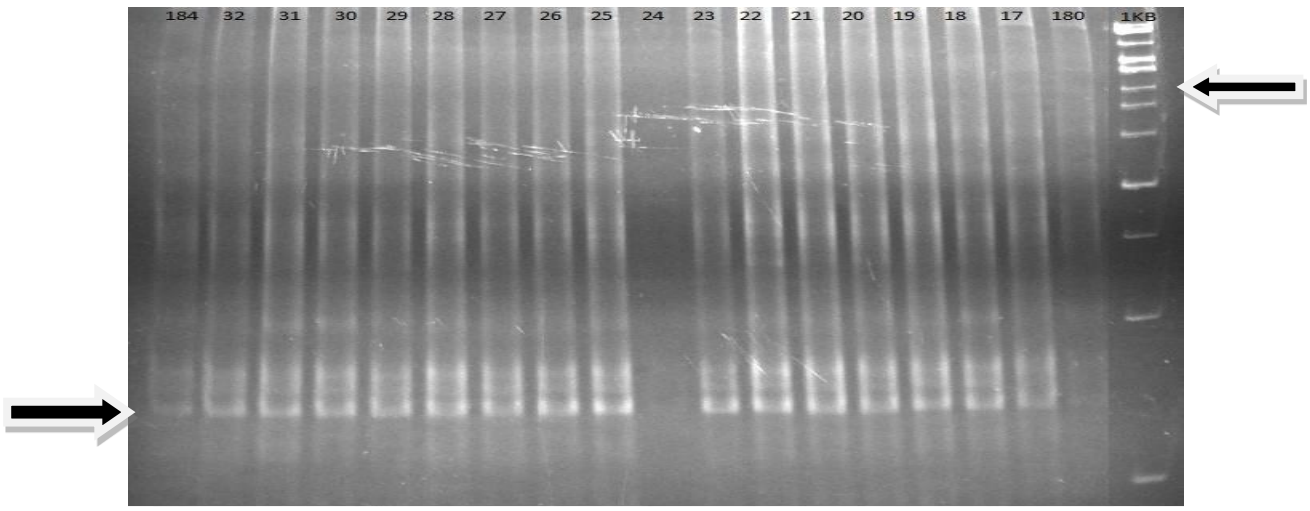

Fig. 1. ISSR–PCR profile visualized on 5% Polyacrylamide gel for primer CA 4
Arrows shows polymorphic and parent specific bands,
184 = Female parent, 180 = Male parent, 17-32 = Hybrid 1 progeny

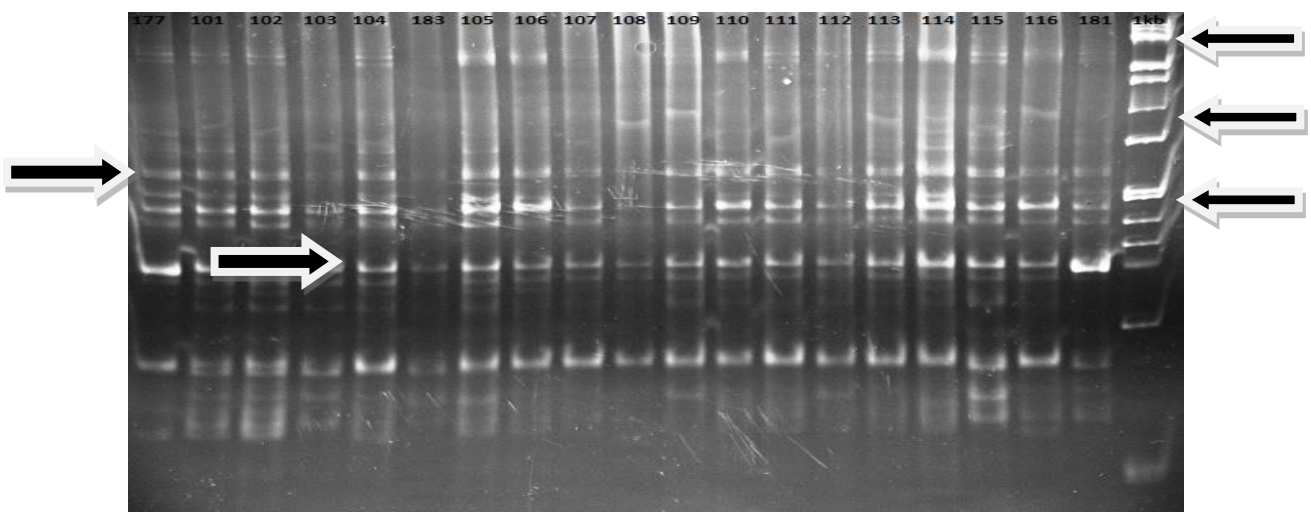

Fig. 2. ISSR–PCR profile visualized on 5% Polyacrylamide gel for primer CA 9
Arrows shows polymorphic and parent specific bands,
177 = Female parent, 183 = Male parent 101-104 = Hybrid 5 progeny, 183 = Female parent, 181 = Male parent of hybrid 6 = 105–116.

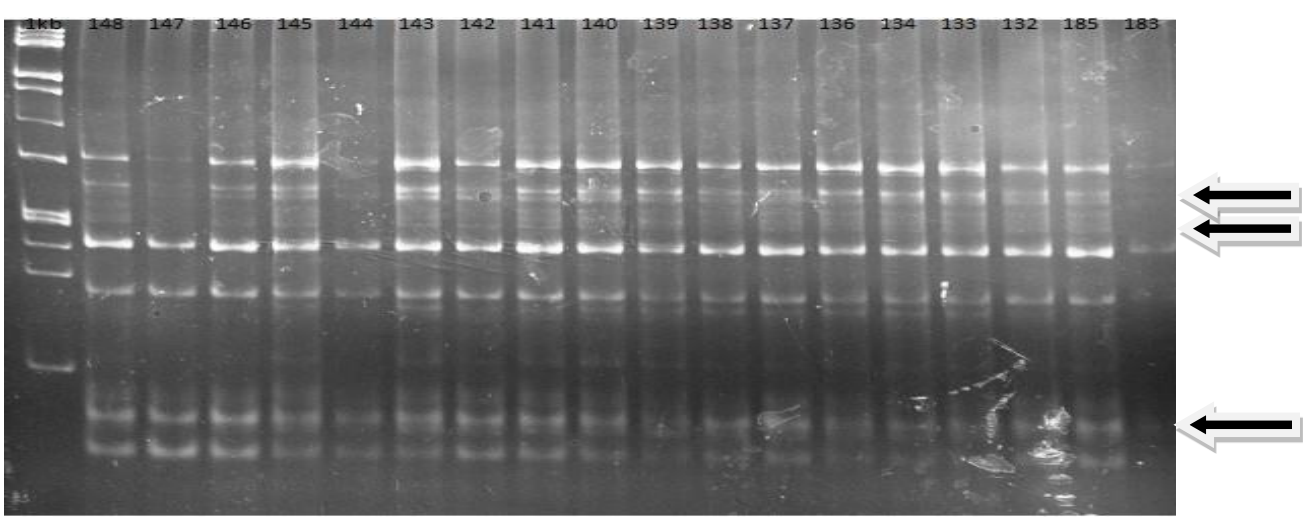

Fig. 3. ISSR–PCR profile visualized on 5% Polyacrylamide gel for primer CA 8
Arrows shows polymorphic and parent specific bands
183 = Male parent, 185 = Female parent 132-148 = Hybrid 7 progeny

Discussion

ISSR markers were used for authentication of 10 hybrids and their parents of *Capsicum annuum* L. The genetic purity of hybridity status of F_1 hybrids can be easily predicted by the comparison of polymorphic parents specific bands between F_1 population and their parents. In addition to DNA based authentication of F_1 hybrids and their parents, morphological analysis was also conducted to support the molecular data in order to assess the genetic purity of hybrids and parental verification in crosses in short time. ISSR PCR analysis revealed as an informative fingerprint process for F_1 hybrid purity with respect to their parents for precise characterization and evaluation of various closely related plant species and may provide better opportunity for selection of superior genotypes for breeding purposes. This research use of ISSR markers for hybrid authentication and identification of their parents will be useful for crop improvement through breeding.

Our findings are in accordance with the studies conducted on different crops like tomato (Rus-Kortekaas *et al*., 1994), mulberry (Srivastava *et al*., 2004), and peanut (Raina *et al*., 2001) in which the ISSR primers showed polymorphism in related cultivars. The generated PCR products for primers CA9, CA 8 and CA 4 gave information of 2 to 4 multi locus profiles, polymorphic and parent specific bands (male parent) which could be visualized on polyacrylamide gels (Figs. 1-3). The ISSR-PCR method was found fit for F_1 hybrid purity because of its highly reproducible results. This has been successfully used in *Capsicum* species by Kochieva *et al*., (2004). The use of ISSR primers also reported to be applicable in the genetic diversity in pepper (Lijun & Xuexiao, 2012).The ISSR primers were used for clear differentiation of disputed chilies samples (Kumar *et al*., 2001).

Our findings are in accordance with the studies conducted on different crops like tomato (Rus-Kortekaas *et al*., 1994), mulberry (Srivastava *et al.,* 2004) and peanut (Raina *et al.,* 2001) in which the ISSR primers showed polymorphism in related cultivars. The 14, 8 and 30 ISSR primers were successfully used to estimate the genetic characterization and generally revealed inter specific hybrid in coffee, bamboo and *tritceae* tribe, respectively (Carvalho *et al*., 2005 and Lin *et al*., 2010). For F_1 hybrid seed purity, the ISSR analysis was employed in artichoke (Bianco *et al*., 2011) in clover and mandarin (Scarano *et al*., 2002). This is the first report on the use of ISSR markers which provide the basis for authentication and identification of each of the progenitors of F_1 hybrids of *Capsicum annuum* L. Moreover, it could be cited as ready reference for chilli crop hybrid development and authentication.

Conclusions

The presented work is of novelty in its nature due to the indigenous development of high yielding hybrids in chillies where most of the hybrid chillies seed is being imported and there is an acute shortage of locally produced hybrid seed. It is concluded from the results of the study that ISSR-PCR based analysis is a valid and reproducible technique of fingerprint screening of F_1 hybrids in terms of its purity for precise characterization. This analysis also testifies the morphological analysis towards hybrid purity.

Acknowledgements

This work on chilli hybrid development and its ISSR-PCR based authentication was conducted in collaboration with the PARC Institute of Advanced Studies in Agriculture, Islamabad and the project entitled "Indigenization of Hybrid Seed Production Technology (IHSPT-PSDP)" funded by Public Sector Development Programme (PSDP), the Government of Pakistan. The authors duly acknowledge the support and time to time help of the Project Director, IHSPT (Dr Muhammad Shafique Zahid) in the commencement of the research work.

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(Received for publication 15 November 2018)