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Original scientific article

ASSESSMENT OF GENETIC PURITY AND EARLINESS IN F_1 AND F_2 POPULATION OF CUCUMBER (*Cucumis sativus* L.) USING SSR MARKERS

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Simple Sequence Repeat (SSR) markers were used to assess the genetic purity and earliness in F_1 and F_2 population of cucumber (*Cucumis sativus* L.) respectively. Experiment was carried out at the Experimental Research Farm of the Department of Vegetable Science, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh (HP) during the years 2015 and 2016. Experimental material used in the study comprised of Khira-75, a commercial variety grown in low and mid hills of the state (Himachal Pradesh) which is late in flowering and genotype PI-618860, an inbred line collected from North Central Regional Plant Introduction Station, USA which is early in flowering and tolerates various biotic stresses. For assessment of genetic purity and earliness the parents Khira-75 and PI-618860 were crossed and F_1 and F_2 population were raised. Genetic purity studies revealed that one marker CSN 160 was observed to be segregating in the parents as well as the population. Further, scoring of earliness in F_2 population revealed promising results using 2 co-dominant SSR00262 and SSR17922 markers, which segregated in the parents and population in correlation with the observed phenotypes.

Keywords: Assessment; cucumber *Cucumis sativus* L.; earliness; genetic purity and SSR markers

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INTRODUCTION

Cucumber (*Cucumis sativus* L., 2n=2x=14) is an economically and horticulturally important crop grown worldwide as a multipurpose vegetable and a better choice to the breeders for using it as a model plant (TANURDZIC and BANKS, 2004; KUMARI *et al.,* 2021). The phenomenon of hybrid vigour is one of the most important genetic phenomena that lead to increase of production of a several crops including cucumber. The hybrid vigour in quantitative genetic is superior of hybrid than the average of its parents or superior of its best parent (BERNARDO, 2002). It is a phenomenon that is specialized in the size of the hybrid, growth rate, and fertility and increases their ability to resist diseases and insects than its parents (SABOUH *et al*., 2010). Many researchers and scientists have worked to discover the phenomenon which associated with first-generation hybrid, including East, Shull and Wright in the *Cucurbitaceae* family which include the cucumber (AL-SAHOOKI, 2006). They found an increase by 24-39% in yield of first generation plants compared with parents with the highest yield (BAIRAGI *et al.,* 2005). Conventionally, genetic/hybrid purity testing is done through grow out test, which is land and labour intensive, time consuming and requires one full season. Due to this, the hybrid seeds are not available for immediate cultivation leading to additional expenditure in storage and hence increased hybrid seed cost. Considering the inherent disadvantages of this test, molecular marker-based seed purity assay could be a better alternative and it is currently receiving more attention (NARESH *et al.,* 2009). A number of DNA fingerprinting techniques are available for detection of polymorphism. Most of the DNA marker assays use polymerase chain reaction (PCR), among them Randomly Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) markers are popularly used for assessing the genetic purity in crop plants. SSR markers are preferred over the dominant markers like RAPD, Inter Simple Sequence Repeat (ISSR) and Amplified Fragment Length Polymorphism (AFLP) due to its co-dominant nature, which helps in unambiguous detection of off types in the hybrid seed lots (SUNDARAM *et al.,* 2008). SSR or Microsatellites are typically the repeat unit of 1-6 nucleotides and its analysis is performed by using pairs of specific primers flanking tandem arrays of microsatellite repeats. The exploitation of the advantages of Marker Assisted Selection (MAS) relative to conventional breeding could have a great impact on crop improvement. The high cost of marker assisted selection will continue to be a major obstacle for its adoption for some crop species and plant breeding in developing countries in the near future. Specific marker assisted selection strategies may need to be tailored to specific crops, and their traits through available budgets. New marker technology can potentially reduce the cost of marker assisted selection considerably. If the effectiveness of the new methods is validated and the equipment can be easily obtained, this should allow MAS to become more widely applicable for crop breeding programmes (LU *et al.,* 2014). High-density genetic linkage maps established using molecular markers, for economically important crops provide a basis for MAS of agronomically useful traits, for pyramiding of resistance genes and the isolation of important genes by map-based cloning strategies. SSR can be successfully used for genetic purity confirmation, which would be a valuable genomic tool for the cucumber breeders (GUO *et al.,* 2007; HE *et al.,* 2007). In Himachal Pradesh (HP), cucumber is grown as a commercial and leading cash crop. The yield may be increased by altering architecture of the genotypes to produce early flowering, gynoecy, multiple branching habits, more number of fruits and multiple disease resistance. These traits play an important role in

cucumber heterosis breeding and identification of markers linked to these characters will facilitate selection of cucumber genotypes in breeding programme. Therefore, it is imperative to study the genetics of some important traits and to identify superior genotypes through marker assisted selection.

MATERIAL AND METHODS

Plant material

Two diverse cucumber genotypes *viz.,* Khira-75, a commercial variety grown in low and mid hills of the state (HP) but late in flowering and genotype PI-618860, an inbred line collected from North Central Regional Plant Introduction Station, USA is early in flowering, gynoecious and tolerates various biotic stresses. The seeds of parents (Khira-75 and PI-618860) were planted in the month of May 2015 to attempt crosses and generate F_1 progeny in open field conditions. The mature fruits of crosses (F_1) as well as selfed (parents) progenies were harvested and seeds were extracted manually. Seeds were dried and then stored in cool place for sowing in the subsequent year. The seeds of the cross *viz.,* Khira-75 × PI-618860 (F_1) and parents were sown in polyhouse in the month of February 2016. Each parent and cross (Khira-75 × PI-618860) were selfed to get sufficient seed of parents and F_2 population in open field condition during next season.

Evaluation of parents and population (F_1 & F_2)

During rainy season (Ist week of July) 2016, seeds of parents and their F_1 & F_2 population were sown with three replications for their evaluation. Row to row and plant to plant spacing of 100 cm × 75 cm was kept in a plot having size 4.0 m × 3.0 m, which accommodated 16 plants per plot. The standard cultural practices as recommended in Package of Practices of Vegetable Crops were followed to raise the healthy crop stand (ANONYMOUS, 2016). Simultaneously, for further molecular studies (confirmation of genetic purity of hybrid and earliness) younger leaves of the parents, F_1 and F_2 population were collected, and genomic studies were performed. The list of primers used has been presented in Annexure1.

Genomic DNA isolation

Genomic DNA from leaves of five-week-old seedlings was isolated following the modified CTAB method (LIU *et al.,* 2003). For each sample, approximately 200-250 mg leaf tissue was frozen in liquid nitrogen and ground to powder. The CTAB solution was added and the DNA samples were incubated for 1 hour at 65°C, with occasional mixing by gentle inversion, after that 1ml of Phenol: Chloroform: Isoamyl alcohol (25:24:1, v/v/v) were added and mixed by inversion to emulsify, spinned at 12,000 rpm for 20 minutes at room temperature, pippetted out gently the aqueous phase without disturbing the interphase to another clean tube. Further 2/3 volume of isopropanol were added and mixed by gentle inversion. Kept at -20°C for 1 hour, then spinned at 11,000 rpm for 15 minutes at 4°C. Collected DNA using a pipette and transferred to another tube. Washed the DNA pellet in 70 per cent ethanol for 10 minutes and spinned at 10,000 rpm for 5-10 minutes at 4°C. Dried the DNA pellet and dissolved in 50 µl TE buffer (pH 8.0) depending upon the yield of DNA.

DNA purification

RNA contaminants in all the samples were digested with 100 µg/ml RNase A for 30 minutes at 37°C. Equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) were added and mixed thoroughly to emulsify. Spinned at 12,000 rpm for 10 minutes and carefully took out the upper aqueous phase. 1/10 volume of 3 M sodium acetate (pH 5.2) were added and mixed thoroughly. Two volume of absolute ethanol (95%) were added and mixed by gentle inversion and kept in ice for 20 minutes. Spinned at 10,000 rpm for 10 minutes at 4°C, DNA pellet was formed and washed it with 70 per cent ethanol. DNA pellet was dried and dissolved in 50 µl TE buffer. Quantification of DNA was done using UV spectrophotometer (Bio-Rad), USA by taking absorbance at 260 nm. Quality of the DNA (*ie* approximate purity of DNA) was estimated by determining the ratio of absorbance at 260 and 280 nm using UV spectrophotometer (Bio-Rad), USA.

SSR markers

SSR primers belonged to the series of CSN, SSR and CSWCT reported by FUKINO *et al.,* 2008; MIAO *et al.,* 2011; LV *et al.,* 2012; ZHANG *et al.,* 2012; ZHANG *et al.,* 2013; LU *et al.,* 2014; YOSHIOKA *et al.,* 2014; SIGVA *et al.,* 2015 were used (Annexure1). These primers were synthesized from Metabion International AG, Germany.

SSR-PCR analysis

SSR-PCR was performed in PTC-100 thermo cycler (MJ Research Inc., USA) following the protocol reported by LIU *et al.* (2007a). The reaction volumes of 25 µl contained 20 ng genomic DNA, 2.5 µl of 10 X Taq buffer, 1.5 µl of 25 mM $MgCl_2$, 0.5 µl each of Forward and Reverse primers (10 µM), 0.5 µl of dNTPs (10mM), 0.25 µl of Taq DNA polymerase (5U/µl), 2.5 µl of template DNA and water to prepare final volume. The PCR program used was as following: initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing depending upon Tm value of primer for 1 minute, extension at 72°C for 3 minutes, and a final extension step at 72°C for 8 minutes, then held at 4°C. The amplification products were separated on 3.5% agarose gels containing ethidium bromide in 1×TAE buffer and photographed under UV light in Gel Documentation System (Bio Rad, USA).

Genetic purity studies

A total of 110 primers were surveyed for screening polymorphism between the two parents *ie.* Khira-75 and PI-618860 and subsequently their random 30 hybrid individuals were selected from 110 individuals of F_1 population. The markers displayed distinct amplification of specific allele in hybrids and parental lines were considered as informative SSR markers. The impurities or the off-types were detected based on deviation in the expected amplification pattern.

Assessment of earliness trait

Among the informative SSR markers revealed as in case of parents and their F_1 population, SSR primers specific to the earliness were further used in case of parents and their F_2

population. In F_2 population, 85 individuals were selected from 336 individuals on the basis of earliness for this purpose.

RESULT AND DISCUSSION

Genetic purity studies were performed based on morphological characters and trait of interest *viz.,* earliness. Full potential of any hybrid can be realized only by using good quality seeds; hence confirmation of genetic purity is an essential requirement for its commercial success. Traditional means of crossing and selection is an expensive, tedious and time consuming procedure which is influenced by many environmental factors (YASHITOLA *et al.,* 2002). Considering the limitations associated with this method, molecular marker-based seed purity assay could be a better alternative (SUNDARAM *et al.,* 2008). Recent developments of molecular techniques and application of molecular markers have brought a new dimension into the traditional area of plant breeding. Molecular markers not only allow the easy and reliable identification of breeding lines, hybrids and cultivars (TABBASAM *et al.,* 2006) but also facilitate the monitoring of introgression, mapping of QTLs, marker assisted selection (MAS) and estimation of genetic diversity (ZHANG *et al.,* 2013). Molecular marker assisted identification is tremendously useful in hybrid seed genetic purity assessment for many traits that are not easily detectable through the visual inspection. PCR based molecular marker technology like SSR (simple sequence repeats), AFLP (amplified fragment length polymorphism), SRAP (sequence related amplified polymorphism), RAPD (random amplified polymorphic DNA) and ISSR (inter simple sequence repeats) has been receiving the attention for this genetic purity assessment purpose. Among them, SSRs are regarded as the most suitable markers with attributes like co-dominance, highly informative and reproducibility for genetic purity assessment in various crops (LIU *et al.,* 2007a; LIU *et al.,* 2007b; LIU *et al.,* 2007c; NARESH *et al.,* 2009; SOLANKI *et al.,* 2010; ZHAO *et al.,* 2012; YU *et al.,* 2013; BORA *et al.,* 2016). A total of 110 SSR primers were surveyed among them, only 45 SSR primers allowed the identification of several markers, which exhibited amplification of alleles 'specific' or 'unique' to a particular parent. The markers SSR05723, SSR23474 and CSN184 amplified maximum number of 4, 3 and 3 monomorphic alleles, respectively, while 12 markers (CSN190, CSN069, CSN061, CSN183, CSWCT28, CSN160, CSN131, CSN126, SSR00116, SSR01331, SSR20852 and SSR10018) amplified 2 polymorphic alleles and rest of the 30 markers showed monomorphic (single) allelic pattern. The polymorphism between the parents was showed by only 5 SSR primers *i.e.,* SSR10018, SSR20852, CSN160, CSN183 and CSWCT28 which could identify and produce unique fingerprints and therefore must be considered as highly informative. The banding pattern obtained with SSR10018, SSR20852, CSN183 and CSWCT28 primers revealed monomorphic amplicons specific to PI-618860 (male parent) in the hybrids. Therefore, only CSN160 was able to differentiate both the parents and hybrids used in the present study wherein Khira-75 (180 bp), PI-618860 (200 bp) and hybrids (both the amplicons) specific uniform banding pattern was obtained across the genotypes showing only one off type (sample no 30) with Khira-75 (female) specific band (Plate 1). List of markers used for hybridity assessment of F_1 individuals have been given in Table 1. Our results are consistent with LIU *et al.* (2007a); LIU *et al.* (2007b); LIU *et al.* (2007c); ZHAO *et al.* (2012) and YU *et al.* (2013) where hybrids showed both the parental alleles indicating heterozygosity. Presence of off types among the hybrid seedlings established the

importance of hybrid purity tests and those false hybrids could be removed at the early stages of plant development and thus reduces the population size which is easy for handling and maintenance of segregating population.

Table 1. Assessment of genetic purity by SSR primers

Sr no	SSR Primer	Sequences	Expected size (bp)	Observed size (bp)		References
				K*	PI*	
1	SSR10018	F:GGGTCTAATATTTGGGGATGG R:GGTTGTTCTTGTGGAATGTG	130	100	140	LV *et al.* (2012)
2	SSR20852	F:GGTTTCCATTGAACTCGTAGC R:GGCTGTCCATTTTGTAGAACC	199	200	215	LV *et al.* (2012)
3	CSN160	F: GTAGCAGAAGCCTCACCGGAGTAA R:CTTGTAGCAGAAGGCTTCCACGTT	210	180	210	FUKINO *et al.* (2008)
4	CSN 183	F: TGGACCACGTGAAAGATTCAGAAA R: GCCTACAACTATCCCAAATGGAGC	215	200	220	FUKINO *et al.* (2008)
5	CSWCT28	F: GAATTCAAAAGCATTTCAAAACTA R: GAATTCATTGGGTTTTTGAACCC	207	210	215	MIAO *et al.* (2011)

K*- Khira-75, PI*- PI-618860

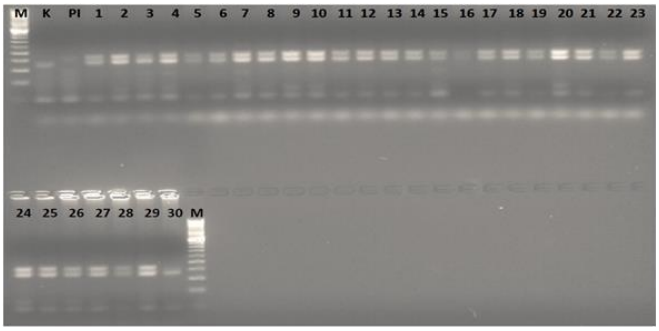

Plate 1. Hybridity confirmation with CSN-160 primer, (Where, M: 50 bp DNA Marker/Ladder; K: Khira-75 (P_1); PI: PI-618860 (P_2), 1-30: F_1 Individuals

Assessment of earliness trait in F_2 population

For the assessment of earliness trait, F_2 population was raised through the selfing of F_1 population. DNA was isolated from the parents (Khira-75 and PI-618860) and 85 individuals of F_2 population selected on the basis of earliness *ie.*, plants were selected and tagged on the basis of appearance of female flower at lower node and number of days required to first harvest of fruits at marketable stage. The highly informative SSR primers based on their polymorphic nature between the parents were considered for confirmation purpose. Therefore, only 5 SSR primers (SSR00262, SSR17922, SSR10018, SSR01331, and CSN160) confirmed the earlier reported findings. Among these SSR00262 and SSR17922 corresponds to earliness (LU *et al.*, 2014), SSR10018 corresponds to peduncle length (SONG *et al.*, 2016), SSR01331 corresponds to horticulturally important traits (MIAO *et al.*, 2011) respectively and no trait linkage data is available in case of CSN160 (FUKINO *et al.*, 2008).The primer SSR00262 generated two

fragments in the parents *i.e.*, 200 bp in Khira-75, 220 bp in PI-618860 and among the F_2 individuals (Plate 2 a and b). LU *et al*. (2014) reported the association of SSR00262 with earliness and the expected size of SSR00262 was 219 bp (Table 2). The primer SSR17922 also generated two fragments in the parents *i.e.*, 190 bp in Khira-75, 200 bp in PI-618860 and among the F_2 individuals (Plate 3 a and b). Lu et al. 2014 reported the association of SSR17922 with earliness and the expected size of SSR17922 was 197 bp (Table 2). So on the basis of earlier reported findings it could be suggested that the earliness trait was present in the parents and among the F_2 individuals. A potential reason for the phenomenon is residual amount of heterozygosity. For the breeding selection is primarily based on phenotypic traits. Therefore the markers in the important gene coding regions of the genome and near locations get fixed quickly for the respective alleles, otherwise markers get fixed at random (LIU *et al.*, 2007c).

Table 2. Informative SSR primers used for assessment of trait of interest in F_2 population

Sr no	SSR Primer	Sequences	Expected size (bp)	Observed size (bp)		CN*	Traits	R*
				K*	PI*			
1	SSR00262	F: CCGTTGGTCTTGGACTCTCA R:TGTAAAAGTGATCAGGAGGGTCT	219	200	220	1	*Ear**	LU *et al.* (2014)
2	SSR17922	F: CATTCTAGGTCAATGAATCGCA R: GCAAAGTTGCCACATTGAAG	197	190	200	1	*Ear**	LU *et al.* (2014)
3	SSR10018	F:GGGTCTAATATTTGGGGATGG R:GGTTGTTCTTGTGGAATGTG	130	100	140	1	*Pl**	SONG *et al.* (2016)
4	SSR01331	F: CGGGATTTACCCCTCACATT R: GTGGGACCGAGAAGTTTGAT	218	200	215	6	*HI**	MIAO *et al.* (2011)
5	CSN160	F:GTAGCAGAAGCCTCACCGGAGTAA R:CTTGTAGCAGAAGGCTTCCACGTT	210	180	210	-	-	FUKINO *et al.* (2008)

K*- Khira-75, PI*- PI-618860, CN-Chromosome number, R-References, *Ear*- Earliness, *Pl*- Peduncle length and *HI*- Horticulturally important traits.

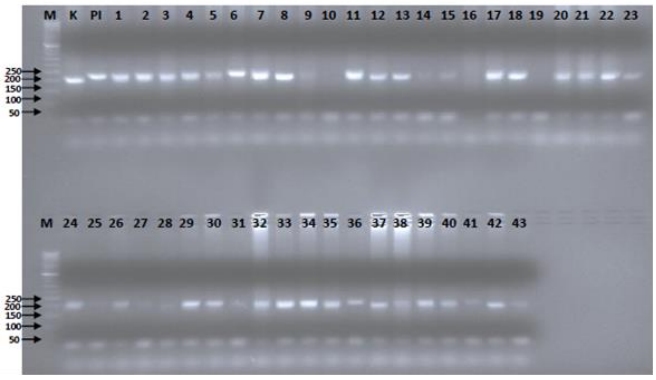

Plate 2a: Amplification profile with SSR00262 primer resolved on agarose gel. (Where, M: 50 bp DNA Marker/Ladder; K: Khira-75 (P_1); PI: PI-618860 (P_2); 1-43: F_2 Individuals)

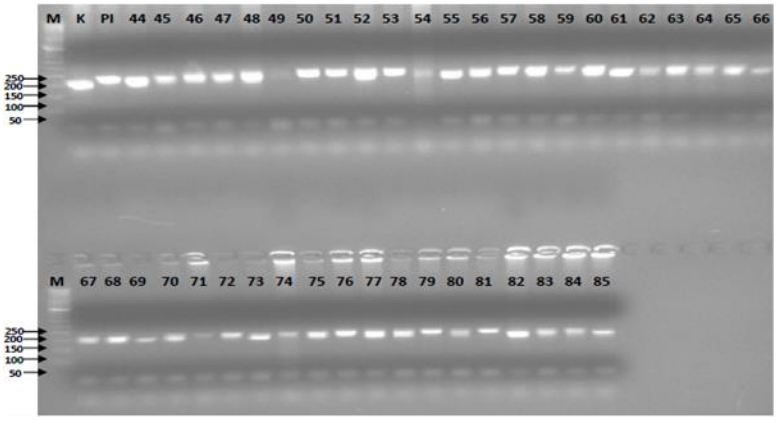

Plate 2b: Amplification profile with SSR00262 primer resolved on agarose gel. (Where, M: 50 bp DNA Marker/Ladder; K: Khira-75 (P_1); PI: PI-618860 (P_2); 44-85: F_2 Individuals)

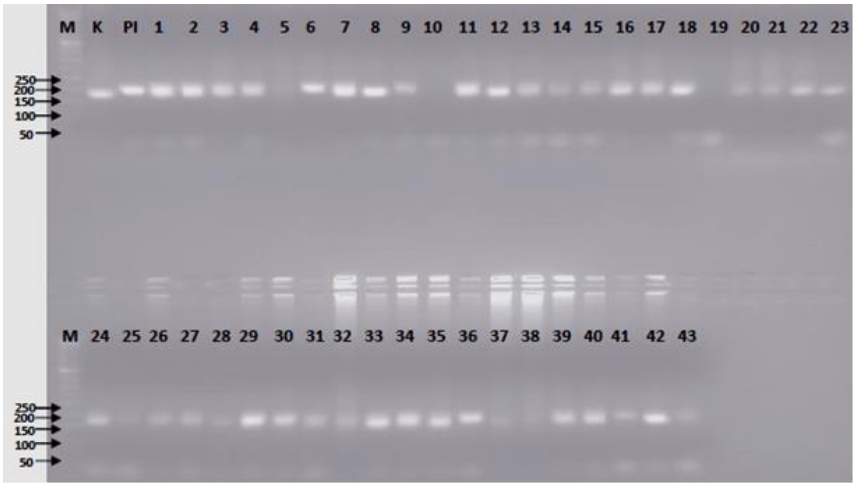

Plate 3a: Amplification profile with SSR17922 primer resolved on agarose gel. (Where, M: 50 bp DNA Marker/Ladder; K: Khira-75 (P_1); PI: PI-618860 (P_2); 1-43: F_2 Individuals)

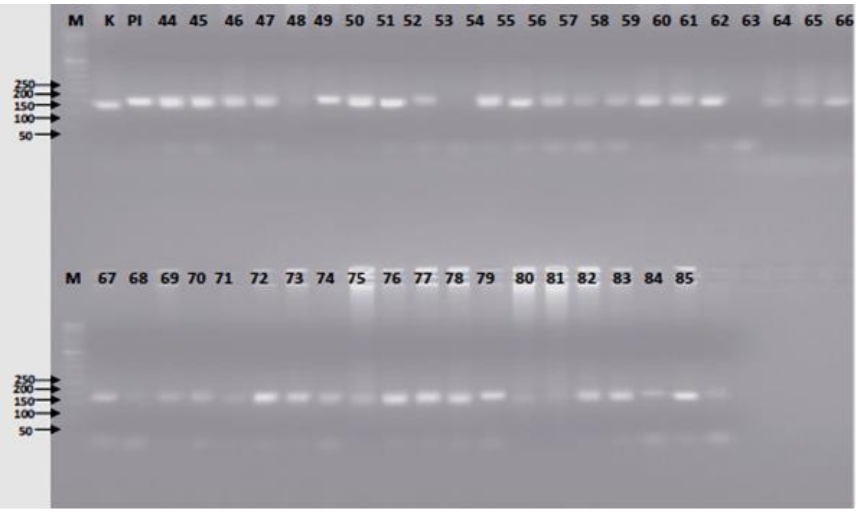

Plate 3b: Amplification profile with SSR17922 primer resolved on agarose gel. (Where, M: 50 bp DNA Marker/Ladder; K: Khira-75 (P_1); PI: PI-618860 (P_2); 44-85: F_2 Individuals)

Other than the targeted trait *i.e.* earliness in the present study, earlier reported SSR primers in cucumber *viz.*, SSR10018 (peduncle length), SSR01331 (horticulturally important traits) and CSN160 showed their presence as revealed in the Table 2. Molecular markers linked to a gene of interest are the milestone and these tags are useful starters for identication of genes (FRANCIA *et al.*, 2005). Therefore, the assessment studies with these SSR markers demonstrated their usefulness in MAS for various horticultural traits in cucumber breeding. The study so conducted yielded strong although preliminary information about genetic purity and earliness. Further, confirmation with respect to chromosome number would be achieved by developing linkage maps on different linkage groups by correlating the phenotypic and genotypic data of desirable traits taken into account in the present study.

CONCLUSION

Assessment of hybrid purity and earliness with codominant CSN160, SSR00262 and SSR17922 markers demonstrated that these markers can be further used for marker assisted selection (MAS) in cucumber breeding.

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PROCENA GENETIČKE ČISTOĆE I RANOSTASNOSTI U F1 I F2 POPULACIJI KRASTAVCA (*Cucumis sativus* L.) KORIŠĆENJEM SSR MARKERA

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Izvod

SSR markeri korišćeni su za procenu genetske čistoće i ranostasnosti u F1 i F2 populaciji krastavca (*Cucumis sativ*us L.), respektivno. Eksperiment je sproveden na farmi za eksperimentalno istraživanje Odeljenja za nauku o povrću, Univerziteta za hortikulturu i šumarstvo dr Iashvant Singh Parmar, Nauni, Solan, Himachal Pradesh (HP) tokom 2015. i 2016. godine. Eksperimentalni materijal korišćen u studiji sastojao se od Khira-75, komercijalne sorte koja se uzgaja u niskim i srednjim brdima države Himačal Pradeš koja kasno cveta i genotipa PI-618860, inbred linije dobijene iz severno-centralne regionalne stanice za introdukciju biljaka , SAD koji rano cveta i toleriše različite biotičke stresove. Za procenu genetske čistoće i ranostasnosti ukrštani su roditelji Khira-75 i PI-618860 i dobijene su F1 i F2 populacije. Studije genetske čistoće otkrile su da je primećeno da se jedan marker CSN 160 javlja kod roditelja i kod populacije. Dalje, ocean ranostasnosti u populaciji F2 otkrilo je obećavajuće rezultate korišćenjem 2 ko-dominantna markera SSR00262 i SSR17922, koji su segregirali kod roditelja i populacije u korelaciji sa posmatranim fenotipovima.

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