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Identification of male sterile (T, S) of fertility (N) cytoplasm by PCR-based molecular markers to access maintainer lines in onion

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ABSTRACT

Purpose: Modern onion breeding is almost completely based on the production of hybrid seed. This project was performed to evaluate the effectiveness of marker-assisted selection (MAS) in identification of the cytoplasmic types and *Ms* locus in 123 onion accessions. **Research method:** Three cytoplasmic markers *cob*, *accD* and *MK* were used to identify the sterility (S) from the fertility (N) cytoplasm and four nuclear molecular markers (*OPT, PsaO, Jnurf-13 and AcSKP1*) were used for genotyping of *Ms* alleles. **Findings:** The results showed that the two *accD* and *cob* markers were quite similar in the detection of the type of cytoplasm with 100% male sterility for male sterile lines and 100% fertility for maintainer lines. The *MK* marker was able to distinguish T-type cytoplasm as well. Based on the results, the frequency of fertility (N) was much more than the frequency of sterility (S and T) cytoplasm found to be 90% in Dorche (pop.1), 100% in Dorche (pop.2) and Kashan based on marker cob and accD and with MK marker, was found to be 80%, 90% and 82% in Dorche (pop.1), Dorche (pop.2) and Kashan, respectively. **Limitations:** In this study, nuclear markers were not successful due to not finding linkage disequilibrium with the *Ms* locus, suggested more markers to be evaluated. **Originality/Value:** Molecuar markers were very suitable for the indentification of S or N lines. Cytotype (N/S) determination of plants by using molecular markers (*cob*, *accD* and *MK*), could easily reduce the population size required for the production of onion hybrid seeds.

INTRODUCTION

Onion (*Allium cepa* L.) as a member of the Alliaceae family is the main vegetable in the world that has been grown and used for more than 5000 years (Tsujimura et al., 2019). The discovery of CMS by Jones and Emsweller (Jones & Emsweller, 1936) has led to the genotypes with improved performance. To date, two CMS cytoplasm types (CMS-S and CMS-T) were recognized. Male sterility due to S-type cytoplasm discovered in the onion cultivar 'Italian Red' is maintained by the sterility inducing cytoplasm (S) and the single nuclear restorer gene *Ms/ms* in its recessive condition (Jones & Clarke, 1943; Jones & Davis, 1944). An S-cytoplasmic plant with a dominant allele at *Ms* leads to male fertility. In T-type cytoplasm at least three independent loci: *aa*, *bb*, and *cc* were identified (Schweisguth, 1973). Plants possessing N cytoplasm are always male-fertile regardless of the genotype at *Ms* (Havey, 2000).

The obtainment of onion hybrids in the mother's parent requires two lines: A-lines as male-sterile lines and B-lines as maintainer lines. Isolation of male-sterile line based on visual/microscopic examination is easy, but identification of maintainer line needs progeny tests that it requires 4–8 years based on the conventional breeding methods and also it is costly (Pike, 1986; Khosa et al., 2016).

Molecular markers can greatly accelerate breeding cycles by replacing time-consuming and laborious progeny testing. Numerous Molecular markers were used for distinguishing normal (N) and sterile (S) cytoplasm (Havey, 1993; Havey, 1995; Sato, 1998; Engelke et al, 2003; Cho et al.,2006; Kim et al. 2009; Kim et al. 2015b; Khar & Saini, 2016; Ferreira et al., 2017; Ferreira et al., 2018). In fact, molecular markers have no effect on shortening the onion seed production period, they can reduce the conventional development of onion hybrids requires 4-8 years for cytoplasm identification and 10-12 years for genome transfer by backcrossing (Havey, 1995; Khosa et al., 2016). The identification of the maintainer line needs progeny tests to do genotyping of the *Ms* locus that is expensive and time-consuming. Markers tightly associated with *Ms* locus were first reported by Gökçe and Havey (2002) followed by others using PCR markers (Kim et al., 2015a; Khar and Saini, 2016; Bang et al.,2011; Kim, 2014; Von Kohn et al., 2013; Huo et al., 2015) and SNPs (Havey, 2013).

The application of marker-assisted selection using the PCR markers in determining types of S/N and T cytoplasm and *Ms* locus were reported in Indian onion germplasm (Khar & Saini, 2016) and Brazilian germplasm (Ferreira et al., 2018), However, no similar studies were found in the literature for the Iranian germplasm, associating marker selection for male-sterile ('A') lines and maintainers of male sterility ('B'). Therefore, the objective of this study was to identify the cytoplasmic types in 123 onion accessions and to genotype them for the fertility restoration nuclear locus (*Ms*) using molecular markers.

MATERIALS AND METHODS

Plant material

The plant materials including the male sterile and maintainer lines (Shahmansouri & Aminpur, 2004; Shahmansouri & Otroshi, 2011) along with the other genetic materials that had been previously identified by the horticulture crops research group in Isfahan Agricultural and Natural Resources Research and Education Center were evaluated (Table 1). In total, DNA of 123 genotypes was isolated for the identification of cytoplasm type and *Ms* locus.

Total genomic DNA was extracted from leaves using a CTAB protocol (Murry & Thompson, 1980). DNA quality was examined by the agarose gel electrophoresis (2%) then

samples were stored at 20°C for PCR amplification using the specific primers (Table 2). PCR conditions for amplification were followed according to Khar and Saini (2016). All PCR products were visualized on 1.5% agarose gels after ethidium bromide staining.

The cytoplasmic type was identified by three molecular markers: The marker *cob* (Sato, 1998) with 180 bp fragment representing the 'N' cytoplasm, while 464 bp fragment showing the 'S' cytoplasm. The marker MKF/MKR1, MKR2 (Kim et al., 2009) with 833 bp fragment showing the 'N' cytoplasm, 833 bp and 628 bp fragments indicating the 'T' cytoplasm, while the 628 bp fragment showing the 'S' cytoplasm. The marker *accD* (Von Kohn et al., 2013) with 375 bp fragment = showing the 'N' cytoplasm; and 420 bp fragment indicating 'S' cytoplasm. Only the marker *MK* can detect T-type cytoplasm.

Four molecular markers were used for identifying the alleles of the male-sterility restorer locus: The marker *jnurf13* (Kim, 2014) with fragments: 241bp for *MsMs*, 229bp and 241bp for *Msms*, 229bp for *msms*; The marker *OPT* (Bang et al., 2011) with fragments: 659bp for *MsMs*, 526bp and 659bp for *Msms*, 526bp for *msms*; The marker *PsaO* (Bang et al., 2011) with fragments: 490bp for *MsMs*, 437bp and 490bp for *Msms*, 437bp for *msms* and the marker MK898/628 primer *FU*898/*SU*628 (*AcSKP1*) (Huo et al., 2015) with fragments: 898bp for *MsMs*, 628bp and 898bp for *Msms*, 628bp for *msms*.

RESULTS

Evaluation of cytoplasm type

PCR markers *cob, MK* and *accD* were used to find the cytoplasm type (Table. 3). All male sterile lines (S1-S9) presented 100% S or T cytoplasm type with three markers. Single plants in male-sterile S2 presented a mixture for cytoplasmic type so that one out of the 5 plants (20%) analyzed presented T cytoplasm type with the *MK* marker (Fig.1). S1, S3-S9 presented a single type (S) of cytoplasm. The observed changes in the frequencies of the 'S' and 'T' cytoplasms for the male sterile lines are such that only the *MK* marker can detect 'T' type of cytoplasm.

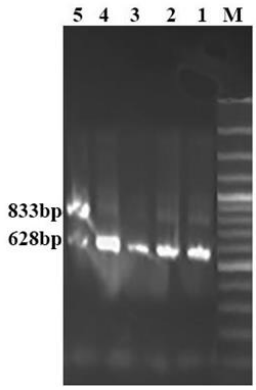

Fig 1. DNA fragments of male sterile lines (S2) of onion amplified with the *MK* marker. Lanes 1,2,3,4 showed 'S' cytoplasm, fragments with 628bp. Lane 5 showed 'T' cytoplasm, fragments with 628bp and 833bp.Lane M = ladder.

Table 1. The list of the genetic material used in the project and the number of plants selected for each sample

Number	Genetic material	Phenotype	Predicted Genotype	Number of plant
1	Male Sterile Line (S1)	Male Sterile	S*msms* or T*msms*	1
2	Male Sterile Line (S2)	Male Sterile	S*msms* or T*msms*	5
3	Male Sterile Line (S3)	Male Sterile	S*msms* or T*msms*	5
4	Male Sterile Line (S4)	Male Sterile	S*msms* or T*msms*	5
5	Male Sterile Line (S5)	Male Sterile	S*msms* or T*msms*	5
6	Back Cross Line (S6)	Male Sterile	S*msms* or T*msms*	1
7	Back Cross Line (S7-S9)	Male Sterile	S*msms* or T*msms*	3
8	Maintainer Line (M1)	Normal	N*msms*	1
9	Maintainer Line (M2)	Normal	N*msms*	1
10	Maintainer Line (M3)	Normal	N*msms*	1
11	Maintainer Line (M4)	Normal	N*msms*	1
12	Maintainer Line (M5)	Normal	N*msms*	1
13	Armaghan Hybrid (A1-A7)	Normal	S or T Cytoplasm	7
14	Baran Hybrid (B1-B6)	Normal	S or T Cytoplasm	5
15	Red Hybrid (R1-R5)	Normal	S or T Cytoplasm	6
16	Seria Hybrid (E1-E8)	Normal	S or T Cytoplasm	8
17	Dorche Hybrid	Normal	S or T Cytoplasm	5
18	Texas Hybrid	Normal	S or T Cytoplasm	5
19	Dorcheh Pop. 1	Male Sterile & Normal	All 3 Types of Cytoplasm	10
20	Dorcheh Pop. 2	Male Sterile & Normal	All 3 Types of Cytoplasm	20
21	Kashan Pop.	Male Sterile & Normal	All 3 Types of Cytoplasm	27
Total				123

Based on three cytoplasmic markers, all maintainer lines were found to have normal (N) cytoplasm and that the classifications of these markers were the same in maintainer lines.

The 26 single plants in hybrids (Armaghan, Baran, Red and Seria) were found to carry sterile (S) cytoplasm based on the cytoplasmic markers *cob* and *accD*, but by the *MK* marker, 20% (1 plants) in Baran hybrid, 17% (1 plants) in Red hybrid, 25% (2 plants) Seria hybrid and 43% (4 plants) in Armaghan hybrid showed 'T' type cytoplasm and others were showed S type cytoplasm, only one plant of Armaghan revealed N cytoplasm. Thus, there was full agreement between the cytoplasmic classification with the two types of male-sterile cytoplasm ('S' and 'T') and the fertile cytoplasm ('N') using the two marker *cob* and *accD*. For the Armaghan hybrid, a difference of 14% (one plant out of 7 plants) was observed between molecular identification of *MK* and two other ones.

For the ten genotypes of Dorcheh and Texas hybrid, a complete agreement was observed in the classification and the frequency of the three types of cytoplasm evaluated with the markers *cob*, *accD* and *MK* was 100% (Table 3).

Table 2. The list of the primers, along with their sequences, used in this study for onion

Primer	Orientation (5'-3')	PCR marker for	Sizes of PCR products (bp)	Reference
cob-N	TCTAGATGTCGCATCAGTGGAATCC	Cytoplasmic	N (180bp) S (464bp)	(Sato, 1998)
cob-S	GTCCAGTTCCTATAGAACCTATCACT			
cob-C	CTTTTCTATGGTGACAACTCCTCTT			
MK F	CATAGGCGGGCTCACAGGAATA	Cytoplasm	N (833bp) S (628bp)	(Kim et al., 2009)
MKR 1	AATCCTAGTGTCCGGGGTTTCT		T (833bp and 628bp)	
MKR 2	CAGCGAACTTTCATTCTTTCGC			
accD F	AGAATGAGGAGCAGGAAAACTCT	Cytoplasm	N (375bp) S (420bp)	(Von Kohn et al., 2013)
accD R	AGTCGTGATTGTTACTCTTAGACCT			
jnurf13 F	TGCAAGCTTGGAACTTACGC	Nuclear *Ms* locus	MsMs (241bp), Msms (229bp and 241bp)	(Kim, 2014)
jnurf1 R	TTGCCAAAGGTTGCAATACA		Msms (229bp)	
OPT	CCTTGGAAAGGCGCAACTAAAGATTTGA	Nuclear *Ms* locus	MsMs (659bp), Msms (659bp and 526bp) Msms (526bp)	(Bang et al., 2011)
OPT	TGTGGCCCAATAATACAAACAAGCAGGA			
PsaO	CCTCATGCTTGCTTGGTCTT	Nuclear *Ms* locus	MsMs (490bp), Msms (490bp and 437bp) Msms (437bp)	(Bang et al., 2011)
PsaO	AAGCGTGATCGATTGTAGGTCCTTT			
FU898	GCAATACACAGCTTCTAGCTGAATT	Nuclear *Ms* locus	MsMs (898bp),	(Huo et al., 2015)
FD898	AACACACACACAGAGTGAGAAATTTTATATAT		Msms (628bp and 898bp)	
SU628	TCTGTGTGTGTGTGTAATTTCTCTG		Msms (628bp)	
SD628	CGGAAGATTAATATTTTGCGTATACAT			

The results of molecular analysis for Dorcheh (population 1) showed a mixture of fertile ('N') and sterile ('S') cytoplasm; 9 plants contained 'N' cytoplasm and one plant had 'S' cytoplasm based on the cytoplasmic markers *cob* and *accD*, while *MK* marker revealed 2 plants with sterile ('S') cytoplasm (Table 3). Therefore, in this population, 10 % was presented cytoplasmic type classification discrepancy, being classified as 'N' by the *cob* and *accD* markers and as 'S' by the *MK* marker. All genotypes in population 2 of Dorcheh, were found to carry fertile ('N') cytoplasm based on the cytoplasmic markers *cob* and *accD*, and by using *MK* marker, 10% (2 plants) sterile ('T') cytoplasm was revealed. Similarly, In Kashan population, all plants (27) were found to carry (N) cytoplasm based on the cytoplasmic markers *cob* and *accD*, while MK marker revealed 18% (5 plants) (T) cytoplasm. In total, the frequencies of the fertile ('N') and sterile ('S' and 'T') cytoplasm changes were 10%, 10% and 18%, for Dorcheh (population 1), Dorcheh (population 1) and Kashan population, respectively using three cytoplasmic markers. Classifications with *cob* and *accD* markers were the same in all populations.

Evaluation of the male-fertility restorer locus (Ms)

The amplicons on the gels were identified for *jnurf13*, *OPT* and *PsaO* markers and detected fragments of the expected sizes according to Kim (2014) and (Bang et al., 2011). For *AcSKP1*, no specific bands were detected.

All the plants of male sterile and maintainer lines, with *jnurf13* and *PsoA* had recessive *Ms* locus (*msms*) as predicted, whereas *OPT* marker was shown the genotype of *MsMs* in male sterile and maintainer lines (Table 4). Hence, discrepancies in predicting male sterility and maintainers in these nuclear markers were observed.

Based on the nuclear PCR marker, *jnurf13*, all single plants in the hybrid of Seria (8plants), Armaghan (7 plants), Baran (5plants), Dorcheh (5plants) and Texas (5plants) contained *MsMs* genotypes; whereas the results of *OPT* marker showed all three genotypes. In Armaghan hybrid, OPT marker predicted 3 plants (43%) as homozygous dominant genotypes, 3 plants (43%) as heterozygous dominant genotypes and 1 plants (14%) with the *msms* genotype. This marker in Baran hybrids revealed that 40% of plants should be homozygous dominant at Ms locus and 20% recessive. In Red hybrids, 40% of plants with *MsMs* genotypes, 34% of plants with Msms genotypes and 16% with *msms* genotypes were released. Nuclear marker OPT in Seria hybrids predicted that 50% genotypes should be homozygous dominant at Ms, 25% heterozygous and 25% homozygous recessive. For Texaz and Dorche only homozygous dominant and heterozygous were identified (Table 4). *PsaO* detected all Armaghan, Baran, Red and Seria hybrids with heterozygous Ms locus (*Msms*). In Dorche hybrid, *PsaO* was showed 20% MsMs, 40% *Msms* and 20% *msms*. But in Texaz hybrid, 40 % of plants with homozygous dominant and 60% of plants with heterozygous was released by nuclear marker *PsaO* (Table 4).

Table 3. Estimation of frequencies of cytoplasm in 123 onion plants

Genetic material	Cytoplasmic markers	Type of Cytoplasm			Genetic material	Cytoplasmic markers	Type of Cytoplasm		
		N	S	T			N	S	T
S1	*accD*	0	1	0	Armaghan Hybrid	*accD*	0	1	0
	cob	0	1	0		*cob*	0	1	0
	MK	0	1	0		*MK*	0.14	0.428	0.428
S2	*accD*	0	1	0	Baran Hybrid	*accD*	0	1	0
	cob	0	1	0		*cob*	0	1	0
	MK	0	0.8	0.2		*MK*	0	0.4	0.6
S3	*accD*	0	1	0	Red Hybrid	*accD*	0	1	0
	cob	0	1	0		*cob*	0	1	0
	MK	0	1	0		*MK*	0	0.16	0.83
S4	*accD*	0	1	0	Seria Hybrid	*accD*	0	1	0
	cob	0	1	0		*cob*	0	1	0
	MK	0	1	0		*MK*	0	0.25	0.75
S5	*accD*	0	1	0	Dorche Hybrid	*accD*	0	1	0
	cob	0	1	0		*cob*	0	1	0
	MK	0	1	0		*MK*	0	1	0
S6	*accD*	0	1	0	Texas Hybrid	*accD*	0	1	0
	cob	0	1	0		*cob*	0	1	0
	MK	0	1	0		*MK*	0	1	0
S(7-9)	*accD*	0	1	0	Dorcheh Pop. 1	*accD*	0.9	0.1	0
	cob	0	1	0		*cob*	0.9	0.1	0
	MK	0	1	0		*MK*	0.8	0.2	0
M1-M5	*accD*	1	0	0	Dorcheh Pop. 2	*accD*	1	0	0
	cob	1	0	0		*cob*	1	0	0
	MK	1	0	0		*MK*	0.9	0	0.1
Kashan Pop.	*accD*	1	0	0					
	cob	1	0	0					
	MK	0.82	0	0.18					

Table 4. Estimation of frequencies of genotypes at *Ms* locus in 123 onion plants

Genetic material	Cytoplasmic markers	Ms locus			Genetic material	Cytoplasmic markers	Ms locus		
		MsMs	Msms	msms			MsMs	Msms	msms
S1	*jnurf13*	0	0	1	Armaghan Hybrid	*jnurf13*	1	0	0
	OPT	1	0	0		*OPT*	0.43	0.43	0.14
	PsaO	0	0	1		*PsaO*	0	1	0
	AcSKP1	-	-	-		*AcSKP1*	-	-	-
S2	*jnurf13*	0	0	1	Baran Hybrid	*jnurf13*	1	0	0
	OPT	1	0	0		*OPT*	0.4	0.2	0.4
	PsaO	0	0	1		*PsaO*	0	1	0
	AcSKP1	-	-	-		*AcSKP1*	-	-	-
S3	*jnurf13*	0	0	1	Red Hybrid	*jnurf13*	1	0	0
	OPT	1	0	0		*OPT*	0.5	0.34	0.16
	PsaO	0	0	1		*PsaO*	0	1	0
	AcSKP1	-	-	-		*AcSKP1*	-	-	-
S4	*jnurf13*	0	0	1	Seria Hybrid	*jnurf13*	1	0	0
	OPT	1	0	0		*OPT*	0.25	0.25	0.5
	PsaO	0	0	1		*PsaO*	0	1	0
	AcSKP1	-	-	-		*AcSKP1*	-	-	-
S5	*jnurf13*	0	0	1	Dorche Hybrid	*jnurf13*	1	0	0
	OPT	1	0	0		*OPT*	0.2	0.8	0
	PsaO	0	0	1		*PsaO*	0.2	0.6	0.2
	AcSKP1	-	-	-		*AcSKP1*	-	-	-
S6	*jnurf13*	0	0	1	Texas Hybrid	*jnurf13*	1	0	0
	OPT	1	0	0		*OPT*	0.4	0.6	0
	PsaO	0	0	1		*PsaO*	0.4	0.6	0
S(7-9)	*jnurf13*	0	0	1	Dorcheh Pop. 1	*jnurf13*	0.3	0	0.7
	OPT	1	0	0		*OPT*	1	0	0
	PsaO	0	0	1		*PsaO*	0.2	0.6	0.2
	AcSKP1	-	-	-		*AcSKP1*	-	-	-
M1-M5	*jnurf13*	0	0	1	Dorcheh Pop. 2	*jnurf13*	0.3	0	0.7
	OPT	1	0	0		*OPT*	1	0	0
	PsaO	0	0	1		*PsaO*	0.5	0.25	0.25
	AcSKP1	-	-	-		*AcSKP1*	-	-	-
Kashan Pop.	*jnurf13*	0.65	0	0.45					
	OPT	1	0	0					
	PsaO	0.55	0.26	0.29					
	AcSKP1	-	-	-					

For *OPT* marker in Dorcheh population1, Dorcheh population2 and Kashan population, only frament of 659 bp was amplified,therefore all genotypes were detected as *MsMs*. For *jnurf13,* in three populations, heterozygote genotype was not shown. In Dorcheh population1 and Dorcheh population2, 30 % of genotypes were homozygous dominant and 70% of plants with homozygous recessive were identified. For Kashan population, 65% genotypes were shown homozygous dominant and 45% of plants with homozygous recessive were released. For *PsaO*, two fragments of 490bp and 437bp appeared,therefore all kinds of genotypes were shown in three populations. In Dorcheh population1, 20% *MsMs*, 60% *Msms* and 20% *msms* were seen. Nuclear marker *PsaO* in Dorcheh population2 predicted that 50% genotypes should be homozygous dominant at Ms, 25% heterozygous and 25% homozygous recessive. In Kashan population, 55% of plants were belonged to *MsMs*, 26% to *Msms* and 29% to *msms* (Table 4).

DISCUSSION

This study strongly reveals that marker-assisted selection (MAS) for cytotype can reduce the investment in the development of the maintainer (B) line in a population. It means decreasing

the number of test-crosses required to isolate maintainers, as only the N-cytoplasmic plants will be carried forward till flowering and used for test crossing with a male sterile (A) line. Many results obtained by the markers used for cytoplasmic identification (Khar & Saini, 2016; Terefe & Tatlioglu, 2003; Santos et al., 2010; Ragassi et al., 2012; Patil et al., 2016) contributing to reduce the number of test crosses required to identify 'A' and 'B' lines.

In this study, male-sterile and maintainer lines presented 100% S or T cytoplasm and 100% N cytoplasm, respectively. The *accD* and *cob* markers in the detection of fertility ('N') and sterility ('S') of cytoplasm were quite similar and showed 100% efficiency. In addition to identify the fertility ('N') and sterility ('S') in cytoplasm, *MK* marker was able to distinguish between two sterility ('S' and 'T') as 100%. These results emphasize the findings of previous studies on these markers (Ferreira et al., 2017). In commercial hybrid seed production, parents with S-type cytoplasm are preferred over T-type due to stability in different environmental conditions (Havey, 2000).

Khar and Saini (Khar & Saini, 2016) used three cytoplasmic molecular markers (accD and cob and MK) to identify cytoplasm and detected that all the markers were equally good in determining the cytoplasm and all of them detected the same type of cytoplasm without any ambiguity. It was observed that accD should be used more for cytoplasm determination because of the relative simplicity of two primers and its visualization (Khar & Saini, 2016).

As in the present study, the proportion of N-cytoplasm was much more than S-cytoplasm found to be 90% in Dorche (pop.1), 100% in Dorche (pop.2) and Kashan based on marker cob and accD and with MK marker, was found to be 80%, 90% and 82% in Dorche (pop.1), Dorche (pop.2) and Kashan, respectively. Since the majority of onion populations possess N cytoplasm; S cytoplasm is likely an alien cytoplasm introgressed into onion (Havey, 1993; Havey, 2000). In fact, numerous polymorphisms in the organellar DNAs of S cytoplasm distinguish it from N cytoplasm (Havey, 2000; Havey, 1995; Sato, 1998; Engelke et al., 2003; Kim et al., 2009). In populations where N-cytoplasm is present in high proportions, the probability of finding maintainers is higher than that of male-sterile plants. In such cases, the frequency of maintainer plants can be estimated by test crossing with a known CMS line in 2 years following the Seed-to-Seed approach. In a study, using the marker cytochrome b (cob), CMS lines were isolated from three open-pollinated onion populations and the frequency of maintainers in any open-pollinated population without performing test crosses were predicted (Malik et al., 2017). In India, the onion cultivars are predominantly open-pollinated, which are primarily N-cytoplasmic in nature (Havey & Bark, 1994). Therefore, the US cultivars like Mountain Denvers, B2215C and Brigham Yellow Globe were decrease due to high proportions of S-cytoplasm, in contrast, the Indian onion populations did not decline significantly (Havey, 1995).

The kind of cytoplasms has been identified in many Brazilian onion populations, with the PCR-based marker system monitoring cytoplasm (Havey, 1995; Santos et al., 2010), suggesting that the N and T-cytoplasms were common in the Brazilian 'Baia Periforme' derived onion populations (Santos et al., 2007). The screening of the cytoplasmic type will be important to develop onion hybrids and to future comparative studies with other onion CMS commercial systems.

In this study, it was observed that all plants in male sterile and maintainer lines matched with the predicted genotypes, this proved that the jnurf13 marker is in linkage disequilibrium with the Ms locus in these varieties. Linkage disequilibrium between the Ms locus and the nuclear markers is essential to determine the minimum distance between markers required for the effective use of these markers in the detection of genotypes. Although the OPT marker at a distance of 1.5 cM, is very closely located to the Ms locus, the result of this study and others researches (Bang et al., 2011; Khar & Saini, 2016) showed no significant linkage

disequilibrium between this marker and the Ms locus, indicating that crossing-over contiguous to the Ms locus mostly occurred throughout the history of onion breeding. Linkage equilibrium between the Ms locus and tightly linked markers was also shown in onion breeding lines bred in the United States (Gökçe & Havey, 2002). Therefore, based on the present results, it would be impossible to predict the correct genotypes of the Ms locus using these closely linked molecular markers from onion germplasm. Although, it is very important to carry out the genotyping of the Ms locus with the use of PCR markers to accelerate the development of onion hybrids. For accurate genotyping of the Ms locus, more tightly linked markers must be developed.

CONCLUSION

Modern onion breeding is almost completely based on the production of hybrid seed. Although the simple PCR-based markers developed in this study failed to predict *Ms* genotypes, the identification of type of cytoplasm, associating marker-assisted selection could substantially reduce the prices of seed hybrid production, making them affordable to producers.

Conflict of interest

The authors declare that they have no conflict of interest to report.

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