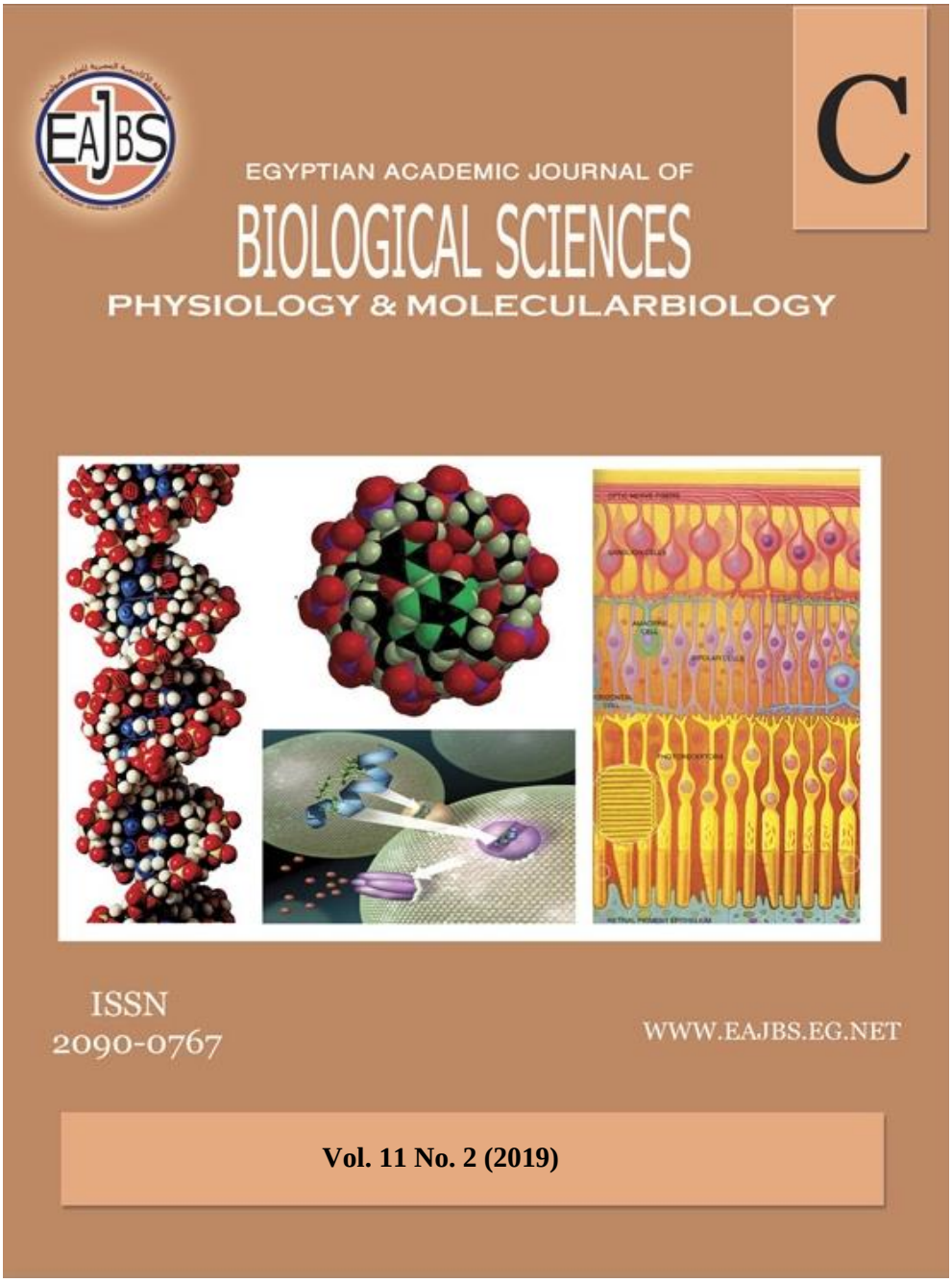

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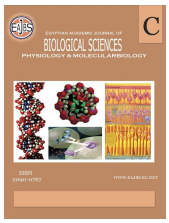

Genetic Variation among Five Egyptian Clover Cultivars Using Random Amplified Polymorphic DNA (RAPD) and Sequence-Related Amplified Polymorphism (SRAP) Molecular Markers

Sawsan S. Youssef [1], Sanaa A. Ibrahim[2], Amany M. Abdel Azim[2], Etr H. K. Khashaba[2], and Ahmed M. El-Sharkawy[1]

1-Department of Genetics, Faculty of Agriculture Cairo University, 12613, Giza- Egypt

2- Plant Protection Research Institute, Agriculture Research Center, Dokki, Egypt.

#E.Mail : ater_hk@yahoo.com

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ABSTRACT

To estimate the genetic variation among five Egyptian clover cultivars, Random amplified polymorphic DNA (RAPD) and sequence-related amplified polymorphism (SRAP) analyses were employed. Sixteen RAPD and six SRAP primers were used in the present study, a total of 34 and 12 bands showed 24.8 and 60% polymorphism percentage. The highest number of RAPD bands was recorded for primers OPE-B-07 (13 bands), followed by OPE-K-04 (12 bands), while the lowest was scored for OPE-B-05 (5 bands). For the SRAP Primers Me1-em2, Me3-em2 and Me3-em4 recorded the highest number of bands (four bands), followed by three bands for Me1-em1and Me3-em3, primer Me3-em1 recorded the lowest band number (two bands only). The dendrogram built based on combined data from RAPD and SRAP analyses clarify the genetic distances among the five Egyptian clover cultivars.

INTRODUCTION

Trifolium alexandrinum, commonly known as Berseem or Egyptian clover (2n=16) is an important winter forage legume in Egypt, Pakistan, Turkey, and the Mediterranean region, this is due to its high product yield and quality (Thalooth, *et al*., 2015), Egyptian clover is considered the "king of forages" because it has been the base of Egyptian crop-livestock farming (Khelil *et al*., 2015). It has enhanced livestock and crop production for centuries in situations where natural grazing land and feed resources are scarce. It is the main winter forage crop conquers about a third of the total cultivated area in winter, which reach more than 1.2 million ha in the Delta and the Nile Valley annually (Zayed *et al*., 2015).

Egyptian clover has two ecotypes of recuperation status after cutting, single cut mower which has not the ability to regenerate after the cutting and multicuts that regenerate from five to six times after cutting (Zayed *et al*., 2015). In the present study, two ecotypes were used, Fahl cultivar as single cut and Serw-1, Helaly, Sakha-4 and Gemmiza-1 as multicuts.

Many genetic markers such as biological markers (isozyme) (Peakall *et al*., 1995), morphological markers (Cheng *et al*., 2011), cytological markers (Bauchan *et al*., 2003) and molecular markers have been carried out to investigate the genetic

diversity. Molecular markers are redundant, highly polymorphic, stable, and allow organism identification in each developmental stage (Agarwal *et al*., 2008; Xie *et al*., 2009).

Random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), inter-simple sequence repeats (ISSR), have been widely used in molecular taxonomy, phylogeny, and genetic mapping (Gasser *et al*., 2006; Mutlu *et al*., 2008; Zhao *et al*., 2009). RAPD has been used for the evaluation of genetic variation (Welsh and McClelland, 1990).

The sequence-related amplified polymorphism (SRAP) system was developed by Li and Quiros (2001) to target overlapping coding and non-coding regions of the genome. It is avowed as a powerful marker system with targeting ORFs used in genetic map construction, cloning, and marker-assisted selection (Li *et al*., 2014). It has been used for studying population structure, genetic diversity and genetic linkage map of plants, such as cotton (Li *et al*., 2007), *Brassica napus* (Sun *et al*., 2007), cucumber (Zhang *et al*., 2010), Piper spp. (Jiang and Liu, 2011), alfalfa (Castonguay *et al*., 2010), and *Vicia faba* (Alghamdi *et al*., 2012), *Silene* (Bargish and Rahmani, 2016).

The objectives of the current study are to assign genetic diversity and variability between five Egyptian clover cultivars using RAPD and SRAP markers and use the combined data to construct a phylogenetic tree.

MATERIALS AND METHODS

Plant Material:

Five Egyptian clover cultivars (*Trifolium alexandrinum*) namely Serw-1, Helaly, Sakha-4, Gimmeza-1 and Fahel were provided by Field Crops Research Institute, Agricultural Research Center- Ministry of Agriculture- Egypt.

RAPD Analysis:

DNA Extraction: Genomic DNA was isolated from young leaves of greenhouse-grown plants using the CTAB method early described in Rogers and Bendich (1985). The quality and quantity of DNA were determined using agarose gel (0.5 %) electrophoresis.

PCR Analysis:

PCR reactions were performed in a total volume of 20 µl containing 10 ng DNA, 10 ng/µl 10 X buffer; 100 µM dNTPs; 50 µM $MgCl_2$, 1 µM of 16 arbitrary 10-mer primers (Operon Technology, Inc., Alameda, CA, USA), 0.5 units of Red Hot *Taq* polymerase (AB gene House, UK) and 10-X Taq polymerase buffer (AB gene House, UK). For DNA amplification Biometra thermal cycler (2720) was programmed as follows: 94 ° C for 5 min followed by 35 cycles 94° C for 1 min, 35 ° C for 1 min, 72 ° C for 1 min and 72 ° C for 7 min. The amplification products were analyzed by electrophoresis in 1 % agarose in TAE buffer, stained by ethidium bromide and photographed under UV light. The sequence of the tested primers is listed in Table 1.

SRAP Analysis:

PCR reactions were performed in a total volume of 20 µl containing 10 ng DNA, 10 ng/µl 10 X buffer; 100 µM dNTPs; 50 µM $MgCl_2$, 10 µM each of forward and reverse primers. PCR program including initial denaturation at 94°C for 5 min. The first five cycles were run at 94 °C, 1 min, 35°C, 1 min, and 72 °C, 1 min, for denaturing, annealing and extension, respectively. Then the annealing temperature was raised to 50 °C for another 35 cycles (Li and Quiros, 2001), the sequence of the tested primers is listed in Table 2.

Table 1: Names and sequences of RAPD primers used to assess the genetic variability among the five clover cultivars

Primer	sequence
OPE A-01	5′- CAGGCCCTTC -3′
OPE N-13	5′- AGCGTCACTC -3′
OPE A-07	5′- GAAACGGGTG -3′
OPE M-15	5′- GACCTACCAC -3′
OPE B-07	5′- GGTGACGCAG -3′
OPE B-17	5′- AGGGAACGAG -3′
OPE F-04	5′- GGTGATCAGG-3′
OPE B-05	5′- TGCGCCCTTC -3′
G OPE -12	5′- CAGCTCACGA -3′
OPE A-11	5′- CAATCGCCGT -3′
OPE K-04	5′- CCGCCCAAAC -3′
OPE M-10	5′- TCTGGCGCAC -3′
OPE N-10	5′- ACAACTGGGG -3′
OPE Q-14	5′- GGACGCTTCA -3′
OPE P-15	5′- GGAAGCCAAC -3′
OPE B-11	5′- GTAGACCCGT -3′

Table 2: Names and sequences of SRAP primers used to assess the genetic variability among the five clover cultivars

Name	Sequence	Name	Sequence
ME1	5´TGAGTCCAAACCGGATA-3´	EM1	5´-GACTGCGTACGAATTAAT-3´
ME3	5′-TGAGTCCAAACCGGAAT-3′	EM2	5´-GACTGCGTACGAATTTGC-3´
		EM3	5´-GACTGCGTACGAATTGAC-3´
		EM4	5´-GACTGCGTACGAATTTGA-3´
		EM5	5´-GACTGCGTACGAATTAAC-3

Band Scoring and Cluster Analysis:

The gel images of RAPD and SRAP were scanned using the Gel Doc 2000 Bio-Rad system and analyzed with Quantity One Software v 4.0.1 (Bio-Rad Laboratories, Hercules, Co. USA). To calculate the pairwise differences matrix and plot the dendrogram among Egyptian clover cultivars the systat ver. 7 computer program was used. Cluster analysis was based on similarity matrices obtained with the unweighted pair-group method (UPGMA) using the arithmetic average to estimate the dendrogram.

RESULTS AND DISCUSSION

RAPD technology is a powerful tool for estimating variance within and among species and populations (Gustine and Huff 1999). In plant genomes redundant phylogenetically conserved and individual-specific products are generated in RAPD reaction (Williams *et al*. 1990, Echt *et al*. 1992). It is assumed that primer-target sites are randomly distributed along the genome and cover both the conserved and hyper variable regions (Caetano-Anollés *et al*. 1991), herewith some RAPD-amplified fragments are polymorphic.

Five clover genotypes were subjected to RAPD analysis using sixteen different random primers. One hundred thirty-seven RAPD markers were gained and out of the 34 were polymorphic (24.8 %) and can be considered as useful RAPD markers for the five clover cultivars used. These amplified fragments ranged in size from approximately 200 to 1200 bp (Fig. 1 and Table 3). The highest number of RAPD bands was recorded for primers OPE-B-07 (13 bands), followed by OPE-K-04 (12 bands), while the lowest was registered for OPE-B-05 (5 bands).

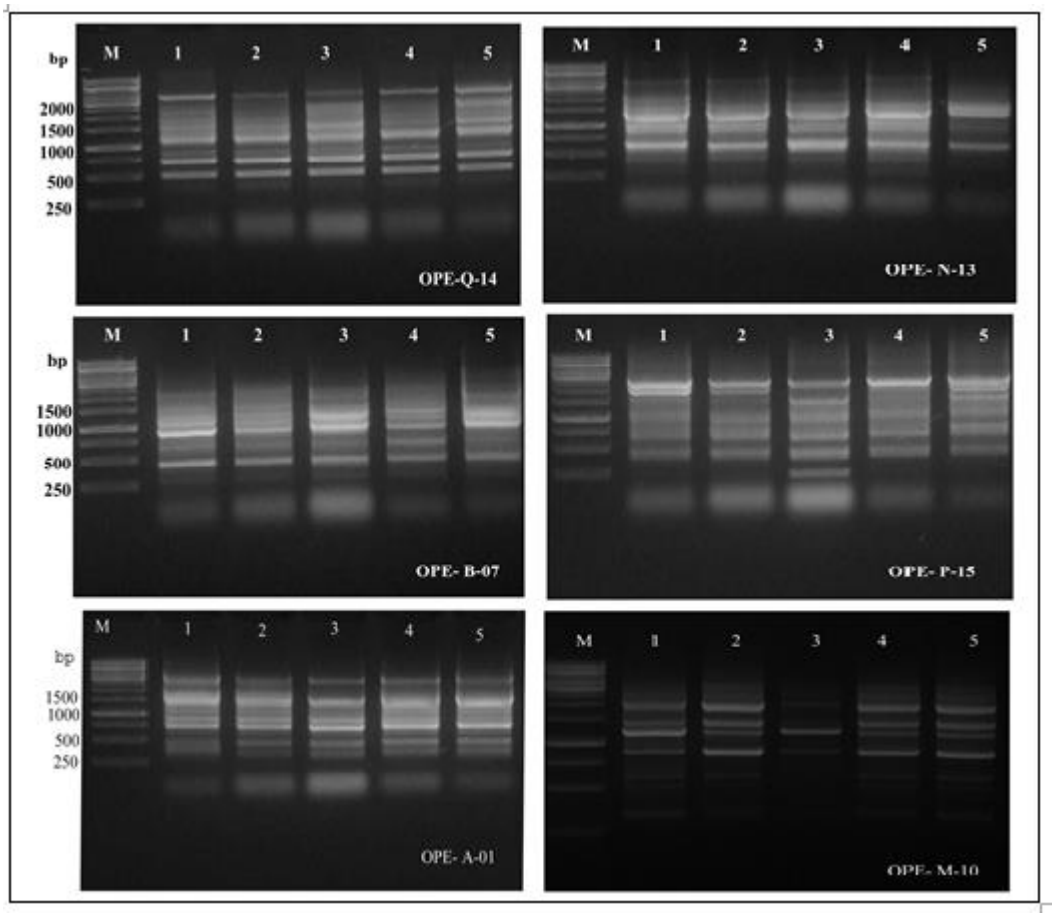

Fig. 1: Genetic polymorphism among clover cultivars as revealed by RAPD analysis. M: 1 kbp plus DNA ladder, 1-5: the clover cultivars Serw-1, Helaly, Sakha-4, Gimmeza-1 and Fahel, respectively.

Table 3 : Primers used in RAPD analysis and their number of bands

Primer	Total scorable band	Band size range	Polymorphic bands	Polymorphism%
OPE A-01	6	400-1500	0	0.0
OPE A-07	9	650-2500	3	33.3
OPE A-11	11	250-1950	6	54.5
OPE B-05	5	500-2500	0	0.0
OPE B-07	13	250-2000	4	30.7
OPE B-11	7	200-1900	1	14.2
OPE B-17	10	300-2500	4	40.0
OPE F-04	9	500-2000	1	11.1
OPE G-12	8	300-1500	2	25.0
OPE k-04	12	400-2500	1	8.3
OPE M-10	8	350-3000	3	37.5
OPE M-15	7	400-2000	2	28.5
OPE N-10	8	250-2000	4	50.0
OPE N13	7	600-2500	1	14.2
OPE P-15	8	250-2500	2	25.0
OPE Q-14	9	450-2500	0	0.0
Total	137		34	24.8

The genotype-specific RAPD markers for the different clover cultivars used are listed in Table 4. The highest number of RAPD specific markers was scored for Fahel (2 markers), while one marker for the other cultivars. These results disagree with Tarrad and Zayed (2009) who using primers for RAPD analysis, revealing the highest similarity of 0.85 between the two varieties Sakha-4 and Gemmiza-1, while the lowest similarity (0.53) was observed between Giza-6 and Helaly. Also, the present results agree with Dolanská and Čurn (2004) who tested Polymorphism of RAPD analysis of various species of *Trifolium* (*T. pratense*, *T. repens*, *T. hybridum*, *T. incarnatum*). Among 60 tested primers, 6 primers gave polymorphic amplification patterns, so they conclude that RAPD analysis showed high interspecies polymorphism. RAPD analysis was performed for better evaluation of the extent and patterns of distribution of RAPD diversity in *T. repens* as outcrossing species. Also the results agree with Zhang *et al*., (2010) who used three RAPD primers analysis for 14 white clover populations that produced polymorphism (96.67%).

SRAP is identified as a new powerful molecular marker method toward its reproducibility, low cost, and little knowledge that is needed for target sequences in a species (Li and Quiros, 2001). Actually, SRAP has been used to assess genetic diversity in many species such as *Medicago sativa* Linn. (Castonguay *et al.*, 2010), *Triticum dicoccoides* (Dong *et al*., 2010), *Panicum virgatum* (Huang *et al*., 2011).

A total of six different SRAP primer pairs were used to amplify polymorphisms using five genotypes from Egyptian clover, twelve bands out of twenty SRAP markers obtained were polymorphic (60%) (Fig. 2,and Table 5). Primers Me1-em2, Me3-em2 and Me3-em4 were recorded the highest number of SRAP bands, followed by three bands for Me1-em1and Me3-em3, while primer Me3-em1 recorded the lowest band number only two bands.

Table 4: Specific RAPD markers for the five clover genotypes

Genotypes	Markers	Total markers
Serw-1	OPE-G-12,850	1
Helaly	OPE-N-10,350	1
Sakha-4	OPE-P-15,500	1
Gimmeza-1	OPE-N-13,950	1
Fahel	OPE-B-17,2500,OPE-K-04,600	2

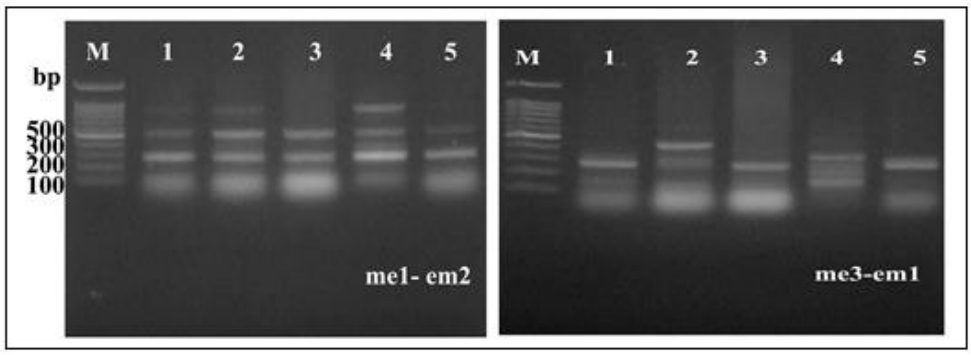

Fig. 2. Genetic polymorphism among Egyptian clover cultivars as revealed by SRAP analysis. M: 100 bp DNA ladder, 1-5: the clover cultivars, Serw-1, Helaly, Gimmeza-1, Sakha-4 and Fahel, respectively.

Table 5. Total number of scorable bands, polymorphism % and a band size of SRAP markers obtained by six primers

Primer	Total scorable bands	Polymorphic bands	Polymorphism (%)	Band size range
Me3-em2	4	2	50	200-550
Me3-em3	3	1	33	100-700
Me3-em1	2	1	50	300-400
Me3-em4	4	4	100	150-450
Me1-em1	3	2	66	190-320
Me1-em2	4	2	50	300-900
Total	20	12	60	

Table 6: Specific SRAP markers for the five clover genotypes

Genotypes	**Markers Total**	**marker**
Serw-1	---------	----------
Helaly	--------	----------
Gimmeza-1	----------	----------
Sakha-4	Me3-em2 (350), Me3-em3(150), Me1-em1(320), Me1-em2 (400)	4
Fahel	Me3-em3 (100)	1

The genotype-specific SRAP markers, the highest number of SRAP specific markers was scored for Sakha-4 (4 markers), while one marker for Fahel is listed in Table 6. Our results agree with Li *et al*. (2010) who suggested that there was genetic diversity among white clover and red clover, which indicate that SRAP technology could be used effectively for clover species and intraspecific genetic relationships, identification and analysis of genetic diversity. Also, the present results matched with Guindon *et al.*, 2016, as their results indicated that (SRAP) represents an efficient tool for genetic analysis of pea (*Pisum sativum*) and could be used to develop a linkage map of a pea. Moreover, since these markers target coding regions of the genome, they can potentially identify markers with inherent biological significance.

Combined Analysis:

The combined analysis with cluster dendrogram revealed two major clusters (Fig. 3). The first cluster has the cultivar Sakha-4, the second cluster is subdivided into two branches the first one has Fahel and the second branch is divided into two branches the first one has Gimmeza-1 and the second one has two branches the first one has Serw-1 and the second branch has Helaly.

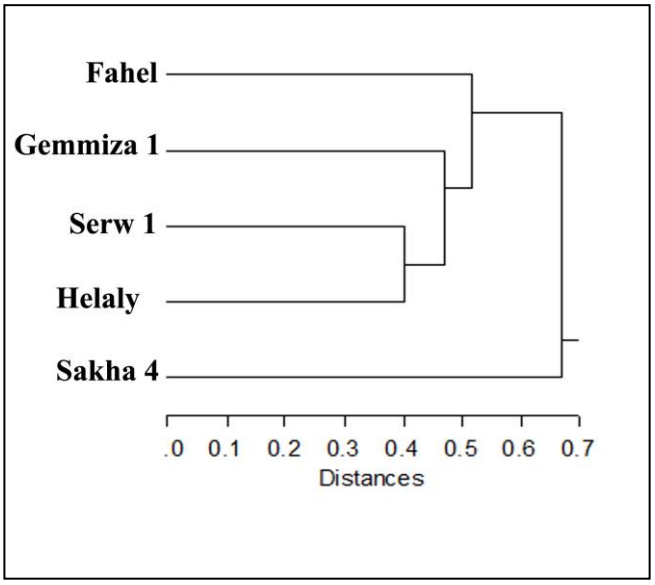

Fig. 3. Dendrogram for the five Egyptian clover cultivars constructed from RAPD and SRAP data analysis using unweighted pair group Arithmetic Average similarity matrix computed according to Dice coefficients

Conclusion:

The present study indicates that RAPD and SRAP can be successfully used as molecular markers for estimating the genetic variability and the genetic relationships among five Egyptian clover cultivars. The genotype-specific molecular markers can be used for providing new information for future breeding programs.

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