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Determination of High Oleic Type and Broomrape Resistant Sunflower Hybrids By DNA Markers

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ABSTRACT

Sunflower is one of the most significant oilseed crops in the world. Sunflower lines with high oleic content have high oxidative stability of its oil. *Orobanche cumana* Wallr., which is a holoparasitic plant infecting the sunflower roots, is one of the limiting factors for sunflower production especially in Eastern Europe and Turkey. Screening for high oleic acid content and broomrape resistant sunflower genotypes by standard methods is time consuming and expensive. Molecular markers associated with high oleic acid trait or broomrape resistance are useful and rapid tool in order to facilitate sunflower breeding program. In this study, two markers were chosen; SSR marker and HO PCR specific fragment for genotyping the 250 sunflower inbred lines for high oleic acid content. According to our results, high oleic acid containing hybrids expressed a specific SSR band at 246 bp, and also HO PCR specific fragment at 870 bp. Also, determination of broomrape resistance for these 250 sunflower inbred lines was done by SCAR and SSR markers. The analyzed SCAR markers (RTS28, RTS29, RTS40 and RTS41) and SSR markers (ORS1036 and ORS1040) were linked to the *Or*5 gene [provides resistance to all five races (A-E)]. According to SCAR and SSR analysis, the studied sunflower inbred lines were resistant to races A-E. These results allowed identification of sunflower hybrids for high oleic acid traits and broomrape resistance by DNA markers.

Keywords: *helianthus annuus*, marker-assisted selection, SCAR, SSR, oleic acid content, *orobanche cumana*

Introduction

Sunflower is one of the most significant oil crops in the world. Sunflower oil contains high level of unsaturated fatty acids (88%); linoleic acid (48-74%), oleic acid (14-40%) and also saturated fatty acids; palmitic acid (4-9%) and stearic acid (1-7%) (Singchai *et al*., 2013; Nagarathna *et al*., 2011). High oleic sunflower production accelerated the consumption for healthy frying oil, and also non-food uses in recent years. Particularly, non-food applications require oleic acid content that is stable and higher than 90% (Vannozzi 2006; Ferfuia *et al*., 2012). Increasing oleic acid content has become one of the significant goals to improve quality of vegetable oil (Lacombe *et al*., 2004). In order to achieve this aim, Sunflower lines and hybrids which have high oleic acid content in their seeds have been obtained by selection programs from HO (High oleic) Pervenets mutant by chemical mutagenesis (Soldatov 1976). The mean content of oleic acid of the seeds from Pervenet population is higher than 65% in comparison to the normal LO varieties which is about 20% (Berville *et al*., 2009). Afterwards, new cultivars with changed fatty acid content were

developed by different researchers (Osorio *et al*., 1995; Velasco *et al*., 2008; Leon *et al*., 2013; Alberio *et al*., 2016; Cvejic *et al*., 2016).

Although the determination of high oleic acid content with analyzing seed oil is easy way, but marker assisted selection studies with tightly linked markers to high oleic trait could speed up breeding process (Dehmer and Friedt 1998). The phenotypic determination (fatty acid analysis) does not allow rapid and early determination of HO genotypes and also cannot provide differentiation of homozygotes from heterozygotes for the mutation. The use of molecular markers has become popular tool for the genetic and breeding studies and it is rapid, cheaper and simple when suitable markers were developed (Varshney *et al*., 2005).Various sunflower lines and hybrids have been studied to distinguish HO genotypes from LO genotypes by different researchers and molecular marker types (Dehmer and Friedt 1998; Schuppert *et al*., 2006; Nagarathna *et al*., 2011; Grandon *et al*., 2012; Singchai *et al*., 2013; Premnath *et al*., 2016; Dimitrijevic *et al*., 2017).

Orobanche cumana Wallr. (broomrape) is a parasitic plant that can lead to advanced losses in yield, in agricultural lands cultivating sunflower, depending on the sunflower varieties and the level of contamination. The numerous and small sized seeds of broomrape causes contamination in sunflower fields quickly. Depending on attack intensity to the field and development stage of sunflower during infection, decrease in sunflower yield could change 5 to 100% (Miladinovic *et al*., 2014). Broomrape is reported by the main sunflower producer countries in the world like Russia, Ukraine, Romania, Bulgaria, Turkey, and Spain, as well as Serbia, Hungary, Moldova, Greece, Tunisia, Israel, Iran, Kazakhstan, China, Mongolia, and Australia (Molinero-Ruiz *et al*., 2009; Pacureanu-Joita *et al*., 2012; Amri *et al*., 2012; Kaya 2014; Miladinovic *et al*., 2014; Marinkovic *et al*., 2014). Eight races (A-H) of *O. cumana* have been reported in Turkey (Kaya 2014).

There are several methods of broomrape control with different level of efficiency (Louarn *et al*., 2016). Sunflower breeders developed *Orobanche*-resistant hybrids from some wild *Helianthus* genus. Wild sunflowers have resistant genes and these genes incorporated into sunflower inbred lines by classical methods. However, broomrape race composition changes rapidly. Breeding resistant sunflower lines to this type of parasitic weed is an urgent and important task for breeders. To accelerate the process of sunflower breeding for resistance to *Orobanche*, collaboration between the breeders from public institutions, universities and private companies should be needed. Since the most reliable method of screening broomrape resistance is the use of molecular markers, various marker types have been used for the selection of resistant genotypes (Imerovski *et al*., 2013; Perez-Vich *et al*., 2013).The objectives of this study were (1) characterization of sunflower hybrids with high oleic acid content and broomrape resistance by DNA markers and (2) test the effectiveness of different marker types for selection of high oleic and broomrape resistant genotypes.

Materials and Methods

For the purpose of screening on high oleic acid and broomrape resistant genotypes, 250 sunflower F_3 individuals were used. Leaves were collected from the field-grown plants, labeled with individual number and stored at -80ºC until further use. i-genomic Plant DNA Extraction Mini Kit was used for DNA isolation from all samples. The quality of DNA was checked by 1% agarose gel electrophoresis, stained with RedSafe Nucleic Acid Staining Solution and visualized by Gel Imaging System Vilber Lourmat Quantum St5. Each of the extracted DNA was diluted as 50 ng per µl and was stored at -20 ºC for later uses.

Genotyping of high oleic (HO) and low oleic (LO) sunflower individuals was performed with two primer pairs; SSR (N1-1F/N1-1R) and HO PCR specific fragment (N1-3F/N2-1R) that were chosen from the patent obtained by Berville *et al*., (2009) (Table 1). Amplified PCR products were controlled by 2% agarose gel electrophoresis and visualized by Gel Imaging System Vilber Lourmat Quantum St5 (Figure 1 and Figure 2). SSR fragments were scored in a Beckman Coulter GenomeLab™ GeXP Genetic Analysis System and fragment sizes were calculated by its Software (Figure 3).Genotyping of resistant (R) and susceptible (S) sunflower individuals was performed with four SCAR and two SSR markers (Lu *et al*., 1999; Lu *et al*., 2000; Tang *et al*., 2003; Iuoras *et al*., 2004; Imerovski *et al*., 2012; Imerovski *et al*., 2013) (Table 2). Amplified PCR products were controlled by 2% agarose gel electrophoresis and visualized by Gel Imaging System Vilber Lourmat Quantum ST5 (Figure 4). SSR fragments were scored in a AATI Fragment Analyzer™ and fragment sizes were calculated by PROSize 2.0 Data Analysis Software (Figure 5 and Figure 6).

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Results and Discussion

The Pervenets mutation was labelled by the polymorphism of the SSR locus located on the Δ12-desaturase gene intron (Berville *et al*., 2009). Berville *et al.,* (2009) reported that 16 SSR motives (16 TTA repeats) associated with the Pervenets mutation and high oleic genotypes have this type of Δ12HOS allele. In our study, according to DNA fragment analysis for SSR locus 246/246 Homozygous, 243/243 Homozygous and 243/246 Heterozygous genotypes were identified (Figure 3). DNA sequence analysis was carried out to confirm repeat motifs corresponding to HO genotypes and 246/246 Homozygous genotypes were evaluated as HO genotypes. In order to confirm HO sunflower genotypes, all studied individuals were screened with HO PCR specific fragment (Berville *et al*., 2009). The Pervenets mutation was labelled by the 870 bp PCR fragment across the 5' insertion point by HO PCR specific fragment (N1-3F/N2-1R) (Berville *et al*., 2009). The results showed that high oleic containing sunflower individuals (HO genotypes) showed a specific band at about 870 bp length which was absent in low oleic (LO) genotypes (Figure 2). Nagarathna *et al*. (2011) studied around 350 sunflower genotypes including RHA-lines, cms lines, inbreds and germplasm lines to screen high oleic genotypes with HO PCR specific fragment and also the determination of fatty acids (linoleic acid, oleic acid, palmitic acid and stearic acid) was performed by gas chromatography. Nagarathna *et al*., (2011) reported that the genotypes having a specific band showed high oleic content and GC results supported the marker analysis results. Singchai *et al*., (2013) studied the developed lines those were used as the representative of low and high oleic acid sunflowers for genotyping by screening thirty seven SSR primers including 34 primers of ORS set, 2 primers of HA set and HO PCR specific primer to identify DNA samples from two lines (high and low oleic acid contents). Grandon *et al*., (2012) studied F_2 mapping population that was obtained from a cross between R285 and R023 with 386 SSR markers for genotyping oleic acid trait and they also determined fatty acid composition by GC. They reported that 82 of analyzed marker will be used for genotyping and selection of HO genotypes. Singchai *et al*., (2013) reported that out of the 37 SSR primers screened for polymorphism, 10 SSR primers including HO PCR specific primer generated differentiating bands between the high and low oleic lines. With the 10 SSR markers they studied, Singchai *et al*., (2013) reported that it is possible to identify the genetic markers linked to high oleic acid trait which may be useful for further sunflower breeding program. Dimitrijevic *et al*., (2017) studied parental lines (high oleic and low oleic), F_1 and F_2 individuals with molecular markers for FAD2-1D sequence. They reported that studied 2 markers enabled the discrimination of genotypes, while one was monomorphic.

The analyzed sunflower hybrids were also controlled by molecular markers in order to determine broomrape resistance. The analyzed SCAR markers (RTS28, RTS29, RTS40 and RTS41) and SSR markers (ORS1036 and ORS1040) were linked to the *Or5* gene [provides resistance to all five races (A-E)]. According to SCAR and SSR analysis, the studied sunflower hybrids were resistant to races A-E. Although the studied 250 sunflower hybrids were divided into three groups according to *Orobanche* inoculation result (*Or6*); Resistant, Tolerant and Susceptible, we have not found the studied SCAR or SSR markers associated with *Or6* in the analyzed sunflower individuals. Imerovski *et al*., (2013) carried out study in order to determine broomrape resistance of 20 cultivated sunflower inbred lines using SSR and RAPD markers. They reported that ORS1036 (240 bp) and ORS1114 (246 bp) primer had unique fragments in lines with *Or6* gene. Previously developed markers for *Or* genes were not always effective for identification of *Or6* genes in different germplasm. Further studies should be carried out to validate different marker types for *Or6* in different genetic backgrounds. After MAS analysis, 26 high oleic type and broomrape resistant sunflower hybrids were selected for further breeding studies. As a conclusion PCR analysis with molecular markers enabling to discriminate HO genotypes or broomrape resistance was easy and reliable method. Consequently, these molecular markers may be used in selection programs to identify genotypes carrying the Pervenets mutation and to discriminate broomrape resistant genotypes. However, these markers need validation in different sunflower populations, hybrids or lines in order to confirm their capability to identify high oleic acid contents, and also to investigate presence of *Orobanche* resistant genes.

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Table 1. Characteristics of markers used to analyze HO and LO sunflower genotypes

No	Primer type	Primer name	Primer sequences (5' → 3')
1	SSR	N1-1F	TTGGAGTTCGGTTTATTTAT
		N1-1R	TTAGTAAACGAGCCTGAAC
2	HO PCR specific prime	N1-3F	GAGAAGAGGGAGGTGTGAAG
		N2-1R	AGCGGTTATGGTGAGGTCAG

Table 2. Characteristics of markers used to analyze resistant (R) and susceptible (S) sunflower individuals

No	Primer type	Primer name	Primer sequences (5' → 3')
1	SCAR	RTS28-F	AGT AGA CGG GCA AAG CGA AAG GAT
		RTS28-R	AGT AGA CGG GTT GAA TAT GTT GAA
2	SCAR	RTS29-F	GCTTCCCCTTAATGATCCGGAAGA
		RTS29-R	GCTTCCCCTTGGCTAGAAGATGAA
3	SCAR	RTS40-F	TCCACCGAGCTACCAGTTCCGGAG
		RTS40-R	TCCACCGAGCGAGCATATTCCGAG
4	SCAR	RTS41-F	TCGTGTTGCTGATCGGAAAGGAAC
		RTS41-R	TCGTGTTGCTCAACAGTGGAGAAT
5	SSR	ORS1036-F	CCCTTTCACTTCCTATTTTCTATTCA
		ORS1036-R	CTAAGAGGGGTCGGTATGATTTC
6	SSR	ORS1040-F	CTGCTGATCGTTTCTTGGATAGA
		ORS1040-R	TGCTAATCCTTCTAATCAACTTCCAC

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Figure 1. Amplified fragments with SSR primer for sunflower individuals

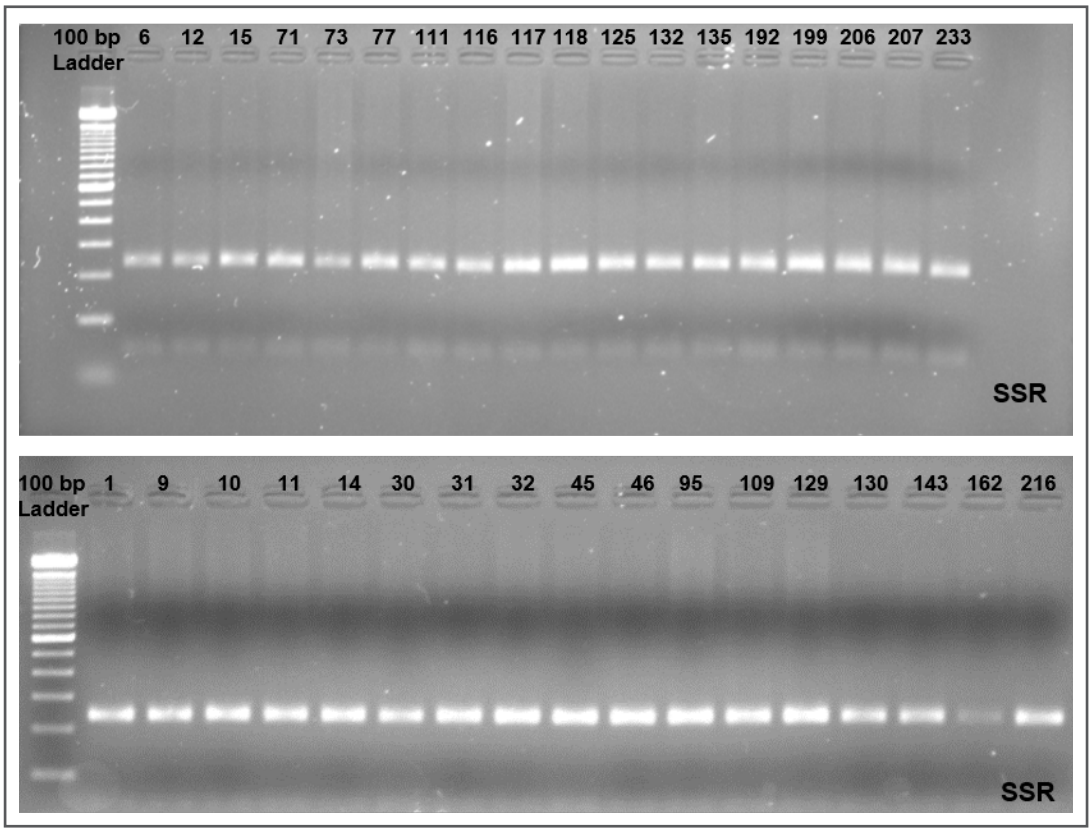

Figure 2. PCR amplification of HO and LO genotypes with HO PCR specific fragment

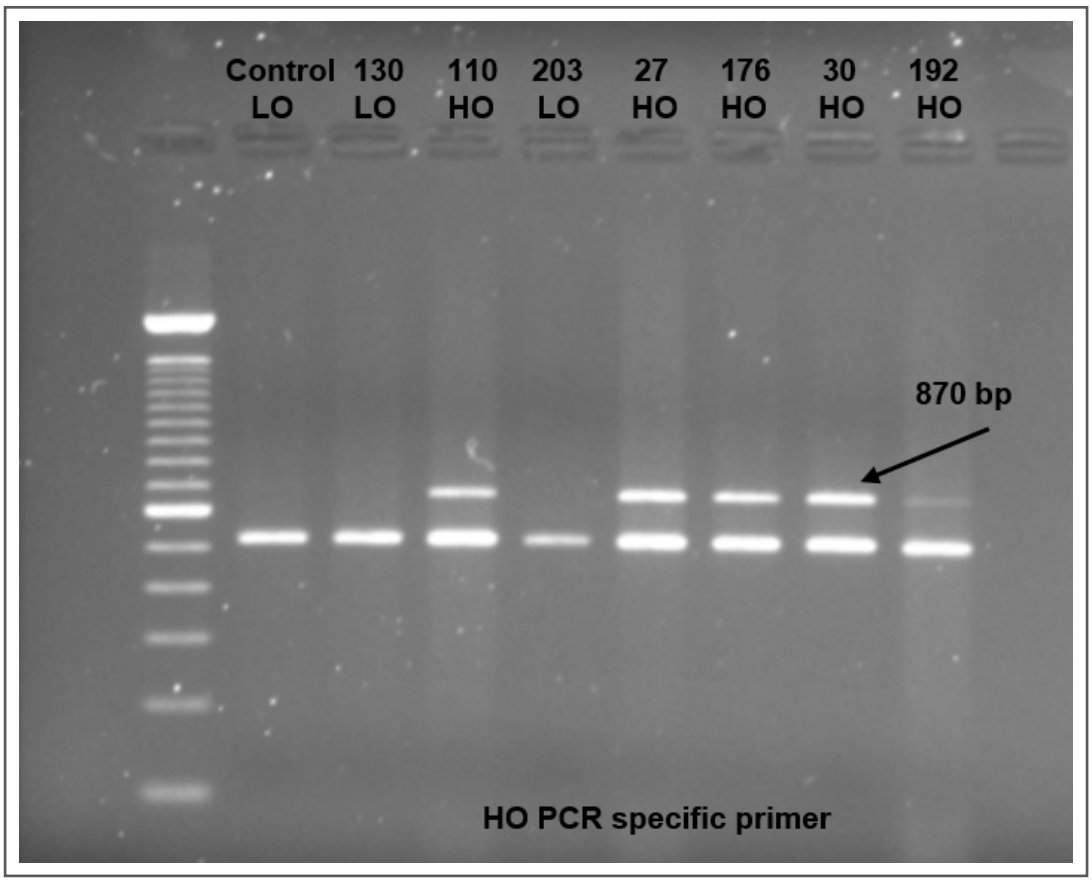

Figure 3. DNA fragment analyses results for SSR (N1-1F/N1-1R) primer

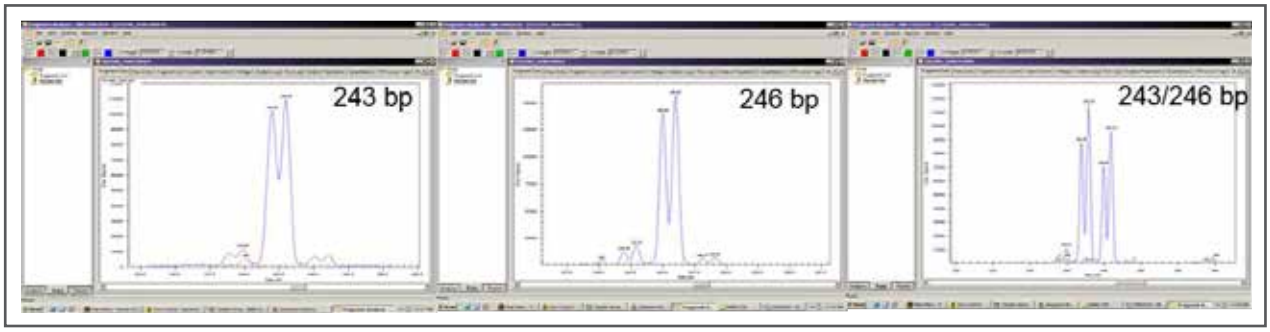

Figure 4. Amplified fragments with SCAR and SSR primers for sunflower individuals

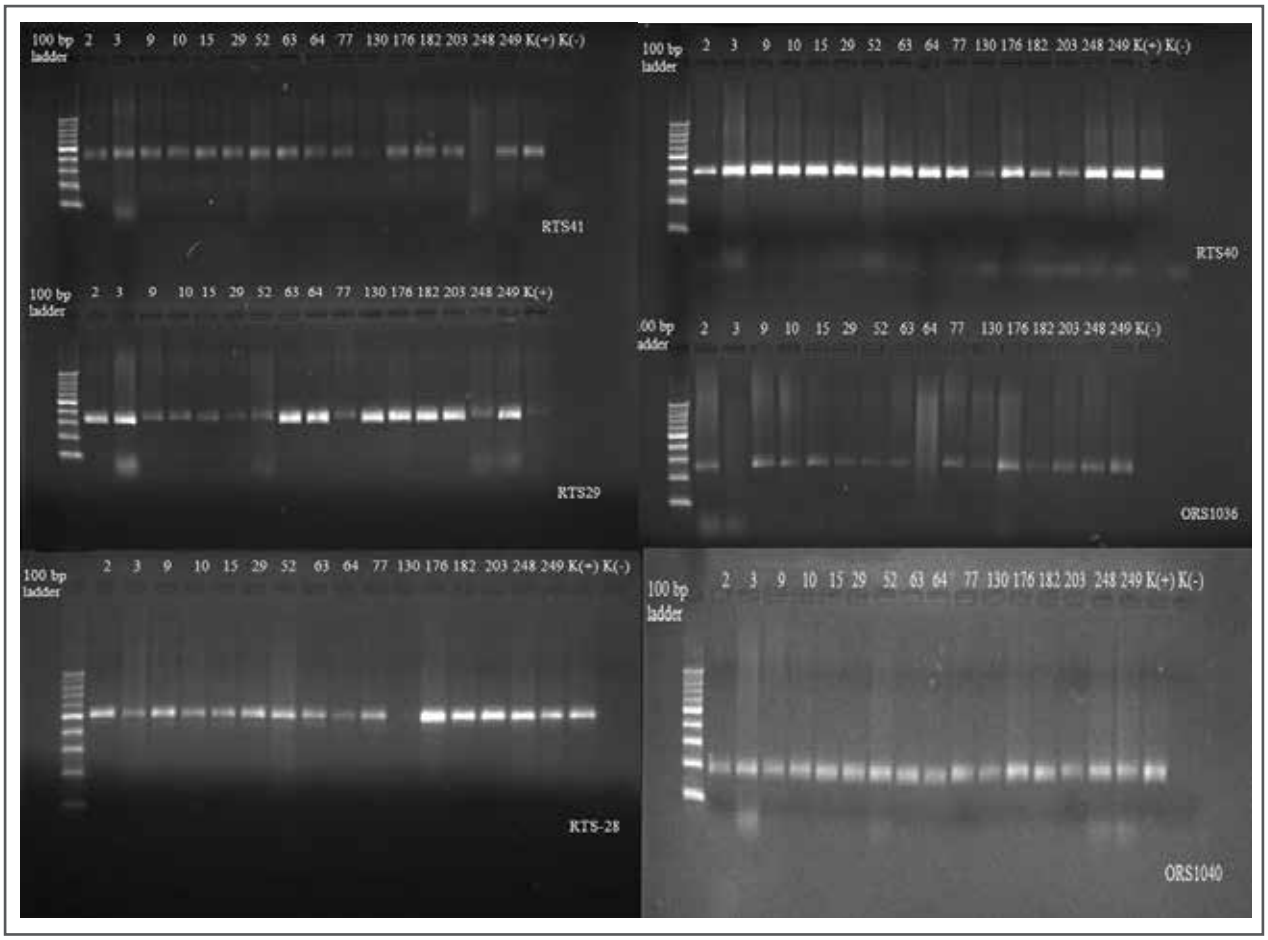

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Figure 5. DNA fragment analyses results for ORS1036 primer

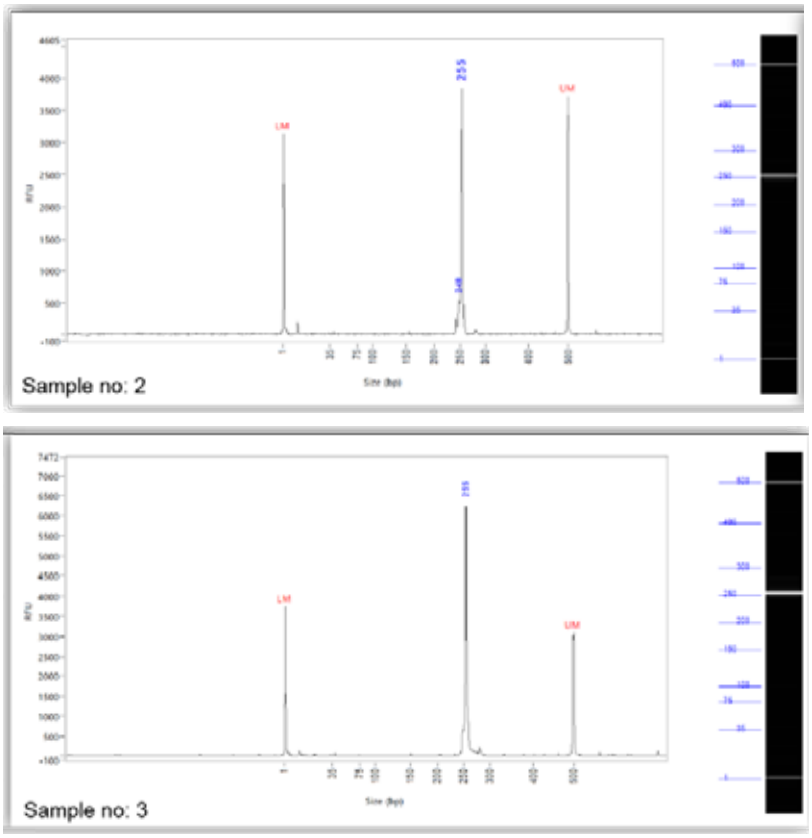
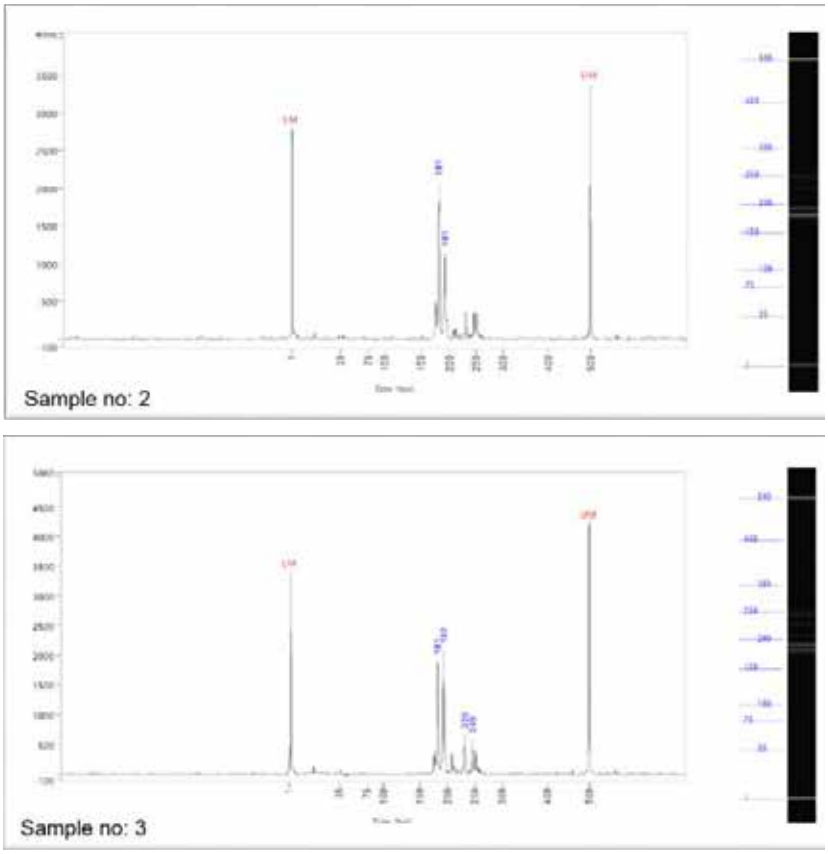


Figure 6. DNA fragment analyses results for ORS1040 primer



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