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Genomic Evaluation of Sunflower Broomrape (*Orobanche Cumana*) Germplasm by KASP Assay

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Abstract: *Orobanche cumana* Wallr. is a holoparasitic plant for only sunflower, hence it is called as sunflower broomrape. Yield loss created by *O. cumana* which is generally 50 % can reach to 100 %. In this study, it was planned to perform molecular characterization of *O. cumana* germplasm as nine locations of Thrace region obtained from Trakya Agricultural Research Institute by using Single Nucleotide Polymorphism (SNP) markers, widely used in plant breeding programs, in Competitive Allele Specific PCR (KASP) assay which is a fluorescent tagged allele specific PCR method based, economic, reliable and easily repeatable genotyping technology. Databases and literature were scanned to spot variations on *O. cumana* genome which is not known clearly. So far, four SSR (Simple Sequence Repeat) marker (*Ocum-197*, *Ocum-006*, *Ocum-023* and *Ocum-151*) regions showing polymorphic pattern were used for searching possible SNPs. Primer pairs were designed for amplification of the regions possibly having SNPs and PCR amplifications with these primer pairs were performed and 1 candidate deletion was detected on the amplicon which was amplified by *Ocum-197* SSR marker. Following, the deletion was converted to KASP primers and KASP assay was performed. The deletion marker, *Del-197*, has grouped the samples from nine locations in the resulting allelic discrimination plot and infestation was performed according to this grouping, As a conclusion, *Del-197* is considered as a selective marker for the ability to rapidly assay allelic variation at DNA markers for *O. cumana* populations that have effects on infestation results were evaluated as races, F, G, H and I in Thrace region.

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Introduction

Parasitic weeds are one of the biggest factors, not for only sunflower, for whole agricultural production in Turkey and in the world (Mohamed *et al*., 2006). *Orobanche cumana* Wallr. as a holoparasitic weed which cannot photosynthesize itself and is completely host plant dependent is regarded as the most important biotic threat for sunflower production by whole countries, especially in Spain, Central and Eastern Europian countries, Turkey, Iran, China, Israel and Kazakhstan, except the countries in North and South America (Mohamed *et al*., 2006; Škorić and Pacureanu, 2010; Molinero-Ruiz *et al*., 2015).

Broomrape infestations are one of the biggest threats for sunflower production. It can spread the area very quickly due to the help of 50,000 to 500,000 of little seeds produced by an individual *O. cumana* plant. Sizes of these seeds are long from 360 µm to 500 µm and wide from 160 µm to 250 µm, their weights are from 1.0 to 2.5 µg (Plaza *et al*., 2004; Škorić and Pacureanu, 2010; Gevezova *et al*., 2012), thus they can disperse by lots of agents such as insects and wind, even attaching on sunflower seeds. Another important property of these seeds is their stability for 15–20 years on soil and germination is occur only in the presence of a host plant (Škorić, 2012).

O. cumana Wallr. was first seen as a parasite on sunflower in Russia in the first half of nineteenth century (Antonova, 2014). Until present time, 8 races of *O. cumana* were reported as capital letters from A to H. Races A and B have appeared different regions of Russia, appearance of race B was also reported in Moldova and Ukraine in 1930s (Duca, 2014; Kaya, 2014; Miladinović *et al*., 2014). Resistance to race B was improved via sunflower hybrids; but this resistance was broken by race C that appeared in Moldova in 1970s (Duca, 2014). During this term, some problems happened like that studies to improving sunflower hybrids which are resistant to a new race resulted in susceptibility to the old races of *O. cumana*, in Russia (Antonova, 2014). In recent past, new and more dangerous races of *O. cumana* which are named as E, F, G and H were identified in southern regions of Russia (Antonova *et al*., 2013). Race F is quite widespread in Thrace region wherein more than half of the sunflower production of Turkey is carried out, race G was also identified in several different locations of the same region. Furthermore, the broomrape was started to appear in sunflower fields of Anatolian region too, in recent years; but the races are not identified yet (Kaya *et al*., 2004; Kaya *et al*., 2012; Molinero-Ruiz *et al*., 2014; Molinero-Ruiz *et al*., 2015).

O. cumana still cannot be controlled and nowadays it is accounted as one of the biggest threats for sunflower production in Europe, Asia and some regions of Africa. Moreover, sunflower broomrape is spreading to new regions day by day and its virulence is increasing with the same speed (Škorić, 2012; Antonova, 2014; Fernández-Martinez *et al.*, 2015). Numerous methods have been and still being studied to explore ways of controlling this parasite. Fumigation, soil solarization, hand weeding, herbicides, crop rotation, insects and fungus feeding on the weed are some examples for these methods. Each of these kind of chemical, physical, biological methods has different disadvantages such as high cost, being environmentally hazardous, yield loss, repetition requirement etc. albeit weed control (Joel, 2000; Aly, 2007). Besides these methods, the best for the sunflower broomrape control is to develop genetically resistant sunflower cultivars in terms of efficiency, reliability and affordability (Molinero-Ruiz *et al.*, 2009; Molinero-Ruiz *et al.*, 2015; Louarn *et al.*, 2016).

About *O. cumana*, it is known that diploid chromosome number is 2n = 38 and the genome size is estimated as 1.42Gb (Piednoël *et al.*, 2012); but no reference transcriptome data or completed genome sequence is known yet (Molinero-Ruiz *et al.*, 2015). Eight races of *O. cumana* have been detected up to date. The genetic identification of *O. cumana* races has not been revealed yet, knowledge on broomrape is even truly insufficient. *O. cumana* race identification is very important for all sunflower breeding countries while it is known that sunflower broomrapes overcome the resistance mechanisms of sunflower, thus a new race of *O. cumana* appears in nearly each twenty years (Antonova, 2014; Kaya *et al.*, 2004; Kaya, 2014). Besides the use of differential sunflower lines, identification of *O. cumana* races by genetic studies can provide great convenience for breeders and scientists working on this topic.

Single nucleotide polymorphism (SNP) is a point mutation as insertion deletion or any change on genome sequences to be used as one of the genetic markers to study genetic diversity among species, populations and individuals and constructing genetic linkage maps. They are co-dominant markers and found abundantly on a genome. Besides, their usage in identification of lots of plant species and lines is also another advantage of SNP markers (Sobrino *et al.*, 2005; Khlestkina and Salina, 2006; Semagn *et al.*, 2006; Ben-Ari and Lavi, 2012).

Competitive Allele Specific PCR (KASP) is one of the uniplex SNP genotyping platforms (Semagn *et al.*, 2014), a homogeneous, fluorescence-based genotyping technology, developed by KBioscience and eventually evolving into a global benchmark technology. The technology is based on allele-specific DNA sequence amplification and fluorescence resonance energy transfer (FRET) cassettes for signal generation (Kumpatla *et al.*, 2012). KASP is an economic,

reliable, easily repeatable, fluorescence tagged allele specific PCR method based genotyping technique which uses two fluorescent dyes for both alleles. It is a very special system for SNP genotyping and is used easily for point mutations with success. In agricultural plant biotechnology field, KASP is a breeder-friendly system (Tan *et al.*, 2017). In this study, determination of SNP markers which can separate *O. cumana* races by using KASP method.

This technology has been successfully applied to the study of humans, animals and plants (Nijman *et al.*, 2008; Bauer *et al.*, 2009; Cortés *et al.*, 2011; Rosso *et al.*, 2011). But, race differentiation of *O. cumana* has not been studied by using KASP, previously. Thereby, in this study, it is aimed to perform searching and evaluation of genomic variations in *Orobanche cumana* germplasm by KASP assay.

Materials and methods

Biological material

Sunflower broomrape (*Orobanche cumana* Wallr.) samples were collected from 9 locations as Havsa (Edirne), Şahinköyü (Malkara, Tekirdağ), Gözsüz Köyü (Malkara, Tekirdağ), Yeniköy (Edirne), İskenderköy (Edirne), Ürünlü (Kırklareli), Budakdoğanca (Edirne), Ballıhoca (Muratlı, Tekirdağ) and Adana by Trakya Agricultural Research Institute, Republic of Turkey Ministry of Agriculture and Livestock General Directorate of Agricultural Research and Policy.

DNA extraction and SSR assay

Genomic DNA extraction was performed by using i-genomic Plant DNA Etraction Mini Kit (iNtRON) according to the manufacturer's instruction manual. 4 highly polymorphic SSR markers (*Ocum-006*, *–023*, *–151* and *–197*) having PIC values higher than 0.6 for Eastern Europe populations previously reported in literature (Pineda-Martos *et al.*, 2013) were used. PCR was performed with the following concentrations of components in the reaction mixture: 1 X PCR Buffer, 3.75 mM $MgCl_2$, 0.2 mM dNTPs, 1.8 µM of each forward and reverse primers, 0.025 U Taq Polymerase and 0.16 ng/µL extracted DNA. Reaction volume was 25 µL. PCR was performed in Veriti Thermocycler (Applied Biosystems) with the cycling conditions: 94 °C – 3 min; 40 cycles of 94 °C – 1 min, 62 °C – 1 min, 72 °C – 1 min; 72 °C – 10 min.

Purification of PCR products was realized by ChargeSwitch-Pro PCR Clean-up Purification Kit (ThermoFisher Scientific) according to the manufacturer's instructions. DNA sequences of purified PCR products were obtained by using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI Prism 310 Genetic Analyzer Sequencing System (Applied Biosystems). Alignment of these sequences was performed by Geneious 10.0.9.

SNP and KASP assay

Ocum-197 SSR marker was converted to KASP primers (Table 1). KASP assay was performed with the following amounts of components in the reaction mixture: 10 µL of 2 X KASP Master Mix, 0.28 µL of KASP Assay Mix and 10 µL of 2 ng/µL extracted DNA (20 ng) as reaction volume became 10.28 µL. KASP Assay Mix contains the allele specific two forward primers and one reverse primer. KASP Master Mix includes FRET cassettes which contains fluorescent dyed molecules (FAM and HEX dyes) besides standard PCR components such as reaction buffer, Taq polymerase enzyme, dNTPs etc. Master Mix which contain FRET casettes should be prevented from the lights because of the unwanted reaction between FRET casettes labeled dyes. KASP assay was performed in StepOnePlus Real-Time PCR Systems Thermal Cycling Block (Applied Biosystems). The thermocycling conditions used for KASP assay were as following: 30 °C – 1 min; 94 °C – 15 min; 10 cycles of 89.2 °C – 20 s, 61 °C – 1 min; 26 cycles of 94 °C – 20 s, 57 °C – 1 min; 30 °C – 1 min.

Table 1: Features of Del-197 primers converted from *Ocum-197* SSR marker.

Del-197 Primers	Primer Sequences (5'–3')	Allele	CG %
Primer FAM	GCAGATCCTCTCTCTCTCTCT	[GAGAGAGAGAGA]	50
Primer HEX	CAGATCCTCTCTCTCTCTCG	[-]	54.5
Common Primer	GACGGCATCATCAATCAATGTAACATGAA		37.9
Amplifying Sequence	GAAAAAGTGACAACTTAATGTAAACCGTATTGCAGAGTAAAAGAGAC		
	GGCATCATCAATCAATGTAACATGAAAATAAAATCGAGAGAGAGAGG		
	A**[GAGAGAGAGAGA/-]***GGATCTGCACTCTGCAGTACCTAGGTGCCT		

* Six GA repeats are deleted from some alleles.

Infestation

The infestation testing was performed under the artificial inoculation, using broomrape seeds collected from nine locations (Havsa, Şahinköyü, Gözsüz Köyü,

Yeniköy, İskenderköy, Ürünlü, Budakdoğanca, Ballıhoca and Adana). In the green house, have been used nine broomrape populations and sunflower genotypes, as differentials for the races (B, C, D, E, F, G, H, I) and for the new races too.

Differential sunflower lines and races they indicate are given in Table 2. All differential sunflower lines and *O. cumana* seeds collected from different regions were provided by TARI.

Table 2: Differential sunflower lines provided by TARI and the races they indicate.

Differential Lines	Race
LC1003A-2	E
16AX25HO/08	G
LC1002-A	D
261-A-A0/07	E-F
1128-A-AO/08	B-C
P64G46	H
13-TR-009	I
LC1093-A	F
9942-A	B
1053-A-A0/08	C

10 differential sunflower lines were planted and inoculated with 1–2 g of *O. cumana* seeds from nine locations and pots were placed into microclimate. Microclimate was adjusted to the temperature of 28 °C and 14/10 hours (day/night) photoperiod. Plants were grown 35 days in the microclimate. 35 days old plants were ripped off from the pots to control the *Orobanche* nodules. Infected *Orobanche* nodules were counted on per plant's roots. Following formulas of frequency (F), intensity (I) and attacking rate (AR) were used to evaluate the infestation as;

F: % of plant with *Orobanche*;

$$F = \frac{\text{The plant number infested by } \textit{Orobanche}}{\text{Total plants in the set}} \text{x}100$$

I: the number of *Orobanche* in one infested plant;

$$I = \frac{\text{Total } \textit{Orobanche}}{\text{Total plants infested by } \textit{Orobanche} \text{ in the set}}$$

AR: the number of *Orobanche* in one plant in the set;

$$AR = \frac{F \text{ x } I}{100}$$

The plants having 0–10 % frequency and 0–1 AR values were accepted as resistant (Vranceanu *et al*., 1980). The plants which had 10–20 % frequency was accepted as tolerant in 2002 evaluations.

Results and discussion

PCR products of *O. cumana* genomic DNA samples with *Ocum-006, Ocum-023, Ocum-151* and *Ocum-197* SSR markers were sequenced and aligned for each marker to observe variations through locations (Figure 1).

No difference as an SNP, deletion or insertion was found in samples amplified with markers *Ocum-006, Ocum-023* and *Ocum-151*. But for *Ocum-197*, one deletion marker was found and this SSR marker was converted to KASP primers (Table 2).

KASP assay

Resulting allelic discrimination plot of KASP assay with *Del-197* is given in the Figure 2.

Each two axes of allelic discrimination plot refer to fluorescent signal emitted by FAM (x-axis) and HEX (y-axis) FRET cassettes indicating allele 1 and allele 2, respectively. Sample locations are seen as gathered into four groups. According to these grouping, İskenderköy, Şahinköyü, Budakdoğanca, Yeniköy and Ürünlü samples were found as homozygous allele 1 as tagged with FAM and containing the sequence of [GAGAGAGAGAGA] for both alleles. Adana and Gözsüz Köyü samples were found as homozygous allele 2 as tagged with HEX and not containing the sequence of [GAGAGAGAGAGA] for both alleles. Ballıhoca has shown a heterozygous pattern as emitting FAM and HEX fluorescent signals for each allele, one containing [GAGAGAGAGAGA] sequence and the other one not containing the repeat sequence. Insufficient flourescent signal was detected from the sample location Havsa. *Del-197* might not have work properly on this sample close to negative controls.

Khera *et al*. (2013), used 96 SNPs to develop KASP assays on 94 genotypes of peanut. They validated 90 of converted KASP assay markers and observed polymorphism with 73 of them. Pariasca-Tanaka *et al*. (2015), used KASP assay in the rice with the aim of developing a high-throughput marker system to specifically detect *Oryza glaberrima*, which is a rice species growing in Africa and tolerant to abiotic stresses like mineral deficiency etc., introgressions in an

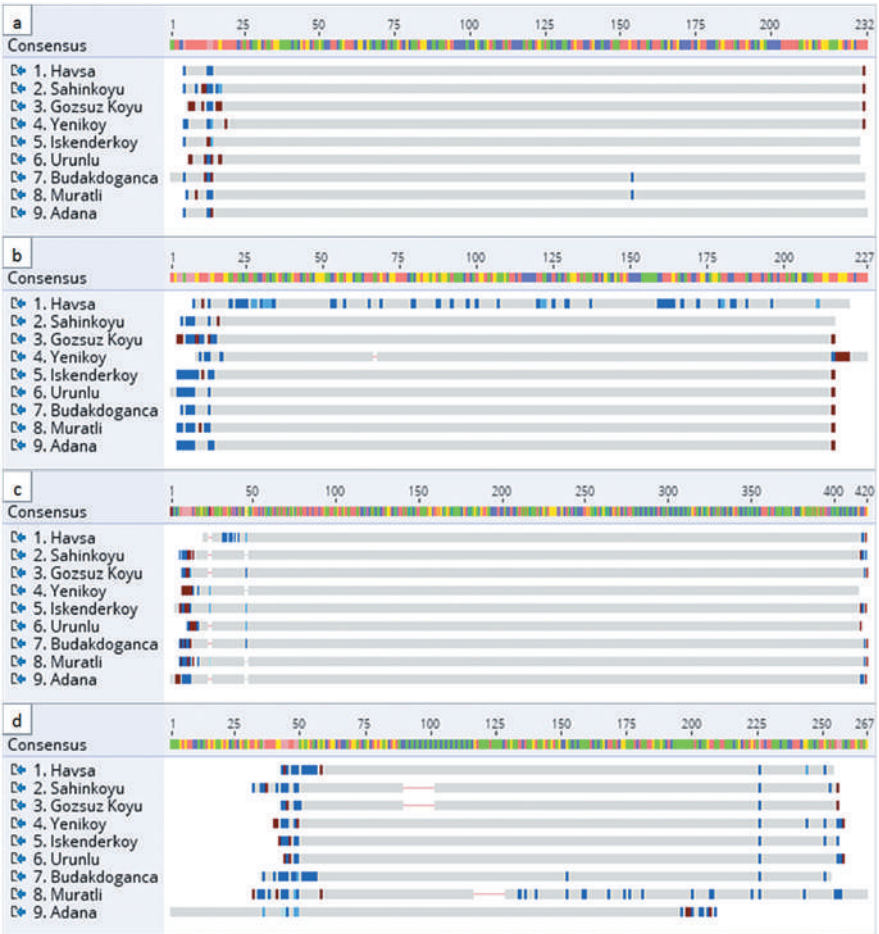

Figure 1: Alignments of PCR production sequences for; **a)** *Ocum-006* (PIC: 0,740; Product Length: 90 bp), **b)** *Ocum-023* (PIC: 0,641; Product Length: 157 bp), **c)** *Ocum-151* (PIC: 0,840; Product Length: 186 bp), **d)** *Ocum-197* (PIC: 0,671; Product Length: 95 bp) SSR markers. Suitable variation was observed only in the alignment of the PCR products with *Ocum-197* SSR marker **(d)** as deletion of 6 GA repeats for 2 samples amongst 9.

Oryza sativa background and 1,540 polymorphic SNPs between *O. glaberrima* versus *O. sativa* were identified with the use of KASP assays. Shi *et al.* (2015), aimed to establish robust marker assays for high-throughput discrimination of soybean cyst nematode (SCN) resistance and to differentiate the sources of resistance in their study by using more than twenty-seven soybean lines which are known phenotypically. After sequencing and SNP identifications KASP

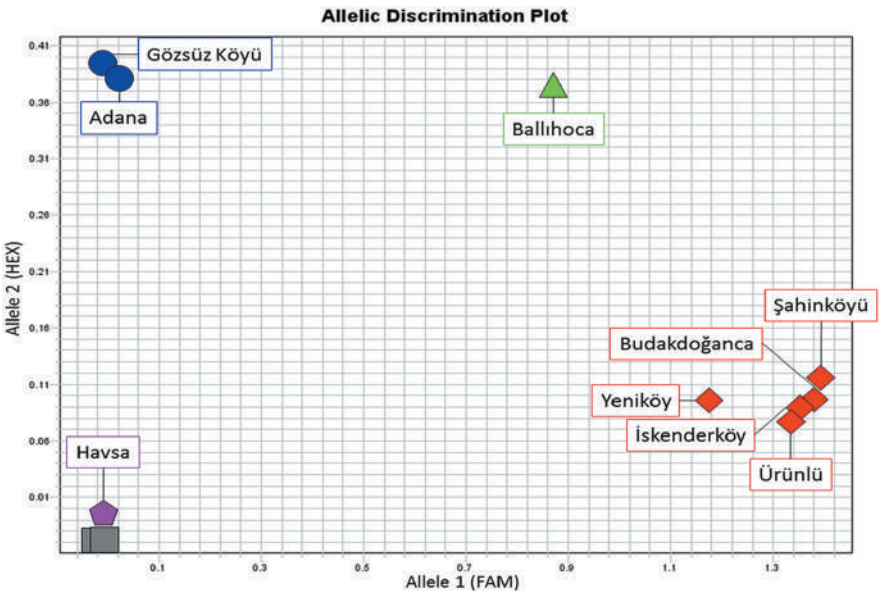

Figure 2: Resulting allelic discrimination plot of KASP assay performed with *Del-197* primers; ◆ as homozygous allele 1/1, both alleles contain 6 GA repeats and emit FAM fluorescent signal; ● as homozygous allele 2/2, both alleles do not contain 6 GA repeats, emit HEX fluorescent signal; ▲ as heterozygous allele1/2, each allele emit one of the FAM and HEX fluorescent signal; ⬟ as not emitting any signal, probably KASP primers could not amplify this sample; ■ as negative control does not contain any DNA sample.

assays were designed for three SNPs which would provide genotype information for the discrimination of SCN resistance and distinguish the sources for most germplasm lines. These three markers were validated for high-throughput marker-assisted selection of SCN resistant plants. Gascuel *et al.* (2016), determined 54 *Pl. halstedii* genes which were assumed as effector genes by scanning transcriptomic data. 22 KASP markers which are highly polymorphic and assumed as functional were tested and genotyped on 35 isolates as 14 reference pathotype and 21 additional isolates collected from different regions of France. 8 of 22 KASP markers were designed for the effectors and eight multi-isolate pathotypes were classified the into six groups by and the eight multi-isolate *Pl. halstedii* pathotypes were classified into six groups by using a combination of eight KASP markers as a set of molecular diagnosis markers for pathotype determination based on effectors. Oueslati *et al.* (2016) studied 46 of 54 clade levels of Aurantioideae subfamily to demonstrate that a molecular taxonomic key, 166

diagnostic SNPs were determined and 40 of these diagnostic SNPs were analyzed in silico. 27 of these markers were converted to KASP markers as diagnostic of 24 clades and tested by genotyping 108 accessions of the Aurantioideae subfamily. As a result of the study, these markers displayed a very fine resolution and high rate of transferability in the Aurantioideae subfamily. They validated the concept that with well-established clades, diagnostic SNPs which can be selected and efficiently converted into KASP markers allowing cost-effective, highly efficient cladistic analysis in large collections at subfamily level. Tan *et al*. (2017), performed a study using KASP with 6 SNP primers linked to streak mosaic virus resistance gene *Wsm2* on a set of 214 F5:7 RILs of wheat derived from a cross of CO960293–2 x TAM 111 (CT111) and resulted as SNP primers differentiated resistant and susceptible wheat genotypes successfully by KASP assay and genetic map of *Wsm2* gene was reconstructed.

There are more studies using KASP assay on a wide range of plants, but there is no previous study on sunflower broomrape using KASP system. In this study, race evaluation of *O. cumana* from 9 locations in Thrace region was performed by using KASP assay and broomrape infestation test. These 9 locations were divided into 4 groups by *Del-197* KASP marker as the result of allelic discrimination plot.

Infestation

The infestation testing was performed under the artificial inoculation, using broomrape seeds collected from nine locations. In the green house, have been used nine broomrape populations and sunflower genotypes, as differentials for the races (B, C, D, E, F, G, H, I) and for the new races too.

Infestation was carried out by using 10 differential lines provided from Fundelae Institute of Romania as LC1003A-2, 16AX25HO/08, LC1002-A, 261-A-A0/07, 1128-A-AO/08, P64G46, 13-TR-009, LC1093-A, 9942-A and 1053-A-A0/08. Beside these differential sets, there are also other differential lines such as sunflower inbred line NR5 differentiate the races A and E (Molinero-Ruiz *et al*., 2006); line L86 carries resistance to race F and susceptibility to races E and G; P96 is resistant to races E and F but susceptible to race G (Molinero-Ruiz *et al*., 2008). At the end of the 35 days infestation process, each grown differential sunflower plant was taken out to observe *O. cumana* roots, seed germination and nodule formation.

Plants were grown in the microclimate for 35 days. And then, plants were ripped off from the pots to control the *Orobanche* nodules. According to F, I and AR calculations, infestation results of 10 differential sets of sunflower with *O. cumana* races were obtained (Table 3).

Table 3: Infestation results of 10 differential sets of sunflower with different *O. cumana* populations.

Differential Lines		Location								
	Race	**İskenderköy**	**Ballıhoca**	**Budakdoğanca**	**Ürünlü**	**Şahinköy**	**Havsa**	**Adana**	**Gözsüz Köyü**	**Yeniköy**
LC1003A-2	E	2	2	10	5	6	23/19	10	3	10
16AX25HO/08	G	3/2	3	10	1	5	28/16	10	4/1	10
LC1002-A	D	½	2	3	6	0	10	8	0	8
261-A-A0/07	E-F	1/0	0	10	4	10	35/32	0/0	1/0	10
1128-A-AO/08	B-C	2	3	7	4	6	3	5	5	10
P64G46	H	0/0	0	0	0	0–2	0/0	0	0	0
13-TR-009	I	0/0	0	0	0	0	0/0	0	0/0	1
LC1093-A	F	0/1	0	10	6	10	12	7	0	10
9942-A	B	2/0	3	10	10	10	0/16	10	2/1	10
1053-A-A0/08	C	6/1	2	4	7	8	18/23	3	1/0	
Results		F	F	G	G	H	G, H, I	G	F, G	G
Final Result		**F**	**F**	**H**	**G**	**I**	**H**	**H**	**F**	**H**

The infestation of 10 differential sets of sunflower with *O. cumana* races revealed that İskenderköy, Ballıhoca and Gözsüz Köyü locations correspond to race F, Ürünlü location corresponds to race G, Budakdoğanca, Havsa, Adana and Yeniköy locations correspond to race H and Şahinköyü location correspond to race I.

Conclusions

In this study, searching and evaluation of genomic variations in *Orobanche cumana* germplasm by KASP assay based on allelic variation was aimed. For this purpose, after the genomic DNA isolation, DNA sequences were amplified with four highly polymorphic SSR markers selected from Pineda-Martos et al.'s study (2013), and PCR products were aligned to search for any variation and one of these markers showed a six GA deletion in two of the samples of nine locations. *Del-197* KASP marker converted from SSR marker used in this study is considered as a selective marker for providing an overview of allelic variation of *O. cumana* races. But only as one marker, *Del-197* is not enough to discriminate the race populations according to allelic variation directly, even though it really shortens the time and gives clues about geographic origins of the samples individually. In this case, the resulting allelic discrimination plot supported the results of the infestation. According to the allelic discrimination plot of the KASP assay with *Del-197, O. cumana* samples from 9 locations of Thrace region were gathered into 4 groups. These groups were evaluated in accordance with the infestation experiment. *O. cumana* samples from İskenderköy, Ballıhoca and Gözsüz Köyü were evaluated as race F, Ürünlü location were evaluated as race G, Budakdoğanca, Havsa, Adana and Yeniköy locations correspond to race H and Şahinköyü locations showed a different pattern in the infestation results, thus these locations were considered as race I (a new race), since it is known that new *O. cumana* races appear in nearly each twenty year.

By this study, new technique was applied to a species which is known scarcely in the manner of genetics. Race differentiation is a very important topic in sunflower breeding. Information about the genetics and evolution of *O. cumana* races year by year is very decisive for development of broomrape resistant sunflower cultivars. KASP assay provides very quick results when standard PCR techniques which require also check the results with electrophoresis and traditional breeding methods by selecting good trait having individuals as parents which take years to obtain the desired trait, are taken into account. Besides, traditional breeding methods are not guaranteed the resistant cultivar

homozygous or heterozygous by only observing phenotypic characters, but KASP assays are dispersive for heterozygosity. Likewise, this method which uses a standard mix for each assay is very economic when economic loss over time was considered during the breeding and harvesting processes after the selection of the right parent plants.

This study was an innovation for sunflower breeding. Only nine locations and only one deletion primer converted from four selected polymorphic SSR markers among 79 were used in this study. Locations which samples were collected, can be diversified for both Thrace and near Adana regions in our country and other countries due to wide spreading of broomrape through the world. With the current technology, after KASP assay, infestation step using a differential set is essential. Concordantly, the futurity of this study should be the search of more markers like *Del-197* by scanning literature more, trying more markers. And trending to next generation sequencing technologies would provide scanning of whole genome by preparing genomic or cDNA libraries of *O. cumana* and finding of any variations like more SNPs or InDels and also more information about *O. cumana*. Combinations of the KASP assay results with newly found markers would be used to will be to better understand the interaction between the sunflower and *O. cumana* and discriminate the exact races. Time consuming infestation step would be unrequired, work load of breeders would be reduced and all processes would be performed in laboratory conditions without any environmental effects.

Our results show that the diversity of broomrape populations needs to be detailed, and new geographical infected areas must be monitored.

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