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Original article

The Study on Determination of Fatty Acid Contents of Some Wild Sunflower Species (Helianthus Spp.)

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Abstract

Wild sunflower species have many sources of genes resistant to abiotic and biotic stresses, broomrape parasite as well as having higher quality traits. The transferring of these useful genes to the cultivated sunflower is so important issue to obtain a continuous resistance and then develop better quality and yielding cultivars in sunflower. The molecular methods in the plant breeding studies shortens the breeding cycles by providing an accurate and effective selection as well as saving time. The study was conducted in wild sunflower garden which set up via a project in previous years in Trakya University Edirne, Turkey. Fatty acid compositions of all wild species materials were determined in Trakya University laboratory for the first time in the world largely via Gas Chromatography (GC). The molecular analysis was performed in the lab to identify individuals with high oleic acid trait containing the homozygous oleic gene with 4 molecular markers (3 INDEL markers F4-R1, F4-R2 and F4-R3) and an SSR marker N1-3F) / (N2-1R HO). Based on GC analysis; no species with a high oleic rate (80% or more oleic acid) was found. However, among the examined wild species, three of them were found having mid oleic acid content (between 60 - 80% oleic acid). Among these species, Helianthus annuus species ranked first with the highest oleic acid content of 77.46%, followed by H. hirsutus with 69.71% and H. floridanus with 67.19%. On the other hand; Helianthus californicus, Helianthus exilis, Helianthus giganteus, Helianthus gracilentus, Helianthus grosseseratus, Helianthus laciniatus, Helianthus mollis, Helianthus neglectus, Helianthus petiolaris, Helianthus petiolaris subsp. petiolaris were determined as high oleic based on molecular 3 INDEL markers. However, these were not determined higher oleic acid content based on GC analysis. In conclusion; there was no genotype containing high oleic acid among the wild species based on GC analysis but high oleic species found in marker analysis; therefore, selectivity of the markers may not be accurate or new markers need to be used for the analysis of further researches.

Key words: Wild sunflower, Fatty Acid, Oleic acid, Linoleic acid, Stearic acid, Palmitic acid, Molecular markers.

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INTRODUCTION

Sunflower grows in all Europe largely as an oil crop as well as in other part of the world. Sunflower prefers mainly because of having 40-50 % higher oil content, growing possibility and higher adaptation capability in different climatic conditions, higher mechanization use, easy production without market problem as well. Furthermore, it is the most preferable vegetable oil in the Europe. Other than oil crops, it uses for confectionery, cakes, birdseed as well as ornamental purposes and fodder and silage crops (Kaya, 2016).

Sunflower (n = 17) belongs to *Helianthus* genus (*Asteraceae*) consisting of 52 species and 19 subspecies, 14 annual & 38 perennials. Its origin is America, so it is a weed in rotation in there. The Spanish travelers collected sunflower seeds from North America in the 1850s then they were firstly grown as ornamentals plant in botanical gardens in Spain, lately delivered in all Europe. North American Indians used sunflower flour to make bread and other foods as well as the durable and ornamental in their lands. Sunflower became an oil crop with great breeding efforts of Russian scientists such as Pustovoit, etc. Sunflower enlarged their areas in Black Sea & other part of the world lately in 1940-1970s after this discovery (Evci *et al*, 2009; Kaya, 2007a, b).

Wild *Helianthus* has huge morphological and genetic variations and so valuable genes for several traits including high seed and oil yield, resistance to abiotic and biotic stresses as well as oil quality. Oil quality in not only sunflower both also in all oil crops is determined by fatty acid contents in the seeds. These are five major fatty acids play important roles in vegetable oil quality as oleic (C18:1), linoleic (C18:2), linolenic (C18:3) stearic (C18:0) and palmitic acids (C16:0). Oleic, linoleic and linolenic ones are unsaturated acids and desired for higher quality oil for consumers but satured acid ones stearic & palmitic acids desired as much as less in vegetable oils. Oleic, linoleic and linolenic are called as Omega 9, 6 and 3 are preferred ones in diets (Kaya *et al*., 2008; Rauf *et al.*; 2017; Askin *et al*,. 2022).

Normal sunflower oil contains an average of 70% linoleic, 20% oleic, 6% palmitic and 4% stearic acid. Frying oils and margarines produced from sunflower oils with medium and high oleic acid are healthier because they have lower amounts of trans fatty acids. Moreover, these types of oils are harder to spoil and have longer shelf lives (Rauf *et al*.; 2017; Askın and Kaya, 2020).

A diverse relationship between oleic & linoleic acids meaning that one increases other decrease. The high oleic acid (HO) trait was first obtained in sunflower by chemical mutation in Russia by Soldatov (1976). In normal sunflower varieties, oleic acid is converted to linoleic acid by the desaturase enzyme, whereas in high oleic varieties, especially immediately after flowering, this enzyme is blocked, resulting in very little linoleic acid production. Beside of the genetic factors, fatty acid content in sunflower is also influenced highly by environmental factors too. Temperature changes

especially night temperatures during the grain filling and stress conditions especially water stresses influence mainly oleic contents of sunflower hybrids (Kaya *et al*., 2009).

Early studies have shown that the oleic acid content defined by the *Ol* genes in sunflower is determined by a single dominant *Ol* gene, with an additional recessive gene (ml) (Miller *et al.*, 1987). Although later studies mentioned three complementary allelic genes *Ol1, Ol2* and *Ol3* (Fernandez - Martinez *et al*., 1989; Demurin *et al.*, 2000; Miller *et al*., 2004), in recent years there are many studies emphasizing that high oleic acid content is determined by a codominant gene called *Ol* and also revealing that this gene is in a partially dominant structure (Pacureanu-Joita *et al*., 2000; Fernandez-Martinez *et al*., 2009). In the market, there are varieties obtained using genetic material with high oleic acid gene from Pervenent hybrids (Demurin and Borisenko, 2011; Evci *et al.*, 2016).

Although the higher content of linoleic acid in sunflower is more suitable for cooking, higher oleic acid ones are more suitable for frying due to higher heat stability at higher frying temperatures. Due to the extensive use of high oleic sunflower oil (over 80 %) for frying, as well as offering the healthier and high quality oil for the consumers and also it is so suitable as energy crop for biodiesel production (Askin *et al*., 2022). However, oleic type sunflower demand has been gradually increasing in recent years in US, France, Australia, Spain, Argentina, Hungary, Germany, etc. but it was just started in Bulgaria, Romania, Turkey Ukraine and others (Kaya *et al*., 2008).

Several sunflower lines and hybrids have been studied for oleic type to differentiate higher oleic from lower genotypes by many researchers by molecular markers (Nagarathna *et al*., 2011; Grandon *et al*., 2012; Singchai *et al*., 2013; Dimitrijevic *et al*., 2017; Bilgen *et al*. 2018a, b). For instance, Nagarathna *et al*. (2011) considered around 350 genotypes including CMS and RHA lines, inbred and germplasm lines to screen on higher oleic acids and she mentioned that HO content genotyping lines gave specific band (at 800 to 900 bp) at PCR specific fragment (N1-3F/N2-1R), and then they confirmed it by fatty acid content utilizing by GC. On the other hand, the polymorphism of the SSR locus located on Δ12-desaturase gene intron displayed in the Pervenet mutated hybrids. Based on SSR fragment analysis, alleles and genotypes determined for SSR (N1-1F/N1-1R) locus in sunflower identify locus 246/246 homozygous, 249/249 homozygous and 246/249 heterozygous genotypes (Lacombe, 2004; Berville *et al*., 2009).

Çolak et al. (2019) carried out a project in Edirne, Turkey with 4 molecular markers as 3 INDEL markers - F4-R1, F4- R2 and F4-R3 and a SSR marker - N1-3F) / (N2-1R HO) shown successfully used to identify individuals with high oleic acid trait. Therefore, these 4 markers were used in the study to determine the genotyping of high oleic (HO) and low oleic (LO) sunflower individuals and to identify wild sunflower species with high oleic ratio, 3 different INDEL markers (F4-R1, F4-R2, F4-R3) to select high oleic character.

Since high oleic in sunflower is an embryo-dependent trait, in the selection to be made for high oleic in breeding studies, it is definitely determined by fatty acid analyses to be performed on the seeds obtained after harvest. This situation leads to the fact that all processes from planting to harvesting are carried out on individuals that are included in sunflower breeding programs and are selected according to phenotype and other characteristics but are not high oleic, and this causes unnecessary labor and waste. If the high oleic (HO) acid characteristic is known at the beginning of the plant's development, such material waste and time loss will not be made and effective and accurate selection can be carried out by selecting only on individuals with high oleic acid. In this context, selection with the help of molecular markers becomes much more important in embryo-related characters such as fatty acids (Evci *et al*., 2009; Evci *et al*., 2016; Kaya, 2007a, b; Kaya, 2016).

MATERIAL AND METHOD

The study was conducted in wild sunflower garden in Trakya University, Edirne, Turkey setting op based on TUBITAK Bilateral Project with Turkey & Bulgaria as Sofia Genetics Institute. Wild sunflower species with more accessions from each species were obtained from USDA Genetic Stocks, Iowa, Ames, USA. Before planting, their seed dormancy was broken firstly & then they were planted them in 2020. The study is covered of screening of wild sunflower species for fatty acid content determination.

Fatty acid contents (Oleic, Linoleic, Stearic, and Palmitic) of sunflower genotypes were determined by Agilent 6850 Gas Chromatography (GC) in Trakya University Food Science Department Lab with HT 88 type colon (Figure 1). A minimum amount of 1 g of sunflower seeds taken from wild sunflower genetic materials was crushed and treated with N-Heptane solution. The seeds of wild sunflower, which had a lot of genetic material, were used with a cold press machine, and the seeds with a small amount were crushed in a mortar and their oils were extracted. Because the seeds of the majority of the existing genetic material consist of very skinny and small seeds, the desired high amount of oil could not be obtained. 2 drops of the extracted oil were placed in the bottle, then 10 ml n-heptane was added to the bottle and then 0.5 ml 2 mol methanol CoH was added. Then, it was vortexed for 2-3 minutes and left for at least 1 hour. Since there was precipitation in the stored tubes, the tubes were opened slowly (without shaking), 2 ml of the solution was withdrawn from the top before it reached the bottom and transferred to 2 ml vials. It was then placed in GC and measured (Figure 2).

Figure 1. The cold press machine to get crude oil from sunflower seeds and obtaining samples for GC analysis

Figure 2. Fatty acid content analysis by Gas Chromatography (GC) in Trakya University

PCR analysis were conducted based on the protocols mentioned the conducted study by Çolak et al. (2019) (Table 1 and 2). SSR fragment analysis were performed to determine high oleic genetic materials via 3 molecular markers as 3 INDEL markers as F4-R1, R2 and F4-R3.

Table 1. PCR analysis protocol

PCR Content (20µl)	Final Amount
Master Mix	10 µl
Primer F	1 µl
Primer R	1 µl
H_2O	6 µl
gDNA	2 µl

Table 2. PCR analysis protocol and cycles and temperatures

Temperature (°C)	Duration	Cycle
95	3 Minutes	1
95	Seconds	35
60	45 Seconds	
72	3.5 Minutes	
72	10 Minutes	1

RESULTS AND DISCUSSION

In the wild sunflower genetic material, fatty acid analyzes were carried out in as many accessions as possible from each species among those whose seeds were available (56 species from 63 wild Helianthus species and subspecies present in the collection). As a result of the GC analysis, no species with a high oleic acid (80% or more) was found. However, among the examined species, three of them were found to have medium oleic acid content (between 60 - 80% oleic acid). These are; wild *H. annuus* ranked first with 77.46% oleic acid, followed by *H. hirsutus* with 69.71% and *H. floridanus* with 67.19% (Table 3).

Table 3. Fatty acid compositions of wild sunflower species according to GC analysis (%).

Species	Accession #	Palmitic C16:0	Stearic C18:0	Oleic C18:1	Linoleic C18:2	Arachidic C20:0	Eicosanoidic C20:1
H. agrestis	3	5,71	4,71	45,76	43,5	0,29	
H. annuus	9	5,24	3,27	77,46	13,55	0,24	0,21
H. anamolus	13	6,38	4,75	42,68	45,65	0,31	0,11
H. argophlyus	17	8,19	7,54	25,52	57,62		
H. atrorubens	23	6,15	3,95	1,69	77,7		
H. bolanderi	29	5,42	4,13	49,65	36,94		
H. californicus	33	6,38	5,09	50,69	37,82		

H. carnosus	35	11,58	4,6	8,56	75,23		
H. cilioris	39	9,39		23,85	66,75		
H. cusickii	40	4,09	2,06	18,94	60,73	0,22	
H. debilis subsp. cucumeriflorus	45	6,72	4,92	18,28	64,81	0,32	
H. debilis subsp. silvestris	49	8,05	6,99	20,94	63,37	0,3	
H. decapetalus	52	4,74	2,9	11,05	69,73		
H. divaricatus	58	5,11	3,09	7,1	84,69		
H. eggertii	65	6,84	5,46	22,92	64,04	0,32	0,12
H. exilis	68	8,3	4,94	21,81	64,93		
H. floridanus	72	5,22	4,2	67,19	22,95	0,3	0,05
H. giganteus	77	5,85	3,15	20,75	70,23		
H. glaucophylus	81	7	4,39	44,89	43,42	0,26	
H. gracilentus	82	6,98	5,64	34,61	52,3	0,33	0,11
H. grossesseratus	88	5,31	3,13	18,61	72,3		
H. heterophylus *	95	0,007		0,02	0,08		
H. hirsutus	100	5,64	3,51	69,71	17,81	0,26	0,18
H. laciniatus	105	5,95	2,49	7,09	82,94		
H. laevigatus *	107	0,05		0,006	0,07		
H. longifolius *	112	0,003		0,005	0,036		
H. maximilliani	115	55,67	44,32				
H. mollis	123	7,73	5,83	29,25	56,56	0,36	0,12
H. neglectus *	129	0,006		0,005	0,06		
H. niveus subsp. Canescans *	131	0,001	0,001	0,003	0,004		
H. nuttalii *	136	0,005		0,016	0,064		
H. nuttalii subsp. Nuttalii *	143	0,004		0,009	0,059		
H. nuttalii.subps rydbergi	144	63,074	36,92				
H. occidentalis	145	6,95	3,13	9,53	80,37		
H. occidentalis subps.occidentalis	147	6,04	2,79	14,31	76,84		
H. occidentalis subps.plantagenius	149	6,86		10,56	82,57		
H. paradoxus	151	8,84	3,82	12,98	74,34		
H. pauciflorus *	155	0,005		0,009	0,085		
H. pauciflorus subps.pauciflorus	156	5,84	2,07	9,98	82,09		
H. pauciflorus subps. subrohomides	159	5,56	3,04	19,1	70,91	0,49	0,24
H. petiolaris subps. fallax	165	5,32	3,29	38,16	53,21		
H. petiolaris subps.	168	0,008	0,006	0,023	0,063		

Petiolaris *							
H. porteri	183	8,95	4,57	1,069	85,4		
H. praecox	184	5,64	4,1	14,68	75,16	0,39	
H. paraecox subps.hirtus	185	5,11	2,61	12,29	37,71		
H. praecox subps. Runyonii *	187	0,007		0,019	0,062		
H. pumilus	191	6,92	4,08	44,9	43,69	0,24	0,13
H. radula	192	5,71	4,71	45,76	43,5		
H. resinosus	201	8,8	5,84	18,55	66,79		
H. salicifolius	206	5,37	4,34	38,69	51,22	0,24	0,11
H. silphioides	208	0,032	0,02	0,31	0,24		
H. simulans	212	5,55	4,65	48,89	40,64	0,25	
H. smithii	214	0,006		0,02	0,092		
H. stromosus	219	4,55	2,67	11,5	80,74		
H. tuberosus	231	7,39	5,35		80,81		
H. winteri	237	6,12	4,99	23,66	65,22		

* Due to being so smaller seeds, there were not obtained oil and fatty acid analysis properly in some wild species

Based on the analysis result, there was no species with high oleic content (80% and above oleic acid) observed. However, three of the examined species were observed to have medium oleic acid content (60-80% oleic acid). From these species, *H. annuus* ranked first with the highest oleic acid content of 77.46%, followed by *H. hirsutus* with 69.71% and *H. floridanus* with 67.19%. The list of individuals used in the project to determine high oleic acid genotypes through molecular analysis is given in Table 4.

Table 4. Wild sunflower species with high oleic acid genotypes based on in molecular analyses.

Pl No	*Species / Wild sunflower accessions*	Accession number
673205	*Helianthus agretis*	3
597890	*Helianthus annuus*	9
468651	*Helianthus argophyllus*	14
468659	*Helianthus atrorubens*	23
673294	*Helianthus bolanderi*	30
649943	*Helianthus californicus*	32
664671	*Helianthus carnosus*	35
531040	*Helianthus cusickii*	40
649870	*Helianthus debilis subsp. cucumerifolius*	47
613754	*Helianthus debilis subsp. silvestris*	49
547169	*Helianthus decapetalus*	52
673143	*Helianthus divaricatus*	63
649981	*Helianthus eggertii*	65

649891	*Helianthus exilis*	68
468720	*Helianthus giganteus*	74
664715	*Helianthus glaucophyllus*	81
673286	*Helianthus gracilentus*	83
547193	*Helianthus grosseserratus*	91
673183	*Helianthus heterophyllus*	95
435703	*Helianthus hirsutus*	97
653545	*Helianthus laciniatus*	102
503228	*Helianthus laevigatus*	107
650000	*Helianthus longifolius*	111
468746	*Helianthus maximiliani*	114
435759	*Helianthus mollis*	120
435769	*Helianthus neglectus*	127
435774	*Helianthus niveus subsp.canescens*	131
650024	*Helianthus nuttallii*	136
531045	*Helianthus nuttallii subsp. Nuttallii*	138
597918	*Helianthus nuttallii subsp. Rydbergii*	144
494592	*Helianthus occidentalis subsp.plantagineus*	148
673253	*Helianthus paradoxus*	151
592353	*Helianthus pauciflorus*	154
650031	*Helianthus pauciflorus subsp.subrhomboideus*	160
597923	*Helianthus petiolaris*	164
503232	*Helianthus petiolaris subsp.petiolaris*	169
673214	*Helianthus porteri*	182
468846	*Helianthus praecox*	184
435855	*Helianthus praecox subsp.hirtus*	185
435847	*Helianthus praecox subsp. Praecox*	186
435853	*Helianthus praecox subsp. Runyonii*	188
650077	*Helianthus pumilus*	191
673184	*Helianthus radula*	197
664672	*Helianthus resinosus*	198
664759	*Helianthus salicifolius*	203
664788	*Helianthus silphioides*	208
664724	*Helianthus simulans*	212
468889	*Helianthus smithii*	214
547223	*Helianthus strumosus*	223
357299	*Helianthus tuberosus*	231
673290	*Helianthus winteri*	237
503285	*Helianthus laetiflorus*	239

F4-R1 Marker

A 653 bp DNA fragment is expected to be amplified in high oleic genotypes with the F4-R1 marker. When the gel images obtained as a result of the study conducted with wild species are examined, 68, 120, 164, 169 (*Helianthus exilis, Helianthus mollis, Helianthus petiolaris, Helianthus petiolaris subsp. petiolaris*) wild species can be considered as high oleic (Figure 3).

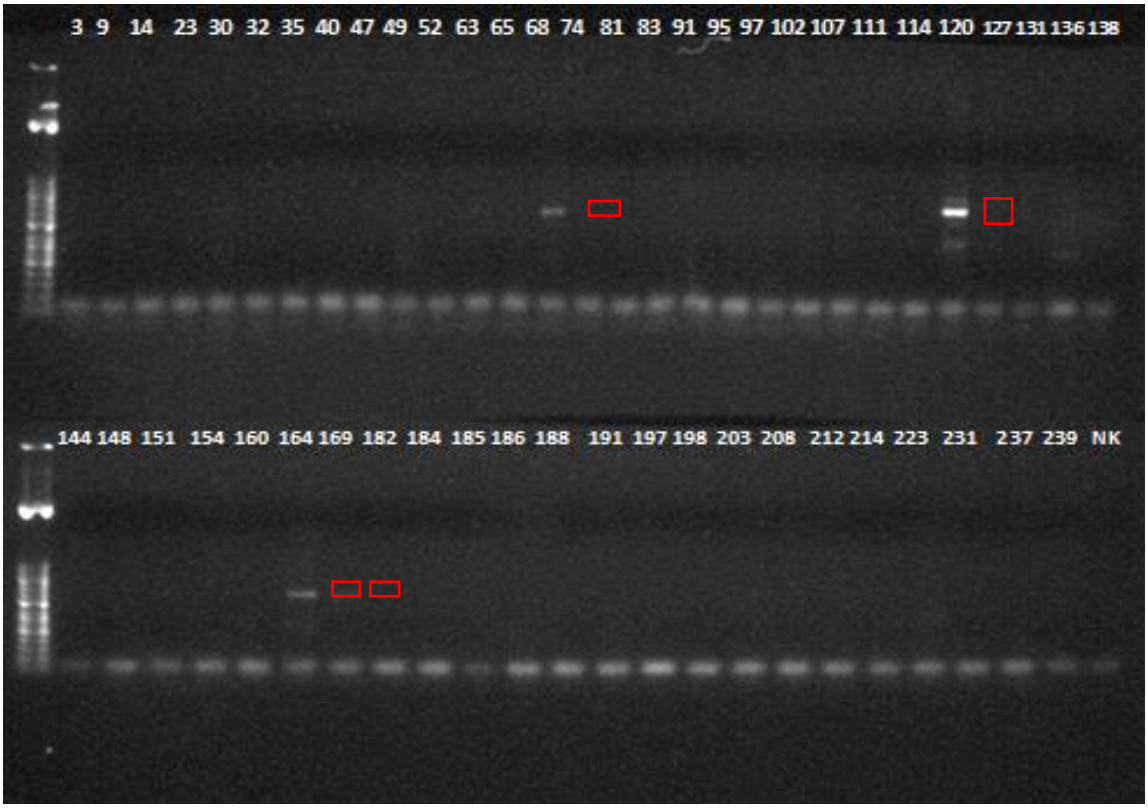

Figure 3. High oleic gel band image with F4-R1 marker.

F4-R2 Marker

A 1259 bp DNA fragment is expected to be amplified in high oleic genotypes with F4-R2 marker. Accordingly, wild sunflower species numbered 83, 102, 127 (*Helianthus gracilentus, Helianthus laciniatus, Helianthus neglectus*) can be evaluated as high oleic (Figure 4).

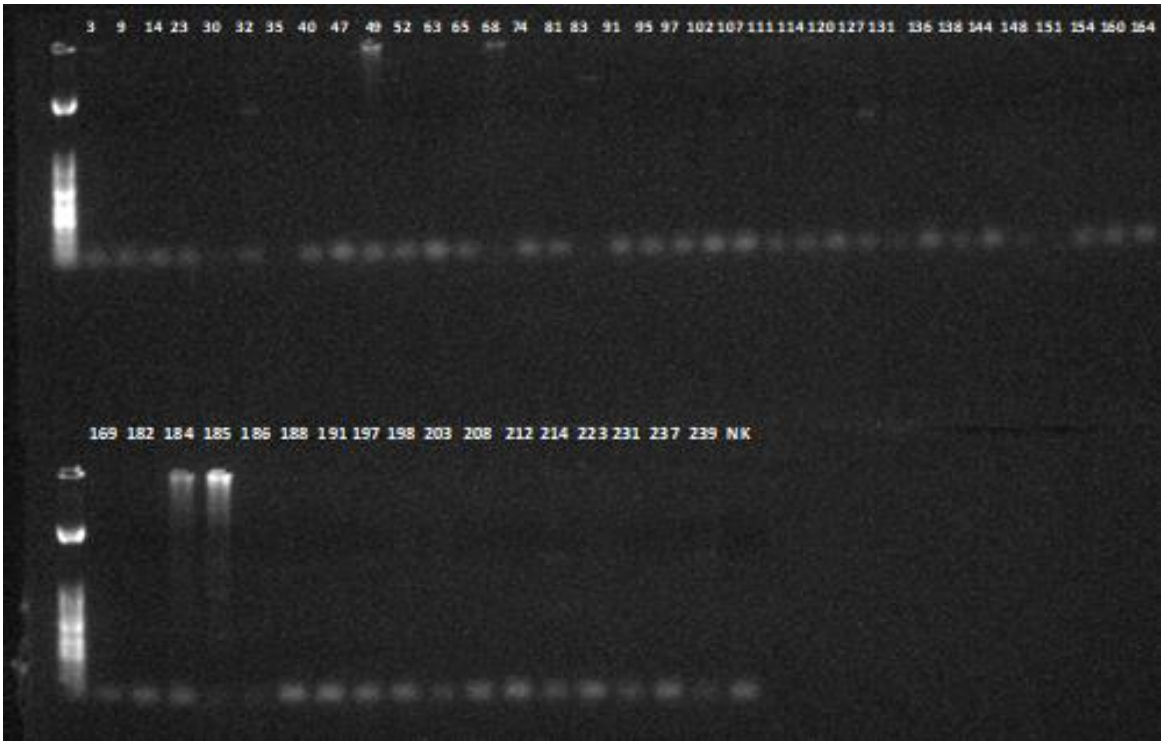

Figure 4. High oleic gel band image with F4-R2 marker

F4-R3 Marker

A 1782 bp DNA fragment is expected to be amplified in high oleic genotypes with F4-R3 marker. In this case, species with sort numbers 32, 68, 74, 91, 164 (*Helianthus californucus, Helianthus exilis, Helianthus giganteus, Helianthus grosseserratus, Helianthus petiolaris*) from wild species could be considered as high oleic.

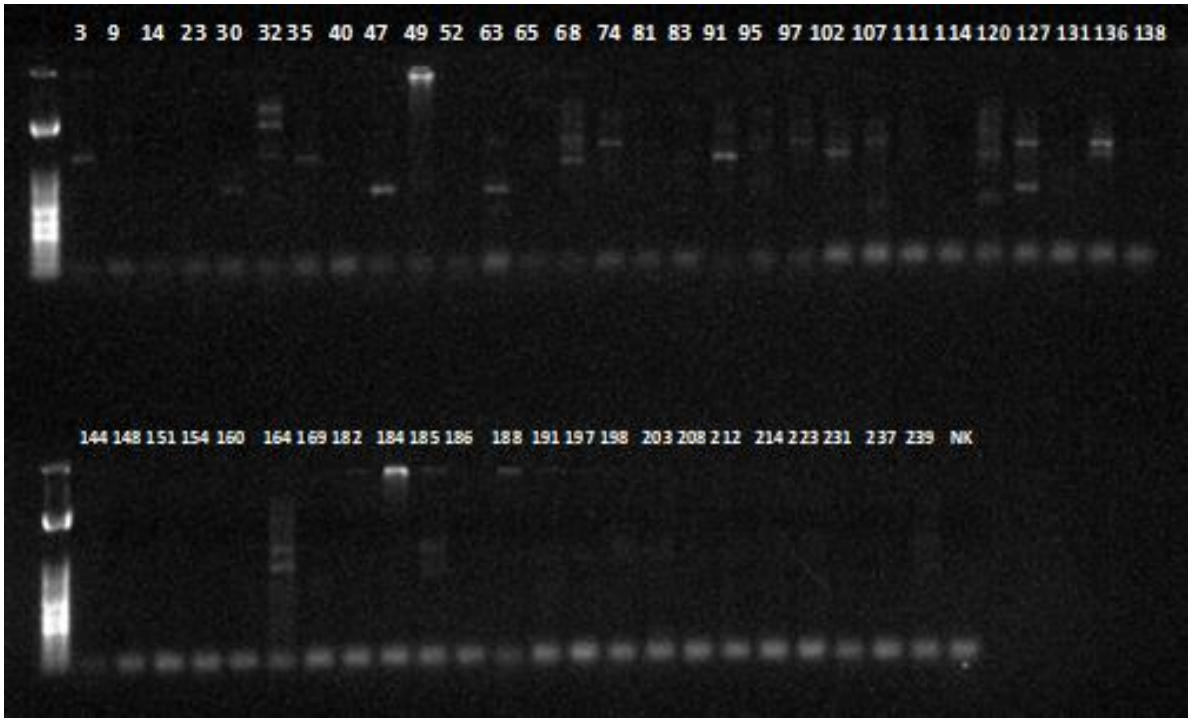

Figure 5. High oleic gel band image with F4-R3 marker.

Based on the result of the study conducted with 3 INDEL markers, species 32, 68, 74, 83, 91, 102, 120, 164, 169 were identified as high oleic according to the accession number. The Table 5 shows the comparison of the species identified as high oleic by molecular markers with the oleic acid amounts measured by GC. However, since there is no genotype containing high oleic acid among the wild species, the selectivity of the markers may not be correct.

Table 5. List of accessions identified as high oleic with molecular markers.

Accession No	Species Name	Oleic acid content C18:1 (%)
32	*Helianthus californicus*	50.69
68	*Helianthus exilis*	21.81
74	*Helianthus giganteus*	20.75
83	*Helianthus gracilentus*	34.61
91	*Helianthus grosseseratus*	18.61
102	*Helianthus laciniatus*	7.09
120	*Helianthus mollis*	29.25
127	*Helianthus neglectus*	0.005
164	*Helianthus petiolaris*	-
169	*Helianthus petiolaris subsp.petiolaris*	0.023

CONLUSIONS

Among the examined wild species, *H. annuus, H. hirsutus* and *H. floridanus* had higher oleic acid content by GC analysis. *H. annuus* species ranked first with the highest oleic acid content of

77.46%, followed by *H. hirsutus* with 69.71% and *H. floridanus* with 67.19%. Based on 3 INDEL molecular marker analysis; *Helianthus californicus, Helianthus exilis, Helianthus giganteus, Helianthus gracilentus, Helianthus grosseseratus, Helianthus laciniatus, Helianthus mollis, Helianthus neglectus, Helianthus petiolaris, Helianthus petiolaris subsp. petiolaris* were determined as high oleic based on molecular markers. However, these were not determined higher oleic acid content based on GC analysis because there was no genotype containing high oleic acid among wild species and the selectivity of the markers may not be accurate on the wild species because of working only Pervenent mutation. Beside, very little amounts crude oil from wild species so it could be also not trustable.

In conclusion; in this study, different results were obtained from GC and marker analysis in terms of high oleic content of the species. Therefore, selectivity of the markers may not be accurate or new markers need to be used for the analysis of further researches. As a result, it is thought that the findings obtained from this pioneering study will contribute to future breeding programs.

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