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Comparison of RAPD and ISSR molecular markers to determine of genetic diversity in weed dodder (*Cuscuta epithymum* L.)

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Key words: Genetic diversity, weed dodder (*Cuscuta epithymum* L), RAPD, ISSR

Abstract

Weed dodder is a perennial herb belonging to the Convolvulaceae family and is widely used as a valuable herbal drug in traditional medicine. In this study, ISSR and RAPD markers were used to evaluation of genetic diversity of 20 genotypes of weed dodder (*Cuscuta epithymum* L.) from Golestan and Mazandarn provinces. 6 ISSR primers amplified 90 bands that 76.33% bands were polymorphism. Average polymorphism information content (PIC) and average marker index value was 0.26 and 20 for ISSR markers respectively. RAPD markers amplified 195 bands that more than 95% bands were polymorphism. Average PIC and average MI was 0.27 and 24.7 for this markers calculated. Cluster analysis based on DICE coefficient and UPGMA algorithm showed that there is wide variation in samples collected. The average of similarity based on ISSR markers was 0.57 and average of similarity based on RAPD markers was 0.45. This high level of diversity could be related to the existents of several sub species in weed dodder (*Cuscuta epithymum* L.) and the high discrimination power of molecular markers that be used in this study.

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Introduction

Dodder is annual herbaceous plant of Convolvulus arvensis (*Cuscutaceae*) family that grows in many temperate and subtropical regions of the world particularly in Iran. Weed dodder can't to produce food because lack chlorophyll and are completely dependent on the host like obligated parasite branches. The plant consists of slender, twining yellow stems with clusters of small white flowers (Parker and Riches, 1993). Swamp dodder (*Cuscuta gronovii*) Grows along stream margins and in areas of continual erosion (Dawson *et al*., 1994). Long-term research studies designed to document dodder germination patterns (Sandler *et al.,* 2001) indicated that 2 or more peaks of dodder emergence occur in any given year in Massachusetts. The plant is immensely used in the system of medicine to treat urination disorder, jaundice, muscle pain and coughs. The seeds are alterative, anthelmintic, carminative, and are widely used in the treatment of bilious disorders. The stems are also used in the treatment of bilious disorders and the whole plant is used as purgative. It is also used internally in treating protracted fevers and externally in the treatment of body pain and itchy skin. The juice of the plant is mixed with the juice of *Saccharum officinarum* which is used in the treatment of jaundice. The crude water extracts of *C. reflexa* exhibited anti-HIV activity which could be due to combinatory effects with compounds of different modes of action (Mahmood *et al*., 1997; Khan *et al*., 2010). The methanol extract of *C. reflexa* exhibited anti-bacterial and free radical scavenging activity (Pal *et al.,* 2006; Uddin *et al*., 2007). The petroleum ether extract of *Cuscuta* and its isolates are useful in the treatment of androgen-induced alopecia by inhibiting the enzyme 5-α-reductase activity possibly because of steroidal constituents (Pandit *et al*., 2008). Another species of the same genus, known as *Cuscuta chinensis* Lam. is also a parasitic plant. The medicinal value of *C. chinensis* is entirely different from that of *C. reflexa*. The stem is used in the treatment of sore heads and inflamed eyes. The seed are aphrodisiac, demulcent, diaphoretic, and hepatic tonic. Total flavones from *C. chinensis* (TFCC) regulate the proliferation and apoptosis of the deciduas and cytotrophoblasts and prevent spontaneous abortions in rat (Ma *et al.* 2008). In this research we want to determine weed dodder genetic diversity in northern of Iran by RAPD and ISSR molecular markers.

Material and method

Biological sampleThe sample of weed dodder (*Cuscuta epithymum* L.) was provided from some part of Golestan and Mazandaran provinces (Table 1). This study performed at plant breeding lab of sari agriculture sciences and natural recourses university (SANRU).

Table 1. The origin and site description of the 20 populations of Weed Dodder

Num	Sample	Origin	E	N
1	G1	Karimabad	53 48' 32''	36 53' 23''
2	G2	Hezarpich	53 45' 33''	36 23' 44''
3	G3	Gorgan.uni	53 22' 12''	36 34' 54''
4	G4	Nodizheh	53 37' 42''	36 34' 54''
5	G5	Kordkoy	52 28' 37''	36 34' 54''
6	G6	Bandargaz	52 30' 60''	36 67' 56''
7	G7	Galogah	53 12' 11''	36 47' 45''
8	G8	Khalilshahr	53 62' 13''	36 04' 59''
9	G9	Zaghmarz	53 23' 12''	36 04' 98''
10	G10	Rostamkola	53 24' 14''	36 01' 02''
11	G11	Neka	53 37' 45''	36 89' 34''
12	G12	Sorak	53 09' 34''	36 08' 23''
13	G13	Semeskandeh	53 64' 45''	36 23' 50''
14	G14	Fajr.sari	52 29' 76''	36 09' 87''
15	G15	Sari.uni	52 13' 09''	36 67' 50''
16	G16	Jourbar	52 11' 04''	36 16' 09''
17	G17	Ghaemshahr	52 25' 22''	36 65' 34''
18	G18	Babol	52 11' 57''	36 09' 44''
19	G19	Amol	52 37' 13''	36 22' 07''
20	G20	Fereidoonkenar	53 60' 29''	36 04' 07''

DNA extraction

The stock solution concentrations were: CTAB 3% (w/v), 1M Tris-Cl (pH 8), 0.5 M; EDTA (pH 8), 5 M

NaCl, absolute ethanol (ARgrade), chloroform-IAA (24:1 [v/v]), polyvinyl pyrrolidone (PVP) (Sigma) and ß mercaptoethanol. All the chemicals used in the experiments were of analytical grade. The extraction buffer consisted of CTAB 3% (w/v), 100 mM Tris-Cl (pH 8), 25 mM EDTA (pH 8), and 2 M NaCl. The PVP and ß-mercaptoethanol were prepared fresh. DNA extraction and purification DNA was isolated from young stem using a modified CTAB (cetyl trimethyl ammonium bromide) method (Doyle and Doyle, 1991).

The fine powder was transferred to the microcentrifuge tube containing freshly prepared 700 µl of extraction buffer (100 mM Tris buffer pH 8. 25 mM EDTA, 2 M NaCl, 3% CTAB and 3% polyvinyl pyrrolidone). The suspension was mixed gently and incubated at 65°C for 20 min with occasionally mixing. The suspension was then cooled to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was centrifuged at 13000 rpm for 10 min. The clear upper aqueous phase was then transferred to a new tube, added with a 2/3 volume of ice-cooled isopropanol was added and incubated at -20°C for 30 min. The nucleic acid was collected by centrifuging at 5000 rpm for 10 min. The resulting pellet was washed twice with 70% ethanol and air-dried under a sterile laminar hood, and the nucleic acid was dissolved in TE (10 mM Tris buffer pH 8, 1 mM EDTA) at room temperature. The contaminating RNA was eliminated by treating the sample with RNase A (10 mg/mL) for 30 min at 37°C. DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260 nm and 280 nm. The quality of the DNA was determined using agarose gel electrophoresis stained with ethidium bromide.

RAPD and ISSR amplification

The RAPD and ISSR reactions were performed according to the method developed by Sadeghi Alikelayeh et al. *(2012). All primers that used in this research provided from Genetics & Agricultural Biotechnology Institute of Tabarestan, Iran (GABIT) (Table 2, 3).*

Table 2. RAPD primers and annealing temperature

Primers	Primer sequence	Annealing temperature (ċ)
OPA-01	CAGGCCCTTC	37
OPA-10	GTGATCGCAG	37
OPB-04	GGACTGGAGT	37
OPB-05	TGCGCCCTTC	42
OPB-20	GGACCCTTAC	40
OPC-08	TGGACCGGTG	37
OPD-02	GGACCCAACC	37
OPD-03	GTCGCCGTCA	40
OPD-05	TGAGCGGACA	40
OPH-01	GGTCGGAGAA	40
OPH-04	GGAAGTCGCC	37
OPH-15	AATGGCGCAG	36
OPH-20	GGGAGACATC	37

Table 3. ISSR primers and annealing temperature

primer	Primer Sequence	Annealing Temperature ('c)
ISSR-4	GAGAGAGAGAGAGAGAGAC	58
ISSR-5	GTGTGTGTGTGTGTGTGTC	53
ISSR-6	GTGTGTGTGTGTGTGTGTT	52
ISSR-8	CTCTCTCTCTCTCTCTG	54
ISSR-11	GAGAGAGAGAGAGAGAC	52
ISSR-13	TCTCTCTCTCTCTCTCC	52

The reactions were carried out in 12.5 µl volumes in a tube using six (ISSR) and thirteen (RAPD) random primers, (GABIT). Each reaction tube contained 10 ng templates DNA, 1.5 mM MgCl2, 300 µM of dNTPs, and 2.5 µL of 1xTaq DNA polymerase buffer, 25 pM primer and 1.5 units of Taq DNA polymerase (Sinagene, Iran) (Table 4).

Table 4. PCR reaction components, the concentrations, a 12.5 µl reaction

Reaction components	Base concentration	Final concentration	A 12.5 µl reaction	19 reaction
Distilled water	---	---	7.29µl	137.9 µl
PCR Buffer	10X	1X	1.25µl	23.75 µl
MgCl2	50Mm	2-1.2 Mm	0.5µl	9.5 µl
DNTPs	20Mm	250 µM	0.32µl	6.08 µl
Primers	10µM	0.75 µM	0.94µl	17.86 µl
Tag DNA Polymerase	5u/µl	1u/µl	0.2 µl	3.8 µl
Template DNA	10ng/µl	---	2 µl	38 µl

Amplification was performed in a DNA thermal cycler (Bioer Thermal Cycler, China), using the following conditions: 95°C for 3 min; 36 cycles at 92°C for 1 min, 52-58°C (ISSR) and 34-45°C (RAPD) for 1 min and 72°C for 1 min; final extensions at 72°C for 5 min. PCR products were resolved on 1.5% agarose gel in 1xTAE buffer. The DNA was stained with 0.5 mg/mL ethidium bromide, visualized and photographed under a UV transilluminator. A sample without template DNA was included as a negative control in each experiment to check contamination. Electrophoretic profile was visualized under UV radiation and photographed with a UV transilluminator. The sizes of DNA fragments were estimated by comparison with standard ladder (1kb; fermentase, Germany) (Table 5, 6).

Table 5. PCR program for amplification of RAPD primers

No.	Steps	Tempe-rature °c	Time (minutes)	Number of cycles
1	DNA Denaturation	94	5	1
2	DNA Denaturation	92	1	35
3	Annealing	35-45	1	
4	Primer Extension	72	1	
5	Primer final Extension	72	5	1
6	Store	4	Long Time	

Table 6. PCR program for amplification of ISSR primers

No.	Steps	Tempe-rature °c	Time (minutes)	Number of cycles
1	DNA Denaturation	94	5	1
2	DNA Denaturation	92	1	35
3	Annealing	52-58	1	
4	Primer Extension	72	1	
5	Primer final Extension	72	5	1
6	Store	4	Long Time	

Statistical analysis

Presence or absence of each band was scored with one and zero for thirteen primers. Then Zero-one matrix was prepared. The total number of bands and polymorphic bands for each primer was calculated with using Total lab software and the percents of polymorphism were calculated using the formula (number of polymorphic bands / total bands). Polymorphism Information Content (PIC) was calculated for dominant markers that the allelic relationship between their bands was unclear with the formula PIC=Σ [2fi (1-fi)]. Dice similarity matrix was obtained using the software NTSYS-pc 2/02 and UPGMA cluster analysis was performed. Cophenetic matrix was calculated to evaluate the adaptation of cluster analysis to the data. Similarity matrices were compared with the cophenetic matrix and cophenetic correlation coefficients were calculated (Peakal and Smouse, 2006).

Result and discussion

Quality of the DNA samples was evaluated using electrophoresis on agarose gel 0.7% (Ladder SM0331) based on a modified CTAB method (Gustine *et al.* 2002) and DNA showed no smear or fracture on the gel (Fig. 1).

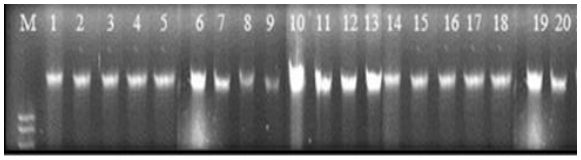

Figure 1. genomic DNA extracted from dodder by CTAB method

RAPD

The results of analyzing 20 genotypes of weed dodder using RAPD marker showed that among the total of 195 scored bands, 172 bands, equivalent to 95% were polymorphic. Average rated bands for each primer was 15 bands and Out of 13.23 bands were polymorphic bands. The maximum numbers of the bands were belonged to the primers OPD-03 and OPH-20 and the minimum numbers of the bands were belonged to the primer OPC-08 (Fig. 2). Respectively the highest percentage of polymorphic bands was for the primers OPD-03 and OPH-20 (100%) and the lowest percentage of polymorphic bands was for the primers OPC-08 (73%). In this study, the average of PIC value was 0.27 for RAPD marker. The maximum amount of PIC was for the primer OPD-02 (0.36) and the minimum amount of PIC was for the primer OPB-05 (0.21) (Table 7).

Table 7. Statistical analysis and results of genetic diversity of 20 populations of weed dodder (RAPD)

Primers	Primer Sequence	Total Band	Polymorphic Band	Polymorphic Percentage	PIC	MI
OPA-01	CAGGCCCTTC	15	12	80	0.24	19.2
OPA-10	GTGATCGCAG	15	13	86	0.25	22
OPB-04	GGACTGGAGT	15	13	86	0.28	24
OPB-05	TGCGCCCTTC	15	13	86	0.21	18.8
OPB-20	GGACCCTTAC	15	12	80	0.25	20
OPC-08	TGGACCGGTG	15	11	73	0.28	20.9
OPD-02	GGACCCAACC	15	14	93	0.36	34.1
OPD-03	GTCGCCGTCA	15	15	100	0.26	26.4
OPD-05	TGAGCGGACA	15	13	86	0.29	25.4
OPH-01	GGTCGGAGAA	15	13	86	0.32	27.5
OPH-04	GGAAGTCGCC	15	14	93	0.24	22.3
OPH-15	AATGGCGCAG	15	14	93	0.31	28
OPH-20	GGGAGACATC	15	15	100	0.32	32.8

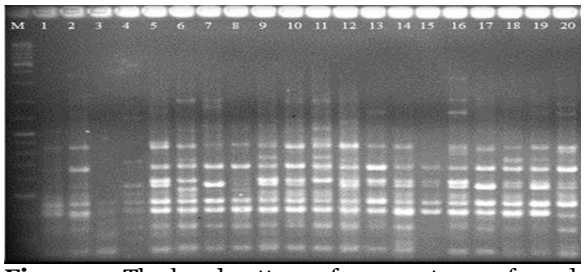

Figure 2. The band pattern of 20 genotypes of weed dodder using OPD-03

The results of similarity matrix showed that the highest genetic similarity (71%) was between the genotypes of Sorak and Semeskandeh and the lowest genetic similarity (27%) was between the genotypes of Hezarpich and Bandargaz. According to the two populations collected from Hezarpich and Bandargaz from Golestan province which are geographically far from each other and Hezarpich has a drier climate

than Bandargaz, therefore it is logical to show the least similarity. Also the geographical proximity of Sorak and Semeskandeh from Mazandaran province is a strong reason for the high level of genetic similarity.

The dendrogram resulting from cluster analysis using UPGMA algorithm and DICE similarity matrix revealed five main groups. The first group consisted of thirteen genotypes from Karimabad, Zaghmarz, Rostamkola, Neka, Sorak, Semeskandeh, Babol, Amol, Fereidoonkenar, Fajr.sari, Sari.uni, Jouybar and Ghaemshahr. The second group comprised two genotypes from Galogah and Khalilshahr. The third group consists of one genotype from Hezarpich. The fourth group consisted of two genotypes from Gorgan.uni and Nodizheh and finally the fifth Group consisted of two genotypes from Kordkoy and Bandargaz. The result of cluster analysis showed that the maximum number of populations were in the first group. As regards, in the RAPD method, the segments of DNA that are amplified are random and the primers are short (8–12 nucleotides) (Doyle and Doyle, 1987), maybe that's the reason why different population from different regions of the North of Iran, are in the same group (first group).The second, fourth and fifth groups contain populations that are located in the areas which are geographically close to each other. According to Fasih *et al.* (2013) result that showed genetic variation does not match with the geographical distribution (Fig. 4)

ISSR

The results of analyzing 20 genotypes of weed dodder using ISSR marker showed that among the total of 90 scored bands, 69 bands, equivalent to 76.33% were polymorphic. Average rated bands for each primer was 15 bands and Out of 11.5 bands were polymorphic bands. The maximum numbers of the bands were belonged to the primers ISSR-13 and the minimum numbers of the bands were belonged to the primer ISSR-04 (Fig. 3). Respectively the highest percentage of polymorphic bands was for the primers ISSR-13 (100%) and the lowest percentage of polymorphic bands was for the primers ISSR-04 (66%). In this study, the average of PIC value was 0.26 for ISSR marker. The maximum amount of PIC was for the primer ISSR-13 (0.32) and the minimum amount of PIC was for the primers ISSR-04 and ISSR-11 (0.23) (Table 8).

Table 8. Statistical analysis and results of genetic diversity of 20 populations of weed dodder (ISSR)

Primer	Primer Sequence	Total Band	Polymorphic Band	Polymorphic Percentage	PIC	MI
ISSR-4	$(GA)_9C$	15	10	66	0.23	15
ISSR-5	$(GT)_9C$	15	11	73	0.28	20
ISSR-6	$(GT)_9T$	15	11	73	0.28	20
ISSR-8	$(GT)_8G$	15	11	73	0.26	19
ISSR-11	$(GA)_8C$	15	11	73	0.23	17
ISSR-13	$(TC)_8C$	15	15	100	0.32	32

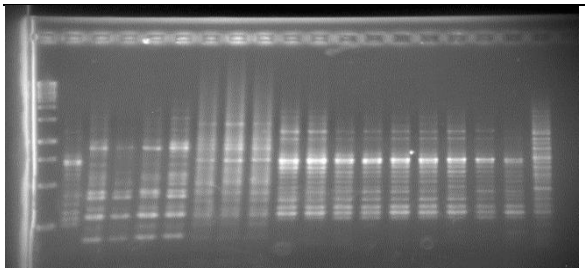

Figure 3. The band pattern of 20 genotypes of weed dodder using ISSR-13

The results of similarity matrix showed that the highest genetic similarity (96%) was between the genotypes of Jouybar and Ghaemshahr and the lowest genetic similarity (25%) was between the genotypes of Hezarpich and Amol. According to the two populations collected from Hezarpich from Golestan and Amol from Mazandaran provinces which are geographically far from each other and Hezarpich has a drier climate than Amol, therefore it is logical to

show the least similarity. Also the geographical proximity of Jouybar and Ghaemshahr from Mazandaran province is a strong reason for the high level of genetic similarity.

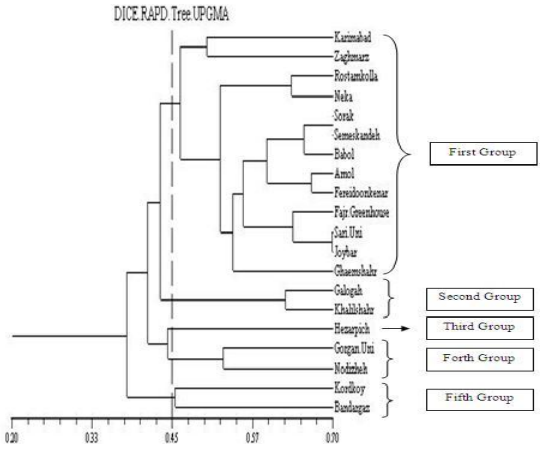

Figure 4. Dendrogram of cluster analysis based on DICE and UPGMA (RAPD)

The dendrogram resulting from cluster analysis using UPGMA algorithm and DICE similarity matrix revealed five main groups. The first group consisted of two genotypes from Karimabad and Fereidoonkenar. The second group comprised thirteen genotypes from Kordkoy, Bandargaz Zaghmarz, Neka, Semeskandeh, Babol, Amol, Fajr.sari, Sari.uni, Jouybar, Ghaemshahr, Galogah and Khalilshahr. The third group consists of one genotype from Rostamkola. The fourth group consisted of two genotypes from Sorak and Nodizheh and finally the fifth Group consisted of two genotypes from Gorgan.uni and Hezarpich. The result of cluster analysis showed that the maximum number of populations were in the first group. As regards, in the ISSR method, the segments of DNA that are amplified are random and the primers are short (8–12 nucleotides) (Doyle and Doyle, 1987), maybe that's the reason why different population from different regions of the North of Iran, are in the same group (first group).The second, fourth and fifth groups contain populations that are located in the areas which are geographically close to each other. According to Fasih *et al.* (2013) result that showed genetic variation does not match with the geographical distribution (Fig. 5).

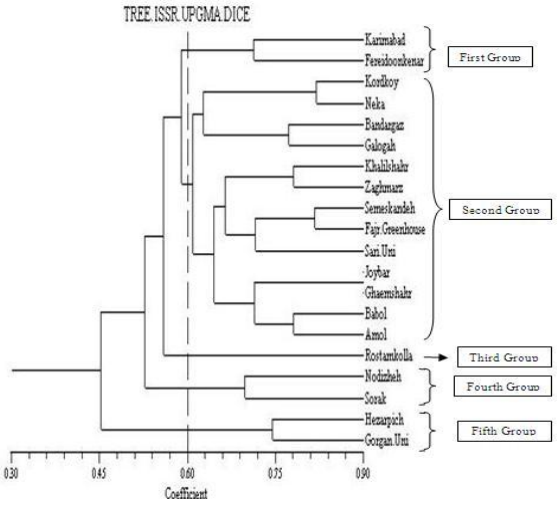

Figure 5. Dendrogram of cluster analysis based on DICE and UPGMA (ISSR)

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