Comparison of ISSR and AFLP markers in assessing genetic diversity among Nettle (*Uritica dioica* L.) populations

Mostafa Haghpanah*[1], Seyyed-Kamal Kazemitabar[1], Seyyed-Hamidreza Hashemi[2] and Seyyed-Mohammad Alavi[2]

[1] Department of Biotechnology and Plant Breeding, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.

[2] Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari Agricultural Sciences and Natural Resources University, Sari, Iran

ABSTRACT: Nettle *(Urtica dioica* L.) is an important medicinal plant which is widely distributed in Mazandaran province North of Iran. In this study, Amplified Fragment Length Polymorphism (AFLP) and Inter-Simple Sequence Repeat (ISSR) markers were used for detection of genetic polymorphism in Mazandaran nettle. Ten AFLP primer combinations and seventeen ISSR markers were utilized. AFLP produced 830 scorable bands out of which 90.21% were polymorphic. ISSR primers amplified 234 bands out of which 181 bands were being polymorphic (77.3%) and average heterozygosity for AFLP and ISSR markers were 0.25 and 0.23, respectively. Marker Index obtains 22.25 for AFLP and 15.57 for ISSR. The number of clusters computed was the same for both molecular markers, with different sample composition. In total, the comparison of two marker systems showed AFLP marker can be useful tool for detection of *U. dioica* genetic diversity.

KEYWORDS: Genetic variation, Medicinal plant, Molecular markers.

INTRODUCTION

The stinging nettle (*Urtica dioica* L.) is a perennial dioecious medicinal plant widely distributed around the world (23, 5) and in Iran, mostly in temperate regions (Mazandaran province). Stace (24) reported two tetraploid forms of nettle (2n = 4x = 48 and 52), with a large genome (2C value = 3.1 pg), where the C (constant value) is the amount of nuclear DNA in the haploid nucleus (4).

Medicinal plants have now become quite popular in both developing and developed countries (21) because of good alternatives or complementaries to synthetic drugs. Understanding the genetic diversity of medicinal plants is the foundation for their optimal exploitation (18). Molecular markers are widespread tools in the study of genetic diversity. Random molecular markers such as random amplified polymorphic DNA (27), inter-simple sequence repeat (29) and amplified fragment length polymorphism (26) are widely used in genetic diversity evaluation of medicinal plants. Genetic diversity information about the nettle plants is limited. Bharmauria *et al.*, used eight RAPD primers to study the genetic relationships among Himalayan nettle and association of diversity and altitude changes (1). On the other hand, many studies indicate that RAPD technique is not a reliable method for measuring variability and determining diversity because of unstable and poorly repeatable (9, 17, 18 and 19). Other random molecular markers such as ISSR and AFLP are more authentic and

*Corresponding author (✉):masoudhgh@gmail.com
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popular in the study of plant genetic diversity. Those markers were widely used in the evaluation of genetic diversity of mulberry (8), *Prunus subgenus* (22), *Populus cathayana* (7, 10) *Tribulus terrestris* (21), *Ocimum* spp. (14) *Phaseolus coccineus* (2) and radish (*Raphanus sativus*) (9) but there is no enough information available to establish *U. dioica's* genetic diversity yet. The aim of this study was to compare AFLP and ISSR molecular marker systems to detect the genetic diversity of three nettle populations in Mazandaran province.

MATERIALS AND METHODS

Plant materials

Leaf samples of each plant were collected from natural populations growing in west (fourteen samples), center (eleven samples) and east (six samples) of Mazandaran province (Table 1).

Table 1. Geographic location of the three studied populations

Population	Geographical origins	Location coordinates	NO. samples
NWM	IRAN, west of Mazandaran Province	36°52.51´, 50°53.02´	14
NCM	IRAN, center of Mazandaran Province	36°34.22´, 51°30.02´	11
NEM	IRAN, east of Mazandaran Province	36°40.55´, 53°35.30´	6

DNA extraction

Genomic DNA was extracted from lyophilized leaf martial following the modified protocol of Doyle and Doyle (3). The qualification and quantification of extracted DNA were determined using agarose gel electrophoresis and spectrophotometry.

AFLP analysis

AFLP was performed according to the Vos *et al.*, protocols (26). Briefly, 250 ng of gDNA was digested by *Eco*RI and *Tru*1I (similar cutting position with *Mse*I) with Tango® 1X buffer (Fermentas) at 37 ºC and 65 ºC, respectively, for 5h and then ligated to related adaptors at 14 ºC for 10h. In order to increase the frequency of correct ligated fragments, pre-amplification was carried out. Pre-amplification PCR was performed with 24 cycles (30s at 94 ºC, 30s at 56 ºC and 60s at 72 ºC). AFLP pre-amplification primers based on *Tru*1I and *Eco*RI adapters had no selective nucleotide at 3' ends. Pre-amplified products were used as template after 10-fold dilution in sterile water for the selective amplification reaction using *Eco*RI and *Tru*1I primers with three selective nucleotides at 3'-end. Ten selective amplification primer combinations were used for analysis (Table 2). Selective amplifications were carried out in a thermal cycler (T-100, BIO-RAD, USA) programmed for 36 cycles, each including a 30s DNA denaturation step at 94 ºC, a 30s annealing step and a 60 s extension step at 72 ºC. The annealing temperature at the initial cycles was 65 ºC and subsequently the temperature declined by 0.7 ºC (touch-down) for the next 12 cycles and then annealing temperature remained at 56 ºC for 23 cycles. PCR product of selective amplifications were separated on 6% denaturing polyacrylamide gel at 100 W using Sequi-Gen GT Sequencing Cell (Bio-Rad). The bands were visualized by staining with silver nitrate.

ISSR analysis

Inter-Simple sequence repeat assay was carried out in 12.5 µl reaction volume which contained 15 ng DNA, 3 mM $MgCl_2$, 2 mM of each dNTP, 10 µM primer, 1 U *Taq* DNA polymerase (Fermentas). The thermal cycler (MJ-Mini, BioRad, USA) program was: 4 min at 94ºC, followed by 35 cycles of 94ºC for 1 min, 55-57 ºC (depending on primer sequence) for 1 min and 72 ºC for 10 min. The last step was 10 min at 72ºC. The amplified products were resolved in 1.5% agarose gel (0.5X TBE), stained with ethidium bromide (10µg/ml) and photographed under UV light. Seventeen ISSR primers were used in this study (Table 3).

Data analysis

The amplification products were scored for the presence (as 1) and absence (as 0) of bands across the genotypes to generate a binary matrix. Co-migrating bands were assumed to be originating from the same genetic locus. The binary matrix was analyzed using the NTSYS-PC version 2.02 software to calculate the similarity matrices and to generate the dendrograms and Mantel test (11). The Mantel test was used to determine the correlation coefficient between similarity matrices and cophenetic correlation values (21). Polymorphic information content (PIC) or average heterozygosity (H_{av}) was calculated with Roldan-Ruiz et al. (20) equation. PIC = 2fi (1 - fi), where fi is the frequency of the polymorphic bands and

Table 2. *Urtica dioica*: summary of AFLP assays

Primer combinations	Total number of bands	Polymorphic bands	Polymorphism (%)
E-ACC/M-CAA	44	39	89
E-ACG/M-CAA	69	60	87
E-AGG/M-CAA	70	64	91
E-CGT/M-CAA	70	60	86
E-TTG/M-CAA	102	96	94
E-AAC/M-CAC	102	91	89
E-AAG/M-CTT	100	89	89
E-ACG/M-CTT	100	76	76
E-AGG/M-CTT	81	61	90
E-CGT/M-CTT	92	86	93
Total (average)	**83**	**72.2**	**90.21**

Table 3. *Urtica dioica*: summary of ISSR assays

Primers name	Sequence	Total number of bands	Polymorphic bands	Polymorphism (%)
issr-2	(GA)9C	7	4	57
issr-5	(GT)9C	5	4	80
issr-6	(GT)9T	18	14	78
issr-7	(GA)8C	7	3	43
issr-8	(CT)8G	6	1	17
issr-9	(AG)8C	21	15	71
issr-10	(AG)8G	6	2	33
issr-11	(GA)8C	9	5	56
issr-12	(GA)8A	36	26	72
issr-13	(TC)8C	8	6	75
issr-14	(TC)8G	8	7	88
issr-15	(AC)8G	8	5	63
issr-16	(TG)8A	13	12	92
issr-17	(AC)8C	29	24	83
issr-18	(ATC)6T	17	13	76
issr-19	(ATC)6C	19	16	84
issr-20	(ATG)6G	17	14	82
Total (average)		**13.8**	**10**	**68**

(1 – fi) is the frequency of monomorphic bands. Average heterozygosity (H_{av}) is obtained by taking the average of PIC values obtained for all the markers and is calculated as:

$$H_{av} = \sum [2 (1- f_i)] / N$$

Multiplex ratio (MR) for each assay was estimated by dividing the total number of bands (monomorphic—m, and polymorphic—p) amplified by the total number of assays (primer combinations employed—n) according to the Powell et al. (17).

$$MR = m + p/n$$

Marker index (MI) was calculated according to the following formula by Powell et al. (17):

$$MI = H_{av} \times MR$$

RESULTS

AFLP analysis

The sizes of AFLP scorable fragments were between 100 and 700 bp, considered to avoid scoring problems (16). Ten AFLP primer combinations produced 830 scorable bands. The number of produced bands for different primer combinations and the percentage of detected polymorphism in *U. dioica* have been summarized in Table 3. The average number of bands per primer combination was 83 and the percentage polymorphism ranged from 94 in primer combinations *E*-ACG/*M*-CTT and *E*-TTG/*M*-CAA to 76 in primer combination E-*ACG*/M-*CTT*.

ISSR analysis

Seventeen ISSR primers produced 234 bands out of which 181 bands (77.3%) were polymorphic. The size range of scorable bands was between 100bp to 2kb. The number of bands for different ISSR primers and percentage polymorphism observed in the *U. dioica* have been showed in Table 3. The average number of bands per primer was 72.2, the percentage polymorphism ranged from 17% for ISSR-8 primer to 92% for ISSR-16 primer and the average percentage polymorphism was 68%.

Statistical analysis

The presence (as 1) and absence (as 0) of bands were scored for all genotypes. The generated binary matrix was used to calculate the similarity matrix based on Jaccard's coefficient (6). Based on genetic similarity data of AFLP and ISSR, the dendrograms were matched together and the major difference was the location of genotypes in each cluster. In other words, both marker systems could have the potential to classify nettle populations. ISSR presents separate clusters with similarity coefficients of 0.98 (Fig.1). In AFLP analysis, the range of similarity coefficients was from 0.86 to 0.88 (Fig. 2). The principal

coordinate analysis of the ISSR (data not shown) revealed that genotypes of each population were grouped together. But the PCoA of AFLP data showed that the genotypes within populations were the most diverse ones. The high-resolution ability of AFLP markers was dependent to produce high number of polymorphic bands (21). These two molecular marker systems were compared (Table 4) on the basis of four different scales (21).Multiplex ratio and the percentage polymorphic band of AFLP have been shown to be higher than ISSR. But the difference in average heterozygosity (H_{av}) of both markers was not significant. Marker index is a measure of overall efficiency of markers. In this study superior MI was observed for AFLP system markers. According to these results, AFLP was a useful tool to establishe *U. dioica* genetic diversity. The Mantel test was used for determination of correlation coefficient between the similarity matrices generated by these markers (11). The coefficient of correlation between AFLP and ISSR was 0.66 (P = 0.01). Multiplex ratio and the percentage polymorphic band of AFLP have been shown to be higher than ISSR. But the difference in average heterozygosity (H_{av}) of both markers was not significant. Marker index is a measure of overall efficiency of markers. In this study superior MI was observed for AFLP system markers. According to these results, AFLP was a useful tool to establishe *U. dioica* genetic diversity. The Mantel test was used for determination of correlation coefficient between the similarity matrices generated by these markers (11). The coefficient of correlation between AFLP and ISSR was 0.66 (P = 0.01).

Table 4. Comparison of various molecular markers in evaluating genetic diversity of *Urtica dioica*

Molecular markers	AFLP	ISSR
Total no. of bands (n)	830	234
Polymorphic bands (p)	722	171
Total no. assays/primer combinations	10	17
Multiplex ratio (n/T)	83	13.8
Percentage polymorphic (%p)	90.21	68
Average heterozygosity (Hav)	0.25	0.23
Marker index (MI) = Hav × MR	22.25	15.57

DISCUSSION

During the course of environmental adaptation process, the isolated plant populations tend to accumulate genetic variations (21). The medicinal plant, *U. dioica* is a perennial herb with production through seeds and rhizomes (25).

Two molecular markers that were used in this study, detected high polymorphism ranged (ISSR 68% and AFLP 90.21%) in nettle plant. Similar result (high percentage polymorphism) was also reported by Sarwat et al. (21).

On the basis of the marker index, AFLP markers were the most informative system compared to the ISSR markers. This AFLP advantage depended on higher number of polymorphic bands and the average of heterozygosity was not significantly different (12). Similar result was

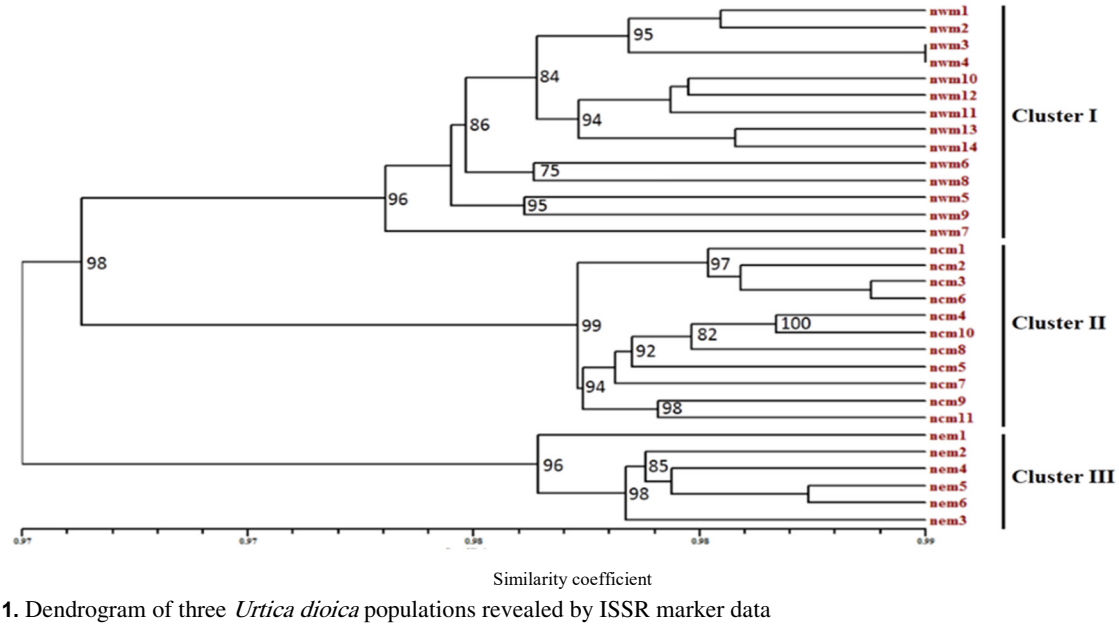

Figure 1. Dendrogram of three *Urtica dioica* populations revealed by ISSR marker data

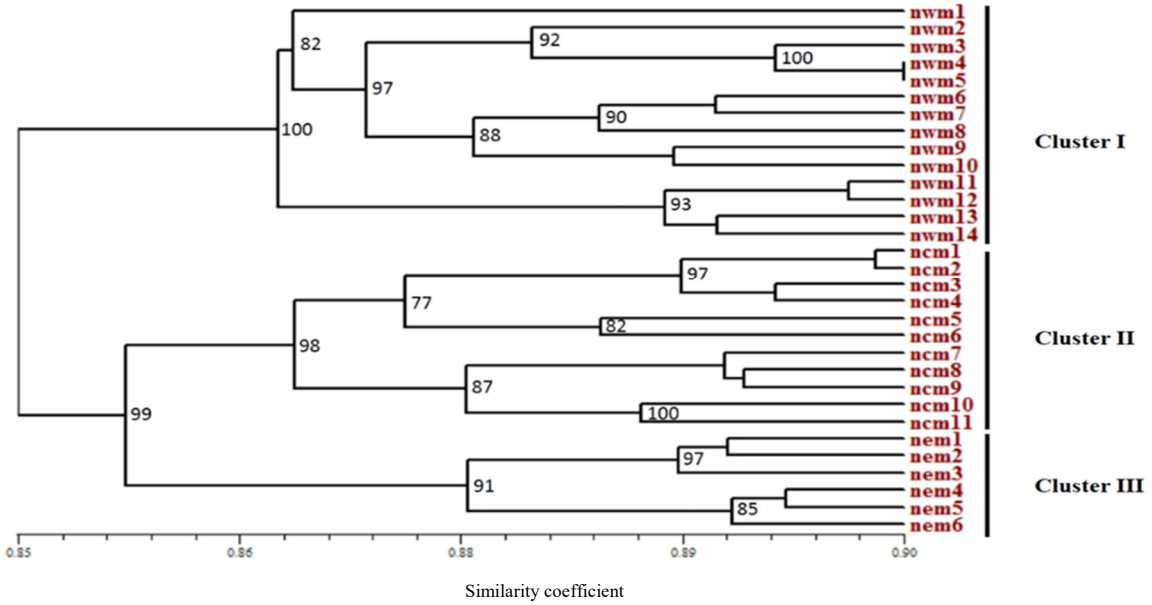

Figure 2. Dendrogram of three *Urtica dioica* populations revealed by data from AFLP markers.

reported by Muminovic et al. (15). Dendrograms generated from AFLP and ISSR marker systems were similar in a number of clusters, but the location of genotypes in the same cluster didn't match together while these different markers, targeted different sequence regions with different mutation rates (13). The cophenetic correlation values of Mantel test were above 0.90 for both marker systems. Overally, we found that AFLP was an efficient marker system for studying genetic variation in the *U. dioica*. This is in agreement with the results of previous studies on medicinal plants. For example, Sarwat et al. (21) found AFLP to be 22.21% more polymorphic than the ISSR in *Tribulus terrestris*. Xiu-qin et al. (28) studied on *Litsea szemaois* and observed AFLP improvement over ISSR in details.

In conclusion, the present investigation revealed that *U. dioica*'s germplasms from the Mazandaran province were mostly diverse. This diversity may depends on cross pollination of nettle plants and discriminatory power of markers. This study can be beneficial for suitable operation of Mazandaran nettle populations.

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مقایسه نشانگرهای ISSR و AFLP در بررسی تنوع ژنتیکی جمعیت‌های گزنه (*Urtica dioica* L.)

مصطفی حق‌پناه*[1]، سیدکمال کاظمی‌تبار[1]، سیدحمیدرضا هاشمی[2]، سیدمحمد علوی[2]

۱. گروه بیوتکنولوژی و اصلاح‌نباتات، دانشکده علوم زراعی، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ایران
۲. پژوهشکده ژنتیک و زیست‌فناوری کشاورزی طبرستان، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ایران
* نویسنده مسئول: masoudhgh@gmail.com

چکیده

گزنه (*Uritica dioica* L.) یکی از گیاهان مهم دارویی بوده که بطور گسترده در استان مازندران (شمال ایران) می‌روید. در مطالعه انجام شده برای اولین بار از نشانگر AFLP و ISSR بمنظور مطالعه چندشکلی ژنتیکی گیاه گزنه استان مازندران استفاده شد. از ده آغازگر AFLP و هفده آغازگر ISSR در این تحقیق استفاده گردید. از مجموع ۸۳۰ باند قابل امتیازدهی نشانگر AFLP حدود ۹۰/۲۱ درصد چند شکل بودند. نشانگر ISSR ۲۳۴ باند تولید کرد که ۱۸۱ باند (۷۷/۳ درصد) آن چند شکل بودند. میانگین هتروزیگوسیتی نشانگرهای AFLP و ISSR به ترتیب ۰/۲۵ و ۰/۲۳ مشاهده شد. شاخص نشانگر بدست آمده ۲۲/۲۵ برای AFLP و ۱۵/۵۷ برای ISSR محاسبه شد. تعداد خوشه‌های حاصله در هر دو نشانگر برابر بود، اما محل قرار گیری افراد درون خوشه‌ها متفاوت بود. در مجموع مقایسه این دونشانگر نشان داد که نشانگر AFLP ابزار مناسب‌تری برای بررسی تنوع *U. dioica* می‌باشد.

کلمات کلیدی: گیاه دارویی، نشانگرهای مولکولی، تنوع ژنتیکی.