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Molecular markers associated with the agronomic traits in the medicinal plant lemon balm

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ABSTRACT. Finding association between molecular markers and agronomic traits provide an excellent tool for indirect selection of a trait of interest in the population. In this study, stepwise regression analysis was used to estimate associations between ISSR and RAPD markers with some agronomic traits in lemon balm ecotypes. The analysis of results revealed significant associations between the traits and some of the studied loci. For all the traits, more than one informative marker was detected. Totally,90informative markers, including 48 ISSR loci and 42 RAPD loci, were identified. The SA-R-10, UBC826-1, UBC812-9, UBC813-10, UBC825-4, OPA-01-15, OPC-04-7 and CS-56-8 markers or fragment showed a significant correlation with Essential oil percentage and controlled 99.8% of the phenotypic variation. These markers are relatively more reliable. Among the RAPD primers, special attention should be drawn to primer SA-R, which had the highest associated fragments with the traits including days for 50% flowering, number of branches per plant, fresh weight and dry weight. Some of ISSR and RAPD markers were associated with more than one trait in multiple regression analysis that may be due to pleiotropic effect of the linked quantitative trait locus on different traits or its linkage to different genes. These primers have been found useful for improved lemon balm.

Keywords: association analyses, informative markers, multiple regression, R square.

Marcadores moleculares associados aos traços agronômicos na planta medicinal erva cidreira

RESUMO. Encontrar associação entre marcadores moleculares e traços agronômicos é uma excelente ferramenta para seleção indireta de um traço de interesse na população. Neste estudo, foi utilizada a análise de regressão *stepwise* para estimar associações entre marcadores ISSR e RAPD com algumas características agronômicas em ecótipos de erva cidreira. A análise dos resultados revelou associações significativas entre os traços e alguns dos loci estudados. Para todos os traços mais de um marcador informativo foi detectado. Foram identificados 90 marcadores informativos, incluindo 48 loci ISSR e 42 loci RAPD. Os marcadoresou fragmentos SA-R-10, UBC826-1, UBC812-9, UBC813-10, UBC825-4, OPA-01-15, OPC-04-7 e CS-56-8 mostraram uma correlação significativa com a percentagem de óleo essencial e controlaram 99,8% da variação fenotípica. Estes marcadores são considerados relativamente mais confiáveis. Entre os *primers* RAPD, destaca-se o *primer* SA-R, que apresentou os maiores fragmentos associados com as características, incluindo dias para 50% de floração, número de ramos por planta, peso fresco e peso seco. Alguns dos marcadores ISSR e RAPD foram associados a mais de um traço na análise de regressão múltipla que pode ser devido ao efeito pleiotrópico do locus de traço quantitativo ligado em diferentes traços ou sua ligação a diferentes genes. Estes iniciadores provaram ser úteis para o melhoramento da erva cidreira.

Palavras-chave: análise associativa, marcadores informativos, regressão múltipla, quadrado R.

Introduction

Lemon balm (*Melissa officinalis*, balm, common balm, or balm mint, is a perennial herbaceous plant in the mint family Lamiaceae and native to south-central Europe, Iran, and Central Asia, but now naturalized in the Americas and elsewhere (Blumenthal, Goldberg, & Brinckmann, 2000). It grows to a maximum height of 70-150 cm (28-59 in). The leaves have a mild lemon scent like mint. During summer, small white flowers full of nectar appear. It is not to be confused with bee balm (genus *Monarda*), although the white flowers attract bees, hence the genus *Melissa* (Dousti, Ramchandani, Barkhordarian, Danaei, & Chiappelli, 2012).The leaves are used as an herb, in teas, and as a flavoring. The plant is used to attract bees for honey production. It is grown as an ornamental

plant and for its oil (to use in perfumery). The tea of lemon balm, the essential oil, and the extract are used in traditional and alternative medicine, including aromatherapy. The plant has been cultivated at least since the 16th century, but reliable medical research is still working to establish the safety and effects of lemon balm (Shan, 2005).

The emergence of morphological traits is a function of plant growth stage. They may be limited in number and are influenced by environmental factors or the developmental stage of the plant (Stuber, Polacco, & Senior, 1999). The shortcomings drove the development of markers based on DNA polymorphisms. These marker types have been superseded by DNA-based methods generate 'fingerprints', which are distinctive patterns of DNA fragments typically subjected to high resolution gel electrophoresis and detected by staining or labeling (Schulman, 2007). Random Amplified Polymorphic DNAs (RAPDs) markers can be defined as DNA polymorphisms produced due to 'rearrangements or deletions at or between two close primer binding sites in the genome'(Welsh & McClelland, 1990, Williams, Kubelik, Livak, Rafalski, & Tingey, 1990). Inter-simple sequence repeat(ISSR) markers are alternatives to the RAPD technique, allowing higher annealing temperatures to be used so it has better reproducibility compared to RAPD. Repeat-anchored or non-anchored primers in polymerase chain reaction (PCR) are used to amplify DNA sequences between two inverted SSR (Zietkiewicz, Rafalski, & Labuda, 1994).

Finding association between molecular markers and morphological traits provide an excellent tool for indirect selection of a trait of interest in the population. This has important applications to the study of relations between molecular markers and agronomic traits, some of which include: the detection and analysis of potential in specific genotypes, collections of germplasm, to identify desirable alleles and to validate of candidate markers linked to quantitative traits (Gebhardt, Ballvora, Walkemeier, Oberhagemann, & Schüler, 2004). Though the mapping Quantitative Trait Loci (QTLs) is well suited for detection of genes associated with the traits, but it is labor intensive, segregating population preparation is both time consuming and costly (Rakshit et al., 2010). To overcome these limitations, multiple regression analysis offer san appropriate method to identify markers associated with the trait. Multiple regression analysis is a statistical process for estimating the relationships among molecular markers as independent variables and morphological traits as dependent variables. It is the way to determine the coefficient of determination R^2; it gives the proportion of the variance (fluctuation) of dependent variable that is predictable from the independent variable(Gomez & Gomez, 1984).

According to study of Khadivi-Khub (2014) by multiple regression analysis, 33 SSR alleles and 135 RAPD fragments were found associated with 14 of affecting fruit traits. Some of SSR and RAPD markers were associated with more than one fruit trait in multiple regression analysis. Marsafari, Mehrabi, and Tahmasebi (2014) investigated the association of 11 morphological traits with molecular markers (ISSR and RAPD) in 15 cultivars of date palm. All regression models were significant for ISSR and RAPD marker and for all traits at 1% level. Of 294 DNA markers (162 ISSR markers and 132 RAPD markers), 173 markers (89 ISSR markers and 84 RAPD markers) with at least one of 11 traits of fruit, stone and tree performance characteristics in both marker systems showed association. Basaki et al. (2011) reported that 14 traits (excluding fruit shape, calyx type, Hull cracking sensitivity and skin color) showed significant association with 14 SSR bands. The association markers explained 2 to 29% of the variation for individual traits.

In this study, it was used multiple regression analysis to identify associations between ISSR and RAPD markers with some agronomic traits in lemon balm ecotypes.

Material and methods

Plant material

In this study, the seeds of 12 lemon balm ecotypes were provided by National Center for Genetic and Biological Resources of Iran, and each ecotype were planted (ten plants per plot) in randomized block design with three replications during 2015 year. Ecotype name, their parentage and releasing centers are given in (Table 1). The data of morphological characters of lemon balm ecotypes which were collected include: Days to 50% flowering (DF), Plant height (PH), Stem diameter (SD), Number of branches per plant (NB), Internode length (IL), Leaf length (LL), Leaf width (LW), Number of nodes (NN), Fresh weight (FW), Dry weight (DW) and Essential oil percentage (EOP).

DNA extraction

The first, second and third leaves from a branch at seedlings stage were used for the DNA extraction, according to the protocol of Murray and Thompson (1980). The quality and quantity of the extracted

DNA was checked using 1% agarose gel electrophoresis and spectrophotometer, respectively. 20 ISSR primers from the University of British Columbia-UBC (Canada), were applied and only 12 of them (Table 3) were amplified and showed polymorphism. Also, 15 RAPD primers were applied; only 10 of them (Table 3) were amplified and showed polymorphism.

Table 1. Geographical origins and code number of lemon balm.

Code	Collection region	Code	Collection region
G1	Tehran-Damavand	G7	Kerman
G2	Ardabil	G8	Esfahan-YazdAbad
G3	Esfahan-Najafabad	G9	South Khorasan-Sarbisheh
G4	Qazvin-1	G10	East Azerbaijan
G5	Fars	G11	Qazvin-2
G6	Hamedan	G12	Gilan-Lahijan

PCR amplifications for RAPD and ISSR were performed by using kits from Sina-Gene in 10 µL volume containing 2 µL of template DNA (5 ng μl^{-1}), 1 µL 10×PCR buffer, 1.2 µL of each primers (5 µM stock concentration), 0.6 µL dNTPs, 0.48 µL of $MgCl_2$ (50 mM), 0.14 µLTaq polymerase (5 U μL^{-1}) and 4.58 µL of sterile nano-pure H_2O. The PCR reaction was performed in a thermal cycler (Applied Biosystems, Germany) at an initial denaturation temperature of 94°C for 5 min, then 35 cycles of 94°C for 30 s, 55°C for 30 s (primer annealing of the most primers), 72°C for 2 min and final extension at 72°C for 5 min.The PCR products of the primers mentioned in the Table 3 were visualized by running at 75 W for 65 min on 1.5% agarose gel electrophoresis in 1 X TBE buffer system, followed by ethidium bromide (0.5 µg mL^{-1}) staining. Fragment size was estimated by using a 100 base pairs (bp) molecular size ladder.

Scoring and data analysis

Average values for all traits in this study were calculated for further analyses. The agronomic traits and molecular markers were considered as the dependent variables and independent variables, respectively. To verify the linear relationship between independent and dependent variables, predict the value of the dependent variable based on the independent variable, remove the variables with negligible effect on the dependent variables and fit the best regression model, stepwise regression was used. The probability level (P) for rejecting any association between a marker and an agronomical trait was 0.01.

Results and discussion

Minimum, maximum, average, standard deviation and coefficient of variation to phenotypic traits are shown in Table 2. According to Table 2, for all evaluated traits, considerable variation among ecotypes was observed. Most of the phenotypic variation among different traits related to 'DW' and 'EOP' traits in the amount of 23.23 and 20.01%, respectively. So, we can say about these traits that there are plant genetic resources for use in breeding programs to improve these traits. The lowest observed variation among traits was for 'DF'. Phenotypic variation coefficient ranged between 2.98 to 23.23% for different traits.

In this study, twelve ISSR and ten RAPD primers were used. The patterns of markers (RAPD and ISSR) were showed in Figure 2. ISSR markers produced a total of 151 bands, of which 106 bands were polymorphic and the average polymorphic loci per primer was evaluated 8.38. RAPD markers produced a total of 175 bands, of which 127 bands were polymorphic and the average polymorphic locus per primer was evaluated 12.7 (Table 3). Twelve ISSR primers created 106 polymorphic bands, among them, UBC813 with 16 bands, and UBC811, UBC815 and UBC817 primers with 15 bands (they had the highest number of polymorphic bands), and UBC825 with 8 bands (they had whish the lowest number of polymorphic bands). Polymorphism percentage ranged from 60 to 80% for ISSR markers with average 69.34%.

In addition, 10 RAPD primers, created 127 polymorphic among them OPA-01with 22 bands and BB13 and OC4 primers with 19 and 18 bands had the highest number of polymorphic bands and OS-03 and OB20 with 14 and 15 bands had the lowest number of polymorphic bands respectively. Polymorphism percentage in the landraces was obtained 71.77 for ISSR markers. Solouki, Mehdikhani, Zeinali, and Emamjomeh (2008) evaluated the genetic diversity of German chamomile ecotypes using 29 RAPD primers. Among the 369 revealed bands, 314 bands were polymorphic. Pirkhezri, Hassani, and Hadian (2010) evaluated 25 German chamomile ecotypes using 18 primers, RAPD and obtained 220 bands among them 93.1% were polymorphic. The average numbers of total and polymorphic bands were obtained 12.2 and 11.4 respectively. Heidary, Marashi, Farsi, and Kakhki (2009) using 4 AFLP primer combinations studied the genetic diversity of barberry and observed 223 bands of which 207 bands were polymorphic. Zhang, Xu, and Li (2010) investigated the genetic variation among *Glycyrrhiza uralensis* ecotypes in northern China using AFLP markers. In this study, 50 individuals from five ecotypes were used. Eight primer combinations totally produced 1025 band of which 57% were polymorphic.

Table 2. Descriptive statistics of studied traits in lemon balm.

Descriptive statistics	Traits										
	DF	PH	SD	NB	NN	IL	LL	LW	FW	DW	EOP
Average	93.67	82.84	3.77	47.64	19.67	5.91	4.48	3.38	484.60	122.26	0.16
Standard Error of average	0.81	2.00	0.13	1.09	0.56	0.14	0.10	0.08	8.13	8.20	0.01
Standard Deviation	2.79	6.94	0.44	3.78	1.94	0.49	0.33	0.27	28.17	28.40	0.03
Variance	7.80	48.15	0.19	14.26	3.76	0.24	0.11	0.07	793.32	806.63	0.00
Range	8.34	19.30	1.20	12.67	5.67	1.30	1.00	0.83	86.43	82.82	0.10
Minimum	89.33	72.00	3.09	41.00	17.33	5.34	3.97	2.93	443.75	88.05	0.12
Maximum	97.67	91.30	4.29	53.67	23.00	6.64	4.97	3.76	530.18	170.87	0.21
CV(%)	2.98	8.38	11.64	7.93	9.86	8.32	7.43	8.06	5.81	23.23	20.01

Table 3. ISSR and RAPD primers with corresponding bands scored with polymorphic bands, observed in lemon balm ecotypes.

	Primers	Primer sequence	No. of Polymorphic bands	Total bands	% Polymorphism	PIC	EMR	MI	Shannon	Nei	Ne
ISSR	UBC811	(GA)8C	12	15	80	0.33	9.6	3.2	0.62	0.43	1.76
	UBC812	(GA)8A	9	14	64.29	0.36	5.79	2.08	0.66	0.47	1.89
	UBC813	(CT)8T	14	16	87.50	0.36	12.25	4.38	0.66	0.47	1.88
	UBC814	(CT)8A	8	12	66.67	0.30	5.33	1.61	0.56	0.38	1.64
	UBC815	(CT)8G	10	15	66.67	0.32	6.67	2.15	0.60	0.41	1.71
	UBC816	(CA)8T	7	10	70	0.36	4.9	1.77	0.66	0.47	1.9
	UBC817	(CA)8A	10	15	66.67	0.35	6.67	2.37	0.65	0.46	1.87
	UBC823	(TC)8C	7	11	63.64	0.37	4.45	1.66	0.69	0.50	1.98
	UBC824	(TC)8G	6	10	60	0.37	3.6	1.33	0.68	0.49	1.95
	UBC825	(AC)8T	5	8	62.50	0.35	3.13	1.11	0.65	0.46	1.86
	UBC826	(AC)8C	8	11	72.73	0.34	5.82	1.98	0.63	0.44	1.79
	UBC876	(AC)8A	10	14	71.43	0.31	7.14	2.21	0.57	0.39	1.66
Average			8.83	12.58	69.34	0.34	6.28	2.15	0.64	0.45	1.82
RAPD	OB20	5' GGACCCTTAC 3'	9	15	60	0.34	5.4	1.85	0.63	0.44	1.8
	OH-04	5' GGAAGTCGCC 3'	11	17	64.71	0.36	7.12	2.56	0.66	0.47	1.9
	OA12	5' TCGGCGATAG 3'	14	18	77.78	0.36	10.89	3.87	0.66	0.46	1.87
	BB13	5' TTCCCCCGCT 3'	17	20	85	0.37	14.45	5.28	0.67	0.48	1.93
	SA-R	5' AGGTCACTGA 3'	10	16	62.5	0.34	6.25	2.10	0.62	0.43	1.77
	CS-56	5' TGGTGGGTCC 3'	12	16	75	0.31	9	2.82	0.58	0.40	1.72
	OC4	5' CCGCATCTAC 3'	15	19	78.95	0.37	11.84	4.34	0.68	0.48	1.94
	OS-03	5' CAGAGGTCCC 3'	9	14	64.29	0.36	5.79	2.08	0.66	0.47	1.9
	OPA-01	5' CAGGCCCTTC 3'	17	22	77.27	0.36	13.14	4.7	0.66	0.47	1.88
	OPC-04	5' CCGCATCTAC 3'	13	18	72.22	0.35	9.39	3.32	0.65	0.46	1.88
Average			12.7	17.5	71.77	0.35	9.33	3.29	0.65	0.46	1.86

Polymorphic information content (PIC) is the equivalent of genetic diversity and shows the resolution of a marker by the number of polymorphic alleles and the frequency of these alleles in the studied population. Polymorphic information content, calculated separately for each primer and the results are presented in Table 3. Polymorphic information content (PIC) ranged from 0.33 to 0.37 and the average of polymorphic information content was calculated 0.34 for ISSR markers. Also, polymorphic information content (PIC) ranged from 0.31 to 0.37 and the average of polymorphic information content was calculated 0.35 for RAPD markers. The highest PIC for ISSR markers were calculated 0.37 for UBC823 and UBC824 primers and for RAPD markers were calculated 0.37 for BB13 and OC4 primers indicating a high efficiency of these markers in differentiating the landraces used in this research. In order to determine the efficiency of markers in showing the polymorphism, MI and EMR were calculated. Among ISSR markers, the highest amount EMR was calculated for UBC813 (12.25) and the lowest one was observed in UBC825 (3.13). Marker index (MI) ranged from 1.11 to 4.38. Among RAPD markers, the highest amount EMR was calculated for BB123 (14.45) and the lowest one was observed in OB20 (5.40). Marker index (MI) ranged from 1.85 to 5.28. Pirkhezri et al. (2010) evaluated 25 German chamomile ecotypes using 18 primers, RAPD evaluated number of effective alleles, Nei's gene diversity and Shannon index for different provinces. The maximum and minimum amount for the number of effective alleles was observed in Khuzestan province (1.657) and Fars province (1.142) respectively. Nei's gene diversity and Shannon index was higher in the ecotypes of Khuzestan province (Nei= 0.528; Shannon= 0.364) and in the ecotypes of Fars province was less than others (Nei= 0.23; Shannon= 0.16).The number of effective alleles was different among the studied markers. The average number of effective alleles was calculated 1.84 in the population and ranged between 1.64-1.98. UBC823, and UBC824, BB13, OC4 had the highest number of effective alleles among the all landraces. Since the number of

effective alleles is one of the important criteria in the selection of appropriate and useful primers, these primers could be used to investigate the genetic diversity of lemon balm ecotypes for the future studies.

The results of stepwise regression analysis revealed a significant association between the traits and some of the studied loci (Table 4). In stepwise regression traits and markers were considered as the dependent variable and the independent variables, respectively. A total of 90 markers (alleles) that were significantly correlated and associated with studied traits, entered the model which some of the markers were involved of few traits and finally 71 markers were effective in phenotypic variation of traits. Other markers have no significant effect on model and therefore we can say that these correlated markers can be used to identify superior genotypes in terms of studied traits. The markers identified were varied from two markers for number of nodes to 11 markers for fresh weight and dry weight. These markers were negative or positive correlated to traits. Other researchers are using regression analysis to identify the relationship between markers and studied traits and used them in breeding program (Rakshit et al., 2010, Basaki et al., 2011, Khadivi-Khub, 2014, Marsafari et al., 2014, Ipek, Seker, Ipek, & Gul, 2015).

Table 4. Stepwise regression analysis of traits (dependent variable) and RAPD and ISSR markers (independent variables).

Traits	Number of informative markers	Informative markers (band or fragment)	R^2 adjusted (%)
DF	7	OPA-01-9, OPA-01-15, OC4-6, OPA-01-1, SA-R-5, SA-R-2, BB13-15	0.930
PH	9	UBC813-5, UBC876-2, BB13-11, UBC813-10, UBC815-8, UBC824-6,OPC-04-13, OC4-6, OPA-01-11	0.989
SD	9	OB20-3, UBC813-13, BB13-14, UBC814-6, UBC812-4, UBC815-5, OS-03-4, OA12-9, UBC826-5	0.974
NB	9	OPC-04-1, SA-R-2, UBC816-4, UBC823-7, UBC813-10, SA-R-4, OC4-15, UBC815-5, UBC812-3	0.961
NN	2	UBC876-10, UBC814-5	0.844
IL	8	OPA-01-9, OPA-01-10, UBC826-2, UBC815-1, OC4-12, OA12-3, OC4-14,UBC823-3	0.981
LL	8	UBC814-1, CS-56-4, UBC823-7, OPC-04-4, OPA-01-4, UBC816-1, UBC813-14, UBC816-4	0.954
LW	8	OS-03-3, UBC811-6, UBC811-7, OC4-6, OC4-5,UBC826-5, UBC876-8, UBC811-2	0.983
FW	11	SA-R-2, UBC824-5, UBC823-1, SA-R-4,OPA-01-1, OH04-9, SA-R-5,UBC876-7, UBC815-2, UBC815-10, UBC817-2	0.985
DW	11	SA-R-2, OPA-01-1, UBC824-5, OA12-9, UBC824-2, OPC-04-2, OB20-1, BB13-12, OPA-01-17, OS-03-5, OPA-01-8	0.992
EOP	8	SA-R-10, UBC826-1, UBC812-9, UBC813-10, UBC825-4, OPA-01-15, OPC-04-7, CS-56-8	0.998

Markers OPA-01-9, OPA-01-15, OC4-6, OPA-01-1, SA-R-5, SA-R-2 and BB13-15 were associated with Days to 50% flowering and could justify 93% of the phenotypic variation. Also, the SA-R-10, UBC826-1, UBC812-9, UBC813-10, UBC825-4, OPA-01-15, OPC-04-7 and CS-56-8 markers showed a significant correlation with Essential oil percentage and controlled 99.8% of the phenotypic variation (Table 4). Calculating standard β can be demonstrated the importance markers of each trait. SA-R-2 marker was the most important markers for dry weight and revealed an increasingly effect. Also, OPC-04-1 marker was an important marker for the Number of branches per plant and had depressing effect (Table 4).According to the standardized β coefficients, some alleles lowering effect and some alleles enhancing effect of the studied traits (Table 4). So, based on the traits type that the increase or decrease of traits are interested, considering to the coefficient standardized β, increasing or decreasing markers can be used to increase or decrease traits in breeding programs.

Identification markers for traits over other markers can be involved in coding region of these traits because entered in the regression model and were explanation of traits variations and showed more variation of these traits. Some of these markers were associated with more than one trait. According to a significant correlation between morphological traits, it can be seen that some of these had very close linkage together or possibly were controlled by pleiotropic effects. Important advantages of association analysis are that in this method, there isn't needed to prepare segregating population which requires more time, although it is better to use a multi-year phenotypic data. On the other hand, crossing over that occurs during preparation of segregating populations, is limited that allows precise positioning does not provide. The efficiency of these methods has been shown for identifying and mapping of controlling gene of Mendelian traits (Breseghello & Sorrells, 2006). It also uses informative markers associated with traits; especially markers are known chromosomal location can be an effective step taken in the initial selection genotypes with high yield. It also can be isolated and cloned the bands of informative markers have been identified that have high R2 from the gel. Then the identified sequences were alignment in databases with existing sequence and candidate genes, which are very similar to markers, were identified. It can also use over the desired sequence to designed primers (SCAR) for interesting traits and be used in marker-assisted selection in breeding programs.

Conclusion

Informative fragments could be successfully cloned and sequenced for polymorphic diagnostic. It is to be hoped that, some of these markers will be used for MAS in future lemon balm breeding programs. In breeding programs, on which crossing between more genetically distant individuals will increase the chance of transgressive segregation in their progeny Therefore, these markers could be used to choose parents for development of the mapping populations. RAPD primer OPC-04-1 and ISSR primer UBC813-5showed fragments with the highest association with the traits. These primers have been found useful for the study of the genetic diversity and association analyses in lemon balm.

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