

First Report of a set of Genetic Identities in *Prunus* Rootstocks by SSR Markers

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ABSTRACT: *Prunus* rootstocks play an important role in modern horticulture and commercial orchards owing to their responsibility for a wide range of characters from compatibility with cultivars to adaptation to biotic and abiotic stresses. In this study, thirty *Prunus* rootstock samples were tested by 25 microsatellite markers in order to identify the genetic identity and relationships among them. 17 SSR markers were useful in the discrimination of the samples on the basis of their unique molecular identities. Samples with similar codes such as (HS-401/HS-402/HS-403), (HS811/HS507/HS737/GF677), (HS126/HS-202), (HS-802/HS602) and (HS522/HS003/HS302) were shown mislabeled trees. Based on partial repeated bisection (RB) data, the samples were grouped into six clusters which the largest cluster contained nine genotypes (all APPL, APU2 and APPU3). The second largest cluster consisted of eight genotypes (all AM, all APL, APU1, APU3 and APH10). APH rootstocks were placed into clusters two, three and six as well as cluster one which included only APPU rootstocks. The highest amount of the average internal similarities (Isim) (0.973) belonged to cluster six, whereas the minimum amount of Isim (0.924) belonged to cluster three. The minimum level of the average external similarities (Esim) was related to groups one (0.664) and six (0.638) indicating, the highest genetic distance from other groups. The genetic identities and relatedness generated in this study provide a standard for further breeding attempts and will be used as a reference the cultivation of these promising newly released genotypes.

KEYWORDS: Promising *Prunus* rootstock, Genetic identity, SSR

INTRODUCTION

Prunus is a genus of more than 400 species of flowering trees and shrubs from the northern regions of the globe. The genus *Prunus* including the cultivated almonds, peaches, plums, cherries, nectarines, and apricots has great economic importance (11). Iran contains a wide biodiversity of various fruit species as well as a large area under

cultivation (48100 hectares) and production (201759 metric tons) of stone fruits, ranking the first producer of *Prunus* species worldwide (8). In nature, there are several species of *Prunus* and their interspecific hybrids which are called rootstocks. Rootstocks have plentiful advantages regarding adaptation to different soil and climatic conditions, diseases

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and insect resistance, the promotion of earlier fruit production and controlling tree growth (2).

In addition, rootstocks obtained from desirable species of *Prunus* are needed for producing commercial orchards. In Iran, the cultivation of this genus as rootstocks is common among commercial orchards because of the ease of the interspecific hybridization and many advantages that are related to parents such as substantial resistance or tolerance to biotic and abiotic stresses (9). On the other hand, cultivar mislabeling (e.g. synonym or homonym trees) and none targeted interspecific crosses within *Prunus* species can dramatically influence on the orchard performance. However, an accurate characterization and discrimination of *Prunus* rootstocks is required in the effective control and utilization of the materials in breeding programs. Traditionally, rootstocks identification relied on morphological traits which were very difficult task because of its high susceptibility to environmental factors and the developmental stage of plants (4). Thus, molecular fingerprinting procedures are becoming practical necessities for the recognition of promising *Prunus* rootstocks and the preparation of genetic identities as a reference to establish more efficient orchards. In the last decades, many different molecular markers such as RAPD (14, 21); ISSR (3, 8); SCAR (12, 14); AFLP (13, 17, 18) and SSR (5, 6, 7, 10, 12, 22, 23) have been extensively used in *Prunus* rootstocks in order to the germplasm identification and evaluation of the genetic variability and relationship. Among these molecular markers, microsatellites are considered as the most reliable molecular marker systems which are used for management and diversity analysis in fruit trees due to their high levels of polymorphism suitable for DNA fingerprinting analysis, high degrees of transferability and reproducibility as well as the co-dominant mode of inheritance (20). However, there is no accurate report on generating the molecular identities of *Prunus* rootstocks. With regard to the phylogenetic studies of *Prunus* rootstocks, the relationships among 29 *Prunus* spp. rootstocks were evaluated resulting in 2 subgroups of *Prunophora* or *Amygdalus* using SSR markers (1). The genetic relationships among 44 clones of

Prunus rootstock were investigated representing the three groups as peach-based rootstocks, Myrobalan-Marianna plums and slow growing plums (2). The goal of this study was 1) to report the first set of unique genetic identities in Iranian *Prunus* rootstocks and 2) to investigate the phylogenetic relationships among the *Prunus* hybrids in order to maintain and conserve the trees and prevent them from being mislabeled in future studies.

MATERIALS AND METHODS

Plant material

The thirty genotypes used in this study were obtained from the germplasms collection kept at the Research Institutes of Tabriz and Karaj in Iran (Table 2). Genomic DNA was extracted from young leaves using Genomic DNA Isolation Kit (CoreBio, Korea). DNA purity and concentration were both checked on 1% (w/v) agarose gels and with a NanoDrop® ND-1000 Spectrophotometer, respectively.

Polymerase chain reaction

We used 25 labeled microsatellite markers which were previously studied by Zeinalabedini et al., (2014) to determine the genetic identities and relationships among collected samples (Table 1). PCR amplification was carried out in the total volume of 15µl containing 1 µl with 10 ng genomic DNA, 0.25 µM of each primer, 1 mM of 40 mM dNTP mix, 0.15 U Taq DNA polymerase, 0.9 mM MgCl₂, and 1 µl 1× PCR buffer. The PCR program contained 1 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 57°C and 2 min at 72 °C, followed by a 4 min extension at 72°C. Amplified PCR products were visualized by electrophoresis on 6.5% acrylamide using DNA analyzer (LI-COR 4300, USA).

SSR marker analysis

To obtain molecular identity for each sample, the alleles were coded for each microsatellite locus. For instance, if a microsatellite marker had three polymorphic alleles, the presence of first allele was given a score of 1, while a 0 denoted its absence. Next, the presence of the second and

Table 1. The characteristic and variability parameters of 17 SSR markers used in this study

Marker name	Sequence (5'→3')	N	H _e	H _o	PIC	Linkage group	Band size (bp)	Reference
UDP96-001	AGTTTGATTTTCTGATGCATCC TGCCATAAGGACCGGTATGT	10	0.856	0.853	0.840	G1	172	Testolin <i>et al.</i> , 2000
UDP96-003	TTGCTCAAAAAGTGTGCGTTGC ACACGTAGTGCAACTGCGC	10	0.856	0.910	0.840	G1	143	Testolin <i>et al.</i> , 2000
UDP96-010	CCCATGTGTGTCCACATCTC TTGATGATCCATGCGTCTC	14	0.906	0.231	0.898	G4	159	Testolin <i>et al.</i> , 2000
UDP96-015	CCTTGACCTATTTGTTGTCGTA ACTAGTCAAACAATCCCCCG	7	0.813	0.00	0.789	G2	131	Testolin <i>et al.</i> , 2000
UDP96-019	TTGGTCATGAGCTAAGAAAACA TAGTGGCACAGAGCAACACC	16	0.334	0.573	0.902	G4	229	Testolin <i>et al.</i> , 2000
UDP98-408	ACAGGCTTGTGAGCATGTG CCCTCGTGGGAAAATTTGA	11	0.881	0.202	0.869	G2	132	Testolin <i>et al.</i> , 2000
CPPCT006	AATTAACCTCAACAGCTCCA ATGGTTGCTTAATTCAATGG	7	0.800	0.865	0.755	G1	253	Aranzana <i>et al.</i> , 2002
CPPCT017	TGACATGCATGCACTAAACAA TGCAAATGCAATTCATAAAGG	2	0.902	0.033	0.894	G4	96	Aranzana <i>et al.</i> , 2002
BPPCT001	AATTCCCAAAGGATGTGTATGAG CAGGTGAATGAGCCAAAGC	3	0.355	0.685	0.326	G2	190	Dirlewanger <i>et al.</i> , 2002
BPPCT002	TCGACAGCTTGATCTTGACC CAATGCCTACGGAGATAAAAAGAC	10	0.884	0.775	0.873	G8	177	Dirlewanger <i>et al.</i> , 2002
BPPCT005	GCTAGCAGGGCACTTGATC ACGCGTGTACGGTGGAT	9	0.864	0.887	0.849	G7	132-148	Dirlewanger <i>et al.</i> , 2002
BPPCT010	AAAGCACAGCCCATAATGC GTACTGTTACTGCTGGGAATGC	10	0.856	0.966	0.840	G6	210	Dirlewanger <i>et al.</i> , 2002
BPPCT024	GAGGAATGTGCCTCTTCTGG CTCCCGTACGCGTTTACC	14	0.909	0.898	0.296	G1	163	Dirlewanger <i>et al.</i> , 2002
M1a	CACGAGGCGCCATTCTACG GTACGACGGGTTTGGCTCA	8	0.802	1.00	0.777	G4	179	Yamamoto <i>et al.</i> , 2002
AM121	TGGTGTGGTGTGTTGTTTGAAG ACCAACTCCATCCACATTTCTC	14	0.889	0.955	0.878	G1	122-140	Hagen <i>et al.</i> , 2004
aprigms18	TCTGAGTTCAGTGGGTAGCA ACAGAATGTGCGTTGCTTTA	13	0.88	0.606	0.874	G6	124-144	D. A. Lalli, 2008
pchgms2	GTCAATGAGTTCAGTGTCTACACTCAA TCATAACATCATTACGCCACTGC	9	0.75	0.853	0.727	G4	122-142	Sosinski <i>et al.</i> , 2000
Mean	-	14	0.805	0.66	0.790	G3	-	-

locus 1				Identification code
Allele 1	Allele 2	Allele 3	Code	
0	2	3	023	023-0030-000
1	2	3	123	123-0030-000
0	0	3	003	003-1030-000
1	2	0	120	120-1030-000
0	2	0	020	020-1000-000
0	0	0	000	000-1000-000
1	0	0	100	100-1000-000

Figure 1. The state of Polymorphic allele encoding of a sample for one locus and the final identification codes for multiple samples.

third alleles were scored 2 and 3 respectively, whereas their absence was 0. The final identification code was characterized based on putting together all codes of microsatellite loci related to each sample (Figure 1). Genetic relationships among *Prunus* rootstocks were evaluated by Partial repeated bisection (RB) cluster analysis (15). RB analyses from $K = 1$ to $K = 10$ clusters were conducted using the software GCluto version 1.0. Simfun (similarity function) was cosine and the number of bootstrap replications was 1,000. For a given K , the clustering solution was evaluated in terms of firstly solution stability and cluster stability, where stability refers to the level of consistency observed across various bootstrap sub-samples of original data ranging between 0 and 1 and secondly the direct observation of higher Isim (Internal similarity) and lower Esim (External similarity) values.

RESULTS

Microsatellite analysis

Based on SSR results, 17 out of 25 SSR markers were successfully used for the identification of genetic identities and relationship among 30 promising *Prunus* rootstock genotypes (Table 2). A total of 171 alleles with an average of 10 per locus were detected. The number of alleles ranged from 2 (for primer CPPCT006) to 16 (for primer UDP96015). Average observed heterozygosity (H_o) was 0.66 per locus ranging from 0 (UDP96015) to 1 (Mla) and

expected heterozygosity (H_e) ranged from 0.79 (UDP96015) to 0.90 (BPPCT010) with an average of 0.79 per locus. PICs ranged from 0.29 (BPPCT010) to 0.90(UDP96015) with an average of 0.77 per locus. Primers UDP96015, CPPCT006 and UDP96003 had the highest PIC values, indicating they were the most informative loci. The results of molecular barcoding are presented in Table 2. Each genotype was exclusively coded based on the most informative primer(s). Figure 2 shows the fingerprints of samples in accordance with the UDP96-015 marker. It is noticed that the SSR markers in the present study were potent molecular tools to detect and discriminate these candidate rootstocks. In addition, most of the genotypes produced unique molecular identities which indicated that the selection and maintenance of trees had been performed accurately except for the samples with similar codes such as (HS-401/HS-402/HS-403), (HS811/HS507/HS737/GF677), (HS126/HS-202), (HS-802/HS602) and (HS522/HS003/HS302) represented as mislabeled trees one of which had to be excluded from the germplasms for future studies.

Cluster analysis

The dendrogram generated from RB cluster analysis divided the 30 *Prunus* rootstock samples into six clusters (Figure 3). Based on the matrix visualization, the largest cluster separated nine genotypes (all APPL, APU2 and APPU3) from the remaining 21 genotypes. The second largest cluster separated eight genotypes (all AM, all APL, APU1, APU3 and APH10). Clusters two, three and six contained only APH rootstocks. Also, cluster one consisted of only APPU rootstocks (Figure 3a,b). In accordance with the mountain visualization analysis, the maximum amount of Isim (0.973) belonged to cluster six, whereas the minimum amount of Isim (0.924) belonged to cluster three. Clusters one and six containing the minimum levels of Esim that is 0.664 and 0.638 respectively, showed the highest genetic distance from other groups (Figure 3c). Our data indicated that the grouping of the *Prunus* rootstocks had concordance with the genealogical data, especially for the genotypes of APH and APPU which were far from each other and close to the other rootstocks of the relatively same species.

Table 2. Molecular identities generated from different primers for 30 *Prunus* rootstocks

No	Sample	Origin	Identification code	Primer name
1	HS703	Almond×Myrobalan (AM1)	00000600000	UDP96-019
2	HS721	Almond×Myrobalan (AM2)	00005000900000	UDP96-003
3	HS704	Almond×Myrobalan (AM3)	003400000	aprigms18
4	HS811	Almond×Peach (APH1)	100-02	CPPCT017+UDP96-015
5	HS-402	Almond×Peach (APH2)	100-00	CPPCT017+UDP96-015
6	HS507	Almond×Peach (APH3)	100-02	CPPCT017+UDP96-015
7	HS-401	Almond×Peach (APH4)	100-00	CPPCT017+UDP96-015
8	HS-403	Almond×Peach (APH5)	100-00	CPPCT017+UDP96-015
9	HS737	Almond×Peach (APH6)	100-02	CPPCT017+UDP96-015
10	GF677	Almond×Peach (APH7)	100-02	CPPCT017+UDP96-015
11	HS314	Almond×Peach (APH8)	100-10	CPPCT017+UDP96-015
12	HS126	Almond×Peach (APH9)	020-02	CPPCT017+UDP96-015
13	HS-202	Almond×Peach (APH10)	020-02	CPPCT017+UDP96-015
14	HS503	Almond×Peach (APH11)	00300070000	UDP96-019
15	HS-802	Almond×Plum (APL1)	00000000100000	BPPCT010
16	HS602	Almond×Plum (APL2)	00000000100000	BPPCT010
17	HS412	Apricot×Plum (APPL1)	1000000090	BPPCT005
18	HS407	Apricot×Plum (APPL2)	1000060000-1030000	BPPCT001+UDP96-010
19	HS409	Apricot×Plum (APPL3)	1030000-1000000090	UDP96-010+BPPCT002
20	HS304	Apricot×Plum (APPL4)	00005600	BPPCT024
21	HS411	Apricot×Plum (APPL5)	1000000	UDP98-408
22	HS706	Apricot×Plum (APPL6)	1000000090	BPPCT002
23	HS405	Apricot×Plum (APPL7)	1000060000-1000000	BPPCT001+UDP98-408
24	HS522	Apricot×Prune (APPU1)	0034000000	UDP96-001
25	HS003	Apricot×Prune (APPU2)	0034000000	UDP96-001
26	HS117	Apricot×Prune (APPU3)	000000080011000	CPPCT006
27	HS302	Apricot×Prune (APPU4)	0034000000	UDP96-001
28	HS702	Almond×Prune (APU1)	00300008000000	pchgms2
29	HS403	Almond×Prune (APU2)	000000009001200	M1a
30	HS-702	Almond×Prune (APU3)	00005000001100	AM121

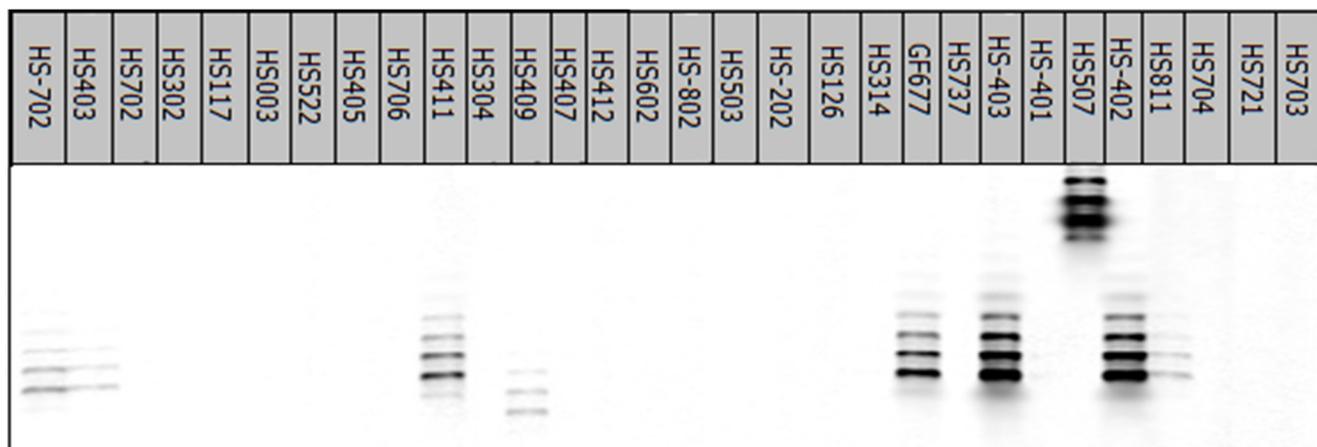


Figure 2. The fingerprints obtained from the UDP96015 marker for preparation of genetic identity

DISCUSSION

One of the main difficulties which orchards growers encounter in Iran is being uncertain of genetic backgrounds of the cultivated stone fruit trees. In many cases, although growers spend much time and high costs of cultivation for a specific cultivar, they suffer from irreversible damages considering the fact that the related cultivar is not the desired one. This causes an extensive non-uniformity of the trees resulting in the reduction of performance in the orchards. Therefore, the preparation of genetic identities of *Prunus* rootstocks makes it possible to produce uniform commercial orchards with more superior germplasms. In this report, 30 *Prunus* rootstock samples were analyzed by 25 microsatellite markers in order to assess the genetic identities and genetic relatedness of these samples.

With respect to PCR analysis, 17 SSR markers indicated that the average number of alleles and the expected heterozygosity were (10) and (0.79), respectively, presenting higher mean values compared to that of reported for SSRs in *Prunus* number of alleles and high level of heterozygosity confirmed the potential of SSR markers to develop genetic resources of *Prunus* rootstocks genotypes. On the basis of the high polymorphism obtained from the present study, it could be assumed that the transferability of SSR markers is applicable for molecular identification of other *Prunus* rootstock species as well. RB analysis showed that the all rootstocks were classified into 6 groups, indicating that the classification of *Prunus* rootstocks based on SSR markers was apparently dependent on their

geological origin. Moreover, the capability of SSR markers for clustering the *Prunus* rootstocks was in agreement with the previous studies reported on cultivated almonds (*Prunus dulcis*) by Zeinalabedini et al. (22) and on peach rootstocks by Bianchi et al. (1). Furthermore, the assessment of genetic relationships among *Prunus* rootstocks based on DNA-based markers can provide proper data for their management and utilization in future rootstock breeding programs (19).

The result of fingerprinting indicated the unique barcodes for each genotype except (HS-401/HS-402/HS-403), (HS811/HS507/HS737/GF677), (HS126/HS-202), (HS-802/HS602) and (HS522/HS003/HS302) with the same barcode which should be removed from further breeding programs. In recent years, few efforts have been made to understand the genetic identity of some stone fruit trees such as almonds and sweet cherries (12) using molecular markers. However, this study presented the first genetic identity of Iranian *Prunus* rootstocks as a standard aimed at improving the management and selection of these promising newly released rootstocks obtained from the present study to guarantee the uniformity in the new orchards and the potentially usefulness of these markers for further breeding researches. In addition, our data provide evidence to protect plant variety patents for breeders and growers can be more confident in their purchases since there is a method to identify and confirm rootstocks in their orchards (11, 16).

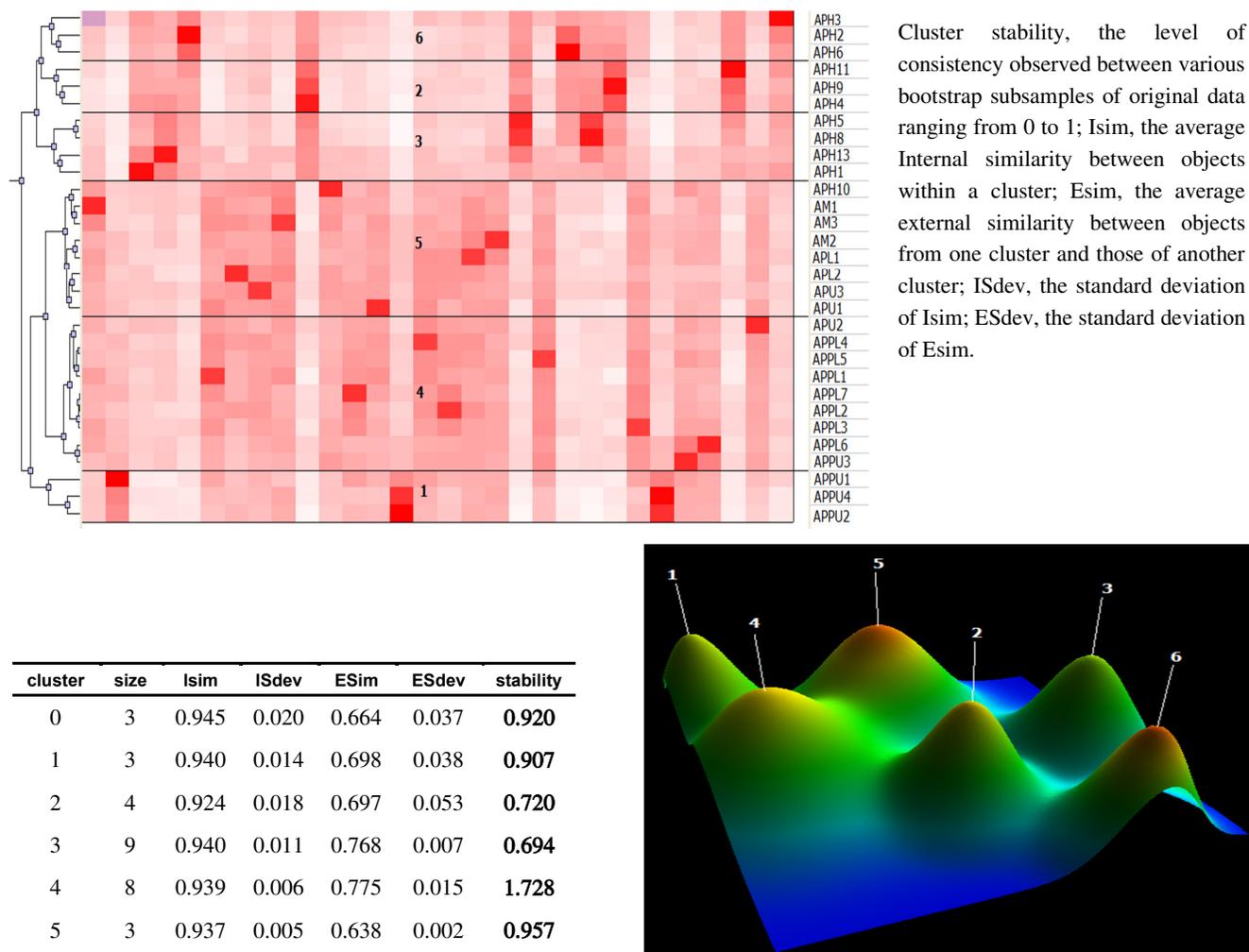


Figure 3. Agglomerative RB clustering method of 30 Iranian *Prunus* rootstocks. GCluto software generates two different visualizations as matrix (A) and mountain (B) for investigating genetic relationship among genotypes.

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معرفی اولین شناسنامه ژنتیکی در دوره های پرونوس با استفاده از نشانگرهای ریزماهواره

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چکیده

دوره های پرونوس نقش مهمی را در باغبانی مدرن و باغداری تجاری به دلیل سازگاری بالای ارقام دوره به تنش های زیستی و غیر زیستی ایفاء می کنند. در این مطالعه، ۳۰ دوره پرونوس با استفاده از ۲۵ نشانگر ریزماهواره جهت تعیین شناسنامه ژنتیکی و بررسی روابط ژنتیکی مورد مطالعه قرار گرفتند. بر اساس نتایج بدست آمده، ۱۷ نشانگر ریزماهواره قدرت بالایی را جهت تمایز نمونه ها بر اساس شناسنامه های مولکولی منحصر به فرد خود نشان دادند. نمونه ها با کد های شناسائی مشابه نظیر (HS401/HS-402/HS-403)، (HS811/HS507/HS737/GF677)، (HS126/HS-202)، (HS802/HS602) و (HS522/HS003/HS302) به عنوان درخت هائی که به طور اشتباه نام گذاری شده اند، شناسائی شدند. داده های حاصل از آنالیز RB نشان داد که نمونه ها در ۶ گروه دسته بندی شدند. بزرگترین گروه شامل ۹ ژنوتیپ (APU2، APPL، APPU3) و دومین گروه بزرگ شامل ۸ ژنوتیپ (AM، APL، APU1، APU3 و APH10) بود. دوره های APH در گروه های ۲، ۳ و ۶ قرار گرفتند و دوره های APPU تنها در گروه ۱ جای گرفتند. بیشترین و کمترین میزان متوسط شباهت داخلی به ترتیب برابر با ۰/۹۷۳ و ۰/۹۲۴ مربوط به گروه های ۶ و ۳ بود. کمترین میزان متوسط شباهت خارجی در گروه ۱ و ۶ به ترتیب به میزان ۰/۶۶۴ و ۰/۶۳۸ دیده شد که بیانگر بیشترین فاصله ژنتیکی در میان نمونه ها گزارش شد. بنابراین، از نتایج بدست آمده از پژوهش حاضر می توان به عنوان یک منبع جهت مطالعات اصلاحی بعدی و کشت و کار دوره های امید بخش در آینده ای نزدیک استفاده کرد.

کلمات کلیدی: دوره های پرونوس، شناسنامه ژنتیکی، ریزماهواره