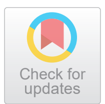

Genetic diversity and nucleotide sequence analysis of *powdery mildew* marker and *Vf2RAD* resistant gene in apple *(Malus domestica)* land races

Análisis de diversidad genética y secuencias de nucleótidos del marcador *mildi polvoso* y del gen de resistencia *Vf2RAD* en variedades locales de manzana *(Malus domestica)*

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SCIENTIFIC RESEARCH

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ABSTRACT

Introduction: DNA sequencing-based methods and nucleotide sequence analysis have become the most common molecular approaches currently used for molecular typing purposes and phylogenetic diversity analysis. **Methods:** In this study, the nucleotide sequence variations of Powdery mildew resistance gene marker (CH03c02) and the apple scab resistance gene (*Vf2RAD)* beside phylogenetic diversity of seven apple landraces have been investigated. The two-locus have been successfully cloned and their nucleotide sequences were determined across all studied landraces. **Results:** Results of sequence alignment of the Powdery mildew resistant locus (CH03c02), compared with that of the published sequence of the same locus of Discovery genotype (HiDRAS), revealed that the nucleotide variations of this locus ranged from 1 to 28 nucleotide substitutions across all seven apple landraces. Whilst, the nucleotide variations of *VF2RAD* ranged from 2-8 nucleotide substitutions across all the investigated landraces. The highest genetic distance (0.062) was between Amara and Barwari. Whereas, the lowest genetic distance (0.0015) was found between each of the Lubnani, Rechard, Ispartal, and the Ahmadagha. The nucleotide sequences of the two loci were concatenated and implemented to build a Neighbor-Joining tree. The seven apple landraces were successfully grouped into two main genetic clusters (C1 and C2) in the phylogenetic tree. **Conclusions:** It can be concluded that the cloning approach used in the current study was found to be very successful and helpful for obtaining the full nucleotide sequences of these two loci. The investigated loci were displayed nucleotide variations among the studied landraces. And, finding of these variations was allowed the distinguishing and discrimination of these landraces.

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INTRODUCCTION

Apple (*Malus domestia*) is considered as one of the most widely distributed and valuable horticultural fruit trees planted in the world due to its wide diversity as well as adaptability to various climatic conditions from high latitude to subtropical areas [1, 2, 3]. Researchers believe that apple is a hybrid species from different progenitor species including; *Malus sieversii*, *Malus orientalis*, *Malus sylvestris*, and *Malus prunifolia*, and its domestication is likely to have occurred many times throughout history [4]. A large diversity, which is the characteristic of the genus *Malus*, might be due to many reasons such as the accumulation of somatic mutations over time which also has been further enhanced by human activities over a long history of cultivation [5]. Estimation of the genetic diversity and phylogenetic relationships among genotypes are important for long-term crop improvement, classification, utilization of germplasm resources and the reduction of plants vulnerability to important crop stresses as well as for breeding purposes [6, 7]. In this point of view, some of the resistant local cultivars (landraces) could serve as a source of biodiversity and many desirable genes which might be used in breeding programs as crossing partners in order to transfer the polygenic resistance and development of new cultivars [8, 9, 10, 11].

In nature, plants are attacked by many viruses, bacteria, fungi, parasitic plants, nematodes and insects either directly or in directly. Until now, different resistance genes for apple scab have been isolated and cloned for selection and breeding purposes. The first molecular markers related to the apple resistance gene *Vbj* (apple scab resistance gene) originating from the crab apple *M. baccata* jackii were presented by [12]. Breeding programs were enriched when resistant genes tos-cab and powdery mildew identified in Czech apple [13].A new apple scab resistance gene *VT57* have been identified from Golden Delicious or Red Dougherty [14]. *HcrVf* type candidate genes have been also identified in a range of *Malu* cultivars using sequence information from the cloned *HcrVf* genes (receptor-like protein) [15].

Cloning and characterization of herbicide resistant, pest resistant, drought and salt tolerance as well as disease resistant genes have been performed in a number of plant species, including both mono- and dicotyledonous plants [16]. Now a days, direct DNA sequence analysis is the most common approach used for investigation of the biodiversity and molecular typing studies [17]. The main objectives of the current study were to identify of two important resistant loci, one locus is a marker for powdery mildew resistance gene (CH03c02) and the other for apple scab resistance gene (*VF2RAD*) among locally cultivated apple landraces. Also, cloning and nucleotide sequence analysis of these tow loci to estimate the nucleotide variations of these two loci among apple landraces as well as assessing the genetic diversity and phylogenetic relationships among them.

MATERIALS AND METHOD

Location of study and Samples collection

The research was carried out in two major parts. The first part including sample collection and DNA extraction was performed at Scientific Research Center laboratories/ College of Science/ University of Duhok from May 2012 to July 2014. The second part was performed at the Faculty of Biology, Medicine and Health laboratories/ Manchester University, UK from September 2014 to September 2016. Apple leaves of landraces varieties were obtained from the Ministry of Agriculture field in Duhok Governorate in Kurdistan region of Iraq. These varieties included (Lubanani, Rechard, Ispartal, Ahmad axa, Amara, Kanisarki and Barwari).

Extraction of Genomic DNA

Apples genomic DNA was extracted from 3g of fresh leave tissues of all samples as described previously [18]. By grounding the tissue to a fine powder using liquid nitrogen, dissolved in pre-heated (60°C) extraction buffer (CTAB) and incubated at 60°C in shaking water bath for 30 min. The mixture was extracted with an equal volume of chloroform/iso-

amyl alcohol (24:1, v/v). The mixture was then centrifuged at 4000g for 30mins. The aqueous phase was transferred to a clean tube and precipitated with 0.66 volume of isopropanol. Precipitated nucleic acids were then dissolved in Tris-EDTA-buffer (TE-buffer). The extracted DNA samples were further purified by transferring 500µl of extracted DNA to an Eppendorf tube and mixed with an equal volume of phenol/chloroform isoamyl and centrifuged at 12000g for 15min. The aqueous layer was again transferred to a new Eppendorf tube and 1/10 of sodium acetate and 2 volumes of absolute ethanol was added, mixed and centrifuged for 5min at 12000g. The supernatant was removed and the pellet dried for 5mins prior to re suspending in 500-750µl of TE buffer and stored at -20°C until further use.

Cloning of Apple Landraces resistance locus (genes)

In the current study, the pJET cloning vector (linearized cloning vector) was used (Promega). This vector contains a lethal gene which is disrupted by a DNA insert into the cloning site. As a result, only cells with recombinant plasmids are able to propagate. Also this vector contains an expanded cloning site. As well as T7 promoter for *in vitro* transcription. Cloning experiments were carried out in several main steps as follow:

PCR amplification

The SSR marker for *powderymildew* resistance gene was amplified across all selected landraces using a primer pair CH03C02 (F: 5'- TCACTATTTACGGGATCAAGCA-3', R: 5'-GTGCAGAGTCTTTGACAAGGC-3'). The apple scab resistance gene (*VF2RAD*) was amplified using a primer pair *VF2RAD* (F: 5'-TCTCAACTTCTTGCACCTAAGC-3', R: 5'-GTGATATTTTGTGAACTGCCC-3'). The amplification reaction consisted of 50µl of 1X one *Taq* quick-load master mix with standard buffer, 2µl of each primer (forward and reverse, 10pmol/µl), 2µl genomic DNA (50ng/µl) and 17µl of nuclease free water. The samples were amplified using a thermocycler machine. The PCR program was as follow: initial denaturation 94°C/45s for 1cycle, followed by 30 repetitive cycles of denaturation 94°C/45s, annealing 58.5°C/45s and extension 72°C/2-3min., and 1cycle of final extension at 72°C for 10min. The products were run on agarose gel to verify the correct PCR fragments.

Ligation of PCR-products

Standard ligation reaction was prepared on ice by mixing 10µl of 2x reaction buffer, 1µl of PCR product, 1µl of blunting enzyme and the volume was completed to 18µl by nuclease-free water. The reaction mixture was vortexed briefly and centrifuged for 3-5s. The mixture was incubated at 70 °C for 5min and then chilled on ice. 1µl of linearized pJET1.2 blunt cloning vector (50ng/µl) and 1µl of T4 DNA Ligase were added to the mixture and centrifuged for 3-5s to collect drops. The ligation mixture was then incubated at room temperature for 5 mins.

Transformation

The ligated samples were used to transform XL10-Gold Ultra Competent Cells (Agilent Technologies). All transformation steps were done on ice. Once competent cells were thawed on ice, they were mixed gently and 50µl of cells were aliquoted into pre-chilled 14ml falcon polypropylene round bottom tubes. 2µl of β-Mercapto ethanol were added to cells and swirled gently. Cells were incubated on ice for 10 minutes and swirling repeated every 2minutes. Then, 1µl of ligated mixture was added to the transformation mixture, swirled gently and incubated on ice for 30 minutes. The tube was heat-pulsed by putting it in 42ºC water bath for 30s. 0.450ml of preheated SOC broth were added to the mixture and incubated at 37ºC for 1hour with shaking at 225-250 rpm. From the previous mixture 100µl were plated on LB- Ampicillin plate and incubated at 37ºC overnight. Next day (in order to select the colony which contain the desired sequence) 10 single colonies from transformed plates were selected and inoculated in 3ml of LB-Ampicillin broth

and incubated overnight at 37ºC with shaking, at the same time selected colonies were streaked on LB-Ampicillin plates for further experiments.

Plasmid DNA extraction and PCR amplification

Plasmid DNA was isolated using QIAprep Spin Miniprep Kit according to the manufacture's instruction. PCR amplification was performed to verify the results of extracted plasmid DNA (Initial denaturation 95ºC/3min.for 1cycl followed by 25 successive cycles (denaturation 94ºC/30s., annealing 60ºC/30s. and extension72ºC/1min.) and final extension 72ºC/10min. for 1cycl).

Sequencing of the selected loci

Three replicates of the extracted recombinant plasmid, each from independent recombinant colonies, have been sequenced at the GATC Biotech. This was performed for each of the investigated loci across all studied landraces.

Data analysis

The nucleotide sequences of both forward and reverse sequences of the two loci were imported, edited, trimmed and verified in Geneious, version R8.1 [(19)] and they were saved in Fasta format. Gaps present in any positions of the aligned sequences were excluded from the analysis. The sequences were aligned for nucleotide sequence analysis and position comparison. Sequence of the two loci were concatenated and used to build a neighbor joining tree.

RESULTS AND DISCUTIONS

Results revealed that the yield of genomic DNA was ranged between 82-1168 ng/µl with an A260/A280 ratio of 1.7-2.0 which considered good-quality DNA. The two loci were successfully amplified, the PCR products were purified and cloned by using pJET1.2 cloning vector. This vector has a lethal gene which is prevent the growth of non-recombinant clones. The lethal gene will be disrupted when a DNA insert is being ligated to the cloning site; as a result, cells with recombinant plasmids will propagate and produce colonies. As such, there is no need for expensive blue/white screening and can be eliminated.

Different markers in different studies have been used to detect apple scab and Powdery mildew resistance genes. RAPD marker OPAT2O450 locus was used with some apple cultivars to reveal the resistance of these cultivars to Powdery mildew. This RAPD marker was developed to SCAR marker which simplified the screening process [(20).] SCAR markers (EM M01 and EM M02) linked in coupling to the *Pl-w* mildew resistance gene were used with some apple cultivars in glasshouse [(21).] SCAR marker was also used by Bus *et al.*,[(14)] for detection of apple scab resistance gene. Apple scab and powdery mildew resistance genes were detected in Czech apple cultivars [(13)]. AFLP-derived SCAR was applied by Huaracha *et al.*, [(22)] in order to narrow down the region of the *Vf* locus for scab resistance gene in apple. Boudichevskaia *et al.*, [(15)] used the same apple scab primer with another two primers to identify apple resistance gene in some apple cultivars. Twenty three pairs of SSR primers were used by [(23)] to build the map of the Rvil (*Vg*) apple scab resistance locus. SSR marker CH03c02 was used to build the map of both *powdery mildew* resistance genes (*Pl1* and *Pld*) in apple in order to calculate the distance between this locus and these genes.

The sequences of the CH03c02 SSR locus were confirmed through aligning them with the same locus of the apple Discovery genotype obtained from HIDRAS (High-Quality Disease Resistant Apples for a Sustainable Agriculture) which was used as a reference sequence. Sequences were aligned to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between these sequences. The alignment of SSR locus (CH03c02), revealed that the nucleotide variations of this locus ranged from 1 to 28 nucleotide substitutions (including 22 trans-version mutations and 7 transition mutations) across all varieties (Figure 1 and Table 1). The lowest nucleotide variation was found between Lubnani and Discovery

genotype, while, the highest nucleotide variation was found between Barwari and Discovery genotype. Results suggested that this locus is highly variable among the studied local varieties. This might suggest that this locus may not be under a high selective pressure as that of the *VF2RAD* locus and it shows more variations and consequently might have a greater evolution rate. As such, they are useful for phylogenetic analysis and/or identification of apple varieties and can be used to discriminate these apple Landraces.

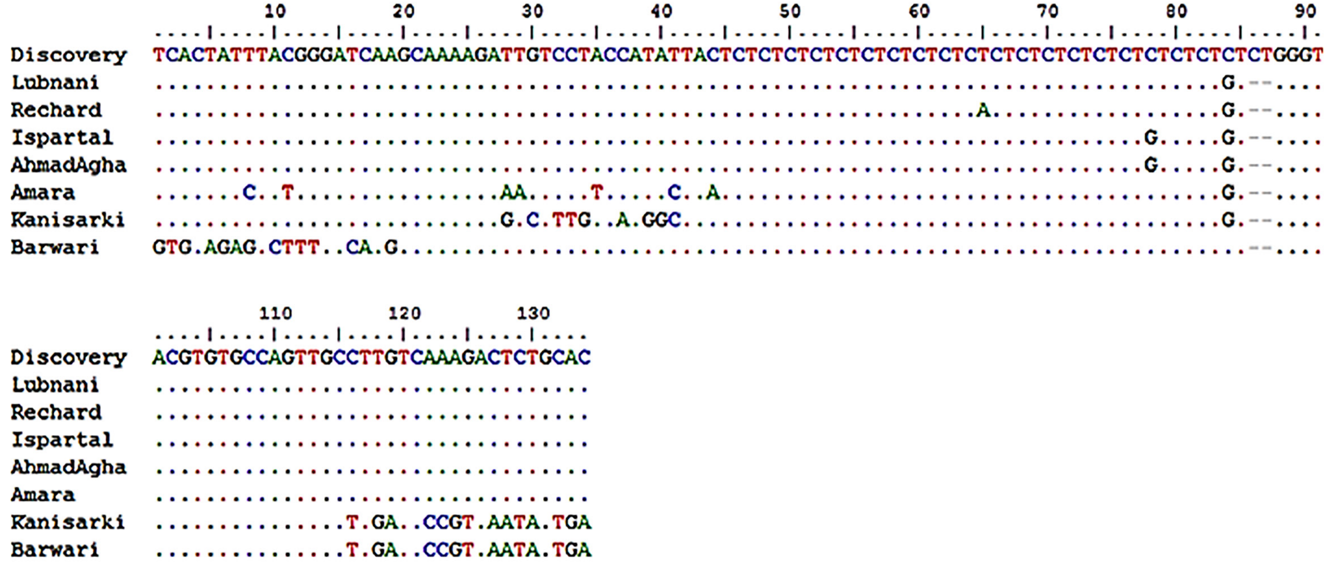

Figure 1. Nucleotide sequence alignments of SSR locus (CH03c02), a marker for Powdery mildew resistant gene, of apple landraces genotypes with out-group (discovery genotypes).

Note: dot marks resemble the same nucleic acid of the first row.

Furthermore, all obtained sequences for the *VF2RAD* in the current study were confirmed by individual BLAST searches at NCBI database at www.ncbi.nlm.nih.gov. Results revealed more than 99% similarity to that of the scab resistance gene sequence of the Discovery apple cultivar which was previously deposited at NCBI database and used in this study as a reference sequence for this locus. In addition, the aligned sequences of *VF2RAD* locus for all studied landraces revealed few nucleotide sequences variations (Figure 2 and Table 1).

The nucleotide variations ranged from 2-8 nucleotide substitutions across all investigated local varieties (including 7 transition and 2 trans-version mutations). The lowest nucleotide variation (2 nucleotides) was found between Kanisarki and Discovery genotype, whereas the highest nucleotide variation (8 nucleotides) was found between Amara and Discovery genotype. These results might suggest that this locus is highly conserved due to its structural or functional important role. Conserved genes have been noticed in other studies on apple such as: *MdML05* and *MdML07* genes in apple could tolerate *Powdery mildew* without any change [(24)], and the gene code for *CPK enzyme* (Calcium-dependent protein kinase) considered as a conserved gene, as it plays important roles in immune and stress signaling, growth and development and hormone responses [(25)].

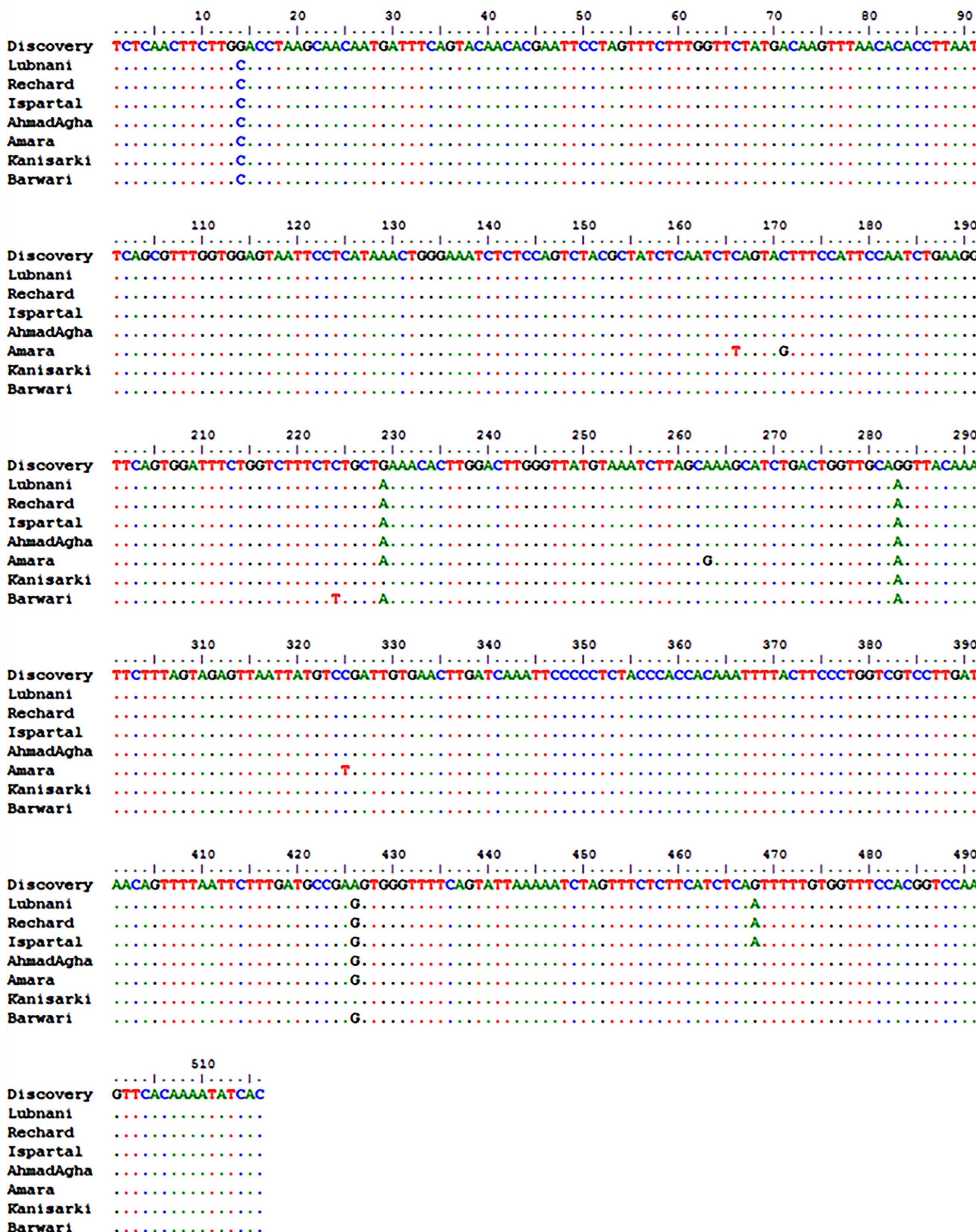

Figure 2. Nucleotide sequence alignments of apple scab resistance gene (VF2RAD) of apple landraces genotypes with out-group (discovery genotypes). Note: dot marks resemble the same nucleic acid of the first row.

Table 1. Analysis of the two loci (VF2RAD gene and CH03c02 SSR locus) of the seven apple landraces.

Sample name	*VF2RAD*gene		SSR locus		VF2RAD + SSR	
	Length of the product(bp)	Variable nucleotide	Length of the product (bp)	Variable nucleotide	Length of the product (bp)	Variable nucleotide
Lubnani	516	5	134	1	650	6
Rechard	516	5	134	2	650	7
Ispartal	516	5	134	2	650	7
Ahmadaxa	516	4	134	2	650	6
Amara	516	8	134	8	650	16
Kanisarki	516	2	134	24	650	26
Barwari	516	5	134	28	650	33

The sequences of both loci were then concatenated using Bioedit program (version 7.2.5) and used to construct the genetic distances and Neiber Joining tree (Figure 3) among these Landraces genotypes in order to allow better discrimination and greater tree robustness. The highest genetic distance was 0.062(93.8%genetic similarity) between Amara and Barwari genotypes. This low rate of genetic similarity might also reflect their environmental requirements and morphological features. For instance; the chilling temperature degree requirement for Barwari genotype is higher than chilling temperature requirement of Amara genotype, as these two genotypes grow in different environment. Also morphologically, the color of Barawari fruit is bright red while Amara fruit has pale red color. The color of leaf in Barwari is bright green smooth, while, Amara leaf has dark green color. In contrast, the lowest genetic distance 0.0015 (99.85%genetic similarity) was found between Lubnani, Richard and Ispartal genotypes and Ahmadagha genotype (Table 2).

Table 2. Sequence distances between the cloned apple landraces with VF2RAD and CHO_3CO_2 primers.

Genotype	Discovery	Lubnani	Rechard	Ispartal	Ahmadaxa	Amara	Kanisarki	Barwar
Discovery	0.0000	0.0093	0.0109	0.0109	0.0093	0.0252	0.0417	0.0533
Lubnani	0.0093	0.0000	0.0015	0.0015	0.0031	0.0188	0.0416	0.0500
Rechard	0.0109	**0.0015**	0.0000	0.0031	0.0046	0.0204	0.0433	0.0517
Ispartal	0.0109	**0.0015**	0.0031	0.0000	0.0015	0.0204	0.0433	0.0517
Ahmadaxa	0.0093	0.0031	0.0046	**0.0015**	0.0000	0.0188	0.0416	0.0501
Amara	0.0252	0.0188	0.0204	0.0204	0.0188	0.0000	0.0533	0.0620
Kanisarki	0.0417	0.0416	0.0433	0.0433	0.0416	0.0533	0.0000	0.0433
Barwari	0.0533	0.0500	0.0517	0.0517	0.0501	**0.0620**	0.0433	0.0000

This high genetic similarity (99.85%) might be due to the presence of some common morphological characters shared among them; including leaf color and shape and fruit color. The sequences were further analyzed to build the genetic relationships, and the genotypes were differentiated into two major clades in the phylogenetic tree, namely C1 and C2 (Figure3). In

the present study discovery cultivar used as outgroup in a separate clade. This was because this cultivar is within the Rosacea family, which is the same family of apple. In the phylogenetic tree construction, it is important to use outgroups which should be a genotype from the closest relatives to the family on the ground, as it may cause polarization (a sharp division) of apomorphic and plesiomorphic [26].

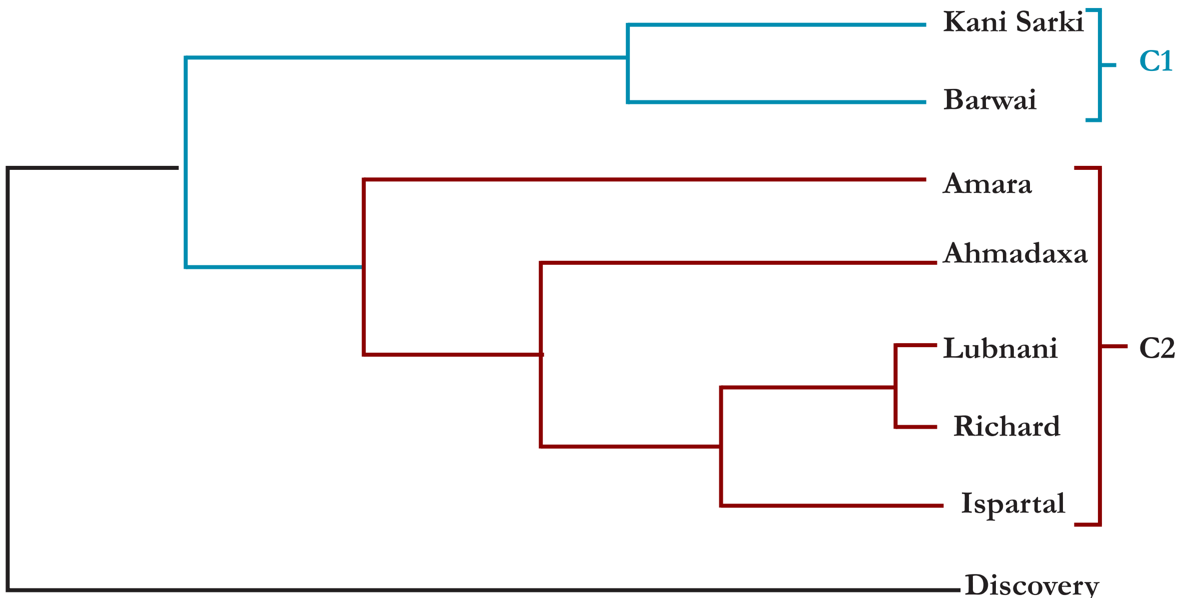

Figure 3. Phylogenetic tree of apple landraces for apple scab and powdery mildew primers constructed according to their alignments.

The C1 clade included Kanisarki and Barwai, whereas, the C2 clade included; Amara, Ahmadagha, Lubnani, Richard and Ispartal. From the phylogenetic tree, it can be concluded that Lubnani and Richard genotypes have the newest common ancestor, suggesting that it is difficult to use these two genotypes for breeding between them. In hybridization program, cultivars which form monophyletic are more preferable than cultivars not forming monophyletic, as they are genetically suitable which increases the efficiency of hybridization and fertility [27].

Different markers in different studies have been used to detect apple scab and Powdery mildew resistance genes. RAPD marker OPAT2O450 locus was used with some apple cultivars to reveal the resistance of these cultivars to Powdery mildew. This RAPD marker was developed to SCAR marker which simplified the screening process [20]. SCAR markers (EM M01 and EM M02) linked in coupling to the *Pl-w* mildew resistance gene were used with some apple cultivars in glasshouse [21]. SCAR marker was also used by Bus *et al.*, [14] for detection of apple scab resistance gene. Apple scab and powdery mildew resistance genes were detected in Czech apple cultivars [13]. AFLP-derived SCAR was applied by Huaracha *et al.*, [22] in order to narrow down the region of the *Vf* locus for scab resistance gene in apple. Boudichevskaia *et al.*, [15] used the same apple scab primer with another two primers to identify apple resistance gene in some apple cultivars. Twenty three pairs of SSR primers were used by Cova *et al.*, [23] to build the map of the Rvil (*Vg*) apple scab resistance

locus. SSR marker CH03c02 was used to build the map of both *powdery mildew* resistance genes (*Pl1* and *Pld*) in apple in order to calculate the distance between this locus and these genes.

CONCLUSIONS

All in all, the cloning strategy used in this study was found to be very successful and helpful for obtaining the full nucleotide sequences of these two loci. Furthermore, the investigated two loci were found to display nucleotide variations among the respected landraces. Finding of these variations was allowed the distinguishing and discrimination of these landraces.

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