The *rbcL* Gene Sequence Variations among and within *Prunus* Species

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ABSTRACT

The objective of this study was to detect the level of SNP variations of *rbcL* gene sequences among and within *Prunus* species including 17 locally cultivated and wild relatives of *Prunus*, and two species of the subfamily *Maloideae* (*Malus domestica* and *Pyrus communis*), as out groups. The *rbcL* sequences were amplified, sequenced, and aligned to determine Single Nucleotide Polymorphisms (SNPs). The *rbcL* gene tree of the samples showed two main clusters. The first included the outgroup taxa (*M. domestica* and *P. communis*); and all *Prunus* samples in the second cluster including *Prunus armeniaca*, which separated in a subcluster. Our results indicate that *rbcL* gene sequence analysis provides a well-defined tool to study relationships within and among *Prunus accessions*.

Keywords: Genetic relationship, Prunus, rbcL, Sequencing.

INTRODCTION

The Rosaceae family contains about 100 genera and 3,000 species (Judd et al., 1999), *Prunus* is the largest genus in the subfamily Prunoideae (Amygdaloideae) including about 250 species (Lee and Wen, 2001), Peach (Prunus persica L.) and almond (Prunus dulcis Mill.; syn. P. amygdalus Batsch) are two commercially grown species that belong to the Prunus genus (Rehder, 1940). The two species have originated in Southeast and Central Asia, respectively (Watkins, 1979), while the wild relatives of Prunus were found growing from eastern China to the Mediterranean Sea (Browicz and Zohary, 1996). According to Rehder (1940), genus Prunus is divided into subgenera of Prunophora (Prunus), Amygdalus, Padus, Cerasus and Laurocerasus. Other authors consider them as a separate genus (McVaugh, 1951). The classification of *Prunus* species is complicated, since closely related taxa often differ by only a single morphological character (Shi *et al.*, 2013). In addition, typical identification requires reproductively mature material that may be available for only a short period of the year (Julian *et al.*, 2009).

Therefore, molecular markers became necessity to study phylogenetic relations of Prunus genus. The Single Nucleotide Polymorphism (SNP) and small Insertion and Deletion (InDels), are the most forms of genetic variations in natural populations. They reflect the results of evolution and adaptation (Yamanaka et al., 2004; Wright et al., 2005). They are frequently used in modern genetics for reverse genetics, linkage analysis, genome-wide association study, genotyping and markers assisted selection. In addition, SNP markers were developed to identify plant diseases resistance (Bakooie et al., 2015).

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Several investigations were carried out to assess genetic variation and phylogenetic relationship among and within Prunus species using isozyme, RFLPs, RAPDs, SSRs and AFLP (Marti nez-Go mez et al., 2003; Mowrey and Werner, 1990). The use of **SNPs** for genetic fingerprinting. parentage verification, and gene mapping was recommended to be applied in the study of genetic diversity of sweet cherry (Prunus avium) and was also proved to be useful in other related species within Amygdaloideae (Marti et al., 2012). At present, techniques for studying the molecular phylogeny and taxonomy of plants rely heavily on chloroplast genome sequence data. This is because the chloroplast genome is haploid with simple and stable genetic structure, where no or very rare recombinations take place, and universal primers can be used to amplify target sequences. In addition, the ease of PCR amplification and sequencing of chloroplast genes using universal primers facilitates phylogeny projects. The chloroplast DNA restriction sites were used to construct the phylogeny of eight cultivated members of Prunus (Badenes and Parfitt, 1995). On the other hand, sequences of chloroplast regions of rbcL, matK, trnL/F, 18S rDNA, and ITS have been used in studies of Rosaceae and Prunus phylogeny (Morgan et al. 1994; Potter et al., 2001,2003, 2007; Bortiri et al., 2001, 2006). Some of these regions were also shown to be informative within subfamilies such as Amygdaleae (Lee and Wen, 2001; Potter et al., 2002), and Maloideae (Potter et al., 2007).

The Ribulose-Bisphosphate Carboxylase/Oxygenase (RuBisCO) is the enzyme that facilitates the primary CO_2 fixation step in the Calvin cycle. The quaternary structure of the enzyme consists of 8 large and 8 small subunits. Sequences of the *rbcL* large subunit have been used to elucidate higher taxonomic relationships in the angiosperms (Olmstead *et al.*, 1992; Chase *et al.*, 1993; Qiu *et al.*, 1993). The large size (more than 1,400 bp) of *rbcL* provides many characters that can be

utilized in phylogenetic analysis. Additionally, the availability of conserved primers allow for rapid amplification and sequencing. The slow synonymous nucleotide substitution rate in chloroplast DNA (cpDNA) compared to nuclear genes, is another reason for the utility of *rbcL* in angiosperm phylogeny studies.

The first suggestions that *rbcL* gene sequence was appropriate in phylogenetic studies were from Ritland and Clegg (1987) and Zurawski and Clegg (1987), and small scale phylogenetic studies based on rbcL sequences were followed (Doebley, 1990; Kim et al., 1992). However, the first phylogenetic collaborative large-scale analysis using *rbcL* sequence data for a broad sampling of seed plants was conducted by Chase et al., (1993). The rbcL gene was also widely used in phylogeny studies of Prunus species (Morgan et al., 1994; Potter et al., 2007; Quan and Zhou, 2011), and was found to be useful for identifying variations among and within the genera and species.

The purpose of this study was to detect the level of *rbcL* sequence variations among *Prunus* species and to produce a phylogeny of genetic tree relationships among *Prunus* species based on *rbcL* gene sequence.

MATERIALS AND METHODS

Plant Material

Plant samples investigated in this study (Table 1) were provided by Cergaya and Hott Agricultural Stations which belong to the General Commission for Scientific Agricultural Researches in Syria. The samples comprised five local varieties of almond (*Prunus dulcis*), seven varieties of peach (*Prunus persica*), two genotypes of the wild relatives of *Prunus orientalis*, *Prunus korshinskyi*, Hybrid GF-677 (*Prunus dulcis* \bigcirc ×*Prunus persica* \bigcirc), one local sample of *Prunus armeniaca*, which belongs to the subgenus *Amygdalus*, one variety of each of *Malus domestica* and *Pyrus*

Species	Varieties name	Origin	Code
Almonds	Shami Furk	Local	P.d_sh
Prunus dulcis	Hama34	Local	P.d_34
	Hama47	Local	P.d_47
	Babnis	Local	P.d_bb
	Oja	Local	P.d_oja
P. persica (L.) Batsch	Gioia	Italy	P.p_gi
Peach and Nectarine	Venus	Italy	P.p_ve
	Caldesi2000	Italy	P.p_ca
	Nectaross	Italy	P.p_ne
	Laure	Italy	P.p_la
	May Crest	Italy	P.p_ma
	Pontina	Italy	P.p_po
Prunus armeniaca. L	Klabe	local	P.a_kl
Prunus korshinskyi	(Rootstock)	local	P.kor
Prunus orientals. Mill	(Rootstock)	Local	P.ori1
Prunus orientals	(Rootstock)	Local	P.ori2
Hybrid GF-677	(Rootstock)	Italy	GF-677
Malus domestica	Starking		M.d_st
Pyrus communis	Koshi		P.c_ko

Table 1. Investigated *Prunus* species and varieties and out group samples and their codes in this study.

communis (from the sub-family *Maloideae*) as out groups.

DNA Extraction, PCR Amplification, Electrophoresis and Sequencing

Young half folded leaves were used for total genomic DNA extraction based on the modified CTAB method (Doyle and Doyle, 1987). Primer pair used in PCR amplification were described by (Shokralla *et al.*, 2010) to amplify about 0.6 Kbp of *rbcL* gene were as follow:

rbcLaF:

^{5′}ATGTCACCACAAACAGAGACTAAAG C^{3′}

rbcLaR:

⁵'GTAAAATCAAGTCCACCRCG^{3'}.

A total volume of 15 μ L of PCR reaction mixture contained the following: 0.15 μ L of 5 U μ L⁻¹ Hot start *Taq* DNA Polymerase (Takara-bio, Japan), 0.25 μ L of 10 mM dNTPs and 0.5 μ L of 50 mM MgCl₂, 0.5 μ L of each primer (10 pmol) (Vienna Biotech, Austria), 1.5 μ L of 10X PCR buffer, 5 μ L of 1 ng μ L⁻¹ DNA sample, and sterile distilled water to adjust to final volume. PCR amplification was performed with a 96 well Veriti thermal cycler (Applied Biosystems, USA) as follows: 95°C for 5 minutes, followed by 40 cycles of 95°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute, and a last elongation step at 72°C for 10 minutes. One microliter of each of the amplified PCR products was tested on 1.5% agarose gel stained with Ethidium Bromide, and visualized under UV for detecting the amplification efficiency.

PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced using the same primers by ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA).

Data Analysis

The obtained sequences were subjected to BLASTn analysis by NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to detect homology of the target gene and species. Editing and assembling of sequences were conducted using BioEdit Sequence Alignment editor (Hall, 1999). Phylogenetic and molecular evolutionary analyses were investigated using MEGA version 5 (Tamura *et al.*, 2011). Bootstrap analyses were used to assess the robustness of the tree with 1,000 replicates (Felsenstein, 1985).

RESULTS

The primers were used on the DNAs of 19 samples, which showed complete

amplifications with a clear band 600 bp which matched the amplified amplicon size, in all samples (Figure 1).

To confirm results and identify the SNPs, sequencing of *rbcL* was conducted in all samples. The SNPs found in our work were both transversion, the substitution of a (two ring) purine for a (one ring) pyrimidine (A/T) and (T/G), and transition, a point mutation that changes a purine nucleotide to another purine ($\underline{A} \leftrightarrow \underline{G}$). Among *P. armeniaca* and all other samples there was one transition (A/G), whereas *P.dulcis* samples showed a transversion SNP (A/T)



Figure 1. An example for PCR amplification using primer pair of *rbcL* F and *rbcL* R. (M) Molecular weight markers; (a) Samples from *P. dulcis* and *P. persica, and* (b): *Prunus* sp. and out group samples.

compared with the rest of the samples, and another clear transversion (T/G) appeared between all *Prunus* sp. and outgroup samples (Figure 2).

The computed nucleotide pair frequencies among all samples showed the presence of three SNPs two transversions and one transition. This data analysis also showed the highest nucleotide changes in *P*. *armeniaca* when compared to the rest of *Prunus* input sequences. However, the analysis showed no nucleotide substitution within each of *P. persica* and *P. dulcis* (Table 2).

The average of the pairwise distance was 0.002, pairwise distance values within each of the group of *P. Persica* and the group of *P. dulcis* and the wild relatives including hybrid GF-677 was zero, whereas the value 0.002 was between *P. persica* and all other samples of *P. dulcis* and wild relatives, including hybrid GF-677. The pairwise distance between *P. armeniaca* and *P. persica* samples was 0.005. The

	•	արութ	արուր	սրուրո	սրուր	սրուր	սրուր	mmun	սրուր	nhini
	<u>.</u>	430	440	450	460	470	480	490	500	510
Malus	domestica	CGCCTCATGGI	ATCCAAGTTG	AGAGAGA <mark>T</mark> AA	ATTGAACAAG	TATGGCCGCC	CTCTATTGGG	ATGTACTATA2	AAACCAAAAT	TGGGGTT
Pyrus	communis									
PE.MA			3		· · · · · [_] · · · ·					
PE.PO			3		· · · · · [_] · · · ·					
PE.LA			3		· · · · · [_] · · · ·					
NE.GI			3		· · · · · [_] · · · ·					
NE.VE			3		· · · · · ⁻ · · · ·					
NE.CA			3		••••					
NE.NE			3		· · · · · [_] · · · ·					
AL.SH			3		· · · · · ⁻ · · · ·				T	
AL.47			3		••••				T	
AL.34			3		· · · · · [_] · · · ·				T	
AL.BB			3		· · · · · [_] · · · ·				T	
AL.OJA	ł		3		••••				T	
P.ORI-	-1		3		••••				T	
P.ORI-	-2		3		· · · · · [_] · · · ·				T	
P.KOR			3		· · · · · [_] · · · ·				T	
GF-677	1				· · · · · [_] · · · ·				T	
P.Arme	eniaca	••••••		• • • • • • • • • • •	••••	••••••	•••••	•••••	· · · · · [_] · · ·	•••••

Figure 2. Partial samples of multiple sequence alignment of *rbcL* gene sequences of the samples sequenced (SNPs are indicated).

Table 2	. Nucleotic	le pair frec	quencies among	the studied	sequences of	the samples. ^a
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Samples	II	SI	SV	R	Total
Within all samples	8.00	1.00	1.00	0.61	10.00
P. armeniaca and the rest of Prunus sp.	3.00	1.00	0.00	0.36	4.00
Wild relatives and P. persica	0.00	0.00	1.00	0.00	1.00
Wild relatives and P. dulcis	0.00	0.00	0.00	nc	0.00
Hybrid GF-677 and P. dulcis	0.00	0.00	0.00	nc	0.00
Hybrid GF-677 and P. persica	1.00	0.00	0.00	0.00	1.00
Within P. persica	0.00	0.00	0.00	nc	0.00
Within P. dulcis	0.00	0.00	0.00	nc	0.00
Within wild relatives	0.00	0.00	0.00	nc	0.00
P. dulcis and P. persica	0.00	0.00	1.00	0.00	1.00

^{*a*} II= Identical DNA nucleotide pairs; SI: Transitional Pairs; SV= Transversional Pairs, R= SI/SV. All frequencies are averages (rounded) over all samples. Analysis conducted in MEGA5 (Tamura *et al.*, 2011).

corresponding value between P. armeniaca and all samples of P. dulcis, and wild relatives, including the hybrid GF-677, was 0.007. The highest value of 0.017 was recorded between Malus domestica and the samples of P. dulcis, and wild relatives, including the hybrid GF-677 (Table 3).

The aligned sequences were used to build a phylogenetic tree using the UPGMA method (Figure 3). Results indicated the presence of two main clusters. The smaller cluster contained plant species Malus domestica and Pyrus communis, species of subfamily Pomoideae, the other samples of Prunus genus (subfamily Prunoideae) were joined in the bigger cluster. The second main cluster was divided into two further subclusters of P. armeniaca (subgenus Prunus) alone, and all other samples of P. persica, P. dulcis. Wild relatives (subgenus Amygdalus) including Hybrid GF-677 joined together in another sub cluster at a high bootstrap value of 95. The subcluster of subgenus Amygdalus, was divided into two branches, the first one contained all P. dulcis samples and wild relatives with hybrid GF-677, the second clad contained P. persica samples (Figure 3).

DISCUSSION

Our study took the advantage of using *rbcL* gene, taking into consideration that the coding rbcL gene is easily amplified and sequenced in most land plants and has an impact in phylogeny investigations by providing a reliable placement of a taxon into a plant family and genus (Kress and Erickson, 2007; Gyulai et al., 2012).

Sequences of *rbcL* marker showed several genetic differences among samples. The interspecific genetic diversity was lower than the intraspecific one, that was in contrary with Mattia et al. (2011) in their previous research on Lamiaceae member. Two SNPs in the *rbcL* region were detected between the three commercial oregano and the other analyzed samples. Whereas, the sequencing of the chloroplast gene *rbcL* of

	M.d_st	P.c_ko	P.p_ma	P.p_p	P.p_la	P.p_g	P.p_ve	P.p_ca	P.p_ne	P.d_s	P.d_47	P.d_34	P.d_bb	P.d_oja	P.ori1	P.ori2	P.kor	Gf-677	P.a_kl
l.d_st	0.000																		
c_ko	0.004	0.000																	
p_ma	0.015	0.013	0.000																
d-d	0.015	0.013	0.000	0.000															
p_la	0.015	0.013	0.000	0.000	0.000														
p_g	0.015	0.013	0.000	0.000	0.000	0.000													
p_ve	0.015	0.013	0.000	0.000	0.000	0.000	0.000												
p_ca	0.015	0.013	0.000	0.000	0.000	0.000	0.000	0.000											
p_ne	0.015	0.013	0.000	0.000	0.000	0.000	0.000	0.000	0.000										
d_s	0.017	0.015	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000									
d_47	0.017	0.015	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000								
d_34	0.017	0.015	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000							
d_bb	0.017	0.015	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000						
d_oja	0.017	0.015	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000					
oril	0.017	0.015	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000				
ori2	0.017	0.015	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000			
kor	0.017	0.015	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
f-677	0.017	0.015	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
a_kl	0.013	0.011	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.000



Figure 3. UPGMA dendrogram of all samples (*Prunus, Pyrus* and *Malus*) using the Maximum Composite Likelihood method. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011). Values presented bootstraps with 1,000 replicates.

Eryngium spp. showed the presence of three SNPs between studied species (Jawdat *et al.*, 2013). This result agrees, to some extent, with our results in the ability of *rbcL* to differentiate between species.

The results of UPGMA cluster analysis were close and agree with some other studies (Morgan *et al.*, 1994; Shaw and Small, 2004; Potter *et al.*, 2002, 2003, 2007; Lee and Wen, 2001) and showed that all samples gathered into groups which matched their taxonomic classification. This reflects the ability of *rbcL* gene to provide high discrimination of taxa at the level of subfamilies, genera, and species.

In our study, *rbcL* sequence variations were high between the outgroup *Malus domestica* and *Pyrus communis* (sub family *Pomoideae*) when compared with other taxa of *Prunus* (subfamily of *Prunoideae*), where the average pairwise distance was 0.002. The sample of *P. armeniaca* (sub genus *Prunus*) has been considerably differentiated by having four variation sites (Table 2) when compared with all samples of *Prunus* and with wild relatives (subgenus *Amygdalus*). Our results support Watkins (1979), Potter *et al.* (2003) and Mallikarjuna *et al.* (2004) studies.

Two groups of taxa including the wild relatives within subgenus Amygdalus formed two separate subclusters, the first one contained P. dulcis samples, the wild relatives and the hybrid GF-677. The second sub cluster contained only Prunus persica samples. Similar diversification was presented in the result of authors sharing some samples with our study (P. dulcis, P. armeniaca, P. persica and some other hybrids) using matk and SSRs, and by using CAPS (Cleavage Amplified Polymorphic Sequence) (Bouhadida et al., 2007).

The complete matching between the wild relatives *P. korshenskyi* and *P. orientalis* and the *P. dulcis* samples (domesticated almond), with the average of pairwise distance of 0.00, may be due to that *Prunus* wild relatives have been used for a long time as a source of genetic pool in almond breeding programs. However, *rbcL* sequence variations between *Prunus dulcis* samples and their wild relatives were not informative

for their identification. Also, this result supports the results of Bortiri *et al.* (2001) and Shaw and Small (2004) in grouping *Prunus dulcis* samples with *P. korshinskyi* and *P. orientalis*.

The GF-677 rootstock is a vegetatively propagated hybrid between the Spanish almonds 'Garfi' [*P. dulcis* (Mill.) D. A., Webb] as the female parent and the North American peach 'Nemared' [*P. persica* (L.) Batsch] as the pollen donor. This hybrid has joined the group of *P. dulcis* and wild relatives in one major sub-cluster. This can be supported by the fact that *rbcL* gene is maternally inherited. Furthermore, a partial sequence may lead to a high match percentage that may not reflect an accurate identification of the query sequence (Schori and Showalter, 2011).

Reliable identification of variation below species level would provide valuable insight into subspecies ranges, and habitat differentiation, additionally, it would help in defining the important variation within species. (Kane *et al.*, 2012).

Peach samples included in this study were vegetatively propagated. Therefore, it is not expected to obtain high variation level in a single gene locus such as *rbcL*. In addition, the fact is that *rbcL* gene is maternally inherited which makes it highly conserved within cultivars belonging to the same species and sharing the same origin of maternally chloroplast genome. The reason along the low evolutionary rate of this chloroplast gene suggests that cultivars from both species (*P. persica and P. dulcis*) included in this study seem to be one taxa in each group.

Finally, a good level of discrimination based on *rbcL* marker was observed between studied species and less or absence of variation within species. The analysis of genetic variations among groups of *Prunus* samples and wild relatives using *rbcL* gene allowed us to cluster successfully all samples supporting their morphological characteristics, and their botanical classification. Although *rbcL* gene sequence was not able to identify cultivars, which belonged to the same species, it is effectively variable at species level and much more detailed work is needed on the complete gene sequence. In addition, using other chloroplast genes such as *matK* or PCR based markers like microsatellites should also be investigated to further clarify genetic diversity of *Prunus*.

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تغییرات توالی ژن *rbcL* در درون و در بین گونه های هلوسا Prunus

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چکیدہ

هدف این پژوهش، بررسی سطح تغییرات SNP توالی ژن *rbcL* در درون و در بین گونه های هلو سا *Prunus* بود و ۱۷ گونه کشت شده محلی و خویشاوندان وحشی هلوسا ها و دو گونه از زیرخانواده (Malus domestica and Pyrus communis) به عنوان گروه پرت (outgroup) را در بر می گرفت. در انجام پژوهش، توالی های *rbcL* تکثیر، توالی بندی و همردیف *rbcL* (gene tree) را در بر می گرفت. در انجام پژوهش، توالی های *rbcL* تکثیر، توالی بندی و همردیف شد تا چندشکلی های نو کلئوئید مجرد (SNP) تعیین شود. نتایج درخت ژنی (gene tree) *nct domestica armeniaca های در خوشه دوم* را نشان می داد. اولین آنها شامل رده گروه پرت *Prunus armeniaca مربو* در خوشه اصلی را نشان می داد. اولین آنها شامل رده گروه پرت (*Prunus communis* و *communis a* مگی در خوشه دوم قرار گرفتند که در یک زیرخوشه جدا شدند. نتایج چنین اشاره داشت که برای بررسی روابط درون گونه و روابط بین گونه ها ، تجزیه توالی ژن*Tbcl* ابزاری به دست می دهد که به خوبی مشخص است و می تواند با موفقیت در ترسیم درخت تکاملی و تبار زایی برای نمونه های ثبت شده گونه هلو سا به کار رود.