**Evaluation of Four Different Protocols for DNA Extraction from Young and Old Carob Leaves**

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**Geliş Tarihi :07.04.2017 Kabul Tarihi :22.05.2017**

**ABSTRACT :** Carob (*Ceratonia siliqua* L.) is an evergreen tree. It naturally grows in forests, but it is also commercially grown in some regions. As it was in other plant species, molecular markers are used to identify genetic diversity in carob. DNA isolation is the most significant factor effecting molecular marker analysis. In this study, four different isolation protocols were used to isolate quality DNA from young and old carob leaves. In young leaves, while the lowest DNA concentrations were obtained from protocol A (5.73±0.89 ng/µl DNA), DNA concentration of Protocol D were significantly higher than other protocols (900.66±23.38 ng/µl DNA). In old leaves, the lowest DNA concentrations were obtained from protocol A (2.9±0.4 ng/µl DNA) and the highest DNA concentrations were obtained from protocol C (11.36±0.8 ng/µl DNA). The DNA concentrations obtained from the freshest tip leaves measured 656.66±36.95 ng/µl DNA. DNA concentration of old leaves significantly lower than young leaves at all protocols. While the lowest A260/280 nm absorbance ratios in young leaves were observed in protocol A (0.76±0.02), the ratio in other protocols were between the optimum values.

**Keywords**: Carob, genetic diversity, DNA, molecular marker

**Keçiboynuzunun Genç ve Olgun Yapraklarından DNA Ekstraksiyonu İçin Dört Farklı Protokolün Değerlendirilmesi**

**ÖZET :** Keçiboynuzu (*Ceratonia siliqua* L. (Cesalpinaceae) herdem yeşil bir bitkidir. Keçiboynuzu doğal olarak ormanda yetişirken bazı bölgelerde ticari olarak da yetiştirilmektedir. Diğer bitki türlerinde olduğu gibi moleküler markırlar keçiboynuzunda da genetik çeşitliliği tanımlamak için kullanılmaktadır.DNA izolasyonu moleküler markır analizlerini etkileyen en önemli faktördür. Bu çalışmada, keçiboynozunun genç ve olgun yapraklarında dört farklı protokol kullanılarak kaliteli DNA elde etmek amaçlanmıştır. Genç yapraklarda, en düşük DNA konsantrasyonu protokol A’dan (5.73±0.89 ng/µl DNA) sağlanırken, protocol D’nin (900.66±23.38 ng/µl DNA) DNA konsantrasyonu diğer protokollerden oldukça yüksek bulunmuştur. Yaşlı yapraklarda, en düşük DNA miktarı protokol A(2.9±0.4 ng/µl DNA) ile en yüksek DNA miktarı protokol C(11.36±0.8 ng/µl DNA) şeklinde saptanmıştır. Genç yapraklardan elde edilen DNA konsantrasyonu 656.66±36.95 ng/µl DNA olarak ölçülmüştür. Olgun yapraklardaki DNA konsantrasyonları genç yapraklardaki bütün protokollere göre oldukça düşüktür. Genç yapraklarda en düşük absorbans A260/280 nm ile protokol A (0.76±0.02) iken, diğer protokollerdeki oranlar optimum değerler arasındadır.

**Anahtar Kelimeler**: Keçiboynuzu, genetic çeşitlilik, DNA, moleküler markır

**INTRODUCTION**

*Ceratonia siliqua* L. (carop; Cesalpinaceae) is an evergreen plant in Southeastern Asia and Mediterranean climates. Carob trees are also encountered over semi-arid sections of South Africa, Mexico, California, Arizona and Australia (Batlle and Tous, 1997). Carob is also naturally encountered in non-arid sections of Morocco at altitudes up to 1150 m (Emberger and Maire, 1941). Carob trees constitute a significant component of vegetation and environment (Batlle and Tous, 1997). Fruits are used as animal feed supplement, as raw material for alcohols, sugars, adhesives and also used in food industry (Carlson, 1986; Tous, 1992). World annual carob production is estimated as 162.000 tons and Spain, Italy, Portugal, Morocco, Greece and Cyprus are the leading countries in this production (FAO, 2012).

Morphological and physiological characteristics are commonly used to characterize and control carob cultivars (Tous and Batlle, 1990; Gharnit et al., 2004). Since these characteristics used in cultivar characterization may be influenced by environmental factors, they may be sufficient in identification of variations among populations (Morsy, 2007).

Isoenzyme markers have initially been used in molecular characterization of several plant species and cultivars. However, their use was quite limited because of low polymorphism levels (Barracosa et al., 1996; Batlle and Tous, 1997). Then, DNA-based molecular markers were started to be used and such markers constituted significant tools to overcome problems encountered in characterization of species and cultivars. DNA markers are obtained from plant tissues and they are not influenced by environmental conditions throughout plant growth stages. Besides the supports provided in plant breeding and classification, molecular markers also provide a reliable tool for taxonomic classification of the organisms (Talhinhas et al., 2003; Guzeldag and Colak, 2007). Among the molecular markers, RAPD technique was reported to provide advantages in molecular analyses on carob trees (Konaté et al., 2007).

Morphological traits and RAPD analyses are widely used in detection of population differences in plants (Crouch et al., 2000, Casiva et al., 2002; Garcia et al., 2002; Samal et al., 2003). RAPDs mostly allow analyses for complex taxonomic relationships and basic information for plant systematic were derived from morphological traits.

It was pointed out in previous studies carried out with DNA markers requiring high quality genomic DNA that DNA extraction methods should be simple, fast and cheap (Ausubel et al., 2002). Although there are several commercially available DNA isolation kits, quite expensive costs of these kits restrict the detailed DNA studies. Moreover, quantity and quality of DNA may vary in different plant tissues, in species with different families and even in samples of different cultivars. Existence of some cell components in extracted DNA samples may hinder the function of molecular reactions (Bushra et al., 1999). The problems created by the tissues of genetic material from which DNA isolated may be eliminated through adjustments made in quantity of chemical used in DNA isolation and the duration of the steps implemented (Lodhi et al., 1994). Therefore, in studies including several numbers of samples, instead of using high-cost laboratory equipment to get quality products, assessment of different methods should be tried.

The methods used in DNA isolation all depend on freeing DNA in extraction buffer through disintegrating cell membrane, protection of endonuclease enzymes with chelating chemicals and DNA purification from protein, RNA, polysaccharides and polyphenols. Since plant leaves have low polyphenol, polysaccharide and other secondary metabolite concentrations, young leaf tissues are commonly employed to get quality DNA (Sytsma et al., 1993). However, it is not always possible to obtain such quality DNA in wild species collected from different locations or in distant relative species. Although materials are able to be stored in silica jells most of the time in such cases, DNA quality and quantity decrease (Akinnagbe et al., 2012). Even when the DNA samples were obtained from young tissues and high quality and quantity DNA were achieved in spectrophotometric analyses, the failures experienced in DNA propagation of certain species may pose a permanent problem (Samarakoon et al., 2013; Aubakirova et al., 2014).

Carob both has a natural spread and is cultivated commercially. This evergreen tree has a spread in Mediterranean region of Turkey. Molecular works have recently been concentrated on carob and commonly leaves and resultant DNA are used as the initial material in these works. Problems are encountered while DNA extraction from several plant tissues. Quality and high concentration DNA may provide significant contributions in molecular works. However, high quality and quantity DNA are not able to be obtained even with the DNA isolations from desired leaved throughout the year.

In this study, 4 different DNA isolation procedures were compared to determine a proper method to obtain high quality DNA from young and old carob leaves through Polymerase Chain Reaction (PCR) analysis.

**MATERIAL AND METHOD**

**Material**

Young and old carob leaves were taken from the fields of Alata Horticultural Research Institute-Mersin, TURKEY. Collected leaf samples were smashed in a mortar for DNA isolation. Four different DNA isolation protocols already used in *Malus sieversii*, *Vitis vinifera*, and *Armeniaca vulgaris* species by Aubakirova et al. (2014) were employed in this study to obtain high quality and quantity DNA with molecular marker analyses.

**Method**

**Protocol A (modified from Edwards et al., (1991)**

About 100 mg leaf powder was supplemented with 1 mL extraction buffer including 200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 250 mM NaCl, 10% sodium dodecyl sulfate (SDS), 10-20 mg polyvinylpyrrolidone (PVP), and 6.5 mM dithio­threitol (DTT). The solution was centrifuged at 15,000 rpm for 2 minutes, then cold chloroform was added at an available volume and resultant solution was again centrifuged at 15,000 rpm for 10 minutes. To settle nucleic acids, cold isopropanol was added at about 60% of available volume and centrifuged at 15,000 rpm for 5 minutes. Then the resultant precipitate was washed through with 500 µL 70% ethanol, dried at room temperature, dissolved again in 100 µL distilled water and incubated at 55 oC for overnight. A second chloroform extraction was performed, 60% of resultant volume was taken, and NaCl was added as to have a final concentration of 0.6 M and centrifuged at 15,000 rpm for 15 minutes. The solution was precipitated with isopropanol, precipitate was washed through 70% ethanol, dissolved in 50 µL distilled water and ultimately supplemented with RNase A.

**Protocol B (modified from Jobes et al., 1995)**

About 100 mg leaf sample was supplemented with 1 mL extraction buffer (100 mM sodium ac­etate, 100 mM EDTA, 500 mM NaCl, 10 mM DTT, 2% PVP (w/v), pH 5.5, 100 mg/mL K proteinase) and incubated at 55 °C for 1 hour through occasional eversions. SDS was added at about 1.5% of the available volume and incubated at 55°C for 1 hour. Then, 5 M sodium acetate was added at 1/3 of available mixture volume, kept at -20 °C for 30 minutes and centrifuged one more time. Cold isopropanol was added at 60% of available volume and incubated at -20°C overnight. Following the centrifuge phase, DNA was dissolved in distilled water and supplemented with a solution including 0.5 volumes of 5 M NaCl and 1.5 volume of isopropanol at about twice of available volume. The resultant solution was then centrifuged again, dissolved in distilled water and supplemented with cold LiCI at 1/3 of available volume against RNA contamination. The solution was incubated at -20 °C for 1 hour and centrifuged again. The resultant precipitate was dissolved in 50 µL distilled water, settled with isopropanol and RNA residues were treated with RNase A.

**Protocol C (modified from Dellaporta et al., 1983)**

About 100 mg leaf sample was supplemented with 1 mL extraction buffer (including 10% SDS, 50 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 8.0, 20 mL/L β-mercapto ethanol, and 10 mg PVP) and the solution was incubated at 65°C for 45 minutes. Sodium acetate was added as to have a final concentration of 1 M and samples were incubated over ice for 20 minutes. Following the centrifuge, DNA solution was supplemented with cold isopropanol at equal volume and kept at 20°C for 1 hour to get a precipitate. The solution was centrifuged again, washed through 70% ethanol and dried at room temperature. DNA samples were dissolved in 100 microliter distilled water, supplemented with RNase A kept in a solution including 0.3 M sodium acetate overnight. Next day, the precipitate was washed through 70% ethanol and the DNA was dissolved in 50 microliter distilled water.

**Protocol D (modified from Doyle and Doyle, 1990)**

About 100 mg leaf sample was supplemented with 1 mL extraction buffer (including 2% Cetyl trimethylammonium bromide (CTAB), 100 mM Tris-HCl, pH 7.5, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 20 mL/mL β-mercaptoethanol and 20 mg PVP ), incubated at 60 °С for 1 hour, supplemented with 600 µL chloroform and centrifuged. Isopropanol was added at equal volume to get precipitate in DNA samples and incubated at -20°С overnight. Following the centrifuge, sample was washed through 70% ethanol, dissolved in distilled water and supplemented with RNase A.

DNA samples obtained from each protocol was electrophoresed in 1% agarose gel containing 1.5% TAE buffer at 80 V for 1-1.5 hours. They were stained with ethidium bromide and imaged under UV light in an EL Imaging System (Bio-Rad, USA) to visualize DNA quality and quantity. DNA of the same samples was also measured in a NanoDrop™ Lite Spectrophotometer and DNA purity was determined based on A260 / 280 nm absorbance ratio. For each parameter, the mean and standard deviation of triplicate measurements was calculated. The statistical significance threshold was set at 95% (0.05). All statistical analyses were performed using SPSS 15.0 statistical software.

**PCR and electrophoresis**

In PCR analyses, 12 RAPD primers were used to determine DNA purity and quantity (Table 1). Amplification reactions for RAPD analyses were performed in 25 µL volume containing 1X PCR buffer(1,5 mM MgCl2, 200 mM dNTPs, 15 ng primer, 0.7 unit Taq DNA polymerase and 10 ng genomic DNA). Initial denaturation phase of DNA amplification reactions was conducted in 40 cycles with incubations at 95 oC for 3 minutes, at 94 oC for 1 minute, at 35 oC for 1 minute, at 72 oC for 2 minutes and at 72 oC for 10 minutes. PCR products were separated in 2% agarose gel with 1X TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA) and run at 115 V for 3 hours. The gel was stained with ethidium bromide and imaged under UV light in a Gel Imaging System (Bio-Rad, USA) and assessments were made based on number and quality of bands of each protocol. To validate proper markers in RAPD analyses, 100 bp standard DNA ladder was used.

Table 1.RAPD primers used in PCR analyses

|  |  |  |  |
| --- | --- | --- | --- |
| **Primers** | **Sequence** | **Primers** | **Sequence** |
| OPAX09 | GGAAGTCCTG | OPAP06 | GTCACGTCTC |
| OPAX08 | AGTATGGCGG | OPAP05 | GACTTCAGGG |
| OPAX07 | ACGCGACAGA | OPAP04 | CTCTTGGGCT |
| OPAX05 | AGTGCACACC | OPAP03 | GTAAGGCGCA |
| OPAX03 | CCAAGAGGCT | OPAP02 | TGGTCATCCC |
| OPAX02 | GGGAGGCAAA | OPAP01 | AACTGGCCCC |

**RESULTS AND DISCUSSION**

A proper DNA extraction protocol is required for DNA marker-based studies in new plant species (Konaté et al., 2007). In this study, quality DNA was tried to be isolated to put forth the genetic similarities in carob trees with natural spread in Turkey. DNA isolations were carried out from the leaves over old shoots and the leaves over young shoots with 4 different isolation protocols to overcome the problems encountered in DNA isolations.

While the lowest DNA concentration in young leaves was obtained from protocol A (5.73±0.89 ng/µl DNA), DNA concentration of Protocol D were significantly higher than other protocols. No statistically significant differences were found between Protocol B and C. In old leaves, the lowest DNA concentration was obtained from Protocol A (2.9±0.4 ng/µl DNA) and the highest DNA concentration was obtained from Protocol C (11.36±0.8 ng/µl DNA). Statistically significant differences were not found among other protocols. DNA concentration of old leaves significantly lower than young leaves at all protocols. Also DNA concentration of fresh leaves measured at Protocol D. DNA concentrations obtained from the freshest tip leaf measured 656.66±36.95 ng/µl DNA. DNA concentrations of fresh leaves were significally higher than old leaves. But this values were lower than young leaves.

A260/280 nm absorbance ratio is the most significant criterion specifying DNA purity. In young leaves, the lowest A260/280 nm absorbance ratio was observed in protocol A (0.76±0.02) and the values in other protocols were between optimum values. In old leaves, while the ratios were low in protocols C and D (<1.5), the values in protocols A and B (respectively 1.91±1.1; 1.75±0.6) were close to optimum values (Table 3). Statistically significant differences were not found among protocols. While absorbance rations of Protocol A and B were optimum values (1.5-2.0); This rations of Protocol C measured lower than optimum values (1.45±0.03). Statistically significant differences were not found among absorbance of Protocols A and B and C in young and old leaves. But absorbance ratios of old leaves in Protocol D were significantly lower than young and fresh leaves(Table 3). There is good level positive correlation (r=0.856) between DNA quantity and DNA quality in young leaves. There is no correlation between DNA quantity and DNA quality in old leaves.

Following the spectrophotometer measurements, same DNA samples were electrophoresed in 1.5% agarose gel (Figure 1). While DNA was encountered in samples in protocol B and D, DNA was not encountered in protocols A and C. The protocol B yielded the best results.

Aubakirova et al (2014) indicated that the extraction protocol decreasing DTT factors and including SDS detergent buffer (protocol A) yielded better DNA concentration and quantity in dry leaf samples of *M. sieversii*. However, such better outcomes were not able to be obtained from young and old carob leaves of the present study. The protocols B and D yielded the best results in young and tip leaves. Compared to the protocol on dry leaves of Aubakirova et al. (2014), the protocol D on young leaves (Doyle and Doyle, 1990) had worse outcomes, but the protocol yielded well outcomes with young and fresh leaves of the present study. Different results may be observed in different plants with different DNA isolation protocols. Such differences between above mentioned studies may be resulted from different species of Aubakirova et al. (2014) (dry and fresh leaves of apricot, apple). Aubakirova et al. (2014) obtained quite well results from protocols B and C and DNA fragmentation was not encountered in both dry and fresh leaves. Protocol B yielded well outcomes in *M. sieversii* leaves. But compared to other protocols, protocol B is a labor-intensive method and thus such labor requirement was considered as a disadvantage of the method (Aubakirova et al., 2014).

PCR analyses were performed on DNA samples obtained from protocols B and D yielding the best results in spectrophotometer measurements and gel images with 12 RAPD primers (Table 1) (Figure 2). Resultant images revealed that RAPD primers successfully worked out in DNA samples obtained from old, young and fresh leaves with protocols B and D (Figure 2). According to PCR analyses on some DNA samples of some primers, some bands were different. Such differences may be related to differences in DNA concentrations.

A study was carried out with 5 different DNA isolation methods used Aubakirova et al. (2014) in bamboo plants and quality DNA was obtained from green leaves compared to fresh leaves because of relationships with high antioxidant level and low secondary metabolite content (Balik et al., 2008). Secondary metabolites stored in vacuoles (Kulkarni et al., 2001) are freed from the genetic materials fragmenting leaf samples with CTAB extraction or SDS-like detergents. When they were freed, polyphenols and melanin with high affinity to nucleic acids are oxidized with atmospheric oxygen to form tannin. In this way, oxidized polyphenols are attached to DNA and they settle with DNA following alcohol supplementation, then they take a brown

color and ends up with high-density solution (Guillemaut and Maréchal-Drouard, 1992). Although there are antioxidants like PVP in lysis buffer, the natural antioxidants play a significant role in prevention or reduction of fragmentation of materials like fats, proteins and nucleic acids (Halliwell, 1990).

Table 2. Spectrophotometric measuremens on quantity of DNA extracted with 4 different isolation protocol(ng/µl DNA)

|  |  |  |  |
| --- | --- | --- | --- |
| **Protocol** | **Young Leaf** | **Old Leaf** | **Fresh Leaf** |
| A | 5,73±0,89 a\*/C\*\* | 2,9±0,4 b\*/B\*\* |  |
| B | 65,01±2,89 a\*/B\*\* | 7,2±2,01 b\*/AB\*\* |
| C | 75,56±15,15 a\*/B\*\* | 11,36±0,8 b\*/A\*\* |
| D | 900,66±23,38 a\*/A\*\* | 5,29±2,81 c\*/AB\*\* | 656,66±36,95 b |

**\*** Different small letters indicate statistically significant differences between leaves.

\*\*Different big letters indicate statistically significant differences between protocols.

Table 3. Spectrophotometric measuremens on purity of DNA extracted with 4 different isolation protocol (A260/280nm absorbance ratio)

|  |  |  |  |
| --- | --- | --- | --- |
| **Protocol** | **Young Leaf** | **Old Leaf** | **Fresh Leaf** |
| A | 0,76±0,02 a\*/B\*\* | 1,91±1,1 a\*/A\*\* |  |
| B | 1,78±0,15 a\*/A\*\* | 1,75±0,6 a\*/A\*\* |  |
| C | 1,63±0,09 a\*/A\*\* | 1,45±0,03 a\*/A\*\* |  |
| D | 1,97±0,03 a\*/A\*\* | 0,88±0,09 b\*/A\*\* | 2,01±0,05 a |

**\*** Different small letters indicate statistically significant differences between leaves.

\*\*Different big letters indicate statistically significant differences between protocols.

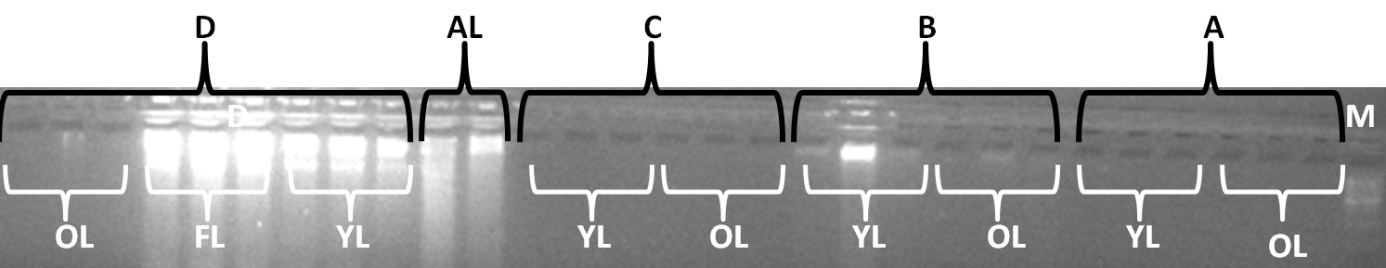


Figure 1. Gel image of Carob DNA Quantity and Quality using 1% agarose gel A, B, C, D: Protocols, AL: Almond DNA, OL: Old leaves, FL: Fresh leaf, YL: Young leaf, M: 100 bp marker

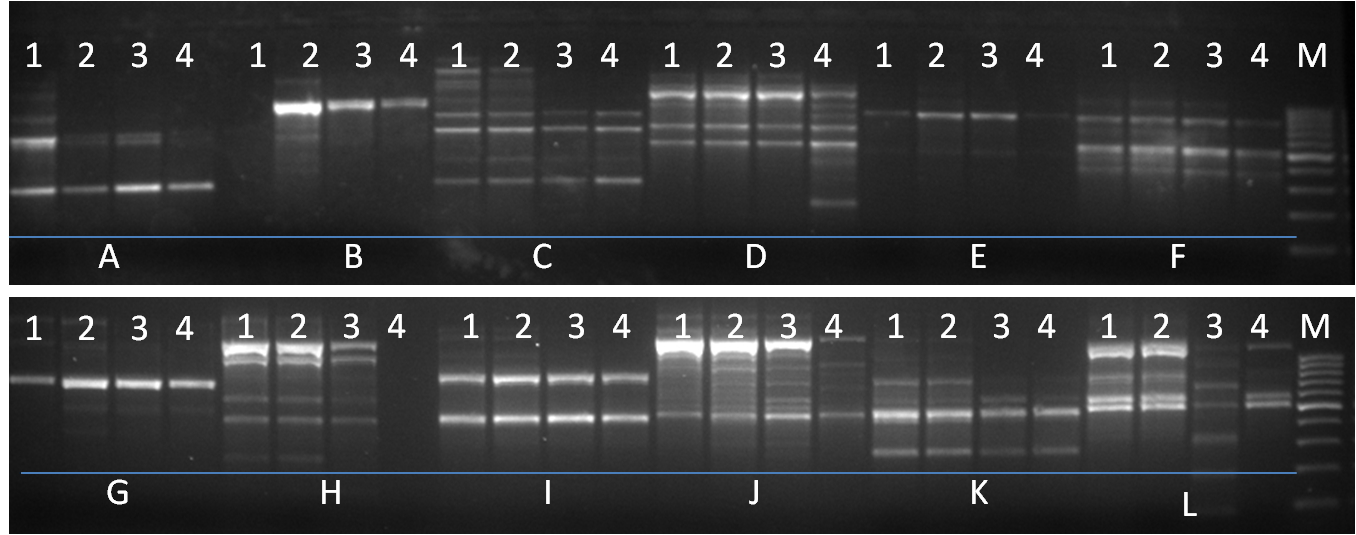


Figure 2. Gel image of Carob DNA PCR products

1. Protocol B Old leaf, 2. Protocol B young leaf, 3. Protocol D Young leaf, 4. Protocol D fresh leaf

A: OPAX09, B: OPAX08, C: OPAX07, D: OPAX05, E:OPAX03, F: OPAX02, G: OPAP06,H:OPAP05, I:OPAP02, J:OPAP03, K:OPAP02, L:OPAP01

Konate et al. (2009) obtained well-quality genomic DNA to be used in PCR analyses with the protocols adopted from Dellaporta et al. (1983), Doyle and Doyle (1987) and Ouenzar et al. (1998) for DNA isolation from carob leaves. The best results were obtained from the method reported by Ouenzar et al. (1998) and supplemented with different concentrations of lysis buffer. With the relevant method, sufficient DNA was obtained from Aïn Safa genotypes (1.765 ng μL-1). DNA/Protein ratio was observed as 1.88. In this study, the protocol of Dellaporta et al. (1983) was used for DNA isolation from old and young carob leaves, bur desired quality DNA was not able to be obtained. The use of young and fresh leaves is ideal for DNA isolation (Sytsma et al., 1993). But, use of dry leaves just shortens the sampling time over large fields. The results on DNA isolation mostly depend on plant species and drying conditions. Therefore, as it was in different species, there is a need for different isolation protocols in close relative species, even in closely-related species (Weishing et al., 1995).

Carob trees are evergreen species. It is possible to leaves over the tree throughout the year. Therefore, it is also possible to perform molecular marker studies through DNA isolation from the leaves throughout the year. However, in periods without spring shoots, leaf samples can be taken only from old shoots. In this study, pure DNA could not be obtained from old leaves through routine DNA isolation. Therefore, 4 different DNA isolation protocols were used to obtain pure DNA especially from old leaves. It was observed in this study that DNA isolation methods and leaf ages had significant effects on DNA quality.

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