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RESEARCH ARTICLE

Comparison and improvement of DNA extraction methods in *Buxus hyrcana*

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ABSTRACT: *Buxus hyrcana* is one of the endangered and evergreen species of the Hyrcanian forests in Iran. The genetic diversity assessment is an essential step towards the conservation of this species. High-quality DNA is required for molecular markers analysis; therefore, we compared different DNA extraction methods on leaf samples of *B. hyrcana*. The quantity and quality of the extracted DNAs were evaluated by spectrophotometry and gel electrophoresis. Also, ISSR (Inter simple sequence repeats) markers were applied on the extracted DNAs to compare their quality for PCR amplification. Results showed that quantity, quality, and PCR efficiency and reproducibility were different for DNA extracted using different methods. The quality of the DNA at the absorbance A260/A280 ratio ranged from 1.02 to 1.97. The highest concentration of DNA measured by spectrophotometry belonged to the Cota-Sanchez extraction protocol (695.3 ng/µl) and the lowest value was obtained with Edward4 method (204.7 ng/µl). The modified Onate method (Onate2) was extracted the highest DNA concentration by comparison of brightness against the DNA ladder. Among the different extraction methods, the good quality and quantity were obtained in extracted DNA for Doyle and Doyle, Cota-Sánchez and modified Onate protocols; the latter method (Onate2) created both good quality and quantity of extracted DNA and operated effectively in terms of cost and time. Onate2 had the best amplification results with ISSR primers.

KEYWORDS: Box tree, DNA quality and quantity, ISSR, PCR

INTRODUCTION

Enlisted as an endangered species, *Buxus hyrcana* is a shade-tolerant and evergreen species in the Hyrcanian forests [1]. Its sustainable conservation is threatened by over-exploitation and the spread of box blight disease which can ultimately destroy its genetic resources in the near future. The assessment of genetic diversity is a primary step towards understanding evolutionary genetic drifts and populations' characterization, especially when considering endangered species. In most genetic studies such as genetic diversity, satisfactory results can be obtained by using molecular markers that require high-quality DNA [2-5].

DNA extraction from plant tissues is more difficult than mammalian tissues, primarily due to the hard cell walls surrounding plant cells [6]. Furthermore, the tissues of woody plants contain different secondary metabolites such as polysaccharides, polyphenols and tannins. Such impurities can cause more serious problems in genomic DNA extraction and downstream processes such as DNA cutting, amplification, and cloning [7, 8]. The contamination and impurities that may exist in the DNA extract can bind tightly to DNA subunits, thereby reducing the presence and detection of polymorphisms between different individuals in a population [9, 10]. Therefore, an ideal DNA extraction method should be fast, simple, affordable, and has few manipulating steps. It should also pose minimum requirement for specialized equipment and hazardous chemicals [11-13].

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Numerous methodologies for DNA extraction from plant tissues have been developed [11, 14-17]. The cetyltrimethyl ammonium bromide and (CTAB) sodium dodecyl sulfate (SDS) methods are commonly used for DNA extraction from different organisms [18]. CTAB method is particularly useful for DNA extraction from plants that produce high amounts of polysaccharides [19]. SDS-based protocols are widely used as an alternative to CTAB, but these may be suitable for a narrower range of species or cell types [20].

Plants of the genus *Buxus* are used in folk medicines to treat wide range of diseases. Therapeutic effects of *B. hyrcana* have been linked to its secondary metabolites including alkaloids and phenols [21, 22]. These compounds can interfere with the DNA extraction procedures. So, an efficient protocol for DNA extraction as well as the optimization of the PCR conditions is required. An optimum DNA extraction method was lacking for molecular analysis of *B. hyrcana*. This study aimed at comparing and optimizing different DNA extraction methods for this species. The quality and quantity of extracted DNA were evaluated, and their effects were assessed in terms of analysis of genetic diversity by ISSR markers.

MATERIALS AND METHODS

Plant material

Bulk young and healthy leaf samples were collected from a *Buxus* tree in Sisangan habitat (Mazandaran Province, Iran, 36°58' N, 51°80' E). The leaf samples were stored frozen at -20°C. Three samples from the same accession were used for DNA extraction.

Reagents and consumables

The necessary materials for DNA extraction were liquid nitrogen, chloroform-isoamyl alcohol (24:1) (CIA), isopropanol, B-mercaptoethanol (BME), sodium acetate (NaOAc), potassium acetate, ammonium acetate, ethanol 70%, 80% and 95%, cetyl trimethyl ammonium (CTAB), sodium dodecyl sulfate (SDS), ethylene diamine tetraacetic (EDTA), Tris-HCl, NaCl, sodium citrate and citric acid

DNA extraction protocols

For DNA isolation, 50 mg of leaf sample was used in each method. The following procedures comprised the process of DNA extraction:

Doyle & Doyle [14]

1. The addition of 500 µl CATBbuffer (0.1 M Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, plus 0.4% BME added just before use) to 1.5 ml tubes containing leaf tissue which had been ground by liquid nitrogen. The ingredients inside the tubes were mixed thoroughly by shaking the tubes well.
2. The incubation of microtubemicrotubes at 65°C for 1 hour and then having the samples vortexed every 15 min.
3. The addition of 500 µl CIA to each microtubemicrotube and then shaking the tubes manually.
4. The centrifuge of samples at 15814 g for 8-10 min, and then considering to pipette off the aqueous phase and transferring it to new microtubemicrotubes.
5. The addition of 0.08 ml of cold 7.5 M ammonium acetate and 0.54 ml of cold isopropanol to each microtube, mixing them well and storing them at -24 °C for 45 min or overnight to obtain a better yield.
6. The centrifuge of samples at 15814 g for 3 min, and then discarding the top liquid and adding 700 µl cold ethanol (70%) to each microtube. The microtubes were inverted once to allow the mixing of ingredients.
7. The centrifuge of samples at 15814 g for 1 min, and then discarding the ethanol and adding 700 µl cold ethanol (95%) to each microtube. The microtubes were inverted once to allow the mixing of ingredients.
8. The centrifuge of samples at 15814 g for 1 min, and then discarding the ethanol, drying the DNA pellet at room temperature and finally re-suspending the pellets in 100 µl TE buffer.

Cota-Sanchez [16]

1. Leaf powder which had been previously ground by liquid nitrogen was added to the 1.5 ml volume of microtubes. Each microtube also received 750 µl CTAB 2X buffer (0.1 M Tris-HCl pH 8.0, 25 mM EDTA, 1.4 M NaCl, 1% CTAB) and 3 µl BME before being mixed well.
2. The microtubes were immersed in a water bath at 60°C for 1 hour. The tubes were inverted manually every 15 min.
3. The addition of 700 µl CIA to each tube, and then their centrifuge at 9240 g for 10 min, followed by the transfer of supernatants to new microtubes.
4. The addition of 0.33 volume of cold isopropanol to each tube and then storing them at -20°C for 1 hour.

5. The centrifuge at 9240 g to 13305 g for 10 min at room temperature, and then discarding the supernatant and drying the pellet at room temperature.
6. Dissolving the DNA pellet at 100 to 200 µl TE buffer and then allowing the samples to remain at 37°C for 30 min.
7. The addition of 200 µl NaOAc 2.5 M and 500 µl cold ethanol 90%, and then storing the samples at -20°C for 30 min.
8. Performing centrifuge at 9240 g to 13305 g for 5 min and then discarding the supernatant of each microtube.
9. Adding 1 ml cold ethanol (70%) to the DNA pellet, performing the centrifuge at 9240 gg for 4 min, and finally pipetting off the ethanol.
10. Drying the DNA pellet at room temperature and re-suspending it in 100 µl TE buffer.

Dellaporta [11]
1. Adding the leaf tissue that had been ground by liquid nitrogen to 1.5 ml microtubes, and then adding 600 µl SDS buffer (0.1 M Tris-HCl pH 8.0, 50 mM EDTA, 0.5 M NaCl, 0.2% SDS, plus 0.1% BME added just before use).
2. Incubating the samples at a water bath of 60 °C for 20 min, adding one third of a volume of potassium acetate, and then mixing them vigorously before placing the tubes on ice for 5 min.
3. Performing the centrifuge at 12000 g for 20 min, transferring the supernatant to new tubes and then adding 0.5 volume cold isopropanol, followed by inverting the tube once so as to mix and store the sample at 4°C for 20 min.
4. Performing the centrifuge at 12000 g for 10 min, discarding the supernatant, and drying the DNA pellet for 10 min.
5. Re-suspending the DNA pellet in 200 µl TE buffer at 65°C for 30 min.
6. Transferring the solution to new tubes and performing the centrifuge at 12000 g for 5 min.
7. Transferring the supernatant to new tubes and adding 0.1 volume of sodium acetate and two thirds of a volume of cold isopropanol, inverting the tubes to be mixed and storing them at 4°C for 1 hour.
8. Washing the DNA pellet with 500 µl cold ethanol (80%) for 10 min and performing the centrifuge again for 1 min.
9. Drying the pellets at room temperature and dissolving the DNA pellets at 100 µl TE buffer depending on the pellet size.

Edward base methods [15]
The method of Edward DNA extraction (Edward1) was followed, although with three forms of modification (i.e. Edward2, Edward3 and Edward4), for DNA extraction (Table 1). The following procedures were used accordingly:
1. Transferring leaf tissue powdered to 1.5 ml micro-tubes, adding 400 µl SDS buffer (0.2 M Tris-HCl pH 8.0, 25 mM EDTA, 0.25 M NaCl, 0.5% SDS) and inverting the tubes for 5 sec.
2. Performing the centrifuge at 12000 g for 1 min, transferring 300 µl of supernatant to new tubes and adding 300 µl cold isopropanol, and then storing the samples at room temperature for 2 min.
3. Performing the centrifuge at 12000 g for 5 min, drying the DNA pellets at room temperature and dissolving the pellets at 100 µl TE buffer.

Onate-Sanchez base methods [17]
The Onate-Sanchez DNA extraction method (Onate1) was also used. It had three modifications (Onate2, Onate3 and Onate4) for DNA extraction (Table 2), according to the following procedures.
1. The grinding of leaf tissue with liquid nitrogen, transferring the leaf powder to 1.5 ml microtubes and adding 300 µl lysis buffer (68 mM Sodium Citart, 132 mM Citric Acid, 1 mM EDTA, 2% SDS). Inverting the tubes for 2 sec and leaving the tubes at room temperatures for 5 min.
2. Adding 200 µl of protein-DNA precipitation solution (16 mM Sodium Citart, 32 mM Citric Acid, 4 M NaCl) to each sample and maintaining it at 4°C for 10 min.
3. Performing the centrifuge at 15814 g for 10 min, transferring the supernatant to new tubes and adding 300 µl cold isopropanol before shaking the microtubes for a thorough mix.
4. Performing the centrifuge at 15814 g for 4 min, discarding the supernatant, washing the pellets with 300 µl ethanol (70%) and then carrying out the centrifuge at 15814 g for 1 min.
5. Discarding the ethanol and placing it at room temperature to dry, and finally adding 100 µl TE buffer.

Table 1. Modified Edward DNA extraction methods.

Methods	Modification	SDS (%)	Additional steps
Edward1	None	0.5	
Edward2	Modified	0.5	Between step 1 and step 2: Add 200 µl CIA and mix well
Edward3	Modified	2	Between step 1 and step 2: Incubate for 10 min in 60°C in water bath
Edward4	Modified	2	Between step 1 and step 2: Add 200 µl CIA and mix well

Table 2. Modified Onate-sánchez DNA extraction methods.

Methods	Modification	Additional steps
Onate1	None	
Onate2	Modified	Between step 1 and step 2: Add 200 µl CIA and mix well
Onate3	Modified	Step1: replace extraction Buffer with SDS buffer (Edward methods)
Onate4	Modified	Step1: replace extraction Buffer with SDS buffer (Edward methods) Between step 1 and step 2: Add 200 µl CIA and mix well

DNA analysis

The quantity and quality of the extracted DNAs were evaluated by spectrophotometry and gel electrophoresis. Also ISSR (Inter simple sequence repeats) markers were applied on the DNAs to compare the quality of DNA for PCR amplification. ISSR molecular markers is efficient and suitable for genetic diversity studies of forest species [23].

The DNA being extracted was processed by electrophoresis on 1% (w/v) agarose gel with SB buffer at 70 V for 45 minutes. All gels were stained with 1x cyber safe and documented with QIAGEN system. Ultimately, the Gel images were analyzed with GelAnalyzer 2010a freeware. DNA concentration was estimated by spectrophotometry and also comparing DNA bands with the ladder band intensity which was then calculated based on ladder concentration [24]. The purity of the extracted DNA was evaluated using the ratios between the absorption at 260 and 280 nm (A260/A280) and at 260 and 230 nm (A260/A230). The A260/A280 and A260/A230 ratios provide indications of protein contamination and carbohydrate contamination, respectively [25].

ISSR analysis

ISSR primers were used for testing the quality and performance of the extracted DNA by PCR amplification. Three ISSR primers (ISSR5, 5'- gtggtggtggtggtggtg -3'; ISSR9, 5'- gagagagagagagagayc -3' and ISSR17, 5'- gaagaagaagaagaagaaaa -3') were used [26]. The primers were synthesized by Bioneer, South Korea. Each PCR (12.5 µl volumes) reaction contained approximately 40 ng of genomic DNA (based on the ladder-compare method), 0.18 µl *Taq* Polymerase (2.5 U/µl, Thermo Fisher Scientific, USA), 1.25 µl 10× PCR reaction buffer, 1 µl dNTP Mixture (2.5 mM), 0.5 µl each primer (10 pM), and ddH2O up to 12.5 µl. The amplification was performed in a Thermal Cycler (Applied Biosystems model 2720). Thermal cycling conditions consisted of a 5 min initial denaturation at 94°C followed by 35 cycles of 30 sec at 95°C, 40 sec at 64.5°C for ISSR5 and 52°C for ISSR9 and ISSR9, and 1 min at 72°C. The final extension was 7 min at 72°C. Subsequently, 5 µl PCR products and 1 µl 6X loading buffer were subjected to electrophoresis on a 1.5% agarose gel with SB buffer at 115 V for 90 min, stained in cyber safe for 20 min, and photographed with QIAGEN system. The gel images were analyzed by GelAnalyzer. The sum pertaining to the intensity of bands, normalized based on the ladder intensity, was used as the measure of DNA amplification quantity.

Statistical analysis

All of the tests were conducted with at least three replicates. The data in this study was recorded as the mean value ± standard error. Correlations among the measured parameters were determined using the Pearson's correlation coefficient by JMP statistical software (Version 4).

RESULTS AND DISCUSSION

Comparing DNA extraction methods

An ideal DNA extraction method should be comprised of limited steps to minimize the experimental error, with minimum use of hazardous chemical reagents and less

Table 3. Compare different extraction methods depending on how much time and resources are needed.

Method	Base	Time required (h)	Cost (EUR per sample)
Cota-Sanchez	CTAB	5:00	1.1
Doyle & Doyle	CTAB	2:15	0.6
Dellaporta	SDS	2:40	0.5
Edward (1~4)	SDS	0:20	0.4
Onate (1~4)	SDS	0:30	0.4

Table 4 The effect of different extraction methods on DNA quantity and quality of *Buxus hyrcana*.

Method	DNA Quantity(ng/µl)				DNA Quality			
	Comparison with DNA ladder		Spectrophotometry		260/230		260/280	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cota-Sanchez	42.6	2.10	695.3	55.9	1.94	0.03	1.74	0.10
Dellaporta	50.2	3.56	240.7	15.9	2.08	0.07	1.94	0.06
Doyle & Doyle	50.5	5.41	356.7	34.8	1.99	0.10	1.97	0.11
Edward1	28.2	2.07	277.3	16.4	0.57	0.01	1.23	0.01
Edward2	27.0	0.83	304.7	39.9	0.59	0.01	1.21	0.01
Edward3	17.9	2.29	275.3	90.6	0.64	0.02	1.02	0.22
Edward4	20.5	0.86	204.7	56.0	0.74	0.12	1.34	0.05
Onate1	50.7	3.04	364.7	61.0	2.96	0.84	1.91	0.07
Onate2	53.8	4.48	342.7	47.4	2.13	0.10	1.90	0.04
Onate3	37.4	5.71	455.3	99.7	1.62	0.10	1.85	0.05
Onate4	38.3	3.71	408.0	84.0	1.98	0.09	1.83	0.07

demand for specialized equipment. It should be fast, cost-effective and straightforward, and produce high-quality DNA suitable for molecular techniques [11, 13]. In this study, different methods were used for DNA extraction from *B. hyrcana*. These methods were varied in their durations of procedure (ranging from 30 min to 5 hours) and their costs (from 0.4 to 1.1 € per sample) (Table 3).

Results showed that the Cota-Sanchez method had the highest yield of extracted DNA based on spectroscopy at 260 nm, as compared to the other methods tested here, while based on the ladder-compare method, Onate2 yielded the highest quantity (Table 4). Cota-Sanchez is a CTAB-based protocol. CTAB, a cationic detergent, help to disrupt plant cell membranes and separate nucleic acids from polysaccharides [27]. In SDS-based methods, such as Onate2, SDS use to aid in lysing cell, followed by adding chloroform-isoamyl alcohol to remove non-DNA biomolecules such as proteins and lipids [28]. The chemical structure of CTAB and SDS may present them more or less effective based on compounds found in plant tissue [29]. DNA yields were affected by surfactants (CTAB and SDS), Tris/HCl, EDTA, and NaCl concentrations [30].

By carrying out the gel electrophoresis of the same volume of extracted DNA (Fig. 1), it was observed that the Cota-Sanchez method produced the highest level of smear compared to the other methods (Fig. 1-e). Degraded DNA, RNA and nucleotides may sometimes interfere with DNA quantification, as when quantifyingby spectroscopy at 260 nm. Similar results have been reported by Holden et al. (2009). It was observed that some DNA extraction methods could calculate the amount of DNA as a higher estimation when using spectroscopy at 260 nm than when arriving at an estimate by the use of PicoGreen fluorescence [31].

At their maximum absorption at 260 nm, nucleic acids (DNA and RNA) represent the amount of DNA and proteins have the highest absorption at 280 nm. The DNA 280/260 ratio check for protein contaminants and should be in the range of 1.7 to 1.9 [32, 33]. According to table 4, the methods of Dellaporta, Doyle & Doyle, Onate1 and Onate2 had ratios of 260/280 that ranged between 1.9 and 1.97. However, the ratio was lower than 1.35 in the modified Edward methods (Edward 1~4). In some medicinal plants, the 260/280 ratio reportedly ranges between 1.7 and 1.9 which are suitable for comprehensive PCR success predictions, even though 260/280 ratios

below 1.3 and above 2.3 indicate a DNA of too poor a quality to be amplified [34].

In general, a high absorption at 230 nm shows the contamination with DNA extraction buffers or with other inorganic materials, which eventually created errors in the PCR results [35]. The 260/230 values for pure nucleic acids are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2 [36]. Apart from the Onate3 and Edward 1~4, other methods had 260/230 ratios higher than 1.8, while the Onate1 showed the highest ratio in between them (Table 4).

The Edwards DNA extraction showed contamination at 280 and 230 nm, indicating the presence of proteins and carbohydrates in extracted plant DNA. In the Edwards method, the DNA is pelleted in the lysis buffer without an intervening organic extraction to push cell debris and other contaminants into a separate phase from the DNA-saturated buffer. These results were consistent with a previous study conducted in *Osmanthus* [29].

Effect of extracted DNA on ISSR results

It was demonstrated that ISSR-PCR method are suitable and sensitive for studying genetic diversity and detect the genetic differences between closer populations in similar habitats [37]. Analysis of genetic diversity relies on high quantity and quality of pure DNA. In PCR-based techniques, including PCR-based markers, the reproducibility of PCR results is essential. Also, PCR amplification itself is known as the best indicator of extracted DNA quality [34]. For these purposes, the DNA extracted from different methods was subjected to PCR

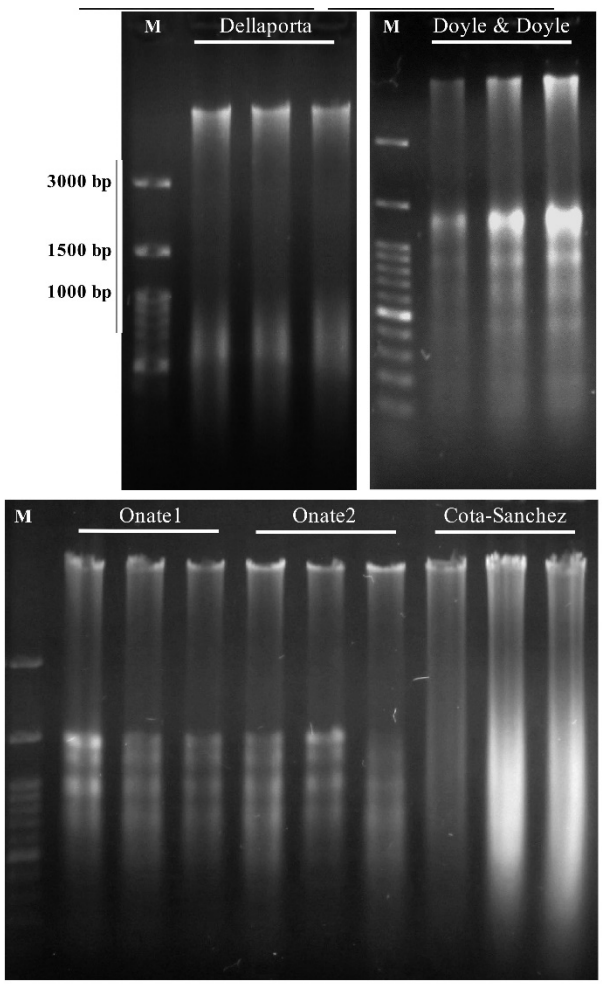

Figure 1. Gel electrophoresis of extracted DNA from *Buxus hyrcana*. DNA extractions using a) Dellaporta, b) Doyle & Doyle, c) Onate1, d) Onate2 and e) Cota-Sanchez methods. M: 100pb DNA ladder.

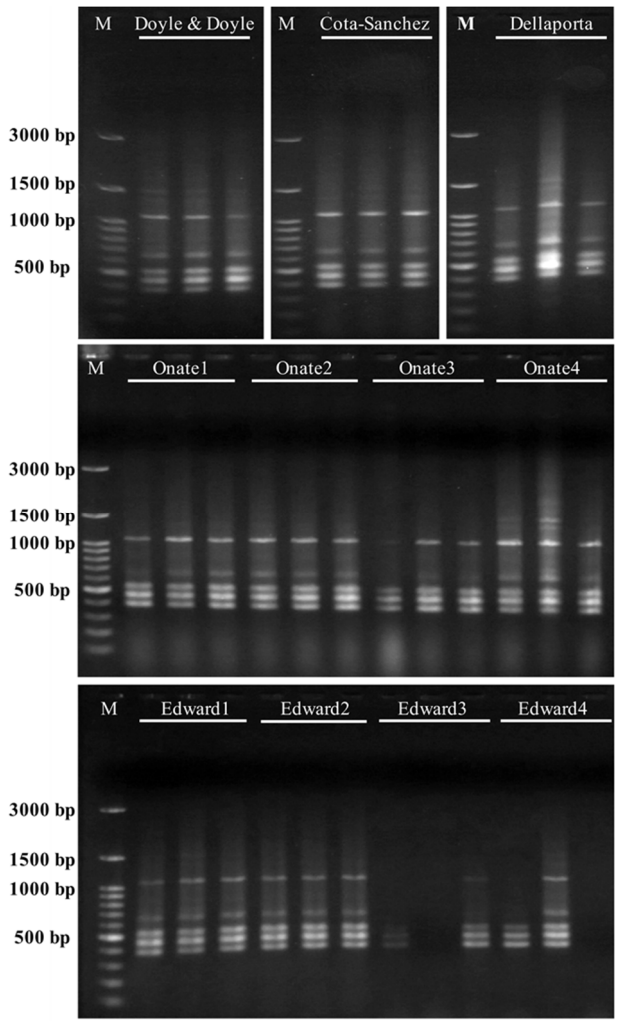

Figure 2. Effect of different extraction method on amplification results of the ISSR5 primer in a single *Buxus hyrcana* tree. M = 100 bp DNA ladder.

Table 5. The effect of different DNA extracted on PCR product concentration and number of bands produced by ISSR primers.

Method	PCR product concentration (ng/µl)		Total number of bands		ISSR5		ISSR9		ISSR17	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cota-Sanchez	25.07	1.84	16.2	0.40	5.50	0.34	6.67	0.33	3.83	0.17
Dellaporta	12.06	1.26	14.2	0.48	5.33	0.33	6.00	0.45	2.83	0.31
Doyle & Doyle	20.11	1.70	15.8	1.24	5.50	0.95	6.67	0.33	3.67	0.33
Edward1	15.32	0.97	14.7	0.67	5.33	0.33	6.67	0.33	2.67	0.33
Edward2	17.84	4.47	12.7	2.33	5.00	0.00	4.67	2.33	3.00	0.00
Edward3	1.31	0.89	4.0	1.53	2.67	1.45	0.67	0.67	0.67	0.67
Edward4	14.46	9.59	7.7	4.98	3.33	2.03	2.33	2.33	2.00	1.00
Onate1	26.11	4.34	16.0	1.15	5.00	0.00	8.00	0.58	3.00	0.58
Onate2	34.16	1.39	17.7	0.33	5.00	0.00	9.00	0.00	3.67	0.33
Onate3	18.75	3.90	16.7	0.88	4.67	0.33	8.33	0.67	3.67	0.33
Onate4	18.54	2.46	15.0	3.21	7.33	1.45	5.00	1.73	2.67	0.33

Table 6. Correlation between DNA quality and quantity parameters with DNA concentration and number of bands.

		1	2	3	4	5	6	7	8	9	10	11
1	PPC	1										
2	S	0.14	1									
3	260/230	0.77**	0.33*	1								
4	260/280	0.72**	0.19	0.82**	1							
5	230	-0.62**	0.33*	-0.73**	-0.58**	1						
6	280	-0.15	0.89**	-0.02	-0.22	0.59**	1					
7	PPC	0.46**	0.34*	0.49**	0.42*	-0.27	0.13	1				
8	TNB	0.55**	0.18	0.56**	0.55**	-0.42**	-0.04	0.73**	1			
9	ISSR5	0.30	0.03	0.33*	0.28	-0.31*	-0.08	0.39*	0.74**	1		
10	ISSR 9	0.57**	0.13	0.54**	0.58**	-0.42**	-0.12	0.71**	0.89**	0.47**	1	
11	ISSR 17	0.44**	0.26	0.46**	0.52**	-0.26	0.05	0.61**	0.71**	0.37*	0.57**	1

*,** have significant in 5% and 1%, respectively. PPC: PCR product concentration, S: Spectrophotometry, TNB: Total number of bands.

amplification by ISSR primers. Results showed that PCR product concentration and band production varied between the extractions methods (Fig. 2). Onate2 had the highest PCR product concentration and a total band number. The next in line were Cota-Sanchez and Doyle & Doyle, which had maximum PCR product concentration. Onate2 had the lowest standard error of the total band number (0.33) (Table 5). Although all the DNA samples were extracted from one plant and all must have had exactly identical genetic data, the band numbers produced by ISSR primers differed per extraction method. These results showed that the extraction method could affect the outcomes of ISSR performance. Similar results were reported by Singh et al. (2013) [38].

Although all PCR reactions used the same amount of DNA (40 ng calculated based on the ladder-compare DNA concentration), the total number of bands and the PCR product concentration had significant positive correlations with the amount of DNA as the estimated based on ladder bands, but not with a concentration estimated by spectrophotometry (Table 6). Thus the calculation of the quantity of DNA, using the absorption at 260 nm, may not be suitable for use in PCR calculation. Also, PCR product concentration had significant negative correlations with DNA absorption at 230 nm and a positive correlation with 260/230 and 260/280 ratio (Table 6). Other experiments showed that EDTA, carbohydrates and phenol, which are known as PCR inhibitors, have absorbance wavelengths near 230 nm [39-41]. These results suggested that, despite usingthe same amount of DNA in each PCR, a higher DNA quantity reaction can have a positive effect on PCR results. On the other hand, when equalizing the amounts of DNA in PCR reactions, low quantity samples transferred more contaminations to PCR reactions than did high quantity samples.

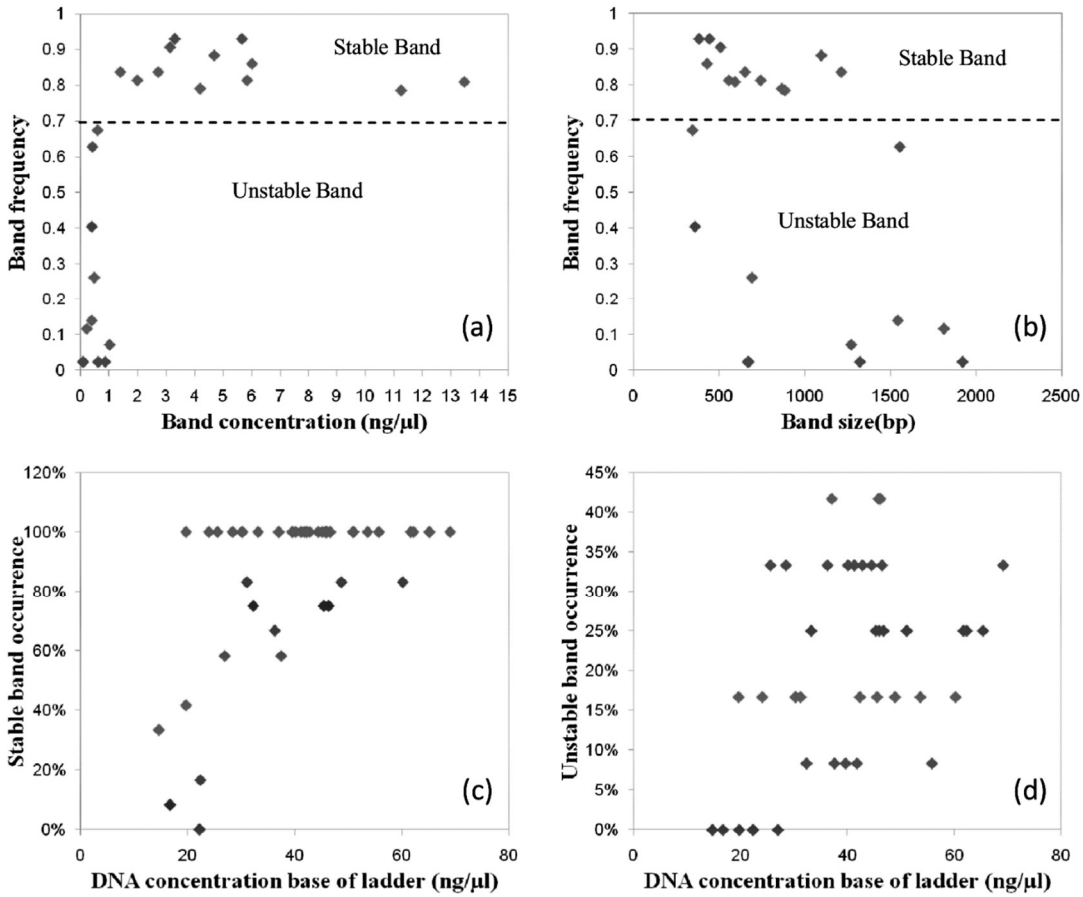

Figure 3. The relationship between ISSR band frequencies with band concentration (a) and band size (b). Effect of DNA concentration on stable(c) and unstable (d) band patterns.

PCR reactions with three ISSR primers on the extracted DNA produced 23 unique bands in total, with a frequency that ranged from 0.02 to 0.93 and sizes that ranged from 341 to 1923 bp. The average band concentration varied from 0.084 to 13 ng/µl. In the scatter plot of band frequency vs. its concentration, two groups of bands were separated. These included the stable bands (with a frequency higher than 0.75 and a concentration higher than 1.5 ng/µl) and unstable bands (with a frequency and a concentration lower than 0.75 and 1.5 ng/µl, respectively) (Fig. 3-A). The band frequency decreased in bands that had sizes smaller than 400 pb or larger than 1250 bp (Fig. 3-B). This may have been affected by PCR reaction and Gel electrophoresis. The PCR extension time limited the product size [42].

When two stable and unstable bands were separated from each other, the occurrence of stable and unstable band patterns varied from 0 to 100% and from 0 to 43% in different extraction methods, respectively (Fig. 3-C, D). In some extractions with DNA concentrations between 20 and 70 ng/µl, the stable band occurrence was 100% and other concentrations correlated positively with stable band occurrence (Fig. 3-C). This positive correlation was also found in unstable band groups (Fig. 3-D).

ISSRs have been successfully used for estimating genetic diversity in trees [43-45]. In genetic diversity analyses, with ISSR markers, PCR bands vary among genotypes by corresponding to the genetic variation among the genotypes. Even when two separate studies are on the same species using the same primers, ISSR banding patterns may vary considerably [46, 47]. This could be due to the effects of using different reagents and settings during PCR analysis [48]. In this study, the results showed that the DNA quality and quantity affect PCR analysis and produce different numbers of bands in one plant by different extraction methods.

Results showed that uniformity in extracted DNA (in terms of both quality and quantity) is more important than DNA quality or quantity alone. Based on these results, the total number of bands correlated with DNA quality and quantity. Therefore, the collections of extracted DNA that appear uniform are capable of producing uniform bands.

The following can be suggested for attempts aimed at reducing errors in the estimation of genetic diversity based on ISSR markers:

1) Using high-quality and reproducible DNA extraction methods.

2) Checking for uniformity in quantity and quality of each extracted DNA.

3) Ignoring ISSR bands with DNA concentrations lower than 1.5 ng/µl (unstable bands) from the analysis of genetic diversity.

4) Removing bands smaller than 400 and larger than 1500 pb from analysis (based on the PCR and electrophoresis condition).

To ensure a more transparent reporting of error rates in genetic diversity research, Crawford et al. (2012) recommended that researchers make detailed reports about the steps taken throughout their experiments, and that reports should preferably contain information about the properties of primers and the PCR reaction protocols associated with each primer [49]. Based on our results, reporting the DNA quality and quantity of each sample can be a valuable approach to reducing the errors in similar studies of this type.

This study suggested that the DNA quality and quantity can strongly affect the ISSR results. Degraded DNA causes the number of loci for ISSR markers to change because DNA is incomplete [50]. Here, the Onate2, Cota-Sanchez and Doyle & Doyle can be used effectively for a better recovery of *Buxus* genomic DNA in terms of higher quality and quantity. These parameters can thus improve PCR applications. But when considering the cost, time and reproducibility, it can be concluded that the modified Onate-Sanchez (Onate2) is the best extraction method for this species of plant. This method showed the highest number of ISSR bands, with the lowest variations, besides having the highest PCR product concentration, not to mention its high speed of operation as it only takes about 30 minutes to complete an extraction.

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مقایسه و بهبود روش‌های استخراج DNA در شمشاد هیرکانی (*Buxus hyrcana*)

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چکیده

یکی از گونه‌های در معرض خطر و همیشه سبز جنگل‌های هیرکانی ایران، شمشاد هیرکانی (*Buxus hyrcana*) می‌باشد. ارزیابی تنوع ژنتیکی مرحله مهمی برای حفظ این گونه می‌باشد. DNA با کیفیت برای بررسی با نشانگرهای مولکولی لازم بوده و بنابراین ما روش‌های استخراج DNA از نمونه‌های برگی *Buxus hyrcana* را مورد مقایسه قرار دادیم. کمیت و کیفیت DNA های استخراج شده با اسپکتروفتومتر و الکتروفورز مورد ارزیابی قرار گرفت. همچنین از نشانگرهای ISSR برای مقایسه کیفیت DNA برای تکثیر با PCR استفاده شد. نتایج نشان داد که کمیت، کیفیت، و کارایی PCR و تکرار پذیری در DNA های استخراج شده با روش‌های مختلف متفاوت بود. کیفیت DNA تعیین شده بر اساس A260/A280 در دامنه ۱/۰۲ تا ۱/۹۷ قرار گرفت. بیشترین غلظت DNA تعیین شده با روش اسپکتروفتومتری به روش استخراج Cota-Sanchez (۶۹۵/۳ ng/µl) تعلق داشت و کمترین مقدار در روش Edward4 (۲۰۴/۷ ng/µl) حاصل شد. در روش مقایسه شدت باند DNA استخراج شده با DNA ladder بیشترین غلظت در روش Onate2 مشاهده گردید. در میان روش‌های مختلف، روش‌های Doyle and Doyle، Cota-Sánchez و Onate تغییریافته منجر به حصول کمیت و کیفیت خوبی از DNA استخراجی شدند که آخرین روش (Onate2) کمیت و کیفیت خوبی از DNA استخراجی را ایجاد کرد و از لحاظ هزینه و زمان کارامدتر بود. Onate2 بهترین نتایج تکثیر با پرایمرهای ISSR را داشت.

کلمات کلیدی: درخت شمشاد، کمیت و کیفیت DNA، ISSR، PCR