Journal of Medicinal Plants Research Vol. 5(3), pp. 332-339, 4 February, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Extraction of genomic DNA from polysaccharide- and phenolics-rich *Ginkgo biloba*

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Accepted 18 August, 2010

One prerequisite to reliable molecular biology work is that the genomic DNA of a sample should be of good quality. The isolation of intact, high-molecular-mass genomic DNA is essential for many molecular biology applications including long Polymerase Chain Reaction (PCR), endonuclease restriction digestion, Southern blot analysis, and genomic library construction. Many protocols are available for the extraction of DNA from plant material. DNA extraction of *Ginkgo biloba* is quite difficult to work on because of the high phenolic and polysaccharide content of its leaves. This study aimed to determine which protocol to use and which part of *Ginkgo* tree is most appropriate to extract good-quality genomic DNA. For this purpose, cetyltrimethylammonium bromide protocol and protocol of commercially available kit by EZ1 Nucleic acid isolation system have been optimized for extraction of genomic DNA from *G. biloba* leaves. Efficient yields of high-quality amplifiable DNA was produced rapidly with kit by EZ1 Nucleic acid isolation method. The purified DNA which has excellent spectral quality was efficiently amplified by 5 arbitrary primers (OPA11-15), and was suitable for long-fragment PCR amplification.

Key words: Genomic DNA, random amplification of polymorphic DNA polymerase chain reaction, DNA extraction, phenolics.

INTRODUCTION

Herbal and aromatic plants are attracting more attention among contemporary plant researchers because some human diseases such as nerodegenerative disease, different types of cancer and diabet associated with free radicals that are scavenged by the plant naturally occuring polyphenolics, phenolics and flavonoids and other secondary metabolites having medicinal properties (Halliwell and Gutteridge, 1989; Vaya and Aviram, 2001; Wada and Ou, 2002). Secondary metabolites is produced by the enzymes such as such as flavanone 3 hydroxylase (F3H), flavonol synthase (FLS) and flavone synthase I (FS I) that are expressed by nucleic acids of the plants (Chua et al., 2008; Reddy et al., 2007). However, the same substances that make the herbal and aromatic plants worthy of such intensive study also may hinder molecular approaches with these plants. Therefore, identification of genomic structure and application of molecular technology would increase and facilitate production of these substances and help to save natural resources. However, molecular studies on plant have some limitations due to the phenolic acids, polyphenolic and flavonoid conents that make difficult DNA extraction. All phenolic compounds and as well as polysaccharides, bind firmly to nucleic acids during DNA extraction and interfere with subsequent reactions (Angeles et al., 2005; Hanania et al., 2004; Puchooa and Khoyratty, 2004). For that reason, extraction of high purity and quantity DNA of the plants is a crucial step for further molecular analyses such as ISSR and random amplification of polymorphic DNA polymerase chain reaction (RAPD-PCR) based on molecular marker and marker-assisted selection (Tanaka and Ikeda, 2002). A number of methods are available and are being developed for the extraction of nucleic acids from the plants (Doyle and Doyle, 1987; Sassa, 2007; Saiyed et al., 2007). Because medicinal and aromatic plants such as *Ginkgo biloba*, and *Echinaceae* are polysaccharide- and polyphenol-rich, it is unlikely that just one nucleic acid isolation method suitable for all plants

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can ever exist (Loomis, 1974). In this study, two extraction methods were carried out for DNA extration from *G. biloba* with different age and growth condition. Freh, frozen, dried and herbarium specimens from *G. biloba* leaves were used for DNA extraction by modified Cetyl trimethylammonium bromide (CTAB) method and EZ1 extraction method. Polymerase Chain Reaction (PCR) amplification was performed by 5 arbitrary primers as OPA11-OPA15 for further analysis. Purity and quality of extracted DNA from four specimens by both methods were compared.

MATERIALS AND METHODS

Plant material

The leaves of *G. biloba L.*(*Ginkgoaceae*) were collected from garden of Antalya Directorate of Foresty (Southern Turkey), June, 2008. The plant was identified by Dr. Osman Tugay. A voucher specimen (O. Tugay 5658 and E. Maltas) is deposited in KNYA Herbarium of the Faculty of Science, Department of Biology, Selcuk University. Leaves of *G. biloba* origined from Japan was provided as herbarium from Faculty of Agriculture, Trakya University. *G. biloba* from Germany and France as young plant was provided by herbal companies, Mine Flora and Fidan İstanbul, respectively.

Genomic DNA isolation from plant tissue

DNA extraction from *G. biloba* leaves was carried out by two methods as CTAB and EZ1 Nucleic acid isolation method. For this purpose, herbarium, dried leaves, fresh and frozen specimens of *G. biloba* leaves were used.

Cetyl trimethylammonium bromide extraction method

Fresh, frozen, dried and herbarium specimens were used for DNA extraction. Total DNA from leaves tissue of *G. biloba* was extracted in CTAB extraction buffer according to the protocol of Doyle and Doyle (1987, 1990) as modified as follows; 500 mg of plant material in test tube are ground with 1.5 ml of preheat CTAB solution (1% CTAB (w/v), 100 mM Tris-HCl (pH 8.0), 20 mM Na_2EDTA, 1.4 M NaCl, 0.5% PVP, w/v). 3 µl 2-mercaptoethanol was added to heterogenous solution and incubated at 65°C for 1 - 2 h. After 1 ml of chloroform-isoamylalcohol (24:1,v:v) was added, mixture was centrifuged for 15 min at 13,000 rpm (rcf 15,700) at 4°C. Supernatant is transferred to new test tube, added 1ml of cold isopropanol and then left it at -20°C overnight. Solution was centrifuged for 15 min at 13,000 rpm (rcf 15,700) at 4°C and remove the supernatant. Pellet was washed with 76% EtOH and then centrifuged for 10 min at 10,000 rpm (rcf 9300) at 4°C. Supernatant was removed and pellet was dried and dissolved in 0.5 ml of 10X TE.

For second purification to remove all phenolic structure onto the extracted DNA, all extraction steps were repeated as follows: 0.5 ml of CTAB (1%, w/v) was added to DNA sample purifed from the plant and incubated at 65°C for 45 min. 0.5 ml of chloroform-isoamylalcohol (24:1,v:v) was added and mixed. After centrifugation for 10 min at 10,000 rpm (rcf 15,700) at 4°C, supernatant was removed and, pellet was added to 1ml of cold isopropanol and left at -20°C for 3 h. Mixture was centrifuged for 10 min at 10,000 rpm (rcf 15,700) at 4°C and the supernatant was removed, again. 0.8 ml of 70% EtOH was added to wash pellet and then centrifugated for 10 min at 10,000 rpm (rcf 9300) at 4°C, After removal of supernatant, DNA pellet was dissolved in 100 - 150 µl of 10X TE. 2 µL RNAase (20 µg ml^{-1}) was added to the DNA sample.

EZ1 nucleic acid extraction method

G. biloba specimens were used for DNA extraction by EZ1 nucleic acid extraction method. And another plant material for DNA extraction is dried *G. biloba* leaf for one year. All samples were used directly for DNA extraction by using EZ1 nucleic acid extraction analyser (QIAGEN, 2007). For this purpose, an extraction method was developed by tissue kit (QIAGEN) and tissue card (QIAGEN) as follows: 50 mg fresh and dry plant material was grinded with liquid nitrogen and remove nitrogen. 190 µl of G2 buffer and 10 µl of proteinaz K was added to grinded plant material and incubated at 65°C for 16 - 36 h. After centrifugation for 5 min at 13,000 rpm, DNA isolation was performed from 200 µl of supernatant by Qiagen tissue kit ve tissue card by using EZ1 Nucleic acid extraction equipment.

DNA quantification

Genomic DNA was quantified by measurement of absorbance ratio at 230, 260, 280 and 320 nm with UV- Spectrophotometer (Shimadzu, Kyoto and Japan). One microliter of DNA sample was submitted to check the quality and quantity of DNA. DNA was also quantified by agarose gel electrophoresis. Ten microliters of extracted DNA (200 - 500 ng) was electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized by UV fluorescence. The quantity and quality of DNA were measured by comparing band intensity with that of standard amounts of DNA.

Total phenolic content

Dried *G. biloba* leaves was extracted with three solvents, methanol, acetone and n-hexane, having different properties. All solvent extraction procedures were applied under same conditions. Each 50 g *G. biloba* leaves were extracted with each solvent at 30°C for 6 h. After resulting extracts were evaporated under vacuum to 10 mL, extract solutions were dried at -50°C in a lyophiliser. Yield of each extracts including methanol, acetone and hexane were determined as 13, 7 and 6% (w/w), respectively. All extracts were kept in the dark at +4°C until used. The concentration of total phenolics was measured by the method described by Singleton and Rossi (1965). In this method, an aliquot of diluted extracts and standard solutions of gallic acid with different concentrations were added to a volumetric flask containing 9 mL of ddH_2O. 2.5 mL of 10% (v/v) Folin and Ciocalteu's phenol reagent and 7.5 mL of 20% (w/v) Na_2CO_3 were added to the mixture and shaked vigorously. After incubation for 120 min at room temperature, the absorbance was recorded at 750 nm. Total phenolic contents of *G. biloba* extracts were expressed as mg gallic acid equivalents (GAE mg/g) for dry extract. All samples were analysed in three replications.

Polymerase chain reaction amplification

PCR amplifications were carried out by 5 arbitrary 10-mer primers (Operon Technologies Inc. Almeda CA, USA) were applied. For the optimization of RAPD-PCR reaction using DNA extracted from various accessions, oligonucleotide primers from OPA series were used for amplification to standardize the PCR conditions (Table 1). PCR reactions were carried out with 200 mg of template DNA in 67

Table 1. RAPD primers used for amplification.

Primer no.	Primer sequence
OPA-11	5′-CAATCGCCGT-3′
OPA-12	5′-TCGGCGATAG-3′
OPA-13	5′-CAGCACCCAC-3′
OPA-14	5′-TCTGTGCTGG-3′
OPA-15	5′-TTCCGAACCC-3′

Table 2. Total phenolic contents of different extracts from *Ginkgo biloba*.

Extracts	Extraction yield (%)	Total phenolic[1](mg GAE/g)
Hexane extract	6	27.6 ± 3.1
Acetone extract	7	59.4 ± 1.6
Methanol extract	13	76.0 ± 2.2

[1]Total phenolic content was expressed as gallic acid equivalent (GAE).Data expressed (P = 0.05) as means ± standard deviation (n = 3).

mM Tris, pH 8.8, 6.7 mM $MgSO_4$, 16.6 mM $(NH_4)_2SO_4$, 1 mM of each dNTP, 250 ng of primer and 1 unit of Taq DNA polymerase in a final volume of 25 µl. Amplifications were carried out in a DNA Thermal Cycler (BioRad) under the following conditions: one initial cycle of 3' at 95°C, 5' at 36°C, and 5' at 72°C followed by 45 cycles of 1' at 95°C, 1' at 36°C and 2' at 72°C, with a final extension period of 15' at 72°C. Tubes containing all reaction components except for template DNA were included as controls. Amplification products were observed in ethidium bromide-stained 1.5% agarose gels following electrophoresis. Three primers of different size were tested and those resulting in visible, reproducible and easily scorable bands were selected. All the reactions were repeated three times.

RESULTS AND DISCUSSION

G. biloba is one of the crucial medicinal plant for centuries (Nakanishi, 2005). It is known that medicinal effect of *G. biloba* is attributed to scavenge or inhibit free radicals related to high phenolic content (Pietta et al., 2000; Goh and Barlow, 2002). Genomic DNA extraction from fresh, frozen, dried and herbarium specimens of *G. biloba* were carried out by a modified CTAB extraction method, and EZ1 Nucleic acid isolation methods in this study. Phenolic contents of *G. biloba* extracts were determined by the method of Folin and Ciocalteu and expressed as gallic acid equivalent (Table 2). In general, the quantity and quality of extracted DNA depends on reagents used for extraction, precipitation temperature and duration. All factors effect on removal of contamination such as proteins, polysaccharides, and aromatic compounds. All methods yielded high-quality and high quantities of DNA. The successful extraction of useful DNA from the plants is associated with all extraction steps for molecular techniques used in the next steps such as PCR amplification, digestion and DNA sequencing. Particularly, the phenolic contents of the plants as well as other substances such as polysaccarides and proteins make diffucult DNA extraction; result in low quality and low quantity DNA (Hanania et al., 2004; Puchooa and Khoyratty, 2004). Several methods of DNA extraction are available (Angeles et al., 2005; Doyle and Doyle, 1987). Unlike in nonplant DNA extraction protocols, methods need to be adjusted to each species and even to each tissue because of the wide range of primary and secondary metabolites in the plants. Current methods produce degraded and denatured DNA or give extremely poor yields. To overcome these difficulties, we modified available DNA extraction methods.

One of the limitations in the extraction steps was that the ground *G. biloba* leaves immediately turned brown upon freezing with liquid nitrogen, indicating the presence of phenolics and polyphenolics in the samples. This brown color was observed in the heterogenous isopropanol-DNA extraction buffer mixture during the course of the DNA extraction process. In oxidized forms, polyphenolics and phenolics covalently bind to proteins and DNA, giving the DNA a brown color and making it useless for most research applications (Katterman and Shattuck, 1983; Angeles et al., 2005). In addition to phenolic compounds in the leaves, polysaccharides also interfere with biological enzymes such as polymerases, restriction endonucleases and ligases (Michiels et al., 2003), result in unsuccesful amplification. The addition of Polyvinylpolypyrrolidone (PVPP) (or its water-soluble counterpart polyvinylpyrrolidone (PVP) has been used to extract genomic DNA from other polyphenol-rich plants such as cotton, sugarcane, lettuce and strawberry (Aljanabi et al., 1999), and grape, apple, pear, persimmon and several conifers (Kim et al., 1997). PVP

Table 3. Amount and purity of DNA from *Ginkgo biloba*.

Origin	Specimens	CTAB method				EZ1 extraction method			
		DNA quantity µg/ml	$A_{260/280}$	$A_{260/230}$	A_{320}	DNA quantity µg/ml	$A_{260/280}$	$A_{260/230}$	A_{320}
Turkey	Fresh	780	1.89	2.30	0.001	1150	1.95	2.20	0.00
	Frozen	775	1.86	2.42	0.001	1112	1.74	2.25	0.001
	Dried	710	1.74	2.53	0.003	934	1.80	2.31	0.002
Germany	Frozen	790	1.81	2.35	0.001	1062	1.81	2.25	0.00
France	Fresh	675	1.80	2.41	0.002	998	1.89	2.22	0.001
Japan	Herbarium	-	-	-	-	852	1.78	2.35	0.005

Data expressed (P = 0.05)as means ± standard deviation (*n* = 3).

forms complex with latex lactones, actucin and other phenolics. The PVP complexes accumulate at the interface between the organic and the aqueous phases by centifugation after addition of chloroform. CTAB binds to fructans and other polysaccharides and forms complexes that are removed during subsequent chloroform extraction (Michiels et al., 2003; Jitu and Kr, 2008). DNA from fresh, frozen and dried leaves of *G. biloba* was extracted by modified protocol of the method of CTAB described by Doyle and Doyle (1990). In this study, removal of the phenolic content in *Gingko* leaves measured by Folin and Ciocalteu method was carried out by addition of PVP to CTAB solution. And 3-mercaptoethanol denaturated sulfate linkage in protein sutructures and make easy removal of proteins from nucleic acid. A second protocol was also processed by CTAB solution including PVP in order to increase purity of extracted DNA by this method. But, a reduction of amount of DNA was observed while DNA quality increased in terms of second protocol. Extraction of fresh, frozen and dried and as well as herbarium specimens of *G. biloba* was also carried out by using EZ1 Nucleic acid isolation analyser (QIAGEN, 2007) for 20 min.

For this purpose, an extraction method was developed by using tissue kit (QIAGEN) and tissue card (QIAGEN) for DNA extraction. Before extraction with tissue kit, proteinase K was used for removal of protein by digestion, and then all specimens were incubated at a period range between 16 and 36 h. Incubation period increased by increasion of the age of *G. biloba* leaves. While fresh and frozen leaves were incubated for 16 h, dried and herbarium materials were incubated at 65°C for 20 and 30 h, respectively. Quantity and quantity of obtained DNA from both extraction method was controlled in terms of absorbance at wavelenght of 260, 230, 280 and 320 nm. While absorbance ratio of A260/A230 indicates impurities caused by coprecipitation of peptides, aromatic compounds, and polysaccharide and/or phenolic complexes, absorbance ratio of A260/A280 indicates presence of dense protein (Table 3). Spectrophotometric analysis at A260/A230 revealed ratio ranged from 2.30 to 2.42. Second protocol of CTAB methods indeed led to a clear reduction of contaminating components such as polyphenolics and polysaccharides during precipitation. DNA yields from *Ginkgo* leaves using the modified CTAB method ranged from 675 ± 79 to 790 ± 132 µg/g fresh tissue with A260/A280 close to 2.00 in Turkey specimens indicating very little contamination of the DNA fraction by polysaccharides, and aromatic compounds. Extraction of herbarium specimen was not managed by modified CTAB method. Compared to EZ1 extraction method, modifed CTAB method yielded smaller amounts of high-quality DNA for all specimens. Amonut of DNA obtained from EZ1 extraction method ranged 1150 ± 126 to 852 ± 85 µg/g fresh tissue. Extraction of the herbarium specimen yielded smaller amounts than those of the other specimens. But, also high-quality DNA from herbarium specimen was obtained. Fresh, frozen and dried leaves appear to be excellent tissue for the extraction of the genomic DNA. So, genomic DNA solutions from *G. biloba* by both extraction methods were of adequate purity and yield for applying PCR, resulting that amplifications of the expected 10 nt primers using the *G. biloba* specific primer pair were detected. When genomic DNA was extracted from *G. biloba* the DNA solutions by CTAB method were of a sufficient purity (A_{260}/A_{280}=1.7–2.0).

In addition, the amplification products from the genomic DNA solutions by CTAB method were not detected. Thus, these results suggested that the desirable genomic DNA was not extracted from *G. biloba* by CTAB method, although, it was possible to extract it by EZ1 nucleic acid DNA isolation method were of a sufficient purity (A_{260}/A_{280}=1.7–1.8). EZ1 Nucleic acid isolation method was found to be extracted rapidly and simply DNA from *G. biloba* with high amount. Therefore, the *G. biloba* DNA extraction procedure is sufficiently efficient and yields adequate amounts of genomic DNA with a sufficient level of repeatability. A DNA extraction by EZ1 Nucleic acid isolation method has been developed and applied successfully to the detection *G. biloba* (Figure 1). But, it has been reported that polysaccarides and phenolics-rich

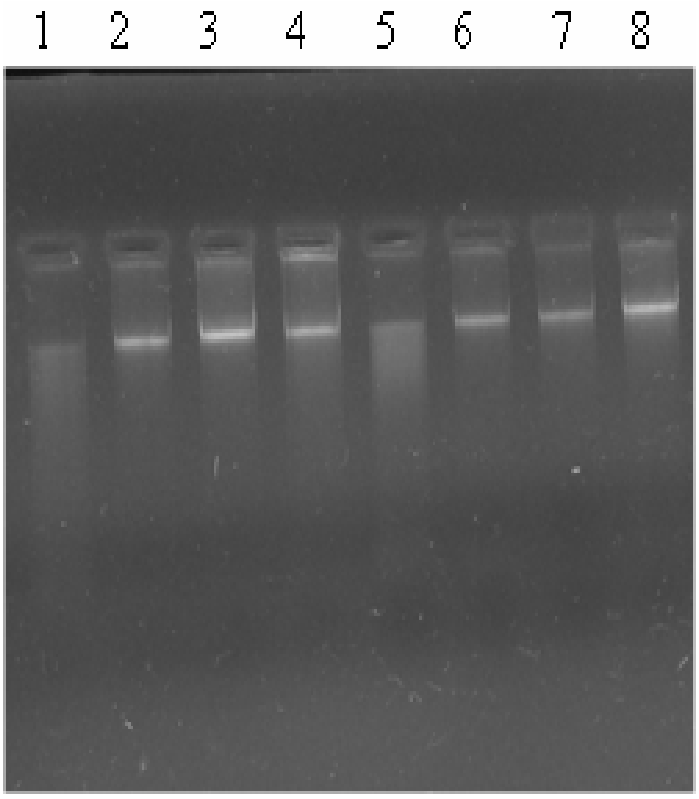

Figure 1. Genomic DNAs, Line 1-4: DNA extraction by EZ1 extraction method: 1; Genomic DNA isolated from herbarium specimen from Japan, 2; Genomic DNA isolated from dried specimen from Turkey, 3; Genomic DNA isolated from fresh specimen from France, 4: Genomic DNA isolated from frozen specimen from Germany, Line 5-8: DNA extraction by CTAB method: **5;** Genomic DNA isolated from fresh specimen from Turkey, **6;** Genomic DNA isolated from fresh specimen from Turkey, 7; Genomic DNA isolated from frozen specimen from Germany, 8: Genomic DNA isolated from fresh specimen from France.

Ginkgo oxidize during DNA extraction and irreversibly interact with proteins and nucleic acids to form a gelatinous matrix. This matrix might inhibit extraction, amplification, and digestion of DNA in the next step. So, DNA extraction have to control by PCR amplifcation and digestion by restriction endonucleases (Xu et al., 2005; Hill-Ambroz, 2002; Skujiene and Soroka, 2003). In this study, extracted DNA from all samples was amplified by using arbitrary primers by RAPD-PCR. RAPD is a common molecular approach employed in DNA fingerprint analysis for genotypic differentiation, molecular taxonomy, and other applications, results in polymorphic bands (Puchooa and Khoyratty, 2004; Paterson et al., 1993). However, RAPD markers allowed identification of the plant species. Our objective was to control purity and amplifiable high-molecular-weight of the extracted genomic DNA from *G. biloba* by amplification with arbitrary primers. Amplification of the extracted DNA by these two methods represented successful DNA extraction that was the crucial step because of levels of phenolic and polyphenolic compounds (Katterman and Shattuck, 1983) and highly acidic (pH 2 - 3) tissue of the plants. Our methods yielded good-quality, high-molecular-weight DNA that was free of contaminants and colored pigments and it was concluded that they could be amplified by means of PCR.

The RAPD procedures were proved to be a useful tool for assessing genetic variability, since band profiles with the five selected primers were reproducible and their patterns of inheritance proved to be Mendelian for a dominant marker. Despite the high proportion of excluded bands, a substantial number of polymorphic markers was detected. Reproducibility of DNA profiles was determined by replicating all RAPD reactions at least three times. The total number of markers was detected among the *G. biloba* genotypes based on RAPD analysis with 5 primers. The number of scorable markers produced per primer ranged from OPA11 to 15 and size of the products

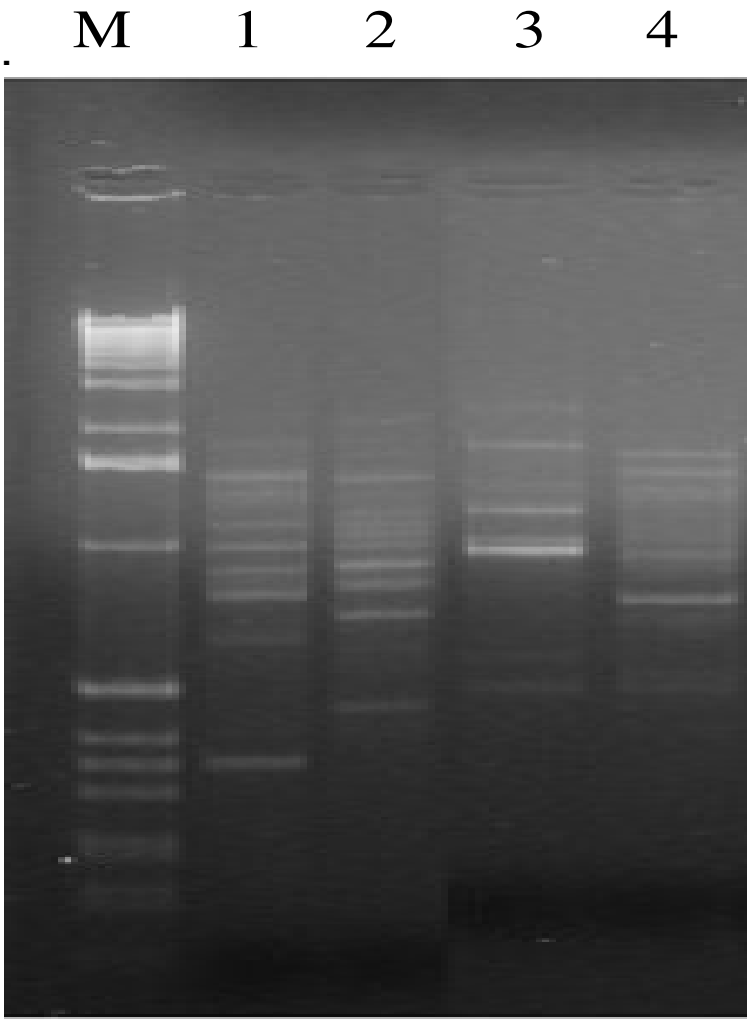

Figure 2. RAPD banding patterns of DNA extracted by EZ1 extraction method, M: Lamda DNA Hind III, Lines 1-4 of PCR product by amplification with OPA-11, 1; Genomic DNA isolated from herbarium specimen from Japan, 2; Genomic DNA isolated from dried specimen from Turkey, 3; Genomic DNA isolated from fresh specimen from France, 4: Genomic DNA isolated from frozen specimen from Germany.

ranged from 1 kb to 10 kb. RAPD marker profiles produced by the primers for all *Ginkgo* genotypes were shown in Figures 2 and 3**.** The reproducibility of the RAPD technique can be influenced by various factors, such as primer sequence, template quality and quantity, the type of thermocycler and polymerase concentration. However, the use of a standardized RAPD protocol can ensure the reproducibility of RAPD patterns. Standard extraction methods resulted in DNA that is difficult to amplify, especially fragments larger than 1 kb, while the DNA extracted by the modified extraction technique is amplifiable by PCR. All the amplified bands by extracted DNA from G*. biloba* by both identified extraction method were identical and similar results were obtained in each repetition of PCR amplification. As a result, the extracted DNA from all four species by four extraction methods was avaliable for digestion and amplification.

Conclusion

The age and growth conditions of the plant material influence the extraction efficiency of high quantity and quality DNA. The extraction of younger *G. biloba* leaves from different origins results in increased yields of high-quality DNA. Unlike DNA from herbarium specimen exhibited lower qunatity than those of younger leaves. The Doyle and Doyle protocol is successfully modified optimized by adding PVP to the extraction buffer. However, DNA extraction from all specimens is also

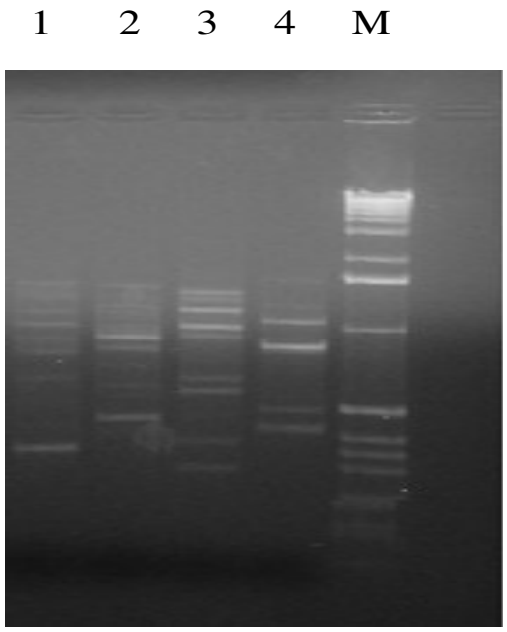

Figure 3. RAPD banding patterns of DNA extracted from dried specimen from Turkey by EZ1 method, M: Lamda DNA Hind III, Lines 1-4 of PCR product by amplification with (1) OPA-12 (2) OPA-13 (3) OPA-14, 4: OPA-15.

successful by tissue card and tissue kit from QİAGEN by using EZ1 automatic Nucleic acid extraction system. Both extraction method permits further genomic analyses.

ACKNOWLEDGEMENT

Authors are very grateful to Scientific Research Foundation of Selcuk University (BAP) for providing foundation with 08101019 of project number. This study is a part of Esra Maltas's Ph.D thesis being continued.

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