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EFFICIENT DNA ISOLATION FROM MOROCCAN ARAR TREE [*TETRACLINIS ARTICULATA* (VAHL) MASTERS] LEAVES AND OPTIMIZATION OF THE RAPD-PCR MOLECULAR TECHNIQUE

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ABSTRACT. *Efficient DNA isolation from Moroccan Arar tree [Tetraclinis articulata (Vahl) Masters] leaves and optimization of the RAPD-PCR molecular technique.* Molecular genetic analysis of Arar tree [*Tetraclinis articulata* (Vahl) Masters] is often limited by the availability of fresh tissue and an efficient and reliable protocol for high quality genomic DNA extraction. In this study, two DNA extraction protocols were specifically developed for extracting high quality genomic DNA from Arar tree leaves: modified QIAgen DNA Kit and protocol developed by Ouenzar *et al*. (1998). DNA yield and purity were monitored by gel electrophoresis and by determining absorbance at UV (A_{260}/A_{280} and A_{260}/A_{230}). Both ratios were between 1.7 and 2.0, indicating that the presence of contaminating metabolites was minimal. The DNA yield obtained ranged between 20 to 40 µg/g of plant materiel. The Ouenzar and collaborators protocol gave higher yield but was more time consuming compared to QIAgen Kit. However, both techniques gave DNA of good quality that is amenable to RAPD-PCR reactions. Additionally, restriction digestion and PCR analyses of the obtained DNA showed its compatibility with downstream applications. Randomly Amplified Polymorphic DNA profiling from the isolated DNA was optimized to produce scorable and clear amplicons. The presented protocols allow easy and high quality DNA isolation for genetic diversity studies within Arar tree.

Keywords. *Tetraclinis articulata*, arar, DNA extraction, leaves, RAPD.

RÉSUMÉ. *Extraction efficace de l'ADN des feuilles du Thuya de Berberie (Tetraclinis articulata (Vahl) Masters) et optimisation de la technique moléculaire RAPD-PCR*. Les analyses en génétique moléculaire chez le thuya de Berberie [*Tetraclinis articulata* (Vahl) Masters] sont souvent limitées par la disponibilité du matériel végétal frais et le temps nécessaire pour l'extraction l'ADN ainsi que par sa qualité. Dans cette étude, deux protocoles d'extraction, à partir des feuilles du thuya, de l'ADN génomique de haute qualité, ont été développés : le Kit Qiagen et le protocole mis au point par Ouenzar *et al*. (1998) modifiés. La qualité et la quantité de l'ADN sont évaluées par électrophorèse sur gel d'agarose et par la mesure de l'absorbance en UV à (A_{260}/A_{280}) et (A_{260}/A_{230}). Ces deux rapports varient entre 1,7 et 2,0 indiquant la faible fréquence des métabolites contaminants. Le rendement d'ADN varie entre 20 et 40 µg/g du matériel végétal. Le protocole de Ouenzar et collaborateurs donne le meilleur rendement d'ADN mais nécessite plus de temps. Néanmoins, les deux protocoles donnent un ADN de bonne qualité utilisable dans les réactions RAPD-PCR. En outre, la restriction enzymatique et l'analyse PCR de l'ADN obtenu ont montré sa compatibilité avec les

applications moléculaires ultérieures. Les paramètres intervenant dans les réactions RAPD ont été optimisés. Les protocoles présentés permettent l'extraction facile de l'ADN de haute qualité nécessaire pour des études de la diversité génétique au sein du thuya.

Mots clés. *Tetraclinis articulata*, thuya, extraction d'ADN, feuilles, RAPD.

INTRODUCTION

Tetraclinis is a genus of evergreen coniferous tree in the cypress family Cupressaceae, containing only one species, *T. articulata* (Vahl) Masters. It is known under the vernacular name of "Arar tree of Berberie". Currently, the range of this species is restricted only to the Mediterranean region. In fact, apart from its two small outlying populations in Malta and in southeast Spain, the Arar tree can be seen as endemic to the Southeast Mediterranean region, Spain and North Africa. The plant grows at relatively low altitudes in a hot, dry subtropical climate. The largest populations of Arar tree in North Africa are found in Morocco with

565 798 ha, which represent approximately 10% of the total forest (Benabid & Fenane, 1999). *T. articulata* is a rustic thermophilous species that thrives in harsh environmental conditions, within the 250-900 mm/year rainfall range (Benabid, 1984). Its optimum altitude is from sea level up to 2000 m (Benabid & Fenane, 1999). The species develops at skeletal as well as deep soils and does not seem to be edaphically very demanding (Guerra *et al*. 1988). It is more frequent in calcareous but also grows in dolomite, granite, or schist soils. *T. articulata* is of high interest, for both the value and diversity of its products (timber, wood tar, firewood, charcoal, sandarac gum, etc.). It is considered as a precious species because its wood is very much admired for its natural beauty and homogeneity and is used for marquetry and cabinet work. Art crafts activities developed around this species generate considerable income for local populations. Unfortunately, drought and high grazing pressure limit the natural regeneration of the species and restrict its distribution range. Assessing the genetic diversity (via morphological, isozymic and molecular markers) within and among populations, according to their ecological distribution, would be required to ensure the *in situ* maintenance of populations and to draw up conservation and improvement strategies. Morphological characters and Random Amplified Polymorphic DNA (RAPD) analysis are broadly used in plant population diversity studies (Crouch *et al*. 2000; Casiva *et al*. 2002; Garcia *et al*. 2002; Samal *et al*. 2003). Mainly, RAPD has allowed the resolution of complex taxonomic relationships. Studies addressing genetic diversity and differentiation among natural Arar tree populations in the Mediterranean countries are very limited. Furthermore, no molecular studies have been carried out so far on this tree. Works using allozyme and RAPD markers have been used to analyse the genetic diversity of some species of *Cupressus* genera (Doulis *et al*. 2000; Bechir *et al*. 2004). Molecular genetic screening techniques require rapid and high quality DNA extraction. This task can be especially difficult for gymnosperms that contain high amounts of polyphenols, resins, and terpenoids (Ziegenhagen *et al.* 1993). Various protocols for DNA extraction have been successfully applied to many plant species (Doyle & Doyle, 1987; Guillemaut & Marechal-Douard, 1992; Ziegenhagen *et al*. 1993). In the present work, modifications of the DNeasy Plant Mini Kit and the protocol of Ouenzar *et al*. (1998) are presented. These modified extraction procedures surmounts commonly encountered problems: yielding genomic DNA of high quality and quantity

from Arar tree leaves. We also optimized RAPD marker technique that would be used to understand the species genetic diversity and population structure.

MATERIALS AND METHODS

Collection and storage of plant material

The Arar tree leaves were collected from an adult tree in Amsitten *Tetraclinis* woodland, Essaouira region, Morocco, quickly frozen in liquid nitrogen, and then placed in -80°C until genomic DNA extraction.

DNA extraction protocols

Two different genomic DNA extraction methods were tested to recover genomic DNA from Arar tree adult leaves.

The first procedure used to extract genomic DNA was adapted from Ouenzar *et al.* (1998) and was carried out as described in Konaté (2007). One gram of fresh or frozen Arar adult leaves were ground in 5 ml of lysis buffer containing 50 mM Tris-HCl (pH 8), 5 mM EDTA (pH 8), 300 mM Mannitol, 0.05% BSA, 1% PEG_{6000} and 0.5% beta-mercaptoethanol. The suspension was transferred in tubes containing 600 µl of SDS (20%) and 400 µl of Sodium Acetate (3 M, pH 8), and incubated at 65°C for 30 min. The DNA was extracted once with chloroform-isoamyl alcohol (24/1) and once with phenol/chloroform/isoamyl alcohol (25/24/1), followed by an additional extraction with chloroform-isoamyl alcohol (24/1). Every extraction was preceded by a centrifugation at 10 000 x g for 10 min. The final aqueous supernatant was recovered in fresh tubes and mixed with an equal volume (v/v) of cold Isopropanol then was incubated at -20°C for one hour. Finally, the mixture was centrifuged at 10 000 x g for 20 min. The resulting pellet was washed twice with cold ethanol (70%), air-dried and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) then treated with K proteinase (20 µg/ml) at 37°C for 30 min. Proteinase K was removed by one extraction with phenol/chloroform/isoamyl alcohol. After centrifugation, nucleic acids were precipitated by adding two volume of absolute cold ethanol and 1/10 of NaCl (5 M), followed by incubation over night at -20°C. The DNA was pelleted by centrifugation at 10 000 x g for 20 min, washed with 70% cold ethanol, air-dried, and then dissolved in 200 µL of TE buffer. The extracted DNA was further treated with 2 µL of RNAase (10 µg/ml) at 37°C for 30 min.

The second procedure assayed to extract genomic DNA from Arar leaves was the DNeasy Plant Mini Kit (QIAGEN). The manufacturer's protocol was applied with some modifications (a, b) that were deemed critical for our material. Fifty milligrams of frozen Arar adult leaves were ground to a fine powder in the presence of liquid nitrogen. The powder was transferred to a pre-chilled microcentrifuge tube containg 400 µl of lysis buffer AP1 and 4 µl of RNase A (stock solution 100 mg/ml). The suspension was vortexed vigorously until a complete emulsion is formed and then incubated for 10 min at 65°C. Clumped tissues were dispersed using a disposable micropestle to allow higher yields of DNA (modification a). 130 µl of buffer AP2 was added to the lysate then the mixture was incubated for 5 min on ice. After centrifugation for 5 min at maximum speed and room temperature, the supernatant was transferred into mini columns filtration "QIAshredder spin column" placed in 2 ml collection tubes using a wide bore pipette and centrifuged for 2 min at maximum speed. The flow-through fraction was transferred into clean eppendorf tube. Occasionally, a light colored cell-debris pellet may appear and must not be disturbed. 0.5 volume of buffer AP3 and 1 volume of ethanol (96–100%) were added to the cleared lysate and mixed by pipetting. 650 µl of the mixture was transferred to DNeasy mini spin column and centrifuged for 1 min at 8 000 rpm. This step was with the remaining sample

and the flow-through and collection tubes were discarded. The DNeasy column was placed in a new collection tube and 500 µl of AW buffer (washing buffer) was added onto the column. After centrifugation for 1 min at 8 000 rpm, the supernatant was discarded. An additional 500 µl of AW buffer was added to column and then centrifuged for 2 min at maximum speed. As modification b, an additional washing step with 500 µl ethanol (96–100%) was applied to avoid coloration of the final eluted DNA. The column was placed in a new collection tube and span for 5 min under a vacuum in order to completely dry the column membrane. This additional step was introduced because it is crucial that no residual ethanol is carried over during elution of DNA from the column. Finally, the DNeasy column was transferred to a 1.5 ml microcentrifuge tube and 100 µl of preheated (65°C) buffer AE (elution buffer) was added directly onto the DNeasy column membrane. After incubation for 5 min at room temperature, the DNA was eluted in buffer AE by centrifugation for 1 min at 8 000 rpm.

DNA quality was examined following electrophoresis on agarose gel. Spectrophotometric analysis was performed at 230, 260 and 280 nm using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Absorbance at 260 nm and the A_{260}/A_{280} ratio provided an estimate of quantity and purity of extracted DNA, respectively.

Restriction analysis and electrophoresis

One microgram of the extracted DNA was analysed by digestion with *AluI*, *BamHI*, *HindIII* and *EcoRI* restriction enzyme according to manufacturer's specifications (Promega, Madison, WI, USA). Effective digestion of DNA by these enzymes was regarded as indicator of absence of polysaccharides (Do and Adams, 1991). The digested genomic DNA was separated on a 0.8 % TBE agarose gel at 80 Volts for 2 hrs, stained with ethidium bromide, and then viewed under UV illumination and photographed using the Oncor-Appligene Imager 2.02. Lambda digested *HindIII* (Promega, Madison, WI, USA) was used as marker.

PCR analysis

To test the capacity of isolated DNA to yield specific products in Polymerase Chain Reaction (PCR), the Internal Transcribed Spacer (ITS) of the nuclear ribosomal DNA (rDNA) was amplified using ITS1 and ITS4 primers (White *et al*. 1990). PCR amplification was carried out using a PCR-2720 thermal cycler (Applied Biosystems) in a 50 µL reaction volume containing: 50 ng DNA template, 1.5 mM $MgCl_2$, 200 µM dNTPs (for each), 0.5 µM of each primer, 5 µL of 10x Taq buffer and 0.6 units of Taq polymerase (Promega). Purified water (25.4 µL) was added to PCR mix (49 µL) to improve success of amplifications. Cycling parameters were 1 cycle of 95°C for 5 min, 35 cycles including 94°C for 30 s, 55°C for 30 s, 72°C for 2 min and a final extension at 72°C for 10 min. Negative controls (no DNA template) were included in all PCR experiments to check for DNA contamination. ITS products were migrated by electrophoresis on 2% agarose gels, stained with ethidium bromide and photographed using the Oncor-Appligene Imager 2.02. Smart ladder (Eurogentec, Belgium) was used as a DNA molecular weight marker.

Optimization of RAPD reaction for Arar tree DNA

For the optimization of RAPD reaction using the DNeasy Plant Mini Kit (QIAGEN) extracted DNA, five decamer primers, OPA-01 to OPA-05 from Operon Technologies Inc. (Promega, Madison, WI, USA), were used. The PCR conditions were optimized by varying the annealing temperature (Tm), the concentration of $MgCl_2$, the units of Taq polymerase and the quantity of the DNA template. The initial

protocol tested for RAPD reaction was the one recommended by the manufacturer of the polymerase. For a reaction volume of 25 µl, the following components were used: 50 ng genomic DNA, 200 µM dNTPs (for each), 0.2 µM Operon primer, 2.5 µl 10x buffer, 4 mM $MgCl_2$ and 0.2 units of Taq polymerase (Promega, Madison, WI, USA). The amplification was performed in a PCR-2720 thermal cycler (Applied Biosystems) according to the following program: 1 cycle of 4 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 40°C and 1 min at 72°C, and finally 1 cycle of 4 min at 72 °C for the final extension. The parameters tested in RAPD-PCR reactions were as follows: 3 different denaturing temperatures (94°C, 97.5°C, 99.9°C), 7 different concentrations of DNA template (5, 10, 20, 40, 70, 100, 200 ng), 9 different concentrations of Taq polymerase (0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2 unit) and 8 different concentrations of $MgCl_2$ (1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 mM). Amplification products were separated by electrophoresis in 1.8 % agarose gels. It was performed in 1xTBE (trisborate-EDTA) running buffer at 100 volts for 3h and then the revelation of RAPD profiles was done by ethidium bromide staining under ultra-violet light. Molecular weights of the amplified products were estimated using 100 bp DNA ladder (Promega, Madison, WI, USA).

RESULTS AND DISCUSSION

Population genetics and phylogenetic analyses of plant species are often limited by time-consuming DNA extractions and purification procedures. In many cases, DNA extraction has nowadays become the time limiting step in such studies. It is therefore desirable to reduce the time as well as number of handling steps in DNA extraction protocols. DNA extraction can be particularly challenging with species and tissues that contain high amounts of polysaccharides or other secondary metabolites such as polyphenols, terpenes and resins. Such problems are frequently encountered with woody plant species, especially with conifers growing in the field such as Arar trees (Ziegenhagen & Scholz, 1998).

PCR parameter	Range tested	Results	Optimum conditions
DNA concentrations (ng)	5, 10, 20, 40, 70, 100 and 200	DNA template of at least 10 ng is necessary for good amplification of PCR products	10 ng
Magnesium chloride (mM)	1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5	Concentration of $MgCl_2$ lower than 3 gave poor PCR amplification	4 mM
Primer concentration (µM)	0.1 and 0.2	Primer concentration lower than 0.2 failed to generate proper amplification products	0.2 µM
Taq polymerase (units)	0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8 and 2	Amount of Taq polymerase lower than 0.8 produced fewer number of bands	0.8 unit

Table 1. Summary of the parameters of RAPD-PCR tested and the optimum conditions selected. *Récapitulatif des paramètres testés de RAPD-PCR et des conditions optimales retenues.*

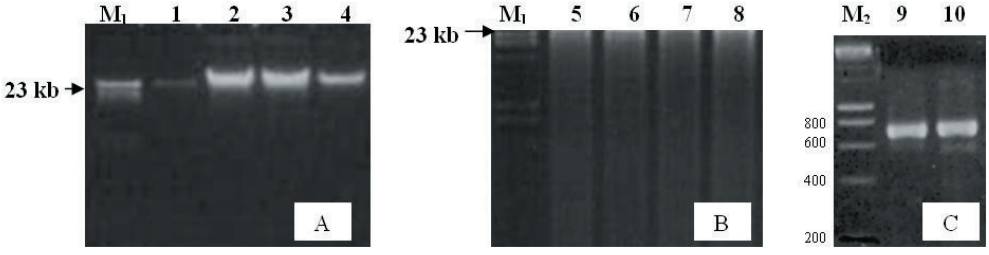

Figure 1. (A) Agarose gel analyses of DNA isolated from Arar tree leaves obtained by the two different procedures; lanes 1 and 4 represent DNA obtained by the DNeasy Plant Mini Kit; lane 2 and 3 indicate DNA samples isolated by the method described in Ouenzar *et al*., (1998). (B) Agarose gel analyses of Arar DNA digested with restriction enzymes. Lane 5, 6, 7, and 8 represent restriction digestion with *AluI*, *BamHI*, *HindIII* and *EcoRI*, respectively. (C) Gel electrophoresis of the amplified rDNA internal transcribed spacer (ITS) region of DNA isolated from Arar tree leaves obtained by the two different procedures. M_1: λDNA/Hind III marker. M_2: Smart ladder marker. *(A) Electrophorèse sur gel d'agarose de l'ADN extrait à partir des feuilles du thuya de berberie obtenu avec 2 différentes procédures; lignes 1 et 4 représentent l'ADN obtenu par le DNeasy Plant Mini kit; lignes 2 et 3 indiquent les échantillons d'ADN isolés par la méthode décrite dans Ouenzar et al., (1998). (B) Eletrophorèse sur gel d'agarose de l'ADN du thuya digéré avec les enzymes de restriction. Lignes 5, 6, 7 et 8 représentent respectivement les produits de digestion avec AluI, BamHI, HindIII et EcoRI. (C) Electrophorèse sur gel des produits d'amplification de l'ADNr de l'espaceur interne transcrit (ITS) de l'ADN isolé à partir des feuilles de thuya de berberie par les 2 différentes procédures. M_1: ADN du marqueur Lambda/HindIII. M_2: marqueur Smart ladder.*

Two different genomic DNA extractions were performed in order to obtain polysaccharide free genomic DNA from Arar tree leaves, namely, the method described in Ouenzar *et al.* (1998) and the QIAgen DNeasy Plant Mini Kit (Valencia, CA, USA). Both procedures were used with some modifications. The steps and reagents of the first procedure are routinely used for plant genomic DNA extraction. For the second procedure, we improved some modifications according to Doulis *et al*. (2000). Leaves should be used fresh, although they are also suitable if frozen at -80°C for many days. The concentrations and purity of DNA obtained from those procedures were determined with spectroscopy and gel-electrophoresis. In addition, the suitability of the genomic DNA obtained was also checked in restriction digestion and PCR-RAPD reactions. The modified Ouenzar *et al.* (1998) extraction procedure produced a relatively high yield product (2 µg/50 mg sample tissue). This procedure is a maxi-scale preparation which is very time-consuming. Its adaptation to the *T. articulata* leaf would probably be due, in part, to its lysis buffer containing the Polyethylene Glycol (PEG_{6000}) and on the other hand, to a long processes of deproteinisation. The PEG is considered by some authors as a chemical agent with a positive and effective impact on the dissolution of the tannin-protein complex responsible for the anchoring of several elements or cellular constituents of certain plants (Jones & Mangan, 1977; Prioli *et al*. 2000). While the average DNA yield by the DNeasy Plant Mini Kit procedure is around 0.8 - 1 µg per 50 mg sample tissue. Doulis *et al*. (2000) reported that the average DNA yield obtained from 100 mg starting material by the DNeasy™ Plant Mini Kit was around 22 µg. Further the procedure can be completed in less than one hour, and more than 10 samples can be processed in a day. Our modified Kit methods are those proposed by Doulis *et al*. (2000). It could be suitable for increasing and standardizing the quality and quantity of genomic DNA extracted.

Arar tree is a recalcitrant species for DNA extraction, but our procedures were capable

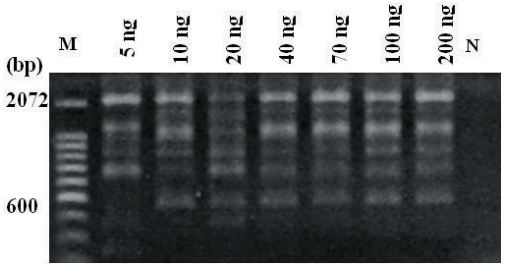

Figure 2. RAPD-PCR profile of seven DNA concentrations ranging from 5 to 200 ng from the same Arar tree obtained by the Operon primer OPA-04 (5'-AATCGGGCTG-3') in 1.8% agarose gel. M: 100 bp DNA ladder. N: PCR negative control (no template DNA). *Profil RAPD-PCR de sept concentrations d'ADN de 5 à 200 ng extrait d'un arbre de thuya de berberie obtenu avec l'amorce OPA-04 (5'-AATCGGGCTG-3') sur gel d'agarose 1.8%. M: marqueur de poids moléculaire (100 pb ladder). N est le témoin négatif amplifié sans ADN.*

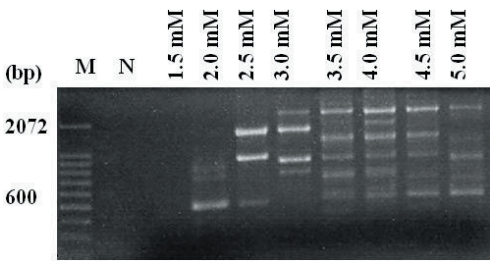

Figure 3. Optimization of RAPD-PCR parameters with eight MgCl2 concentrations using the Operon primer OPA-04 and 10 ng of Arar tree genomic DNA. M: 100 bp DNA ladder. N: PCR negative control (no template DNA).*Optimisation des paramètres RAPD-PCR avec huit concentrations de $MgCl_2$, obtenu avec l'amorce OPA-04 et 10 ng d'ADN génomique du thuya de berberie. M: marqueur de poids moléculaire (100 pb ladder). N est le témoin négatif amplifié sans ADN.*

of extracting, high-quality genomic DNA as indicated by the absorbance ratio $(A_{260})/(A_{280})$ of approximately 2.0. In addition, digestion reactions with four different restriction enzymes, *AluI*, *BamHI*, *HindIII* and *EcoRI*, suggest that the extracted DNA was of low polyscacharide contaminats (fig. 1A and 1B). Further, PCR amplification of the ITS region of the ribosomal DNA using the extracted Arar tree genomic DNA as template yielded a DNA fragment of 700 bp (fig. 1C) suggest that the DNA extracted is amenable to PCR-based studies of Arar tree, such as RAPD and AFLP.

RAPD is a technique, which identify genomic variations within species (Welsh & McClelland, 1990; Williams *et al.* 1990). Although RAPD-PCR approach is potentially a very powerful technique for intra-species identification, fingerprint profiles must be consistently reproduced. Additionally, this technique is highly sensitive to minor alterations in the reaction conditions (Yu & Pauls, 1992; Bowditch et al. 1993). In this study, we optimized RAPD-PCR reaction conditions for Arar tree. A summary of the parameters tested and the corresponding results is presented in table 1. The effects of the template DNA quantity, $MgCl_2$ concentration, primer concentration, Taq polymerase concentration and the initial denaturation temperature, on RAPD-PCR were investigated. Optimum conditions were chosen that give more discriminatory band profiles. In previous studies, the amount of template DNA strongly were found to influence the outcome of the RAPD-PCR reaction and for most plants species, more than 30 ng/25 µl was needed to give the premium amplification (Caetano-Annoles *et al.* 1991; Henegariu *et al*. 1997). In the present study, identical RAPD profiles were found when DNA amounts varied from 10 to 200 ng. DNA concentration of 10 ng/25 μl was found optimum. Figure 2 represents the effect of seven different DNA concentrations of RAPD banding pattern. Similar observations have been reported for cypress DNA (Doulis *et al*. 2000).The fact that even quantity as small as 10 ng/25 ul reaction gave a nice banding pattern in our RAPD-PCR reation is another indication that presence of polysaccharides, which are common contaminants in DNA preparations, were minimal in our DNA extraction method. The second parameter we optimized was the

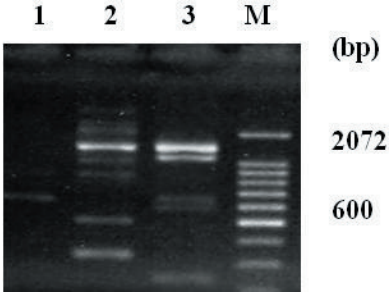

Figure 4. RAPD-PCR profile of Arar tree DNA (10 ng) in 1.8% agarose gel with three different initial denaturation temperatures: (1) 94°C, (2) 97.5°C and (3) 99.9°C. M: 100 bp DNA ladder. N: PCR negative control (no template DNA). *Profil RAPD-PCR de l'ADN du thuya de berberie (10 ng) sur gel d'agarose 1.8% obtenu avec 3 différentes températures de dénaturation initiale: (1) 94°C, (2) 97.5°C et (3) 99.9°C. M: marqueur de poids moléculaire (100 pb ladder). N est le témoin négatif amplifié sans ADN.*

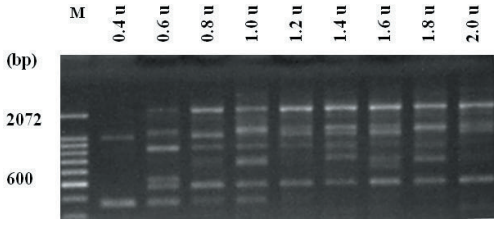

Figure 5. RAPD-PCR profile of Arar tree DNA (10 ng) using nine different amounts of Taq DNA polymerase using the Operon primer OPA-04 (5'-AATCGGGCTG-3'). M: 100 bp DNA ladder. *Profil RAPD-PCR de l'ADN du thuya de berberie (10 ng) avec neuf quantités différentes d'ADN polymérase de Thermus aquaticus obtenu avec l'amorce OPA-04 (5'-AATCGGGCTG-3'). M: marqueur de poids moléculaire (100 pb ladder).*

concentration of $MgCl_2$ in the PCR buffer. Eight different concentrations were investigated: 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 3.5 mM, 4 mM, 4.5 mM and 5 mM. Magnesium is an essential component of PCR reactions and can affects the quality of RAPD profiles (Munthaly *et al.* 1992). Magnesium Chloride is known to affect primer annealing and template denaturation, enzyme activity and fidelity and primer-dimer formation (Saiki *et al*. 1988). Among the 8 different $MgCl_2$ concentrations used in our RAPD reactions, 4 mM $MgCl_2$ was found to be optimal for generating the most reproducible and complex fingerprint profiles from Arar tree (fig. 3). The oligonucleotide primers OPA-01 to OPA-05 generate relatively more diverse and reproducible genomic fingerprints for a number of plant species and were used for the optimization of RAPD parameters for cypress (Doulis *et al.* 2000). We used the same primers to optimize the primer concentration for RAPD-PCR analysis of Arar tree. Results showed that 0.2 µM of the primer generated distinct and reproducible DNA fingerprints profiles for all Arar tree samples, without any detectable primer artifacts.

Initial denaturation temperature is one of the most important parameters that need adjustment in the PCR. An incomplete denaturation results in renaturation of strands, hence, a reduction in yield of amplification and reproducibility (Innis *et al*. 1990). The normal range of denaturation temperature is 94°C - 100°C which is sufficient for the denaturation of DNA of complex and large genomes such as those of conifers. To test if the variation of the denaturing temperature affects the outcome of RAPD-PCR results of Arar tree, we tested three different temperatures: 94°C, 97.5°C and 99.9°C, and we found that an initial denaturation temperature of 97.5°C for 4 min gave the best results (fig. 4). A similar result has been reported by Doulis *et al*. (2000) in another coniferous tree, cypress.

Since the initial use of a thermostable polymerase for PCR amplification (MuIlis & Faloona, 1987), a large number of variants of Taq (e.g. ampliTaq) and thermostable polymerases from other organisms (e.g. *Thermus brockianus*, *T. flavus*, *T. litoralis*, *T. thermophilus*, *Thermococcus litoralis*, *Thermotoga maritima*) have been isolated and

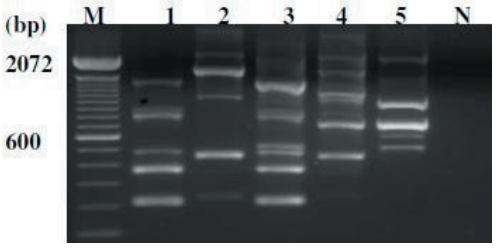

Figure 6. A Representative agarose gel fractionation of RAPD amplification products from Arar genomic DNA (10 ng) derived from a single Arar tree using the Operon primers: OPA-01 (1), OPA-02 (2), OPA-03 (3), OPA-04 (4), OPA-05 (5). Amplification products were fractionated in a 1.8% agarose gel. M: 100 bp DNA ladder. N: PCR negative control (no template DNA). *Représentation sur gel d'agarose du produit d'amplification RAPD à partir d'ADN génomique (10 ng) d'un arbre de thuya obtenu avec les amorces: OPA-01 (1), OPA-02 (2), OPA-03 (3), OPA-04 (4), OPA-05 (5). Les produits d'amplification sont séparés sur gel d'agarose 1.8%. M: marqueur de poids moléculaire (100 pb ladder). N est le témoin négatif amplifié sans ADN.*

shown to be suitable for PCR amplification of DNA. In the current study, we tested several amounts of Taq polymerase (Promega, Madison, WI, USA) ranging from 0.4U to 2U and found that 0.8 U of Taq polymerase in a 25 µL reaction volume was sufficient to produce a good DNA amplification (fig. 5).

The optimized reaction conditions for RAPD-PCR of Arar tree produced clear, scorable amplified products suitable for RAPD applications (fig. 6) using 5 different primers. The number of bands of each primer ranges between 6 bands (OPA-01) to 8 bands (OPA-04). Each primer produced amplicons between 0.2 kb in (OPA-01) to 3.0 kb (OPA-02).

CONCLUSION

The present study on development of protocol for isolation of high purity DNA and optimization of RAPD conditions is the first report in the species *T. articulata*. The two different extraction methods, used with some modifications, gave a DNA of quality suitable for RAPD-PCR analysis. The protocol described in Oenzar *et al*. (1998) gave higher DNA yield compared to QIAgen. The standard reaction developed included: 10 ng of DNA template, 4 mM $MgCl_2$, 0.2 µM primer, 200 µM dNTPs (for each) and with 0.8 U of Taq polymerase per 25 µl PCR reaction. The protocols described here and the optimized RAPD parameters constitute a strong beginning for future molecular characterization and genetic improvements studies in Arar tree.

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