DNA ISOLATION AND AMPLIFICATION IN OAK SPECIES (*Quercus* spp.)

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Abstract: *Two DNA isolating protocols were tested and compared at species from Quercus genus for their ability to produce DNA in good yield and purity. Both procedures were adapted to the conditions of our laboratory. The DNA resulted from isolation was amplified through polymerase chain reaction. The best results were obtained by using strong dilutions (1:100) which very likely reduce the amount of inhibitors such as tannins, phenols and polysaccharides. At this dilution, the success of DNA amplification was almost 95% for long fragments from chloroplast DNA and almost 100% for short fragments from nuclear DNA such as SSRs (simple sequence repeats).*

Key words: *DNA isolation, PCR, Quercus spp.*

1. Introduction

The quality of DNA is essential for any genetic analysis based on molecular markers. A high purity of DNA stock solution ensures the success of DNA amplification during the Polymerase Chain Reaction (PCR). The protocols that use plant tissue for DNA extraction have derived from those used for animals. In comparison with the herbaceous species, trees' buds and leaves, particularly those of oaks (*Quercus* spp.) contain large quantities of phenols, tannins and polysaccharides [1]. These secondary metabolites cannot be eliminated during the DNA isolation and can inhibit the activity of DNA polymerase during PCR [7]. A good quality of DNA corresponds to a low concentration of PCR inhibitors.

Nearly all molecular genetic markers are based on PCR technique. In this reaction, a thermo-resistant DNA polymerase is used for the amplification of the target DNA. Initially, this enzyme was isolated from the bacterium *Thermus aquaticus* which can live in thermal wells at high temperatures (95 °C). This *Taq* DNA polymerase is able to survive during the DNA denaturation, the first step of PCR. DNA and primer concentration, the presence of inhibitors, temperature rate ramp are among the factors which influence the outcome of the reaction. In each laboratory, protocols have to be adjusted to the existing thermocyclers and consumables.

There are different protocols for DNA isolation starting from plant material (e.g. Dneasy Plant Mini Kit - Qiagen, Hilden, Germany and ZR Plant/Seed DNA Kit - Zymo Research, U.S.A). The Qiagen kit or CTAB (Cetyl Trimetyl Ammonium Bromide) method [5] adapted for isolation of DNA from oaks [6] delivered a good quantity and quality of DNA.

The aim of this paper was to compare

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two different DNA isolation protocols after adaptation to the conditions of our laboratory and to test the amplification of short and long DNA fragments via PCR in several oak species native to Romania. As a short DNA fragment (approximately 200 bp) we selected a simple sequence repeats (SSRs) locus. The larger fragments (approximately 2000-4000 bp) were amplified from the chloroplast DNA (cpDNA).

2. Materials and Methods

Two standard protocols, Dneasy Plant Mini Kit (Qiagen, Hilden, Germany) [11] and ZR Plant/Seed DNA Kit (Zymo Research, U.S.A) [12], were tested. Winter buds were used in both cases as starting materials. We tested on fresh buds, collected in the same day with the isolation, and on buds kept for several months in a freezer at –60 °C. The usage of winter buds is recommended because they contain more DNA and have a low concentration of phenols and tannins. For the isolation we removed the scales of the buds and extracted the apical meristem. We avoid including any wood into the isolation tube because this tissue has a high concentration of metabolites. The wet weight of material was between 75 and 90 mg in both protocols.

The Qiagen procedure requires grinding of the tissues in the presence of liquid nitrogen. Keeping the material in liquid nitrogen improves the breakage of cell membranes. We eliminated this step because the procurement of liquid nitrogen was not possible. The material was grinded in 2 mL - Eppendorf tubes with the help of a Tissue Lyser Ruptor. For incubation, we used a Memmert water bath.

For quantification of the stock DNA solution we run horizontal electrophoresis in 1.5% agarose gel with 2 µL of DYE (loading buffer) combined with 5 µL of DNA. SERVA DNA Standard 1000 and 5000 bp DNA ladder was used. After 50 minutes at 100 V, the gels were stained with GelRed Nucleic Acid Gel Stain (Biotium) and visualized in an ultraviolet light chamber (GelDoc-It, UVP Imaging system).

PCR was carried out in two thermocyclers (Eppendorf and Corbett) in 0.2 mL individual tubes and 96 well plates. For a short DNA fragment, we selected microsatellite locus ssrQpZAG9, which was for the first time characterised in *Q. petraea* [9]. This locus was sequenced in several oak species [3]. Four longer fragments originating from cpDNA trnD/trnT (DT), psaA/trnS (AS), psbC/trnD (CD) [4], respectively trnT/trnF (TF) [10] were amplified. This large chloroplast fragments were used for identifying cpDNA haplotypes across Europe [8]. For both types of fragments the PCR was performed in a 15 µL volume containing 2 µL template DNA (approximately 1.2-1.6 ng), 7.5 µL Master Mix, 3.5 µL H_2O and 0.5 mM (1 µL) of each primer. The PCR profile for the amplification of the SSR locus was as follows: 3 minutes of denaturation at 94 ºC followed by 32 cycles of 50 s denaturation at 94 ºC, a 50 s annealing step at 56 ºC, a 1 min elongation step at 72 ºC and a final extension step at 72 ºC for 10 min. The PCR profile for the amplification of the cpDNA fragments was as follows: 4 minutes of denaturation at 94 ºC followed by 30 cycles of 50 s denaturation at 94 ºC, a 50 s annealing step at 58 ºC, a 2 (4) min elongation step at 72 ºC and a final extension step at 72 ºC for 10 (15) min.

3. Results and Discussion

3.1. DNA Isolation

Because of the difficulty of getting liquid nitrogen, we adapted the Qiagen protocol by freezing the plant material until disruption at 60 °C. For a better grinding with the

TissueLyser, we used two tungsten carbide beads with 3 mm in diameter instead of one. The collection tubes together with the tungsten carbide beads and the adaptors were kept at low temperatures until shortly before the disruption.

We started grinding the plant material in tubes with conic base. The resulting amount of DNA was very low because nearly all of the material remained intact at the tip of the tubes. By contrast, when using tubes with hemispheric base the amount of DNA was substantially higher. Moreover, an additional step of centrifugation after the grinding helped the detachment of the plant material from the cap of the tubes. This step does contribute to a better mixing of lysat with Buffer AP1 and RNase a solution. We have also observed that a longer time of incubation (12-14 minutes as compared to the 10 minutes specified in the standard protocol) increases the amount of DNA. A longer incubation will help the breakage of cell and nuclear membranes. The temperature of incubation was the same (65 °C) as well as the remaining steps of the standard protocol.

The DNA yield was 60-80 ng/µL as estimated by agarose gel electrophoresis. The elution volume was 2 x 100 µL. The total DNA yield was estimated at 6-8 µg in each elution. Though the DNA yield was very similar, the second elution showed very little degradation as compared to the first one (Figure 1).

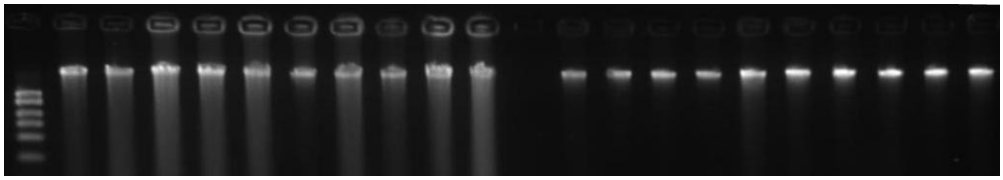

Fig. 1. *DNA isolated from ten different oak individuals by using Qiagen procedure* (*first elution - left and second elution - right*)

An advantage of the Qiagen procedure was the relatively short period of time necessary to obtain the DNA in comparison of the classical protocols based on DNA washing and purification with different solutions (PVP, EDTA, chloroform, isoamyl alcohol, ethanol in different concentrations etc.).

The Zymo Research protocol supposes grinding of the plant material with the lysis buffer and numerous beads of quartz in the tubes provided by the kit. As in the case of Qiagen procedure, the disruption of the material was carried out in the TissueLyser. However, the first results were not satisfactory, very likely due to the beads' diameter and weight. The beads were too small and did not have the power to crush the tissues. Therefore, the protocol was improved by replacing the quartz beads with those used in Qiagen procedure (two tungsten carbide beads with 3 mm in diameter). Examples of DNA extracted from oaks by using Zymo Research procedure is shown in Figure 2.

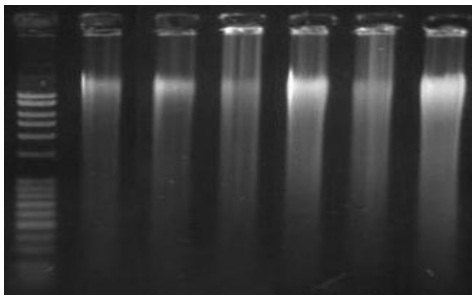

Fig. 2. *DNA isolated from eight different oak individuals by using Zymo procedure*

Similar results were obtained in a comparative analysis of different DNA extraction protocols in which. DNA from many herbaceous plants but also from ligneous plants including one oak species (*Q. robur*) was isolated [2]. The Qiagen protocol has showed the best result in *Q. robur*.

3.2. Polymerase Chain Reaction (PCR)

Despite our efforts to get a very high DNA purity, several impurities such as proteins, polysaccharides, phenol, tannins and salts were very probably present in the stock solution. Even though PCR is a robust procedure, because it consists of many cycles of enzymatic reactions, it is sensitive to these impurities. However, when simple sequence repeats (SSRs), fragments up to 200 bp were amplified in our oak species, the rate of success was almost 100% (Figure 3). The situation was totally different for long cpDNA fragments. When the same standard DNA dilution (1:20, 2 µL of DNA stock solution in 38 µL of RNase-free water) was used, we had almost no product or products of very low intensity. The results were very similar irrespective of the primer pairs used into the reaction. By testing different DNA dilution prior to PCR we realized that a very strong dilution (1:100) would be optimal for all four cpDNA primer pairs (Figure 4).

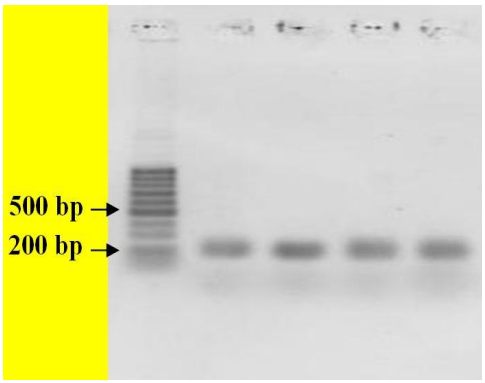

Fig. 3. *Amplification of SSRs in four oak individuals. The length of the fragments is approximately 200 bp*

At this dilution, the PCR products are uniform and show the same intensity in all samples (Figure 5). At small dilutions of DNA (e.g. 1:20), very likely, the enzyme inhibitors still have a high concentration. Another plausible explanation is that the proportion of DNA is too high, and primers and free-nucleotides are not in a sufficient quantity for completing the reaction.

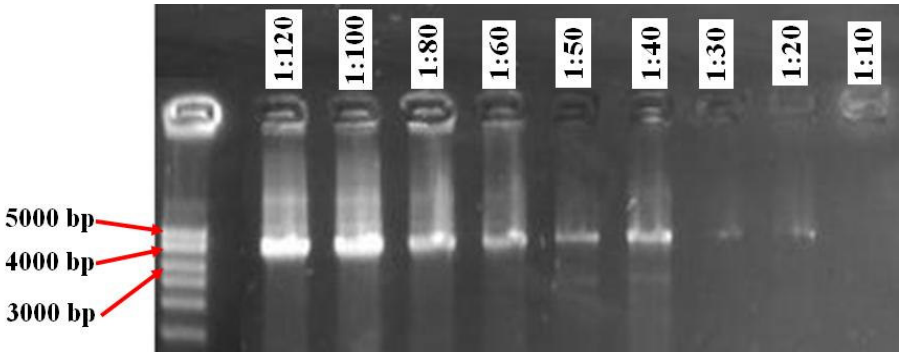

Fig. 4. *The effect of DNA dilutions on PCR amplification of the chloroplast region psaA/trnS. The length of the fragment is approximately 4000 bp. DNA from the same Q. robur individual was used in all cases*

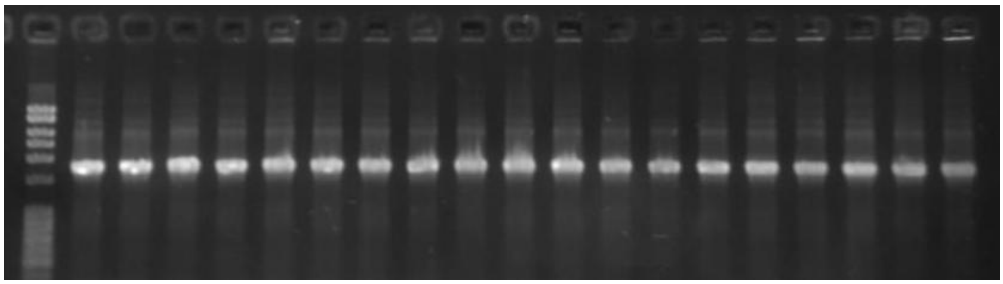

Fig. 5. *Longer PCR products for trnD/trnT cpDNA fragment in different Quercus species* (*DNA dilution 1:100*)

Gradient PCRs were performed on both thermocyclers. No significant differences were observed between the two types of PCR machine.

The optimization process continued with the number of cycles, duration of denaturation and elongation. We also tested different types of DNA polymerase and associated buffers (Qiagen Taq PCR Master Mix, Qiagen HotStarTaq DNA Polymerase, GoTag Hot Start Polymerase Promega, and GreenDreamTaq DNA Polymerase Fermentas). For the SSR locus, we have always got very good products. The type of the polymerase enzyme seems to have very little influence on the efficiency of PCR. On the contrary, the type of polymerase played a major role for the amplification of the large non-coding cpDNA regions. The best results, under the present circumstances, were obtained for PCR using GreenDreamTaq DNA Polymerase from Fermentas.

4. Conclusions

The yield and purity of DNA was very similar in all native oak species we tested: *Q. robur, Q. petraea, Q. pedunculiflora* and *Q. cerris*, respectively. As expected, we obtained larger amounts of DNA from fresh winter oak buds than from buds frozen for a long time at –60 °C. However, the differences were small. The use of buds collected in early spring, shortly before flushing, is not recommended. In this case, the DNA showed a higher degradation. The Qiagen procedure delivered good results without using liquid nitrogen for freezing the plant material prior to disruption and homogenization step.

Shorter DNA fragments like SSRs could be amplified via PCR without difficulties in all samples. In order to successfully amplify large regions from the chloroplast DNA, serial dilutions proved to be very helpful. A 1:100 DNA dilution seems to be the optimal starting template for PCR in our case. In this way, the effects of impurities present in our DNA could be overcome. The amplification of larger DNA fragments was more sensitive to the type of polymerase used.

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