DNA extraction and amplification from Pinaceae dry wood

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Abstract

Wood constitutes the unique source of DNA in dead trees, but extraction of adequate quality DNA from dry wood is usually challenging. However, many different molecular studies require the use of such DNA. We have standardized and validated a modified CTAB protocol to isolate DNA from dry wood from *Abies pinsapo* and *Cedrus atlantica* species. Due to the degradation and very little DNA that is normally present in the wood from dead trees we have developed a PCR based test to certify the quality of the extracted samples. In the present study, we have proved too the effectiveness of this methodology to isolate DNA from conifer dry wood samples of sufficient quality to perform further molecular genetic experiments.

Keywords: : *dry wood, DNA isolation, CTAB, 18S, Abies pinsapo, Cedrus atlantica.*

Introduction

DNA isolation from wood of dead trees has not been studied extensively so far. Several studies about wood DNA isolation have been published with different purposes: increasing the knowledge of ancient DNA, development of forensics applications, identification of samples, control of timber, etc. As consequence, DNA has been isolated from different sources: fresh wood (White et al., 2000; Jiao et al., 2012; Fatima et al., 2018), fossil plants (Liepelt et al., 2006; Sønstebø et al., 2010; Parducci et al., 2018) or dry and processed wood (Asif & Cannon, 2005; Jiao et al., 2012, 2015; Almeida de Souza et al., 2017).

In this work, two species belonging to the Family Pinaceae have been used as models to test the effectiveness of a modified CTAB method (based on Kistler 2012) for dry wood DNA extraction in conifers: *Abies pinsapo* Boiss (Spanish fir) and *Cedrus atlantica* (Endl.) Manetti ex Carriere (Atlas cedar). Fresh wood from *Pinus sylvestris* L. (Scots pine) was used too as a positive control of the DNA extraction protocol.

A. pinsapo is a relict Mediterranean fir, endemic from southern Spain and northern Morocco, very drought sensitive (Linares et al., 2011a). Nowadays, it is classified as an endangered species (IUCN, 2018). On the other hand, the Atlas cedar is also an endemic conifer tree, located in northern Morocco and Algeria, over almost 130,000 Ha (Cheddadi et al., 2009). Several studies have also stated that Atlas cedar is highly vulnerable to drought (Cheddadi et al., 2009; Linares et al., 2011b). In these species, we are interested in comparing the genomes of surviving trees with those of dead trees regarding climate change.

In this work, 71 wood samples were used to perform DNA extraction. A total of 26 dry wood samples of *A. pinsapo*, which were obtained from Sierra de las Nieves Natural Park (Málaga, Spain), and 29 dry wood samples of *C. atlantica* from the High Atlas and Ifrane National Parks (both located in North Morocco). Besides, 19 samples of fresh wood of *P. sylvestris* from Picos de Urbión Natural Park (Soria, Spain) were used to compare the

DOI:10.2478/sg-2019-0010
edited by the Thünen Institute of Forest Genetics

differences between the isolation of DNA in dry and fresh woods.

Firstly, we tried several plant DNA isolation kits with unsuccessful results in our dry wood samples. Then, we tested several manual methods, and finally selected a CTAB method to extract DNA from seeds (Kistler, 2012) to be proved in our material. After different adaptation experiments, we ascertained that a pre-treatment of wood samples was needed. First, it was necessary to remove the bark and sand from the surface with sandpaper. Then, wood shavings were obtained by means of the use of a scalpel, which was sterilized with ethanol 70 %. After that, wood was macerated by using a sterile pestle and liquid nitrogen until turning the samples into powder, which was placed into 2 mL tubes. Next, four to six tungsten balls were added and a fine powder was obtained by the use of Tissue Lyser II of Qiagen® for 4-6 minutes. This complete pre-treatment protocol is required to achieve a successful extraction from dry wood samples.

Subsequently, 100 mg of wood powder were used to carry out the DNA extraction with the method described by Kistler (2012). Regardless of the species, in the wood of dead trees there are only traces of degraded DNA. In our hands, such vestigial DNA could not be observed in 1 % agarose gels. In addition, as expected, analysis of dry wood DNA with Nanodrop™ showed spectrophotometric curves very far from the observed in the case of good-quality DNA (Figure 1). Then, any valuation of DNA via Nanodrop™ would give us invalid results. Consequently, we developed a method to determine the quality of the isolated DNA, which was based on a PCR amplification of an 18S ribosomal gene fragment. Briefly, we assumed that a positive PCR amplification of this 18S DNA fragment means that such sample is useful for any further genetic studies.

of DNA extracted from wood were added. Finally, the PCR programme used has the following steps: 3 minutes at 94°C; 35 cycles of: 1 minute at 94°C, 1 minute at 58°C and 01:20 minutes at 72°C; and 8 minutes at 72°C.

At that point, we tested this PCR protocol in our samples (Figure 2). As expected, a 100 % success rate in fresh wood from Scots pine was obtained (19/19). In the case of the dry wood samples, the results were variable. On one hand, for Atlas cedar 21 samples out of 29 were positively amplified (72.4 %). On the other hand, 14 out of 26 *Abies pinsapo* samples were positives too (46.2 %). Moreover, some of the samples that showed negative results in this first experiment were extracted again with positive results in the following amplification of the 18S fragment.

In addition, as proof of the usefulness of these isolated DNAs, a mtDNA SSR marker (*nad5-4*, Liepelt et al., 2002; Cinget et al., 2015) was positively genotyped in the isolated samples that showed a previous positive amplification of the 18S fragment. This PCR of the SSR was carried out in the same conditions that we described above, except the annealing temperature that was 55°C instead of 58°C.

Due to low quantity and huge degradation of DNA templates, the difficulties in amplifying DNA from wood is expected to be high and especially, when wood from dead trees is used. For this reason, designing short size amplicons are the best way to get successful amplifications in this kind of samples from wood. In addition, ome of the samples that were not isolated in the first experiment could be successfully extracted in subsequent experiments. This fact shows that in most cases is possible to obtain useful DNA traces from wood samples (fresh or dry).

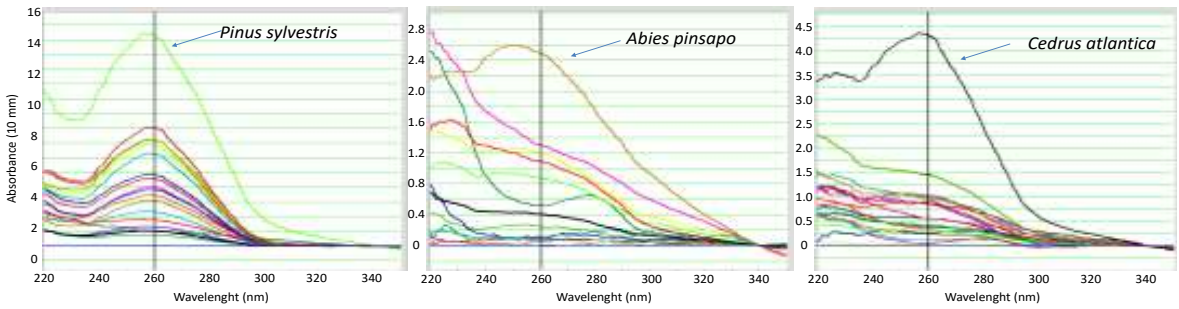

Figure 1
Spectrophotometric curves obtained via Nanodrop™ of the DNA isolated from the different samples of the three species. Arrows indicate the spectrophotometric curve of a DNA extracted from leaves of each species. Clear differences can be appreciated between control DNA (leaves) and the wood samples. Also, differences between dry wood (*A. pinsapo* and *C. atlantica*) and fresh wood (*P. sylvestris*) are evident

PCR amplification of this 18S 75-bp fragment was carried out in a final volume of 15 µl of the reaction mix, which was composed of: 7.5 µl of Master Mix of *DNAmplitools* (Biotools), 7.5 pmoles of each 18S universal primer (5´-CGCGAGAAGTCCACTAAACC-3' and 5'-CCTACGGAAACCTTGTTACGA-3´; Osakabe et al., 2013), 3 µl of sterile deionised water and 1.5 µl

In summary, DNA from three forest species of Pinaceae Family has been isolated using a modified CTAB protocol. Moreover, the obtained DNA was functional, as it has been shown by PCR amplifications (18S fragment and mtDNA SSR). Thus, this extraction procedure of conifer wood samples isolates DNA traces to perform further molecular genetic research, even when trees are dead. For

this reason, our results would contribute, among others, to open unexplored paths in the research of the molecular basis of conifers response to changes in environmental conditions, such as recent climate change.

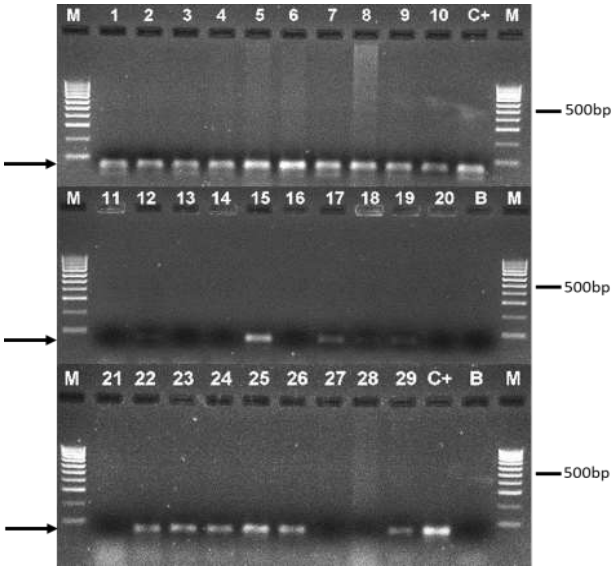

Figure 2
PCR of an 18S ribosomal gene fragment of 75 bp. Amplification products were analysed by electrophoresis in 2.5 % agarose gels. Lanes 1-10: DNA from fresh wood of *P. sylvestris*; Lanes 11-20: DNA from dry wood of *A. pinsapo*; Lanes 21-29: DNA from dry wood of *C. atlantica*; C+: DNA from leaves of each species; B: Blank; M: 100 bp ladder (Biotools). Arrows indicate the 18S PCR fragment.

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