Flexible DNA isolation procedure for different tree species as a convenient lab routine

Tobias Bruegmann*, Matthias Fladung, and Hilke Schroeder*

Thuenen Institute of Forest Genetics, Sieker Landstrasse 2, 22927 Grosshansdorf, Germany

* Corresponding authors: Tobias Bruegmann, Email: tobias.bruegmann@thuenen.de; Hilke Schroeder, Email: hilke.schroeder@thuenen.de

Abstract

DNA isolation is a fundamental technique for all molecular biology laboratories. Depending on the plant species, DNA isolation can be challenging. In particular, adapted protocols rarely exist for tree species which are not used as standard model organisms. Here, we describe a flexible DNA isolation protocol that works for 59 tree species in a modular system. It is based on an ATMAB-containing extraction buffer to which proteinase K and/or boric acid are added, depending on the plant species. Subsequent purification steps include one or two precipitations with dichloromethane and, depending on the tree species, an optional sodium acetate precipitation. Using leaf material of a hybrid poplar clone from *in vitro* culture, it was determined that higher amounts of DNA could be isolated with this material than from field leaves. Starting from leaf material, DNA isolation for difficult cases was achieved with cambium or root tissue. This protocol was used to extract DNA for subsequent PCR amplification. Markers for cpDNA, mtDNA, and genomic DNA were used for standardized testing.

Introduction

DNA isolation from plant material is one of the most basic techniques in molecular genetic laboratories worldwide. It is applicable to many plant species including trees like poplar, beech, oak, and many conifer species. In particular, for the latter plant species, the Thuenen Institute of Forest Genetics, Grosshansdorf, Germany, has established a thriving standard protocol over the years. For this protocol, the published work of Doyle and Doyle (1990) and Dumolin et al. (1995) have been fundamental, however, many improvements have been made to include as many tree species as possible. For reasons of reproducibility, therefore, citing both publications does not seem to be sufficient. It is thus appropriate to publish the institute's standard operating procedure (SOP) for isolating total DNA from different tissues of several tree species. Commercial kits for DNA isolation are often not adapted to the specific requirements of tree tissues as they can contain high amounts of disturbing secondary plant compounds such as polyphenols and/or polysaccharides.

The protocol described here is based on the deep-frozen homogenization of the plant material and the use of alkyl-trimethyl-ammonium bromide (ATMAB) as extraction buffer. ATMAB acts as a detergent and polyvinylpyrrolidone (PVP) is supposed to remove phenolic compounds from the extract as it forms hydrogen bonds with the phenols. Dithiothreitol (DTT) is a reducing agent and denatures proteins. It substitutes the ß-mercaptoethanol (ß-ME) originally used by Doyle and Doyle (1990). DTT is less volatile compared to ß-ME and is therefore preferable for laboratory use. All other components are aimed to maintain appropriate conditions for the DNA. In extended protocols, either proteinase K is added which supports the protein degradation or boric acid which might support the removal of carbohydrates from the solution.

Genomic (gDNA), chloroplast (cpDNA) and mitochondrial (mtDNA) DNA are isolated with the described protocol. The obtained DNA is available for various applications and downstream analyses, e.g., next generation sequencing (NGS). DNA from this isolation routine has already been used for numerous studies and various tree species (Blanc-Jolivet et al. 2018; Brenner et al. 2019; Bruegmann et al. 2019; Bruegmann and Fladung 2013; Bruegmann and Fladung 2019; Müller et al. 2020; Schroeder et al. 2012; Schroeder et al. 2016; Schroeder et al. 2017; Schröder et al. 2019).

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To provide a flexible protocol for a simple laboratory routine, we include a wide range of utilized tree species, the use of plant material from *in vitro* culture, different amounts of plant material in the extraction batch, and possible modifications. Some developments have been made for challenging plant species and proposed as alternative protocols for adequate DNA yield (SOP A-C). In order to prove the functionality of the extracted DNA, we tested its usability in PCR amplification for all plant species investigated in this study. For this purpose, we selected *psbD* for cpDNA and *cox1* for mtDNA. The markers were chosen because they have been developed in our institute for the differentiation of high taxonomic levels (as conifers and angiosperms) and have already been validated with a broad range of species. Thus, the quality of PCR results can mainly be traced back to the extraction method. Furthermore, *cox1* has already been discussed for reasons of barcoding (Hebert et al., 2003; Vijayan and Tsou, 2010). We chose the internal transcribed spacer (*ITS1*) as a marker for the nuclear DNA (Álvarez 2003; Baldwin 1992; Baldwin 1993; Fladung et al. 2015).

Material and Methods

Sample material and preparation

To demonstrate the wide range of applications, 59 tree or shrub species were selected from 19 families, in some cases additional different subspecies, varieties, or cultivars. The plants were mainly harvested in August and September 2020 from the *in vitro* culture, the greenhouses, or the Tannenhöft arboretum of the Thuenen Institute of Forest Genetics in Grosshansdorf, Germany (Grosshansdorf, Germany). Only the material from *Ailanthus altissima* has been sampled in a wild occurrence in Hamburg (Germany), *Quercus mongolica* is from the research project "white oaks", and *Ulmus minor* from the institute's nursery. To compare different applied parameters like tissue weight, cultivation method, or incubation times, we used the poplar model clone INRA 717-1B4 (*Populus* × *canescens*, Leple et al. (1992)) as it is available *in vitro* and is therefore a nearly unlimited tissue source.

The fresh plant material (120 mg, unless otherwise stated) was frozen in a 2 mL tube by liquid nitrogen. For leaf tissue, approx. 320 mg ceramic beads (mix of 1.4 mm ceramic beads from Qiagen, Hilden, Germany; and 2.8 mm beads from Biolab Products, Bebensee, Germany) were used for grinding with a bead ruptor ("Bead Ruptor Elite", Omni International, Kennesaw, GA, USA). Two bead ruptor runs for 20 s at 3.55 m/s with nitrogen cooling between the runs are necessary for complete homogenization. As needle tissue is usually harder, the needles were cut into approx. 5 mm pieces prior to homogenization. Two stainless steel beads (diameter 4 mm) were given to the cut needles. With the swing mill "MM300" (Retsch, Haan, Germany) the tissue was homogenized twice for 2 min at 20 Hz with nitrogen cooling between the runs. After complete homogenization, the tubes were refrigerated until used for extraction or kept in liquid nitrogen for subsequent use.

The cambium samples were taken in October 2020. Punched pieces with a diameter of 2 cm were taken from six selected tree species (four deciduous and two coniferous species) growing in the institute's arboretum: *Acer campestre*, *Fagus sylvatica*, *Quercus robur*, *Salix caprea; Picea abies*, and *Pinus sylvestris*. The cambium was separated from the bark with a scalpel, homogenized as described for leaf material, and used for DNA isolation.

Root samples were also taken in October 2020 from potted *Ulmus minor* and *Fraxinus excelsior* plants from the institute's nursery. The roots were processed like needles as described above.

DNA isolation

The extraction buffer contained 2 % ATMAB (w/v), 1 % PVP K30 (w/v), 1.4 M NaCl, 20 mM EDTA II and 100 mM Tris-HCl. For the purpose of long storage, it was autoclaved at 121 °C for 20 min.

1 mL of 55 °C pre-warmed extraction buffer was given to the homogenized and still cold plant material. The mix was vortexed immediately and vigorously until a homogeneous suspension was obtained. The ceramic or steel beads left in the tubes are helpful for vigorous mixing. Due to the warm buffer and the motion, the extract thawed and was ready for the further handling. 50 µL of 1 M DTT (dissolved in 10 mM NaAc, pH 5.2) was added to each sample. The tube was vortexed shortly. Starting with the addition of DTT, all steps are done under a fume hood. The mix was incubated for 1 h at 55 °C shaking with 300 rpm. Afterwards the samples were cooled on ice for 10 min. A total of 400 µL dichloromethane were added to the sample for the precipitation. The tube was inverted several times and afterwards centrifuged 20 min at 16,200 × g and 4 °C ("Heraeus Fresco 21" centrifuge, ThermoScientific, Waltham, MA, USA). The aqueous upper phase was transferred in a new tube and mixed with 600 µL 2-propanol (cooled at -20 °C). We recommend that the ceramic beads be discarded and the steel balls reused after cleaning. During the incubation at -20 °C (time span variable, 30 min recommended), nucleic acids precipitated and are isolated by 10 min centrifugation at 16,200 × g and 4 °C. The supernatant was discarded and the nucleic acid pellet was washed with 1 mL 70 % ethanol cooled at -20 °C with a subsequent centrifugation of 20 min at 16,200 × g and 4 °C. After discarding the supernatant, the pellet was air-dried and eluted with 50 µL 1× TE buffer containing RNase A. The sample was incubated for 30 min at 37 °C and afterward stored at -20 °C for long term. The quantification measurements were done with a NanoDrop One (ThermoScientific, Waltham, MA, USA). A bullet point protocol is provided in the supplementary material.

The mentioned TE buffer with RNase was prepared with 10 mM Tris and 1 mM EDTA-Na_2 (Sambrook et al. 1989). The pH was adjusted to 8.0. Ten µL RNase A (100 mg/mL) were added to a final volume of 100 mL buffer.

Modifications of the isolation protocol

Modifications were introduced for challenging species for which DNA could not be extracted with the SOP (Fig. 1).

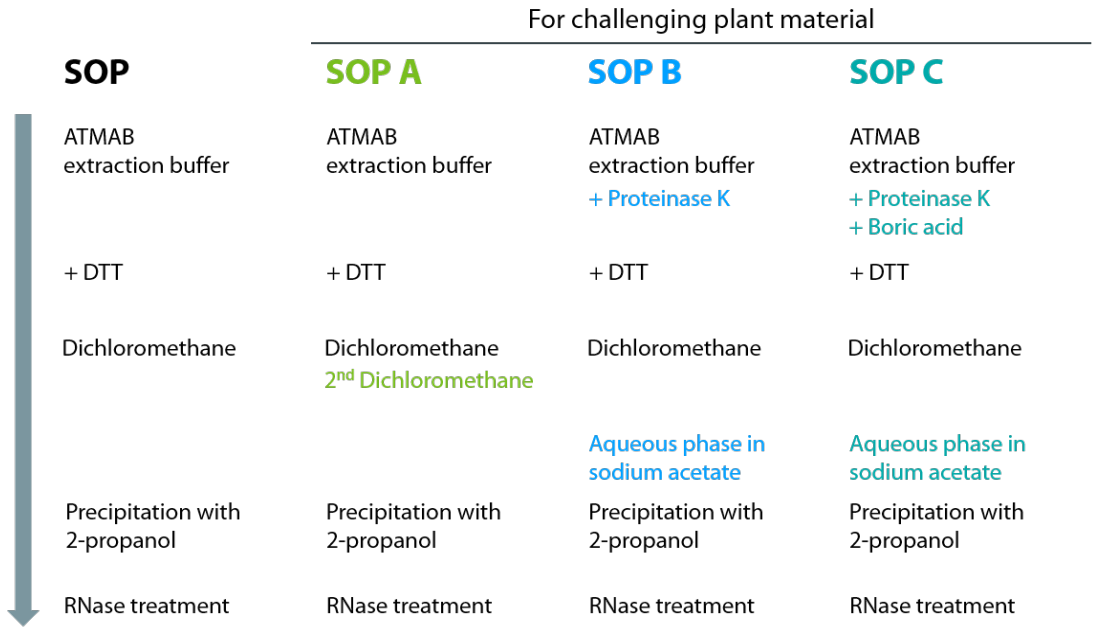

Figure 1
The standard operating procedure (SOP) was modified in three different protocols for recalcitrant plant material. SOP A was carried out if the phase separation was insufficient and is just like the SOP but extended with a second dichloromethane purification. SOP B contained proteinase K in the extraction buffer. In addition, the upper, aqueous phase from the dichloromethane purification was given in sodium acetate and subsequently precipitated with 2-propanol. SOP C corresponds to SOP B, but to the extraction buffer boric acid is also added.

Modification A (SOP A) was used when no clear phase separation was visible after shaking out with dichloromethane, for example due to a slimy interphase. A larger volume was taken from the top and again mixed with dichloromethane and shaken out. The obtained aqueous phase was processed as described in the standard protocol.

Modification B (SOP B) was used for leaves of *Ailanthus altissima* to get the best results. Additionally, it was tested with leaves, cambium, and roots from *Ulmus minor* as well as cambium and roots from *Fraxinus excelsior*. Here, the ATMAB buffer was supplemented with 1 µL proteinase K (stock concentration 20 mg/mL, New England Biolabs, Frankfurt/Main, Germany) and 600 µL of the obtained aqueous phase from the separation with dichloromethane were given into 225 µL sodium acetate (stock concentration 3 M, pH 5.2). All other steps correspond to SOP.

Modification C (SOP C), an extension of SOP B, was used for leaves of *Quercus mongolica* and *Quercus turneri* as well as cambium and roots of *Ulmus minor*. In this case, 1 mL ATMAB extraction buffer was supplemented with 1 µL proteinase K (stock concentration 20 mg/mL) and 2 % boric acid (v/v). Six hundred µL of the obtained aqueous phase from the separation with dichloromethane were transferred into 225 µL sodium acetate (stock concentration 3 M, pH 5.2). The incubation time with 2-propanole for DNA precipitation was 30 min.

For *Ulmus minor*, extraction buffers with 3 M or 4 M NaCl were tried to reduce the carbohydrates in the extracts. The further processing corresponds to the mentioned SOPs. A high salt concentration may avoid the simultaneous precipitation of carbohydrates with the DNA (Souza et al. 2012).

For seven tree species (*Ailanthus altissima*, *Alnus incana*, *Betula maximowicziana*, *Castanea sativa*, *Fraxinus excelsior*, *Quercus mongolica*, and *Sorbus aucuparia*) we tested whether DNA extracts could be optimized with the use of phenol/chloroform and chloroform to shake out the nucleic acids. For this purpose, after phase separation with dichloromethane, the upper phase was transferred to 1 vol. phenol/chloroform and shaken for 10 min. The mixture was then centrifuged for 10 min at 16,200 × g and 4 °C. The upper aqueous phase was transferred to 1 vol. chloroform and centrifugation was repeated. The resulting upper phase was further processed according to SOP.

Marker description and PCR amplification

Four primer combinations were used to test the quality of the DNA isolation, one each belonging to the cpDNA, and the mtDNA, and two belonging to the nuclear genome. The cp and mt primers were developed during the research project "Wood DNA barcoding" conducted at the Thuenen Institute of Forest Genetics, Grosshansdorf, Germany, aiming to amplify a broad range of species. Using the primers NL_psbD_F1 (5′ AAT AAA CGT TGG TTA CAT TTC TT 3′), and NL_psbD_R1 (5′TTC ATG AGG CTG ATC TTG AG 3′) a 225 bp long fragment was amplified. The PCR product amplified with the primer combination NL_cox_F1 (5′ GCC CTT AAG TGG TAT TAC CAG 3′), and NL_cox_R2 (5′ TAA GCA TCT GGA TAA TCT GGA AT 3′) has a length of 251 bp.

For amplification of the genomic *ITS* region, the primer combination "ITS1 and ITS2" as well as "ITS3 and ITS4" as described in Baldwin (1992; 1993) were used.

The PCR reactions contained 20 ng template DNA, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 1 unit Taq polymerase (DNA Cloning Service, Hamburg, Germany), and 1× concentrated PCR buffer in a total volume of 25 µL. PCR was carried out in a PCR Thermocycler (Sensoquest, Göttingen, Germany) with a pre-denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45 s, suitable annealing temperature for each primer combination (50 °C for *psbD*, 55 °C for *cox1*, and 52 °C for *ITS*) for 1 min, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 5 min.

Electrophoresis

For quality control of genomic DNA samples, an 0.7 % agarose TBE gel was used in electrophoresis according to Sambrook et al. (1989). PCR products were detected on an 1.5 % agarose TBE gel. The prepared gel was mixed with 2.5 µL Roti Gel Stain (Carl Roth, Karlsruhe, Germany) per 100 mL gel for UV visualization. As DNA ladders, SmartLadder (Eurogentec, Lüttich, Belgium) and 50 bp DNA Ladder (NEB, Frankfurt am Main, Germany) were used. 5 µL DNA were mixed with 2 µL 1× Orange G loading dye (from prepared 6× stock with 30 % sucrose and 1 % Orange G, Sigma-Aldrich, St. Louis, MO, USA) and given to electrophoresis until adequate separation. Finally, the DNA was UV-visualized with an INTAS documentation system (Intas, Göttingen, Germany).

DNA isolation from 59 plant species

From leaf tissue of 59 woody plant species (including certain cultivars or subspecies in some cases), DNA could be successfully isolated with SOP or modified SOP A-C (Tab. 1).

The species *Ailanthus altissima*, *Alnus incana*, *Betula maximowicziana*, *Fraxinus excelsior*, *Quercus mongolica*, *Sorbus aucuparia*, *Thuja occidentalis*, and *Ulmus minor* turned out to be challenging, probably due to high amounts of secondary metabolites as phenols and carbohydrates. The DNA extracts were unsatisfactory with the SOP. The obtained extract of *Ulmus minor* was slimy. All mentioned plant extracts had a dark color, indicating contaminations from secondary metabolites. Thus, further modifications were tested.

DNA isolation from the recalcitrant tree species

Quercus mongolica is known to be a recalcitrant tree species to work with, which is due to secondary plant compounds in the leaves. Seven different methods were tested for this tree species, including additional methods with phenol/chloroform purification described in the appendices. The best result in DNA isolation was obtained with boric acid and proteinase K and sodium acetate precipitation at the end (SOP C, Tab. 2). The inclusion of phenol/chloroform leads to either lower yield or poorer purity values when sodium acetate is omitted at the end. This procedure can therefore not be recommended.

DNA from leaves of *Ailanthus altissima* could not be satisfactorily isolated using the SOP. However, good values in yield and purity were obtained with proteinase K in the extraction buffer (SOP B, Tab. 3). DNA isolation also worked well with SOP C, with slightly poorer purity values.

If the DNA isolates contain carbohydrates, some publications recommend the use of a high salt concentration in the extraction buffer. This was exemplarily tested with leaf tissue of *Ulmus minor*. The extraction buffer was prepared with 3 M and 4 M NaCl. However, the purity values could not be improved. If leaf material turns out to be challenging, root or cambium tissue should be used instead, if possible.

In vitro material and free area grown material

Leaf tissue from either *in vitro* or greenhouses are suitable for DNA isolation. To estimate limits concerning the applied plant tissue weight, we tried DNA isolations with different amounts of leaf tissue from the poplar hybrid clone INRA 717-1B4 (*Populus* × *canescens*). Overall, the isolation worked with each amount of tissue (Tab. 4). Working with *in vitro* tissue, the DNA yield was increased proportionally with the applied fresh weight up to 11,701 ng/µL. Such a strong increase could not be observed for greenhouse leaf tissue.

Variation in incubation time

Leaf material (120 mg) from the *P.* × *canescens* clone INRA 717-1B4 was used to investigate the effect of precipitation time in 2-propanol on DNA yield. An incubation period of 0.5, 1, 2, 3, or 16 h was tested. *In vitro* material appears to be insensitive to longer incubation times. The yield of DNA isolation from greenhouse material becomes poorer with longer incubation time, so 30 minutes incubation time is recommended (Fig. 2).

DNA isolation from cambium

Cambium from *Acer campestre*, *Fagus sylvatica*, *Pinus sylvestris*, *Picea abies*, *Quercus robur*, *Salix caprea* (with 50 mg cambium tissue), *Fraxinus excelsior,* and *Ulmus minor* (both with 120 mg cambium tissue) was used for DNA isolation. DNA isolation was possible for all species tested. The SOP worked for most species, only *U. minor* needed a modified protocol because of the poor purity values (Tab. 5). SOP C was used with proteinase K in the extraction buffer for the cambium of this species. However, this modification is not always advisable: SOP C was also tested with *F. excelsior*, but this resulted in poorer values in yield and purity. In addition, SOP B was tested with *U. minor* and *F. excelsior*, but was inferior to the aforementioned protocols (Fig. 3).

DNA isolation from roots

Root tissue may also be considered for DNA isolation, especially in young plants. This becomes particularly relevant when secondary plant compounds in the leaves interfere with DNA isolation and cambium cannot be harvested. Our isolation protocols were tested with the two difficult tree species *Fraxinus excelsior* and *Ulmus minor*. For *U. minor*, the protocol variant SOP C with boric acid was the best in terms of purity, but here the DNA concentration was the lowest (Fig. 4). A higher DNA concentration was obtained with SOP. Before using SOP, however, it should be clarified here whether the poorer purity values have a negative effect. For *F. excelsior*, SOP was best because the DNA concentration was highest here and

Table 1
From 59 different tree species, partly including subspecies, varieties, and cultivars, attempts were made to isolate DNA from leaf material and partly also from cambium or root tissue. PCRs were performed for the amplification of the four marker gene regions *psbD*, cox1, ITS1_2 and ITS3_4 to check the functionality of the isolated DNA. Abbreviations: SOP – standard operating procedure, x – tested, + – worked well, (+) – worked sufficiently, - – did not work, yes – PCR product amplified, (yes) – PCR product amplified but only faint, no – no PCR product amplified.

		Tissue			DNA isolation				PCR			
Family	Species	leaf	cambium	root	SOP	SOP A	SOP B	SOP C	psbD	cox1	ITS1_2	ITS3_4
Pinaceae	*Abies grandis*	x				+			yes	yes	yes	yes
Pinaceae	*Abies nordmanniana*	x				+			yes	yes	yes	yes
Pinaceae	*Abies numidica*	x			+				yes	yes	yes	yes
Pinaceae	*Larix decidua*	x			+				yes	yes	yes	yes
Pinaceae	*Larix gmelinii*	x				+			yes	yes	yes	yes
Pinaceae	*Picea abies*	x				+			yes	yes	yes	yes
Pinaceae	*Picea abies*		x		+				yes	yes	no	yes
Pinaceae	*Picea abies cv. aurea*	x			(+)				yes	yes	yes	yes
Pinaceae	*Picea orientalis*	x				+			yes	yes	yes	yes
Pinaceae	*Pinus contorta*	x			+				yes	yes	yes	yes
Pinaceae	*Pinus mugo*	x			+				yes	yes	yes	yes
Pinaceae	*Pinus ponderosa*	x				+			yes	yes	(yes)	yes
Pinaceae	*Pinus pumila*	x			+				yes	yes	yes	yes
Pinaceae	*Pinus sylvestris*	x			+				yes	yes	yes	yes
Pinaceae	*Pinus sylvestris*		x		(+)				yes	yes	no	yes
Pinaceae	*Pseudotsuga menziesii*	x			+				yes	yes	yes	yes
Pinaceae	*Tsuga heterophylla*	x				+			yes	yes	yes	yes
Cupressaceae	*Chamaecyparis pisifera*	x			+				yes	yes	no	no
Cupressaceae	*Sequoiadendron giganteum*	x			(+)				yes	yes	yes	(yes)
Cupressaceae	*Thuja occidentalis*	x			(+)				yes	yes	yes	yes
Taxaceae	*Taxus baccata cv. adpressa*	x			(+)				yes	yes	yes	no
Taxaceae	*Torreya californica*	x			(+)				yes	yes	yes	no
Ginkgoaceae	*Ginkgo biloba*	x				+			yes	yes	(yes)	no
Magnoliaceae	*Liriodendron tulipifera*	x			+				yes	yes	yes	(yes)
Platanaceae	*Platanus occidentalis*	x			+				yes	yes	yes	yes
Hamamelidaceae	*Hamamelis japonica var. flavo-purpurascens*	x			+				yes	yes	yes	yes
Salicaceae	*Populus × canescens*	x			+				yes	yes	yes	yes
Salicaceae	*Populus euphratica*	x			+				yes	yes	yes	yes
Salicaceae	*Populus tremula*	x			+				yes	yes	yes	yes
Salicaceae	*Populus tremula × P. tremuloides*	x			+				yes	yes	yes	yes
Salicaceae	*Populus trichocarpa*	x			+				yes	yes	yes	yes
Salicaceae	*Salix viminalis*	x			(+)				yes	yes	yes	yes
Fabaceae	*Robinia pseudoacacia*	x				+			yes	yes	yes	yes
Rosaceae	*Sorbus aucuparia*	x			-	(+)			no	no	no	no
Rosaceae	*Malus domestica*	x			(+)				yes	yes	yes	yes
Ulmaceae	*Ulmus minor*	x			-	-	-	-	no	no	no	no

Table 1 Continued

		Tissue			DNA isolation				PCR			
Family	Species	leaf	cambium	root	SOP	SOP A	SOP B	SOP C	psbD	cox1	ITS1_2	ITS3_4
Ulmaceae	*Ulmus minor*		x		-		+	+	yes	yes	(yes)	yes
Ulmaceae	*Ulmus minor*			x	-		(+)	(+)	yes	yes	(yes)	yes
Betulaceae	*Alnus glutinosa*	x			+				yes	yes	yes	yes
Betulaceae	*Alnus incana*	x			-	(+)			no	no	no	no
Betulaceae	*Betula maximowicziana*	x			-	(+)			no	no	yes	yes
Betulaceae	*Betula pendula*	x			+				yes	yes	yes	yes
Betulaceae	*Carpinus betulus cv. albo-variegata*	x			+				yes	yes	yes	yes
Betulaceae	*Carpinus betulus quercifolia*	x			+				yes	yes	yes	yes
Betulaceae	*Corylus avellana*	x			(+)				yes	yes	yes	yes
Fagaceae	*Castanea sativa*	x			-	(+)			no	(yes)	yes	yes
Fagaceae	*Fagus sylvatica*	x			+				yes	yes	yes	yes
Fagaceae	*Fagus sylvatica*		x		+				yes	yes	yes	yes
Fagaceae	*Fagus sylvatica purpurea*	x			+				yes	yes	yes	yes
Fagaceae	*Quercus imbricaria*	x				+			yes	yes	yes	yes
Fagaceae	*Quercus mongolica*	x				-		(+)	yes	yes	yes	yes
Fagaceae	*Quercus palustris*	x				+			yes	yes	yes	yes
Fagaceae	*Quercus robur*	x				+			yes	yes	yes	yes
Fagaceae	*Quercus robur*		x		(+)				yes	yes	(yes)	yes
Fagaceae	*Quercus rubra*	x				+			yes	yes	yes	yes
Fagaceae	*Quercus turneri (Q. robur × Q. ilex)*	x						+	yes	yes	yes	yes
Fagaceae	*Quercus turneri cv. pseudo-turneri*	x			+				yes	yes	yes	yes
Juglandaceae	*Juglans cordiformis*	x			(+)				yes	yes	yes	yes
Juglandaceae	*Juglans regia*	x			+				yes	yes	yes	yes
Sapindaceae	*Acer campestre*	x				+			yes	yes	yes	yes
Sapindaceae	*Acer campestre*		x		+				yes	yes	yes	yes
Sapindaceae	*Acer negundo*	x			(+)				yes	yes	yes	yes
Sapindaceae	*Acer platanoides*	x				+			yes	yes	yes	yes
Sapindaceae	*Acer rubrum*	x			(+)				yes	yes	yes	yes
Sapindaceae	*Aesculus × carnea*	x			(+)				yes	yes	yes	yes
Sapindaceae	*Aesculus turbinata*	x			(+)				yes	yes	yes	(yes)
Simaroubaceae	*Ailanthus altissima*	x				(+)	+	+	yes	yes	no	no
Paulowniaceae	*Paulownia tomentosa*	x			+				yes	yes	yes	yes
Oleaceae	*Fraxinus excelsior*	x			-	(+)			no	(yes)	no	no
Oleaceae	*Fraxinus excelsior*		x		(+)		(+)	(+)	yes	yes	yes	yes
Oleaceae	*Fraxinus excelsior*			x	-		(+)	-	no	no	no	no
Araliaceae	*Kalopanax septemlobus*	x			-	(+)			no	no	no	no

Table 2
Quantity and quality values for different isolation modifications for *Quercus mongolica*. The best results of DNA isolation from *Q. mongolica* leaves were obtained with SOP C (samples 3a and 3b), which involves the use of boric acid and proteinase K in the extraction buffer, and sodium acetate precipitation.

Sample number	Boric acid	Proteinase K	Dichloro-methane	Sodium acetate	DNA concentration [ng/µL]	Purity A260/A280	Purity A260/A230
1a	X	X	X		4,449.48	1.72	0.99
1b	X	X	X		3,576.37	1.79	1.26
2a	X		X	X	3,030.20	1.88	1.64
2b	X		X	X	3,224.31	1.88	1.59
3a	X	X	X	X	3,669.15	1.92	1.78
3b	X	X	X	X	3,172.42	1.94	1.99

Table 3
DNA isolation from leaves of *Ailanthus altissima.* This worked best with SOP B (samples 2a and 2b). SOP A leads to failure and a poor result and is therefore not recommended.

Sample number	Protocol	DNA concentration [ng/µL]	Purity A260/A280	Purity A260/A230
1a	SOP A	-	-	-
1b	SOP A	873.69	1.56	1.32
2a	SOP B	3,676.87	1.93	1.99
2b	SOP B	2,547.38	1.93	2.02
3a	SOP C	2,909.50	1.99	2.21
3b	SOP C	2,655.38	1.98	2.11

Table 4
DNA yield increases with the amount of material used when *in vitro* material of the poplar clone INRA 717-1B4 is used. This effect is not as strong with material from the greenhouse. In comparison, more DNA can be isolated from the *in vitro* material.

Tissue type	Fresh weight [mg]	DNA concentration [ng/µL]	Purity A260/A280	Purity A260/A230
In vitro	30	537.39	2.03	2.25
In vitro	60	2,456.90	2.06	2.26
In vitro	90	2,958.26	2.08	2.11
In vitro	120	4,956.01	2.00	2.20
In vitro	150	5,174.45	2.06	2.13
In vitro	180	11,701.45	1.92	2.26
Greenhouse	30	1,163.42	2.03	2.23
Greenhouse	60	1,920.71	2.07	2.27
Greenhouse	90	2,335.29	2.07	2.26
Greenhouse	120	2,899.74	2.05	2.15
Greenhouse	150	2,607.83	2.05	2.20
Greenhouse	180	2,983.33	2.04	1.98

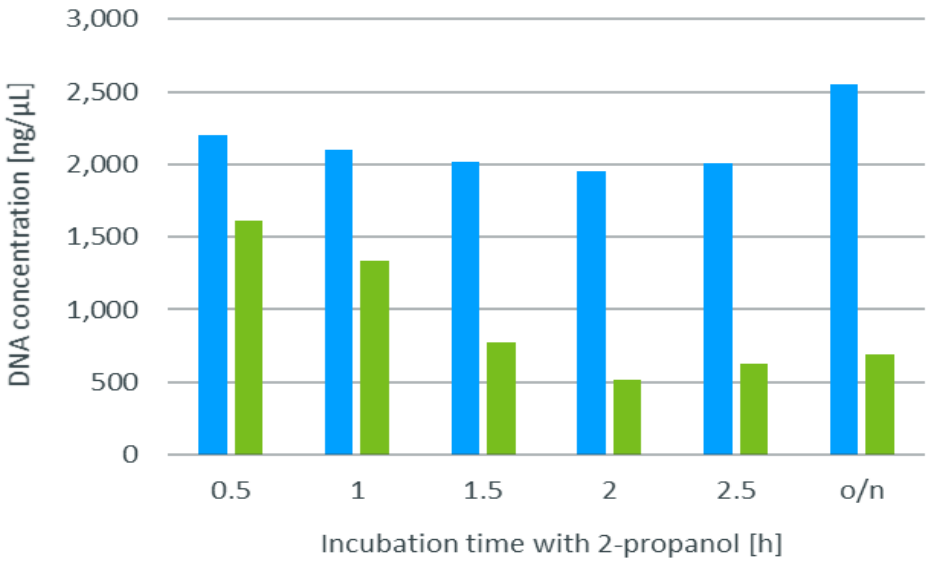

Figure 2
Duration of precipitation in 2-propanol. On poplar leaf material from *in vitro* culture (blue), the duration does not have a strong effect. Here, incubation can also be carried out overnight (o/n). For leaf material from the greenhouse (green), the incubation time has a strong effect, so that these samples should only be incubated for 30 minutes.

Table 5
DNA isolation from cambium for different species. Up to approximately 700 ng/µL DNA was isolated with the unmodified SOP from 50 mg cambium tissue harvested from six Central European tree species. Two independent isolations were performed for each species.

Sample number	Species	DNA concentration [ng/µL]	Purity A_{260}/A_{280}	Purity A_{260}/A_{230}
1a	*Acer campestre*	709.57	1.97	2.17
1b	*Acer campestre*	593.33	1.99	2.18
2a	*Fagus sylvatica*	291.13	1.97	1.90
2b	*Fagus sylvatica*	379.69	1.98	1.94
3a	*Picea abies*	320.75	1.92	1.41
3b	*Picea abies*	439.61	1.96	1.93
4a	*Pinus sylvestris*	335.74	1.92	1.48
4b	*Pinus sylvestris*	459.48	1.92	1.53
5a	*Quercus robur*	634.39	2.20	0.81
5b	*Quercus robur*	552.41	2.05	0.98
6a	*Salix caprea*	207.10	1.92	1.80
6b	*Salix caprea*	184.00	1.87	1.58
7a	*Fraxinus excelsior*	3,075.10	2.11	2.16
7b	*Fraxinus excelsior*	4,107.69	2.11	2.18
8a	*Ulmus minor*	852.95	1.56	0.74
8b	*Ulmus minor*	648.67	1.34	0.53

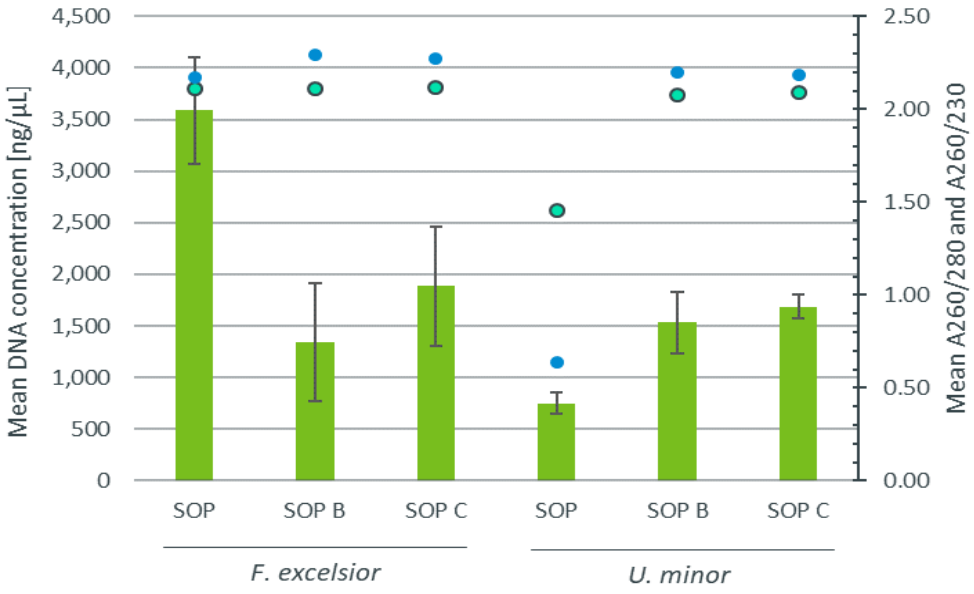

Figure 3
DNA extracted from cambium with different SOPs. For 120 mg cambium tissue of *Fraxinus excelsior* and *Ulmus minor*, the SOP and its modifications B and C were tested with two independent isolations each. The best results were obtained with SOP for *F. excelsior* and with SOP C for *U. minor*. Green dots with black outline – A_{260}/A_{280}, blue dots – A_{260}/A_{230}.

modifications to the protocol did not result in significant increases in purity. The slightly brownish coloration of the DNA extract could not be removed by any of the protocols.

DNA can be used for subsequent PCR amplifications

The PCR amplification for all four applied markers worked for 42 species out of the 59 species used for PCR amplification in total. For five further species, three markers worked well and only one of the markers worked less well. There were few species (five) for which only either the cp and mt or the nuclear genes could be amplified. Last but not least for only three species neither of the PCR markers resulted in an amplification product (*Alnus incana*, *Kalopanax septemlobus*, and *Sorbus aucuparia*) (Tab. 1).

So, except for four species, all species from which the DNA had a good quality after isolation with either SOP or SOP A (marked in green in Tab. 1) resulted in PCR products for all applied markers. For the remaining four species, the cp and mt markers worked and one or both of the nuclear markers failed. For the eight species for which we only got intermediate or bad DNA quality (marked in yellow or red, respectively, in Tab. 1), positive PCR results were rare.

Even for the three most challenging species *Quercus mongolica*, *Ulmus minor* (but only when using cambium instead of leaves), and *Ailanthus altissima*, we got PCR results when using the modified protocols SOP B or SOP C.

Discussion

In this study, we present the applicability of a DNA isolation method and modifications for special requirements in a broad range of tree species. Mostly single individuals of shrubs, conifers and deciduous tree species have been chosen to isolate DNA from different tissues to demonstrate the vast reach of the method. Only a few samples per species (only one sample in the ideal isolation procedure) were tested because the focus of this analysis was on the broad applicability of the isolation protocol. From 54 individual samples tried with the standard operating procedure (SOP), already 42 showed quality and quantity results good enough for amplification of three or four of the applied markers. For further 17 samples, the first slight modification (SOP A) worked well. Astonishingly, all conifer species are present in this group, although it is mentioned that the leathery material of conifers is challenging for DNA isolation (Barzegari et al. 2010). In the used deciduous species all samples from three families (Salicaceae, Juglandaceae and Sapindaceae) already yielded sufficient results with SOP or SOP A.

Five of the species from different families needed the SOP B or SOP C for a sufficient DNA quality and quantity for subsequent results for at least some of the applied markers in PCR amplification. For two of these species no results could be achieved in the subsequent PCR when using leaf material. But, for these two species PCR amplifications were possible using DNA isolated from cambium. Overall, the modified protocols lead to a very good success, e.g., even for *Q. mongolica*, as has

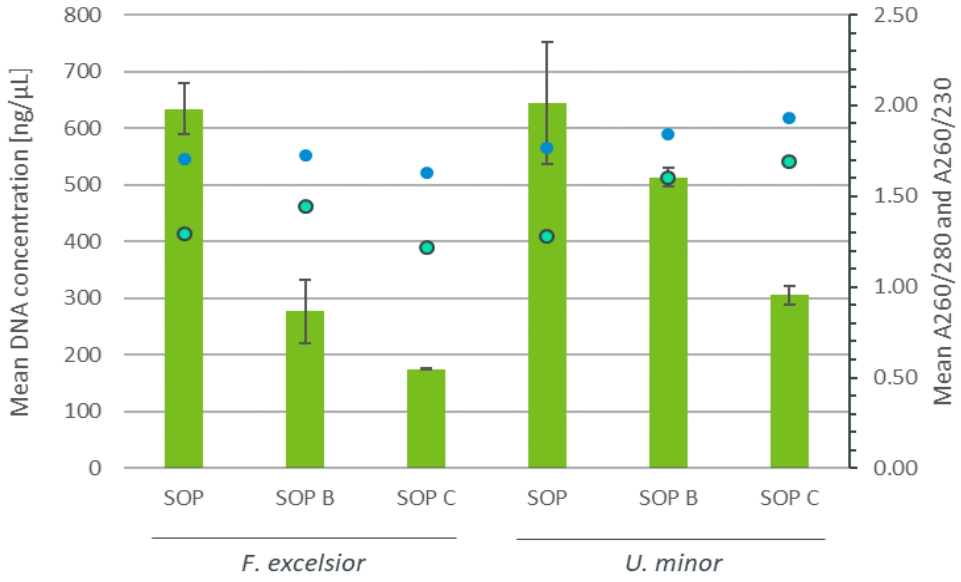

Figure 4
DNA extracted from root material with different SOPs. For 120 mg root tissue of *Fraxinus excelsior* and *Ulmus minor*, the SOP and its modifications B and C were tested with two independent isolations each. The best results were obtained with SOP for both species. Green dots with black outline – A_{260}/A_{280}, blue dots – A_{260}/A_{230}.

already been shown in Schröder et al. (2019). *K. septemlobus* is the only tree species tested for which we could not isolate appropriate amounts of high-quality DNA using any of the protocols.

Interestingly, some of the applied modifications for recalcitrant species did not work. For example, a phenol/chloroform step used for *Q. mongolica* and *U. minor* led to no improvement in DNA quantity and quality. Although it worked well for other different challenging material as forensic samples (e.g., Köchl et al. (2005)), environmental DNA (e.g., Renshaw et al. (2015)), or faeces (e.g., Janabi et al. (2016)).

The DNA isolation protocols described here have even led to several successfully performed next generation sequencing analyses where high-quality DNA is crucial. For instance, we used DNA isolated according to SOP from *Fagus sylvatica* for Illumina MiSeq (Mader et al. 2020) and from *Pinus cembra* for Illumina HiSeq analyses (Schott et al. 2019). Also for the slightly more difficult species *Quercus robur* or the recalcitrant species *Q. mongolica*, respectively, we could isolate appropriate amounts of high quality DNA according to SOP A or B for Illumina MiSeq (Schroeder et al. 2016) and for the targeted genotyping by sequencing NGS method SeqSNP (Degen et al. 2021).

Overall, we interpret these results as an indication for the broad range of applicability of the described SOPs.

The DNA isolation SOPs have been tested for leaves, cambium, roots and *in vitro* material. Remarkably, for *Picea abies* and *Pinus sylvestris* DNA extracted from cambium was of lesser quality than from leaf material. But, for most deciduous species, such as *Ulmus minor* and *Fraxinus excelsior*, it was converse. Thus, for some recalcitrant species, such as *Ulmus minor* and *Fraxinus excelsior*, the quantity and quality values are much better when using cambium than leaf material. Consequently, one can assume that some of the PCR-disturbing ingredients in these species exist only in leaves but not in cambium. In tropical species, better results were achieved when using cambium instead of leaves, because PCR inhibitors are known to be frequently present especially in tropical tree species (Colpaert et al. 2005). So, we also recommend the use of different material for species – as far as possible – when facing problems during DNA isolation.

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Overview DNA isolation protocol

Preparation of chemicals and solutions

Extraction buffer

The extraction buffer contains:

2% ATMAB (w/v), 1% PVP K30 (w/v), 1.4 M NaCl, 20 mM EDTA II, 100 mM Tris-HCl.

Autoclave the buffer for 20 min at 121 °C for long storability.

SOP B: Add 1 µL proteinase K (stock 20 mg/mL) per sample to the extraction buffer.

SOC C: Add 1 µL proteinase K (stock 20 mg/mL) per sample and 2% boric acid (v/v) to the extraction buffer.

DTT solution

The utilized DTT (dithiothreitol) solution is 1 M DTT, dissolved in 10 mM NaAc, pH 5.2.

TE buffer with RNase A

The TE buffer is prepared with 10 mM Tris and 1 mM EDTA-Na_2 and adjusted to pH 8.0. 10 µL RNase A (100 mg/mL) is added to a final volume of 100 mL buffer.

Sodium acetate solution

For SOP B and C, sodium acetate solution is necessary. The 3 M sodium acetate solution is adjusted to pH 5.2.

Protocol overview

	For challenging plant material		
SOP	**SOP A**	**SOP B**	**SOP C**
ATMAB extraction buffer	ATMAB extraction buffer	ATMAB extraction buffer + Proteinase K	ATMAB extraction buffer + Proteinase K + Boric acid
+ DTT	+ DTT	+ DTT	+ DTT
Dichloromethane	Dichloromethane 2nd Dichloromethane	Dichloromethane	Dichloromethane
		Aqueous phase in sodium acetate	Aqueous phase in sodium acetate
Precipitation with 2-propanole	Precipitation with 2-propanol	Precipitation with 2-propanol	Precipitation with 2-propanol
RNase treatment	RNase treatment	RNase treatment	RNase treatment

Isolation procedure

Preparation

- Pre-warm extraction puffer to 55 °C
- Prepare new tubes and cool the centrifuge, at the latest during the incubation on ice.
- Make sure that 2-propanol and ethanol are available at -20 °C.

Tissue homogenization

- Prepare recalcitrant tissue by manual comminution.
- Homogenize the tissue with ceramic beads or steel balls in a beadmill or swingmill homogenizer until the tissue is completely pulverized.

Isolation

- Add to the sample: 1 mL pre-warmed extraction buffer
- Add to the sample: 50 µL DTT (1 M)
- Vortex shortly
- Incubation: 1 h, 55 °C, shaking with 300 rpm
- Incubation: 10 min, on ice
- Add to the sample: 400 µL dichloromethane
- Invert the sample tube several times
- Centrifugation: 20 min, 16,200 × g, 4 °C
- Transfer the aqueous upper phase in a new tube
  - *Optional for SOP A: Repeat the previous 4 steps starting with addition of dichloromethane*
  - *Optional for SOP B + C: Transfer the aqueous phase in 225 µL sodium acetate solution (3 M, pH 5.2)*
- Add to the sample and mix: 600 µL 2-propanol, cooled at -20 °C
- Incubation: 20 min, -20 °C
- Centrifugation: 10 min, 16,200 × g, 4 °C
- Discard the supernatant
- Wash the pellet with 1 mL 70% ethanol, cooled at -20 °C
- Centrifugation: 20 min, 16,200 × g, 4 °C
- Discard the supernatant, pipet residues
- Let the pellet air-dry
- Elution of the pellet with 50 µL 1× TE buffer, containing RNase A
- Incubation: 30 min, 37 °C
- Storage or measurement