High-molecular-weight DNA extraction for broadleaved and conifer tree species

Melina Krautwurst[1], Annika Eikhof[1], Sylke Winkler[2], Daniel Bross[1], Birgit Kersten[1]*, Niels A. Müller[1]*

[1] Thünen Institute of Forest Genetics, 22927 Grosshansdorf, Germany
[2] Max-Planck Institute of Molecular Cell Biology and Genetics, 01307, Dresden, Germany

* Correspondence authors: Niels A. Müller, Email: niels.mueller@thuenen.de; Birgit Kersten, Email: birgit.kersten@thuenen.de

Abstract

Plant genome sequencing based on long reads has increasingly been applied also to tree species in recent years. A crucial step in these genome projects is the successful extraction of high-molecular-weight DNA in high quality and sufficient quantity, which is imperative for long-read sequencing. The extraction of high-molecular-weight DNA in trees has been limited and difficult to conduct. To achieve successful extraction, modification of the protocol for tree species is necessary. Here, we present a protocol for high-molecular-weight DNA extraction from the broadleaved tree species *Fraxinus excelsior* and from the conifer species *Taxus baccata*. The protocol is based on nuclei isolation and is divided into two main steps, i.e. nuclei separation based on the nuclei isolation buffer and DNA extraction using the Nanobind® plant nuclei kit by Pacific Biosciences (PacBio). The protocol can be applied to different tree species to obtain high-molecular-weight DNA in high quality, which can be used for Oxford Nanopore Technologies (ONT) and PacBio sequencing. ONT sequencing using four high-molecular-weight DNA preparations from *Fraxinus excelsior* resulted in different read length N50 values (12.91-38.19 kb) and total base output (5.81-23.17 Gb), emphasizing the complex nature of the sequencing pipeline of high-molecular-weight DNA. PacBio HiFi sequencing of *Taxus baccata* produced circular consensus sequencing reads with an average length of 12.9 kb and an N50 of 13.59 kb. Altogether, this study presents a protocol to obtain high-molecular-weight DNA from different challenging tree species and discusses several important points that can be considered when adapting the protocol to additional species.

Keywords: *High-molecular-weight DNA extraction, long-read sequencing, Oxford Nanopore Sequencing, PacBio Sequencing, Tree genomics*

Introduction

The past 20 years have seen increasingly rapid advances in the genomics field, especially through the impressive development of high-throughput DNA sequencing technologies, ranging from short-read (e.g., Illumina) to long-read sequencing technologies. Advanced long-read sequencing technologies include single-molecule real-time sequencing developed by Pacific Biosciences (PacBio) and (ultra-)long-read nanopore sequencing developed by Oxford Nanopore Technologies (ONT). Long-read sequencing is fundamental to modern genome studies (Pucker et al. 2022). The according technologies require high-molecular-weight (HMW) DNA of excellent quality and usually relatively high quantity. Each long-read sequencing platform has different requirements for DNA extraction. While some studies advocate avoiding guanidine lysis buffer, phenol, and chloroform purification for PacBio high-fidelity (HiFi) sequencing (Nishii et al. 2023; PacBio 2022b), others have reported successful genome assemblies using DNA extracted with these methods (Grohme et al. 2018; Jebb et al. 2020). The rationale behind these divergent practices may stem from considerations such as potential toxicity associated with specific extraction components. For ONT, the purity of the DNA can influence pore activity and sequence quantity (Fleck et al. 2022; La Cerda et al. 2023; Oxford Nanopore Technologies 2023).

Understanding the nuances of DNA extraction protocols is crucial for optimising sequencing outcomes and elucidating their impact on downstream analyses. Over the years, multiple protocols have been published for extracting HMW DNA from plants, using different approaches mostly optimized for only one sequencing platform (Aggerwal et al. 2022; Li et al. 2020; Schenk et al. 2023). The approaches to extract HMW DNA range from nuclei isolation followed by DNA extraction to direct precipitation of DNA with Cetyltrimethylammonium bromide (CTAB)–based methods or a combination of both. Establishing one protocol that fits all plant species may be difficult because

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of the diversity of cellular components in different species and tissues. Trees, for example, are rich in polyphenolic compounds, polysaccharides, tannins, and other secondary metabolites (Couch and Fritz 1990; Formato et al. 2022). The combination and concentration of these compounds vary significantly among tree species (Huang et al. 2023; Witzell and Martín 2008). These compounds affect DNA in different ways during extraction (Healey et al. 2014). Oxidized and co-precipitated DNA and/or nucleases and physical shearing can result from insufficient removal of these compounds. To regulate the negative impact of such compounds individually, the adaptation of the following steps/components in the HMW DNA extraction protocol is essential: the grinding process, washing buffer, centrifugation force and lysis. This indicates the need for establishing protocols that are suitable for recalcitrant tree species and applicable for ONT and PacBio HiFi sequencing.

The protocol developed here – using broadleaved and conifer tree species – seeks to obtain HMW DNA (after nuclei isolation) for multiple purposes and is feasible to be applied to different tree species. This protocol is based on HMW DNA preparation trials using two tree species with different genome sizes and secondary metabolite compositions: common ash (*Fraxinus excelsior* L.), and European yew (*Taxus baccata* L.). The prepared HMW DNA of the deciduous broadleaved tree species *F. excelsior* was sequenced with ONT, and that of the conifer species *T. baccata* was sequenced with PacBio HiFi. The methodological approach was separated into two main steps: (1) cell wall disruption and nuclei release. Removing debris, nuclei separation with an osmotic nuclei isolation buffer (NIB) and nuclei isolation; (2) lysis of the nuclear membrane and extraction of DNA using the Nanobind® plant nuclei kit by PacBio (Figure 1). For the presented protocol, the average cost per sample is about 40 €, and compared to other protocols of HMW DNA extraction and sequencing preparation (Zerpa-Catanho et al. 2021), it is relatively time-efficient (1-2 days).

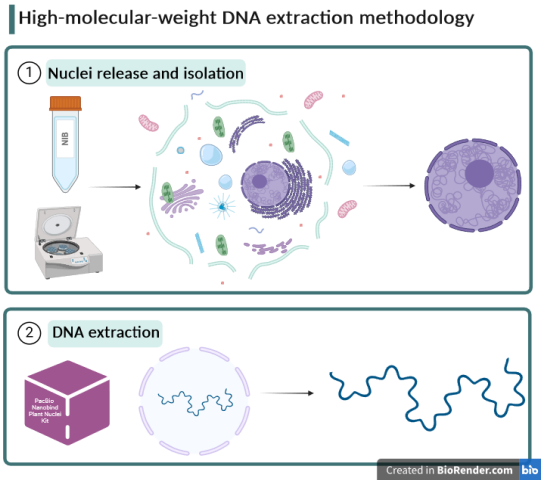


Figure 1
Schematic overview of the high-molecular-weight DNA extraction. (1) Cell wall disruption breaking of cell organelles, and nuclei separation with osmotic nuclei isolation buffer (NIB) and centrifugation. (2) Lysis of nuclear membrane and extraction of DNA using Nanobind® plant nuclei kit (PacBio).

Materials and methods

Biological samples

As starting material, 1-2 grams of plant tissue, stored at -80 °C or fresh, were used. The amount of plant tissue required depends on the tree species and the sample. Two grams of *F. excelsior* leaf material resulted in the highest HMW DNA quantity. For *T. baccata*, 1-2 grams of needles were used as starting material.

Reagents, equipment and supplies for HMW DNA extraction

Table 1-4 list the reagents, equipment, and supplies essential for conducting the HMW extraction.

Buffer preparation for HMW DNA extraction

For HMW DNA extraction, the five buffers described below must be prepared in advance: 10x HB (Homogenization Buffer Stock), 1x HB (Homogenization Buffer), TSB (Triton-Sucrose-Buffer), Nuclei Isolation Buffer (NIB), and Sorbitol wash buffer. 10x HB, 1x HB and TSB were used as stock solutions and stored at 4 °C in a glass stock bottle. The NIB should not be older than one day. Owing to the aggregation of PVP360, a longer stirring time should be planned. Sorbitol wash buffer should be made fresh before every extraction. The amount of sorbitol wash buffer required depended on the efficiency of the washing steps. Some tree species seem to require fewer washing steps than others.

10x HB (Homogenization Buffer Stock), 500 ml

Reagent	Amount	Final concentration
Trizma	6.06 g	0.1 M
KCl	29.8 g	0.8 M
EDTA (0,5 M)	100 ml	0.1 M
Spermidine	1.28 g	17 mM
Spermine	1.74 g	17 mM

Add the reagents to a clean beaker and stir until dissolved; fill up to 500 ml with pure water in a glass stock bottle and set to pH 9 with 10 M NaOH (storage up to one year at 4 °C).

1x HB (Homogenization Buffer), 1 l

Reagent	Amount
Buffer 10x HB	100 ml
Sucrose	171.2 g

Add the reagents to a clean beaker, stir until dissolved, and fill up to 1 l with pure water in a glass stock bottle (stored for up to three months at 4 °C).

TSB-Buffer (Triton-Sucrose Buffer), 100 ml

Reagent	Amount
Triton X-100	20 ml
Buffer 10x HB	10 ml
Sucrose	17.1 g

Add the reagents to a clean beaker, stir until dissolved, and fill to 100 ml with pure water in a glass stock bottle (stored for up to one year at 4 °C).

Table 1
List of reagents.

Reagent	Source
Trizma® hydrochloride solution	Sigma-Aldrich
Potassium chloride (KCL)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA) 0.5 M pH 8.0	AppliChem
Spermidine trihydrochloride	Sigma-Aldrich
Spermine tetrahydrochloride	Sigma-Aldrich
Sucrose	Duchefa Biochemie
Triton X-100	Sigma-Aldrich
Polyvinylpyrrolidone (MW ~360 kDa, PVP360),	Sigma-Aldrich
Polyvinylpyrrolidone 1% (MW ~ 40 kDa, PVP40)	Sigma-Aldrich
Tris	Roth
Sorbitol	Sigma-Aldrich
β-mercaptoethanol, 14 M	Merck
Nuclease-free water	Invitrogen
Water for HPLC (filtered 0.1 µM)	ChemSolute
Ethanol (96-100%)	Roth
Isopropanol (100 %)	Roth
Qubit 1x dsDNA BR Assay kit	Thermo Fisher
Nanobind® plant nuclei kit	PacBio
Short Read Eliminator (SRE <25 kb) Kit	PacBio

Table 2
Equipment and other supplies.

Reagent	Source
Ice	X
Liquid nitrogen (LN2)	X
DNA LoBind tubes, 1.5 ml	Eppendorf
Wide bore 200 – 1000 µl pipette tips	Axygen
Micro-centrifuge, Fresco 21	Thermo Fisher
Mini-centrifuge	Sprout
Centrifuge, Mega Star 600	VWR
Steriflip 20 µm pore size, 50 mL process volume	Millipore
50 mL conical tubes	Sarstedt
Tube rotator, shaker, platform rocker or tumbling rocker	Biometra
Vortex shaker	neoLab
Thermo-Shaker	BioSan
Hula mixer or multi rotator	biolab
Magnetic tube rack, DynaMag®-2	Invitrogen
Nanodrop 1000 spectrophotometer	Peqlab
Fluorescent DNA quantification, Qubit™3 fluorometer	Invitrogen
Small nylon or synthetic paintbrush	X

Table 3
Equipment for using a pestle and mortar.

Reagent	Source
Pestle and mortar	X
Ice	X
Glass beakers, of different sizes	X
Magnetic stir bar	X
Stainless steel laboratory spoon	Thermo Fisher

Table 4
Equipment for using TissueRuptor.

Reagent	Source
TissueRuptor II	Qiagen
TissueRuptor II disposable Probes	Qiagen

NIB-Buffer (Nuclei Isolation Buffer)

Reagent	Amount (50ml)	Amount (100ml)
Buffer 1x HB	48.75 ml	97.5 ml
Buffer TSB	1.25 ml	2.5 ml
PVP360	0. 5 g	1 g

Add the reagents to a clean beaker and stir until dissolved (storage at 4 °C).

Sorbitol wash buffer

Reagent	Stock	10 ml	20 ml
100 mM Tris	1 M	1 ml	2 ml
0.35 M Sorbitol	-	0.64 g	1.28 g
5 mM EDTA	0.5 M	100 µl	200 µl
1% PVP40	-	0.1 g	0.2 g
1% ß-mercaptoethanol (just before use)	-	100 µl	200 µl

Add the reagents to a clean beaker and stir until dissolved.

CAUTION: ß-mercaptoethanol is toxic, only use under fume hood.

Procedure for nuclei isolation and HMW DNA extraction

The protocol for nuclei isolation is based on a related protocol used for nuclei isolation from *Abies alba* needles for HMW DNA preparation and PacBio sequencing (Kersten et al. 2022), which was further modified and adapted to the tree species analyzed in our study. The protocol for HMW DNA extraction represents an adaptation of the following protocol: 'Extracting HMW DNA from plant nuclei using Nanobind® kits' (PacBio 2022a), where only a few steps have been adapted. The protocol, including nuclei isolation and DNA extraction, takes 2-3 days. Isolation and extraction can be conducted on the same day or two separate days. If the protocol is performed in two days, the extracted nuclei pellet can be stored at -80 °C. After the extraction, the DNA must be incubated overnight at room temperature (12 h or longer). A detailed visualisation of the individual steps of the nuclei isolation and DNA extraction is shown in Figure 2.

Nuclei isolation

Total duration 3 – 4 h

1. Place three tubes of 50 ml on ice. Chill the centrifuge for conical tubes to 4 °C.

2. Start with preparing the NIB and sorbitol wash buffer.

   Owing to the aggregation of the NIB, it is best to prepare it one day in advance.

3. Place NIB and sorbitol wash buffer without β-mercaptoethanol in precooled 50 ml tubes on ice.

Nuclei Isolation with pestle and mortar

30 - 40 min

a. Precool pestle, mortar, and laboratory spoon on ice.

b. If fresh leaves or needles are collected, cut material with a sterile scalpel into pieces smaller than ~0.5 cm^2 and shock freeze in liquid nitrogen.

   The cutting process should be done quickly.

c. Before transferring the plant material into the mortar, add liquid nitrogen into the mortar to additionally lower the temperature of the equipment. Dip the pestle and laboratory spoon into liquid nitrogen that was added to the mortar. Everything needs to be as cold as possible.

   Thawing the plant material during the grinding process can strongly influence the extraction results. If the plant material starts sticking to the equipment it is not cold enough.

d. When all liquid nitrogen is evaporated in the mortar, start adding the plant material.

e. Grind with pestle for at least 20-30 min, and add liquid nitrogen in between to cool plant material and equipment.

   Add the liquid nitrogen slowly, adding it too fast can lead to overflow of the plant powder. To ensure that the mortar is kept cold, it can be placed into a box of ice during the grinding process.

f. After 20 min the material should be a fine powder. Transfer the powder to a glass beaker and add a mix stir bar. Add 10 ml sorbitol wash buffer (without ß-mercaptoethanol). Transfer the glass beaker to a container or petri dish with ice. Mix on magnetic stirrer for 10 min.

g. Transfer the sample to a precooled 50 ml conical tube.

Isolation with TissueRuptor

10 min

a. Keep the frozen plant material cold as long as possible– transfer plant material to an empty precooled 50 ml conical tube. If plant material is larger than ~0.5 cm^2 mince it with a mini pestle or scalpel until material is smaller than ~0.5 cm^2.

b. Add 10-20 ml precooled sorbitol wash buffer (without ß-mercaptoethanol) to plant material. Keep the tube cold the whole time.

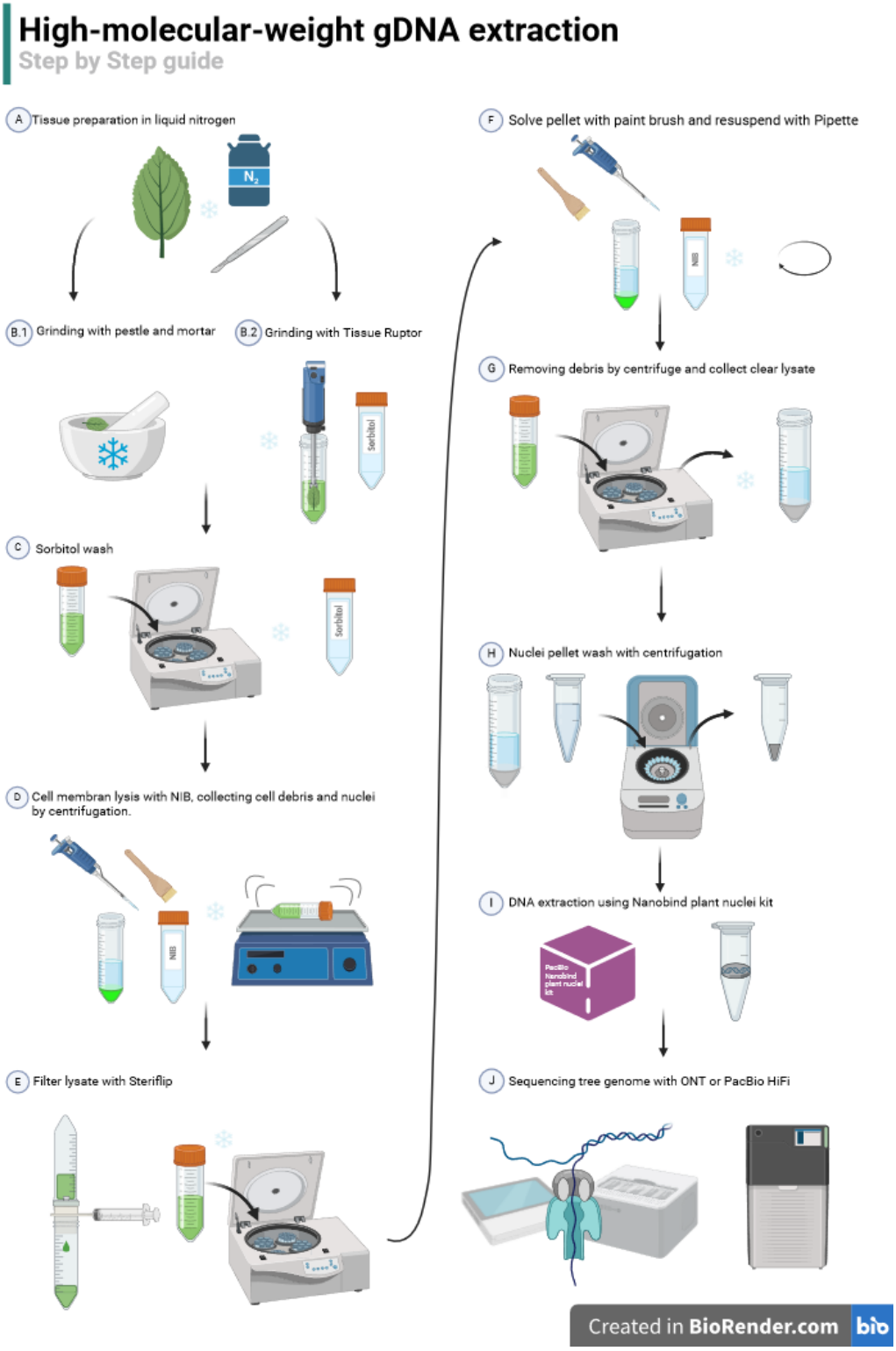


Figure 2
Illustration of the steps involved in the high-molecular-weight DNA extraction based on nuclei isolation and extraction.

The quantity of sorbitol buffer depends on the amount of plant material.

c. Pull over TissueRuptor Disposable Probes on TissueRuptor device. Mix 2-4 times for 30 sec on max speed. Move through the tube. The plant material should be homogeneous and no bigger plant material pieces should be visible.

Mix no longer than 30 sec, buffer can foam intensely. The foam should not reach the end of the probes of TissueRuptor; there can be a risk of damaging the device. The foam is not a concern for nuclei isolation.

4. Incubate for 5 min on ice.

5. Centrifuge for 15 min at 3,000-10,000 x g (depending on genome size) at 4 °C.

3,000 x g are recommended for tree genome size >1 Gb. For tree genome sizes < 1 Gb or unknown genome sizes 7,000 x g are recommended. For *F. excelsior* the centrifugation speed was lowered to 5,000 x g from the recommended speed. Too fast centrifugation can result in damaging the isolated nuclei.

Species	Centrifuge spin speed	Haploid genome size
Fraxinus excelsior	5,000 x g	0.867 Gb
Taxus baccata	3,000 x g	ca. 10.5 Gb

6. Repeat until the supernatant is clear or only a hint of green remains.

7. If repeat is necessary: discard supernatant, add 1 ml precooled Sorbitol and resuspend the pellet using a small paintbrush. Add again 10 ml sorbitol with ß-mercaptoethanol and mix with a P1000 pipette until the solution is homogenous.

Depending on the pellet's consistency, suspension with a paintbrush is optional. Light shaking can be performed instead.

8. If the supernatant is clear, discard the supernatant and add 1 ml NIB (add ß-mercaptoethanol to NIB, use 125 µl ß-mercaptoethanol per 50 ml NIB), resuspend the pellet using a small paint brush and mix with a P1000 pipette until the solution is homogenous. Fill up to 15 ml with NIB.

CAUTION: The procedure should be further performed under a fume hood to avoid inhalation of ß-mercaptoethanol.

9. Place the tube on ice on the tube rotator, shaker, or platform rocker for 15 min. Horizontal shaker on 150 rpm or a platform rocker at max speed.

10. Use a Steriflip to filter the lysate. Approximately 10-15 ml of lysate should be left after filtering.

Depending on the consistency of the lysate, the filter of the Steriflip can get clogged. Additional buffer or/and using a second Steriflip can be an option.

11. Centrifuge for 10 min; spin speed depends on the genome size of the tree species (step 6).

12. Discard supernatant and, add 1 ml precooled NIB and, resuspend the pellet using a small paint brush and mix with a P1000 pipette until the solution is homogenous. Fill up to 10 ml with NIB.

The pellet mostly consists of nuclei at this point, mixing with the pipette should be done gently and not more than 5-7 times.

13. Centrifuge at 4 °C for 10 min; again, spin speed depends on the genome size of the tree species (step 6).

14. Repeat steps until the supernatant is clear, 4 - 7 times.

Nuclei can be damaged by repeating the centrifugation step too often. Examples of nuclei pellet shades can be found in Supplementary Figures 1 - 4.

Species	NIB wash step repeats
Fraxinus excelsior	5-7
Taxus baccata	4

15. Discard supernatant; add 1 ml precooled 1x HB to the pellet.

16. Solve the pellet with a paintbrush and resuspend with P1000 pipette.

17. Transmit 1 ml nuclei suspension into a 1.5 ml DNA LoBind microcentrifuge tube.

18. Centrifuge at 7,000 x g for 5 min at room temperature. To avoid overheating, it is best to use a cooling centrifuge and set it to 18-22 °C.

19. Discard supernatant. Continue with Nanobind® plant nuclei kit DNA (PacBio® 102-302-000) extraction protocol or freeze nuclei pellet in liquid nitrogen and store at -80 °C.

DNA extraction

Only those steps were listed that have been adapted from the original protocol 'Extracting HMW DNA from plant nuclei using Nanobind® kits' by PacBio (PacBio 2022a). This protocol consists of sixteen steps. Researchers interested in the detailed protocol are directed to the original source.

Total duration 3 - 4 h

1. Consistency of nuclei pellet can differ between tree species. *F. excelsior* nuclei pellet can be viscous. The nuclei pellet of *T. baccata* is highly viscous and of jelly-like consistency. The nuclei lysate can get stuck in the pipette tips (wide bore tips) due to viscosity. Mixing by vortexing and light stirring should be conducted for the efficiency of lysis.

   For an efficient lysis, the amount of Proteinase K, RNase A and PL1 can be increased. For *T. baccata*, it can be necessary to double the amounts of reagents.

3. Mixing step is important, additionally mixing with the vortexer is necessary.

4. Incubation on a ThermoMixer can be expanded to 2 h or more. After 15 min 5x 1 sec pulse mix on vortexer, invert gently every 30 min. If the lysate is less viscous, the rpm can be lowered (approximately 450 rpm).

   Mixing with a vortexer after a 15 min period can be critical for the DNA quality.

Species	Incubation time
Fraxinus excelsior	2 h
Taxus baccata	1 – 1.5 h

8. Adaptation of HulaMixer settings, DNA can be sticky and the Nanobind disk should not get stuck at the bottom or top of the tube.

   Observe mixing on the HulaMixer closely, to adapt if the Nanobind disk is stuck.

Step	Setting	Time (s)
Rotation	9 rpm	OFF
Tilting	30°	12
Vibration	2°	1

11. Repeat the washing step 3-5 times until the DNA is clear.

Quantity and purity (A260 /A280 and A260 /A230 ratios) measurement.

If required, follow the Short Read Elimination Kit protocol by PacBio (cat.no. SS-100-101-01, since March 2023: SKU 102-208-300). If HMW DNA concentration is sufficient, continue with ONT library preparation or PacBio protocol. The SRE aims to reduce the number of short DNA fragments. Short genomic DNA fragments have a higher binding preference than long fragments to the flow cell pores or SMRT cell ZMW in single molecule sequencing platforms such as ONT and PacBio. This higher preference for short fragments can shift the mean fragment length or N50 towards shorter reads.

Estimating DNA fragment size distribution through gel electrophoresis

For a rough estimation of the DNA fragment size, agarose gel electrophoresis with Marker Lambda Mix 19 (Fermentas #SM0231) can be performed, bearing in mind the inherent limitations of this method for precise sizing. The Marker Lambda Mix 19 was prepared using 2 µl Lambda Mix, 2 µl 6x loading dye (Fermentas #R0611) and 8 µl H2O. The mix was denaturated at 65° C for 5 min and stored on ice afterwards. The samples were prepared using 2 µl HMW DNA, 2 µl Orange G and 5 µl H2O. Agarose gel was prepared at a concentration of 0.3 % in 0.5x TBE and ran at 100 V in 0.5x TBE for 5h.

Characterizing DNA fragment size distribution through precision analysis with the Agilent FEMTO Pulse System

To examine the DNA's size and integrity, we used the FEMTO Pulse device (Agilent Technologies), which applies pulse field gel electrophoresis. HMW DNA was separated using the Genomic DNA 165 kb Kit (Agilent Technologies, FP-1002-0275) according to the manufacturer's instructions (https://www.agilent.com/cs/library/usermanuals/public/quick-guide-fp-1002-gdna-165-kb-kit-SD-AT000141.pdf).
In brief, each DNA sample was equilibrated to room temperature for at least 30 min and then diluted with 0.25x TE buffer to a final concentration of 125 – 250 pg/µl by performing serial dilution steps. Between each step, a 30-minute interval incubation at room temperature was applied. 2 µl of final diluted samples and the HMW ladder were taken into a FEMTO pulse run for 1.5 hours. These pulse field conditions allow the separation of HMW DNA fragments up to 165 kb. The visualization and analysis of the FEMTO Pulse runs were carried out with the Prosize software tool (Agilent Technologies).

Oxford Nanopore Sequencing Setup

The input DNA mass can influence the outcome as well, which means choosing the right sequencing kits and amount of DNA is crucial. For ONT the input mass for the flow cells varies, depending on the ligation sequencing kits. When using the ligation sequencing kit SQK-LSK110 the input mass is 150-300 fmol (ca. 1.5-3 µg) DNA for the flow cell R10.4.1 and 100-200 fmol (or 1.5 - 3 µg) DNA for the flow cell R9.4.1 (which will be soon discontinued). Ligation sequencing kit SQK-LSK114 is only compatible with the R10.4.1 flow cell; the HMW DNA input mass for this combination is 100-200 fmol (ca. 1 µg) DNA. The purity should be measured using Nanodrop (A260/A280 of 1.8 and A260/A230 of 2.0–2.2). All DNA input for sequencing and purity are recommended by the manufacturer.

PacBio high-fidelity sequencing setup

PacBio HiFi libraries have been prepared making use of the most recent protocol (Preparing whole genome and metagenome libraries using SMRTbell® prep kit 3.0). gDNA has been sheared with the MegaRuptor™ device (Diagenode) to fragment sizes of 20 kb. The final PacBio libraries have either been size selected for fragments larger than 8 kb with the Blue Pippin™ device or alternatively by AMPure beads (Beckman) as

described in the Pacbio library preparation guide. Input into the library preparation protocol was 3 ug of sheared DNA. PacBio requires a minimum of 2-3 µg of DNA per 1 Gb of genome length. Pacbio HiFi libraries were loaded on the SEQUEL2 device on SMRT cells with 8 million zero mode waveguides (ZMW) and run with the SEQUEL II sequencing kit 2.0 for 30 hours. For circular consensus sequence (CCS) calling, either the standard PacBio SMRTlink pipeline for CCS calling could be used or, alternatively, the deep consensus tool (Baid et al., 2023).

Results

HMW DNA concentration and quality

For a reliable estimation of HMW DNA concentration, it is necessary to measure DNA yield three times for every sample from the top, middle and bottom of the sample. Yield can differ up to 10-20 ng/µl between the fractions. The main reason for the fluctuation is the inhomogeneity of the HMW DNA in the tube. The DNA yield measured by Nanodrop and Qubit should ideally be within a 1:1 to 2:1 ratio (Nanodrop/Qubit). DNA yield was measured before and after applying the short read eliminator kit (PacBio).

Fragment size distribution of the HMW DNA of Fraxinus excelsior

The DNA of *F. excelsior* extracted with our protocol was tested on the ONT MinION sequencing platform. Four samples were sequenced on four flow cells, as outlined in Table 6, aiming to provide a comprehensive understanding of their respective characteristics. For all four *F. excelsior* samples, the short read eliminator was employed before library preparation, and the sequencing was performed with an R9.4.1 flowcell (MinION) and the SQK-LSK110 ligation sequencing kit from ONT (Input DNA for the flow cell in Table 6). Notably, samples I and II underwent identical protocol steps including grinding with the TissueRuptor (Table S.1). Nevertheless, these samples exhibited different read N50 values, with 12.91 kb and 17.88 kb, respectively. The total output for sample I was 23.17 Gb (26.1X genome coverage), while sample II yielded 16.45 Gb (18.5X genome coverage), highlighting the considerable genomic content captured in both cases. Sample IV, representing a different genotype, followed the same isolation and extraction steps as samples I and II. Despite a good N50 value of 24.71 kb, it generated the lowest output with of 5.81 Gb (6.5X genome coverage; Figure 3). Sample III, while exhibiting the highest N50 value of 38.19 kb, also had low output (7.54 Gb, 8.5X coverage) (Table 6).

Fragment size distribution of the HMW DNA of Taxus baccata

Using the presented protocol, HMW DNA was extracted from 12 needle samples (1-1.6 grams material) of *T. baccata* for PacBio HiFi sequencing (Tables 7 and 8). The fragment size distribution of the DNA was assessed by the FEMTO Pulse system (Agilent) (Figure 4, Figures S.5-S.15). The average DNA fragment size, as measured by the Agilent FEMTO Pulse System and/or gel electrophoresis, was >30 kb.

Successful PacBio HiFi sequencing depends on long and intact DNA without contaminants such as secondary metabolites that might interfere with sequencing polymerase and/or the SMRT cell loading by sticking to the native DNA molecules. To test for these requirements, not only the size and integrity of the DNA is analyzed by pulse field gel electrophoresis, but also the purity of the DNA is determined spectrophotometrically. The A260/A280 and A260/A230 ratios should be in the range of 2. Another indication of high-quality DNA is the comparison of concentration measurements by Nanodrop and Qubit (Table 7). This ratio is preferably between 1 and 3.

For PacBio sequencing in general, and especially considering the large genome size of *T. baccata* (circa 10.5 Gb), it is recommended to add a size-selection step to the PacBio HiFi library preparation, e.g., with a BluePippin device (SAGE). The size selection removes short fragments of a given size range. Especially for large genome assemblies, it is highly recommended to sequence long and continuous fragments with high accuracy. These highly precise and continuous reads increase the chance to span repetitive regions in large genomes, which will provide better contiguity across the genome. In addition, a size selection by preparative pulse-field gel electrophoresis adds another purification step to the HiFi library preparation protocol. Although the DNA is enriched for longer fragments, this step comes with the caveat of DNA input. The HiFi library preparation includes a mechanical shearing step to achieve a defined library size. In addition, some shearing cannot be avoided when pipetting long gDNA, and some gDNA nicking occurs. Depending on the size and integrity of the input gDNA, about 20-50 % of the HiFi library gets lost during the size selection by preparative gel electrophoresis. However, this loss of the HiFi library comes with the big advantage of a HiFi library that contains only fragments larger than, e.g. 10 or 12 kb, depending on the size exclusion. Figure 5 shows the result of a representative PacBio HiFi run based on a HiFi library generated from *T. baccata* HMW DNA that was prepared according to our protocol. The average length of the generated CCS reads of this SMRT cell is 11.9 kb, with the majority of CCS reads larger than 8 kb (Figure 5). The median QV score of these CCS reads is Q37. The total yield of CCS reads of this SMRT cell was 30.6 Gb (Figure S.16).

Table 8 shows further PacBio HiFi sequencing results based on deep consensus (Baid et al. 2023) calling of 24 runs with libraries based on samples 2 and 12 (Table 7), with an average N50 HiFi read length of 13.59 kb and a mean yield of HiFi reads of 32.05 Gbps. These findings underscore the importance of size selection in PacBio HiFi library preparation, particularly for larger and more repetitive genomes, facilitating efficient contig generation and the need for high-quality, high-molecular-weight DNA to reach good-quality results.

Table 5
High-molecular-weight DNA extraction quality and quantity values after application of the protocol to the two tree species *Fraxinus excelsior* and *Taxus baccata*. For each measurement 1 µl DNA was used.

	Before Short Read Eliminator				After Short Read Eliminator			
	Nanodrop (ng/µl)	A260/A280	A260/A230	Qubit (ng/µl)	Nanodrop (ng/µl)	A260/280	A260/A230	Qubit (ng/µl)
Fraxinus excelsior	120-900	1.60-1.90	1.50-1.80	300-500	25-175	1.30-1.90	1.40-2.00	40-420
Taxus baccata	220-800	1.70-1.80	1.50-1.90	200-500				

Between 6-12 different HMW DNA preparations were considered for each of the tree species. Detailed values of each sample of *Fraxinus excelsior* are presented in supplement table-S3. For detailed values of *Taxus baccata* see table 7. HMW DNA extraction from *Fraxinus excelsior* is indicated for sequencing with Oxford Nanopore. The *Taxus baccata* HMW DNA extraction (without short-read elimination) is indicated for PacBio HiFi sequencing.

Table 6
Comparative analysis of high-molecular-weight DNA sequencing results in *F. excelsior* genotypes using Oxford Nanopore Technology. The DNA concentration measurement with Nanodrop and Qubit is the mean out of three measurements (top, middle, bottom). All DNA samples were stored at -80 °C, samples I, II and III have been stored for 5 months and sample IV for 12 months. The input DNA volume for ONT was adjusted according to Qubit measurement to achieve an input DNA amount of about 100-200 fmol DNA.

Sample	Genotype	Tissue-weight (g)	Nanodrop (ng/µl)	A260/280	A260/A230	Qubit (ng/µl)	Input (µl)	N50 (kb)	Bases (Gb)	Genome coverage
I	A	2	36.3	1.92	2.12	42.4	23.5	12.91	23.17	26.1X
II	A	2	56.9	1.76	1.88	69.2	14.45/14.5/18.0	17.88	16.45	18.5X
III	A	1	43.0	1.26	0.56	41.0	24.3	38.19	7.54	8.5X
IV	B	2	59.5	1.61	1.40	83.8	14.23/28.6	24.71	5.81	6.5X

A flow cell for sequencing was used 1-3 times for one sample depending on DNA quality and quantity (row input (µl)).

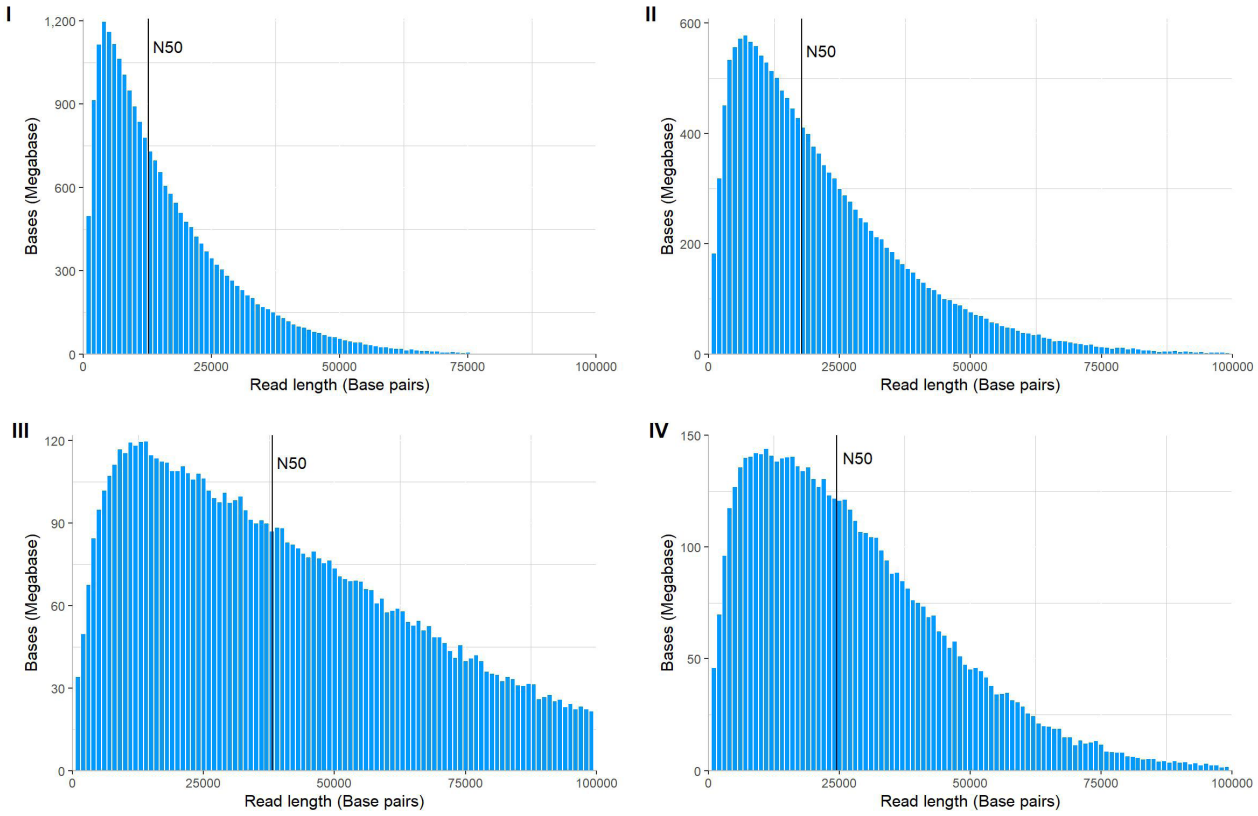


Figure 3
Sequencing results of *Fraxinus excelsior* DNA using the Oxford Nanopore Technologies MinION platform. The bar plots illustrate the read length distribution for the four samples, vertical lines highlight the N50 values. The Y-axis represents the cumulative number of bases in Megabase pairs (Mb) and the X-axis the read length range in base pairs (bp).

Table 7
Details of 12 high-molecular-weight DNA extractions from needles of two *Taxus baccata* individuals.

ID	Genotype	Starting weight (g)	Nanodrop (ng/µl)	A260/A280	A260/A230	Qubit (ng/µl)	total amount (µg)	GQN (50kbp)
1	A	1.0	521.50	1.69	1.29	356	17.80	2.7
2	A	1.0	160.50	1.86	1.69	124	6.20	6.2
3	B	1.0	375.90	1.81	1.82	288	14.40	3.7
4	A	1.0	518.24	1.75	1.49	339	16.95	5.8
5	A	1.0	379.43	1.79	1.34	194	9.70	5.6
6	A	1.3	524.15	1.77	1.51	405	20.25	6.4
7	A	1.3	197.46	1.80	1.62	155	7.75	4.7
8	B	1.2	438.42	1.76	1.56	316	15.80	5.9
9	B	1.2	529.68	1.77	1.68	376	18.80	3.7
10	B	1.4	387.51	1.80	1.69	230	11.50	6.1
11	B	1.5	368.18	1.79	1.63	253	12.65	6.1
12	B	1.6	691.32	1.82	1.82	422	21.10	3.9

NanoDrop measurements were performed on sample parts taken from the middle of the tube. m denotes the total amount of extracted DNA based on Qubit concentration measurements. GQN (genome quality number) estimates the proportion of DNA that is contained in fragments longer than 50,000 bp based on FEMTO Pulse results on a scale from 0-10. Before GQN measurements, samples were frozen at -20°C for 1-3 months. HMW DNA extraction exact steps for each sample are detailed in Table S2.

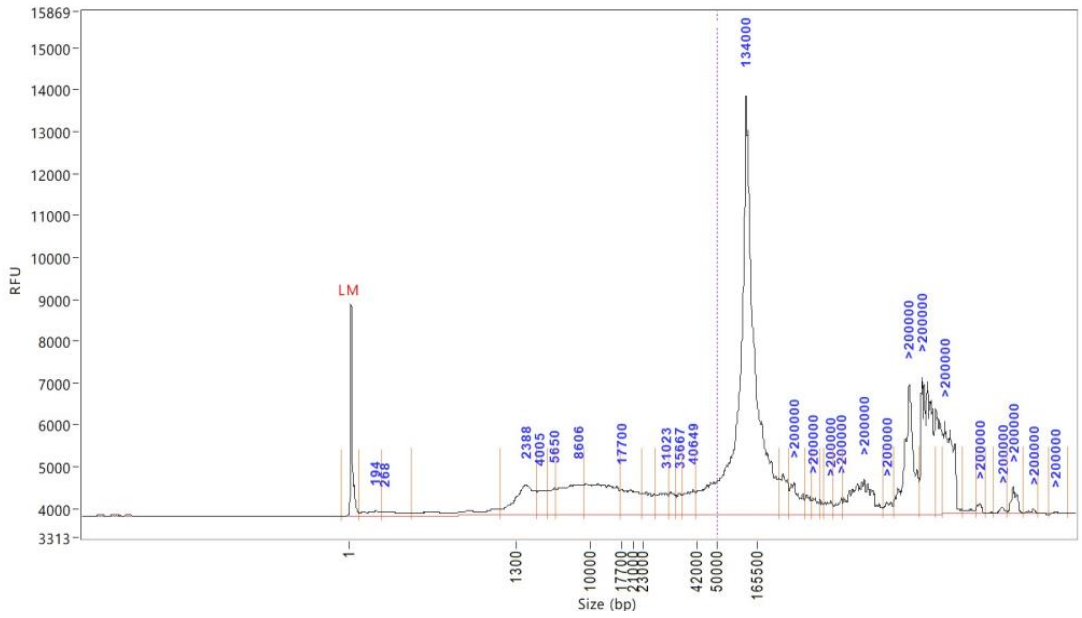


Figure 4
FEMTO Pulse DNA sizing QC analysis of *Taxus baccata* sample 2 (see Table 7). RFU = relative fluorescence unit.

Discussion

Limitations and critical steps in the procedure

The presented results are based on five to twelve successful HMW DNA preparations of the two tree species, *F. excelsior* and *T. baccata*. Our proposed methodology can likely be adapted to other tree species. Notably, various improvements contribute to its overall effectiveness.

Quality of plant material

The sample material quality is essential. The timing of plant material harvest stands as a crucial determinant, varying significantly among species. It arises due to diverse factors such as life cycle variations, needle or leaf characteristics, and other species-specific considerations (Formato et al. 2022; Gaytán et al. 2022; Lin et al. 2024). For *F. excelsior*, young tree leaves a month after flushing did not work well for HMW DNA

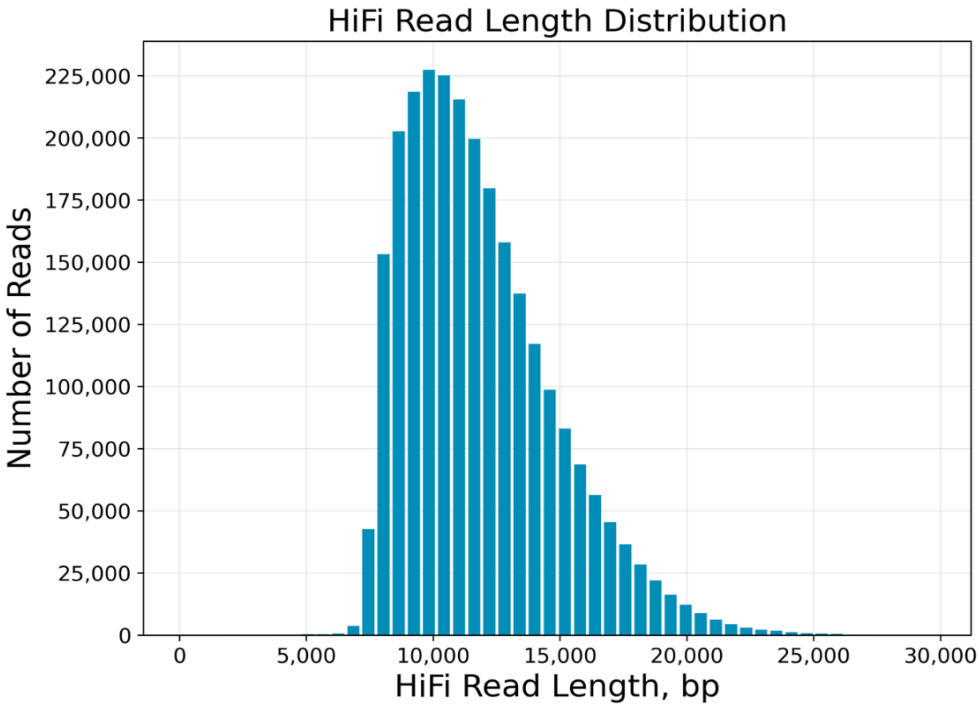


Figure 5
Sequencing results of *Taxus baccata* DNA using PacBio HiFi sequencing (Run ID 21, Table 8). The histogram shows the number of circular consensus sequencing (CCS) reads (Y-axis) of a given length range (X-axis). The average length of the CCS reads of this SMRT cell is 11.9 kb with the majority of CCS reads larger than 8 kb. HiFi reads provide base-level resolution with 99.9 % single-molecule read accuracy. A description of the HiFi read generation is found here: https://www.pacb.com/technology/hifi-sequencing/. CCS reads have been called with the PacBio SMRT link pipeline (SMRTlink version 11.0.0.146107).

extraction with our protocol. The material was difficult to wash with NIB. Presumably, secondary metabolites, whose composition depends on the age of the leaf, could be a cause (Jouve et al. 2007). The harvest between June and July produced acceptable results for *F. excelsior*. *T. baccata* was more flexible with the harvest time. In general, if the trees are cultivated in a greenhouse, it is important to keep them as healthy as possible and away from plants with any disease.

Quantity of plant material

The quantity of plant material used affects the extraction results. We used different amounts of input material for the two species. Increasing the amount of *T. baccata* needles resulted in a drastically higher DNA yield. The resulting nuclei pellet was difficult to handle during lysis, particularly in resuspension, filtration, and mixing steps. This trend was observed consistently. For other plant species and protocols, input material can be as high as 5-10 grams (Kang et al. 2023; Li et al. 2020; Zerpa-Catanho et al. 2021). Depending on the plant used, the amount needs to be adapted. While increasing the amount of material may lead to higher DNA yield in some cases, as observed in *T. baccata*, it does not necessarily guarantee the same outcome for all species or sample types. Zerpa-Cetanho et al. (2021) mentioned that the white colour of the nuclei pellet can be an indicator of the number of nuclei. If the nuclei pellet is not white, this may indicate that not enough nuclei are available.

Grinding

The grinding is one of the most delicate steps of the protocol. The grinding process can cause abrasion of the DNA, and bruised and thawed leaves produce polyphenols that corrupt the results, and DNA fragment length can be influenced (Couch and Fritz 1990; Kang et al. 2023). The grinding tool used has the biggest impact. We observed an increase in DNA yield after using the TissueRuptor, with an increase between 100-600 ng/µl compared to the results using mortar and pestle; however, the TissueRuptor may negatively affect fragment length. The grinding process with mortar and pestle involves several steps, and the quality and quantity of DNA output can suffer. The temperature, the time of grinding and the right magnitude of sample material influence the grinding success. Other options were tested, since a TissueRuptor is not always available and expensive. A beater mill or similar devices that are often used for grinding plant material for DNA extraction seem to be an option. Unfortunately, both species showed similarly deficient results. Mostly, the amount of plant material and the division into separate 1.5 ml tubes and the cooling process seem to be

Table 8
HiFi yield, the mean HiFi read length and the N50 of HiFi reads of *Taxus baccata*. The deep consensus tool was used to calculate the HiFi reads. The first part of the Library ID refers to the sample ID of the input material (see Table 7).

Run ID	Library ID	Yield HiFi reads (Gbp)	Mean length of HiFi reads (kb)	N50 of HiFi reads (kb)
1	12-1	34.55	11.01	13.39
2	12-1	39.08	13.30	14.86
3	12-1	39.06	13.20	14.75
4	12-1	39.32	13.04	14.69
5	12-1	37.50	12.76	14.45
6	12-1	35.42	11.59	13.91
7	12-1	34.81	12.01	14.13
8	12-1	32.93	10.73	12.93
9	12-2	32.58	11.33	13.59
10	12-2	34.07	10.85	13.58
11	2-1	21.72	10.02	13.02
12	2-1	27.73	10.94	13.76
13	2-1	19.54	11.35	14.41
14	2-1	27.61	11.72	14.49
15	2-2	27.61	12.08	12.33
16	2-2	29.71	12.90	13.17
17	2-2	33.40	13.44	13.80
18	2-2	33.57	13.82	14.25
19	2-2	30.28	14.01	14.49
20	2-2	27.75	12.23	13.17
21	2-3	34.51	11.89	12.13
22	2-3	32.40	11.86	12.11
23	2-3	32.92	12.07	12.34
24	2-3	31.27	12.07	12.35

an issue. In the context of Oxford Nanopore sequencing, it has been observed that the grinding process significantly impacts read length distribution and N50 values. Notably, the use of mortar and pestle grinding has demonstrated optimal results in this regard (La Cerda et al. 2023). However, in other protocols based on CTAB and other tree species (e.g., *Populus tremula*), the use of cyclone mills or similar devices worked well for HMW DNA extraction and long-read sequencing (Inglis et al. 2018; Müller et al. 2020). Some authors recommend keeping the time of grinding to a minimum of 2 min and using mechanical separation devices for best results (Jones et al. 2023; Kang et al. 2023; Schenk et al. 2023).

Application of PVP

PVP can reduce the number of polyphenolic compounds that contaminate the extracted DNA and reduce browning of DNA (Loomis and Battaile 1966; Rogers and Bendich 1985). PVP causes foaming of the NIB and sorbitol wash buffers, which affects handling during the grinding process and washing steps. We used PVP360 for the NIB and PVP40 for sorbitol wash buffer. Other protocols use PVP10, which resulted in higher DNA yield (Carey et al. 2023; Schenk et al. 2023). Further, for the CTAB extraction method no PVP is included in the NIB but only used in the CTAB solution. In most CTAB protocols, purifying steps with chloroform are added, which removes the carryover of contaminants (Russo et al. 2022; Zerpa-Catanho et al. 2021). Additionally, there is the option to use PVPP (Polyvinylpolypyrrolidon) as a substitute (Porebski et al. 1997; Schenk et al. 2023).

Centrifuge speed

The genome size determines the optimal centrifuge force. Out of the two tested species, *T. baccata* had the largest haploid genome size (10.5 Gb). Here, the best extraction results were achieved using 3,000 x g. For *F. excelsior* (genome size of 0.867 Gb), the centrifugation speed was lowered to 5,000 x g from the recommended speed (7,000 x g for genome size <1 Gb). The quantity of DNA increased after that adaption.

DNA 'jellies'

Another aspect observed with both species is the formation of DNA 'jellies' after completing the nuclei extraction. The DNA 'jellies' are supposed to disperse overnight. Trials to leave the DNA resuspended over 24 h to 72 h did not make a difference; the 'jellies' dispersion was incomplete. The 'jellies' have been a problem for DNA yield measurement during the cleaning steps and when proceeding with the Oxford Nanopore ligation sequencing kit. One reason for the 'jellies' can be a DNA-phenol complex (Japelaghi et al. 2011).

Quality and quantity of DNA

Other protocols recommend the increase of proteinase K during nuclei lysis if the A260/280 is too low (Zerpa-Catanho et al. 2021). Lysis period varies between different protocols; a longer lysis can increase the quality and quantity of extracted HMW DNA. Kang et al. (2023) tested multiple factors which can influence the DNA results (CTAB method used). One was the pipetting during the process. They recommend minimizing the pipetting and using wide-bore tips during the process. In another study, they tested different incubation times and temperatures using the CTAB method on herbarium leaf tissue. They concluded that lower or higher temperatures and shorter incubation times resulted in DNA being less fragmented and less contaminated (Carey et al. 2023). Different approaches are proposed for purification, such as DNA precipitation or liquid phase extraction. They suggest using solid phase extraction (high salt gel electrophoresis trap), which separates the DNA with electrophoretic mobility (Kalendar et al. 2021).

In a comparison of the output of the four *F. excelsior* samples that have been sequenced with ONT, sample III had the most controversial output (Table 6). The N50 is high with a value of 38.19 kb, but a low output with 7.54 Gb and 8.5X coverage. Interestingly, sample III was the only one for which mortar and pestle were used for grinding, indicating that the use of the TissueRuptor might compromise fragment length but increase data output. The utilization of the different grinding method may have also adversely affected the purity of the DNA in sample III, leading to failure to meet the quality criteria for ONT sequencing. This may be another reason for the lower output of that sequencing run. Overall, the flow cell quality, e.g. such as the number of active pores over the sequencing run, can affect the outcome.

Conclusion

Protocols for extracting high-molecular-weight DNA from various species are becoming more applicable. Yet, a noticeable gap persists for perennial species such as trees, which often have a large repertoire of secondary metabolites. Excellent quality HMW DNA is essential for long-read sequencing and subsequent de novo genome assemblies, which have been increasingly applied in recent years. This study addressed key aspects of HMW DNA extraction for broadleaved and conifer tree species. While the study could benefit from a higher number of repetitions, our protocol's implications are nevertheless relevant. It opens avenues for extracting high-quality HMW DNA from tree species for Oxford Nanopore and PacBio HiFi sequencing. In addition to its application in long-read sequencing for genome assembly, our nuclei extraction protocol has demonstrated versatility in other genomic techniques. For instance, nuclei extracted using our protocol were successfully utilized for chromatin conformation capture using the ARIMA Hi-C protocol, allowing for comprehensive exploration of the three-dimensional chromatin structure in *T. baccata*. Overall, this study underscores the importance of the quality and quantity of the plant material and choosing the right grinding technique and centrifuge speed. Our protocol for extracting high-quality genomic material from two tree species has the potential to be adapted to various other tree species in future studies.

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