UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Escola Tècnica Superior d'Enginyeria Agronòmica i del Medi Natural

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Escuela Técnica Superior de Ingeniería Agronómica y del Medio Natural

Optimización de la extracción de ADN de alto peso molecular de plantas silvestres para la obtención de genomas de referencia de alta calidad

Trabajo Fin de Grado

Grado en Biotecnología

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CURSO ACADÉMICO: 2023/2024

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Optimizing the extraction of high molecular weight DNA from wild plants to obtain high-quality reference genomes

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Palabras clave: ADN; Alto peso molecular; NGS; Contaminantes; Extracción

Resumen

La extracción de ADN de alto peso molecular (HMW) es un paso necesario para la generación de un genoma de referencia. Si bien se ha optimizado para varias especies de plantas modelo como Arabidopsis thaliana u Oryza sativa, aún presenta muchos desafíos para muchas plantas no modelo. La mayoría de las plantas son ricas en polisacáridos y metabolitos especializados como polifenoles y taninos que dificultan la extracción de ADN. La situación se agrava cuando se trabaja con plantas recolectadas de forma silvestre, ya que las plantas pueden haber sufrido estreses ambientales durante su vida que aumentan la presencia de estos compuestos especializados que dificultan la extracción de ADN de alta calidad. En este trabajo, hemos probado varios protocolos de extracción en seis especies de plantas mediterráneas (*Limbarda crithmoides, Pistacia lentiscus, Phillyrea angustifolia, Reseda alba, Reseda barrelieri* y *Reseda hookeri)* recolectadas en El Saler (Valencia) que tienen características que las hacen recalcitrantes a la extracción de ADN (p. ej., altas concentraciones de polisacáridos). Posteriormente, los resultados se han evaluado mediante métodos estándar de espectrofotometría, fluorometría y electroforesis en gel de agarosa con el objetivo de evaluar qué método puede ser más adecuado para la extracción de alta calidad y cantidad de ADN de alto peso molecular para cada una de las especies. Se probaron seis protocolos de extracción, incluidos métodos de extracción directa (CTAB, SILEX, DNAbsolute, CTAB modificado) y métodos de aislamiento de núcleos (PacBio, CellLytic). La efectividad de los protocolos varió significativamente entre especies debido a sus metabolitos y propiedades fisiológicas únicas. El aislamiento de núcleos de PacBio combinado con los kits Nanobind o PanDNA produjo la mayor cantidad y pureza de ADN para P. angustifolia, adecuado para la secuenciación de lectura larga. Otras especies produjeron rendimientos y pureza de ADN bajos, lo que a menudo requirió una mayor optimización.

Keywords: DNA; High molecular weight; NGS; DNA; Contaminants; Extraction

Summary

High molecular weight (HMW) DNA extraction is a necessary step for the generation of a reference genome. While it has been optimized for several model plant species such as *Arabidopsis thaliana* or *Oryza sativa*, it still presents many challenges for many non-model plants. Most plants are rich in polysaccharides and specialized metabolites such as polyphenols and tannins that make DNA extraction difficult. The situation is aggravated when working with plants collected from the wild, since plants may have suffered environmental stresses during their life that increase the presence of these specialized compounds that hinder the extraction of high-quality DNA. In this work, we have tested several extraction protocols in six Mediterranean plant species (*Limbarda crithmoides, Pistacia lentiscus, Phillyrea angustifolia, Reseda alba, Reseda barrelieri* and *Reseda hookeri*) collected in El Saler (Valencia) that have traits that make them recalcitrant to DNA extraction (e.g., high concentrations of polysaccharides). Subsequently, the results have been evaluated by standard methods of spectrophotometry, fluorometry and agarose gel electrophoresis with the aim of evaluating which method may be more suitable for the extraction of high quality and quantity of HMW DNA for each of the species. Six extraction protocols were tested, including direct extraction methods (CTAB, SILEX, DNAbsolute, modified CTAB) and nuclei isolation methods (PacBio, CellLytic). The protocols' effectiveness varied significantly across species due to their unique metabolites and physiological properties. The PacBio nuclei isolation combined with Nanobind or PanDNA kits yielded the highest DNA quantity and purity for *P. angustifolia*, suitable for long-read sequencing. Other species produced low DNA yields and purity, often requiring further optimization.

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Relationship between the work and the Sustainable Development Goals of the 2030

	High	Medium	Low	Not applicable
SDG 1. End poverty				X
SDG 2. Zero hunger				X
SDG 3. Health and well-being				X
SDG 4. Quality education				X
SDG 5. Gender equality				X
SDG 6. Clean water and sanitation				X
SDG 7. Affordable and clean energy				X
SDG 8. Decent work and economic growth				X
SDG 9. Industry, innovation and infrastructure				X
SDG 10. Reducing Inequalities				X
SDG 11. Sustainable cities and communities				X
SDG 12. Responsible production and consumption				X
SDG 13. Climate action		X		
SDG 14. Life underwater				X
SDG 15. Life of terrestrial ecosystems		X		
SDG 16. Peace, justice and strong institutions				X
SDG 17. Partnerships to achieve goals.				X

The study involves the collection and analysis of various non-model plant species, some of which are endemic or have unique adaptations to high temperatures, drought and saline soils. By developing optimized protocols for DNA extraction, the research facilitates the creation of reference genomes for these species. This is crucial for biodiversity conservation, as it allows for the accurate identification, cataloguing, and monitoring of plant genetic resources. Improved genetic understanding aids in the conservation of threatened species and ecosystems, ensuring that they are preserved for future generations.

The study contributes to SDG 13 by enhancing our understanding of plant resilience to climate stress, which is vital for developing strategies to mitigate the impacts of climate change on ecosystems. It supports SDG 15 by aiding biodiversity conservation, ecosystem restoration, and sustainable land management through improved genetic research and data on non-model plant species.

Relación entre el trabajo y los Objetivos de Desarrollo Sostenible del 2030

	Alto	Medio	Bajo	No procede
ODS 1. Fin de la pobreza				X
ODS 2. Hambre cero				X
ODS 3. Salud y bienestar				X
ODS 4. Educación de calidad				X
ODS 5. Igualdad de género				X
ODS 6. Agua limpia y saneamiento				X
ODS 7. Energía asequible y no contaminante				X
ODS 8. Trabajo decente y crecimiento económico				X
ODS 9. Industria, innovación e infraestructuras				X
ODS 10. Reducción de desigualdades				X
ODS 11. Ciudades y comunidades sostenibles				X
ODS 12. Producción y consume responsables				X
ODS 13. Acción por el clima		X		
ODS 14. Vida submarina				X
ODS 15. Vida de ecosistemas terrestres		X		
ODS 16. Paz, justicia e instituciones sólidas				X
ODS 17. Alianzas para lograr objetivos.				X

El estudio implica la recolección y el análisis de varias especies de plantas no modelo, algunas de las cuales son endémicas o tienen adaptaciones únicas a altas temperaturas, sequías y suelos salinos. Al desarrollar protocolos optimizados para la extracción de ADN, la investigación facilita la creación de genomas de referencia para estas especies. Esto es crucial para la conservación de la biodiversidad, ya que permite la identificación, catalogación y monitoreo precisos de los recursos genéticos de las plantas. Una mejor comprensión genética ayuda a la conservación de especies y ecosistemas amenazados, asegurando su preservación para las generaciones futuras. El estudio contribuye al ODS 13 al mejorar nuestra comprensión de la resiliencia de las plantas al estrés climático, lo cual es vital para desarrollar estrategias para mitigar los impactos del cambio climático en los ecosistemas. Apoya el ODS 15 al ayudar a la conservación de la biodiversidad, la restauración de los ecosistemas y la gestión sostenible de la tierra mediante una mejor investigación genética y datos sobre especies de plantas no modelo.

INTRODUCTION

1.1. Context: Reference Genomes and Biodiversity

As of 2024, we are currently in an ongoing human-caused sixth mass extinction, which is accelerating (Ceballos et al., 2015; Cowie et al., 2022; Diamond, 1989; Dirzo et al., 2022; Wake & Vredenburg, 2008). The loss of biodiversity is one of the most serious ecological problems we face, as extinction is permanent, and each species has its own unique role in the ecosystem. When species disappear, so do their genes, interactions, phenotypes and a whole array of characteristics.

Plants are an integral part of ecosystems, and it is likely that any impacts on global plant health and diversity would rapidly affect other organisms, hence the conservation of plant species should be a primary concern (Tylianakis et al., 2008). However, plants species are poorly covered in The Red List (Bachman et al., 2019; Humphreys et al., 2019) and are often underappreciated in terms of their effect on the ecosystem (Heywood, 2017) and impact on global health (L. Hefferon, 2012)

Based on current estimates (IUCN, 2024), 26,276 plant species out of the 66,535 evaluated are currently threatened (39.5%). However, it is likely that such numbers are underestimates, given that many more species are probably already at risk because of their narrow ranges and hence are less likely to have been identified and formally described (Pimm & Raven, 2017).

This includes some species listed in this work such as *Limbarda crithmoides, Reseda hookeri, Reseda alba and Reseda barrelieri*, which are not evaluated on the IUCN RedList (IUCN, 2024).

To face this urgent crisis, a way to aid the conservation of threatened species is conservation genomics. Genomic data can help in monitoring biodiversity, taxonomic identification and can be integrated into several conservation strategies (Theissinger et al., 2023). The availability of reference genomes of non-model and endangered species substantially improves the use of genomics in biodiversity conservation (Formenti et al., 2022).

A “reference genome” is a representative example of a species´ genome. They are typically derived from a single individual and provide standards for comparison with the genomes of other individuals of the same or of related species. In its most complete form, a reference genome includes the complete nucleotide sequence and also structural information (Kress et al., 2022). Reference genomes provide a framework to map intra and interspecific variation and to characterise biodiversity and identify genetic variants. Their analysis can give place to the identification of new genes, regulatory elements, regions of low heterozygosity, patterns of linkage disequilibrium and more elements (Theissinger et al., 2023).

One of the main ways reference genomes are used for conservation efforts is in the selection of breeding specimens for repopulation. Annotated genomes provide information about inbreeding depression (detrimental fitness effects associated with inbreeding) and regions of low heterozygosity. By localizing loci contributing to inbreeding depression with runs of homozygosity (ROH), selective breeding can be used to minimize inbreeding depression and its negative consequences (Theissinger et al., 2023). This can lead to better conservation policies and more effective management of genetic resources (Aitken & Bemmels, 2016).

The availability of the reference genome has had a positive impact in conservation strategies of several endangered species. For example, the Tasmanian Devil (*Sarcophilus harrisii*) is an endangered Australian marsupial, threatened by the Tasmanian devil facial tumour disease (DFTD). The reference genome for this species has been crucial for understanding DFTD and the management of the species in the wild (Brandies et al., 2019). Another example is the European beech (*Fagus sylvatica L.*), which is threatened by the recent increase and prolonged periods of droughts in Central Europe. The reference genome of this species allowed for the selection of drought-resistant individuals for conservation and breeding programs (Pfenninger et al., 2021).

Several biodiversity genome projects have arisen in the last few years to build annotated reference genomes capturing as much diversity as possible. Such are the EarthBioGenome project, which aims to assemble representative genomes for all known eukaryotic species, currently estimated to be ~1.5 million (Lewin et al., 2001) and the 10KP project (Cheng et al., 2018). The 10KP project is structured as an international consortium that aims to sequence complete genomes from more than 10,000 plants and protists, in order to build to annotated reference genomes for a member of every genus of the Viridiplantae. Information obtained from such reference genomes will enable further studies of phylogeny, origin and diversification of specific traits and correlation between genomic and morphological changes (Cheng et al., 2018). More specifically, by obtaining the reference genomes of plants, we can learn more about the effects of their unique mating systems and structural variation on genomic diversity (Exposito-Alonso et al., 2020).

To be able to generate reference genomes, first the genomic DNA must be isolated and purified to later be sequenced, assembled and annotated. Thanks to the recent advances in third generation sequencing technology, longer fragments of DNA can be read, which facilitates the generation of chromosome-assembly reference genomes and assembly and annotation of repetitive sequences, which is often highly challenging with short reads. These technologies require high molecular weight DNA (fragments of 50 kbp or longer) of high quality for the generation libraries and high-quality reference genomes (Mitchell et al., 2023).

In this work, the DNA of wild species of recalcitrant plants from different families including the Asteraceae (*Limbarda crithmoides)*, Oleaceae (*Phillyrea angustifolia*), Anacardiaceae (*Pistacia lentiscus*) and Resedaceae (*Reseda alba*, *R. hookeri* and *R. barrelieri*) is extracted aiming to obtain DNA of enough quality and length for long-read sequencing and generation of reference genomes in the context of the Catalan Initiative for the Earth Biogenome Project (https://www.biogenoma.cat/en/home/).

The Resedaceae, containing 6 genera and about 85 species, are widely distributed in the Mediterranean and Southwestern Asian areas, with a major biodiversity centre of species diversity in the Mediterranean basin (Martín-Bravo et al., 2007). There are three related species native to the Spanish Mediterranean area with interesting evolutionary properties. *R. hookeri* is critically endangered and considered extinct in Catalonia and in danger of extinction by the Valencian catalogue of "Threatened Flora Species" (Bravo et al., 2018). *R. hookeri* inhabits sandbanks and coastal cliffs with substrates of volcanic origin and *R. barrelieri* is supposed to be a tetraploid species

adapted to limestone habitats. These two species co-habit with a wide-range species. Finally, *R. alba is* characterized as an octoploid (Çilden & Yıldırımlı, 2021), and can be found on dry rocky ground on fields and roadsides (Reseda alba L. (n.d.).

Limbarda crithmoides (L.), formerly known as *Inula crithmoides (L.)*, is a halophyte, also adapted to maritime meadows and saltwater marshes, it inhabits the coasts of the Mediterranean Basin (Bucchini et al., 2015). It is rich in specialized metabolites of interesting properties such as compounds with antibacterial, antifungal, antioxidant and anti-inflammatory activities (Abdel-Wahhab et al., 2008; Bucchini et al., 2013, 2015; Faten Omezzine, 2011; Giamperi et al., 2010; Zurayk & Baalbaki, 1996)).

Pistacia lentiscus is a common shrub in the Mediterranean Basin (Specht & Moll, 1983), and its ability to sprout after fire and to protect soil from erosion makes it valuable for management and reforestation programmes under Mediterranean climatic conditions (Garcia-Fayos & Verdu, 1998). Another species evaluated in this work is *Phillyrea angustifolia*, an evergreen shrub also typically found in dry and warm areas of the Mediterranean Basin (Álvarez et al., 2019; Gucci et al., 1997), that showed leaf reflectance and photo- and antioxidant protection in field-grown summer-stressed individuals, which is an adaptation that helps them survive stressing drought, and high irradiance and temperatures of the summer in the Mediterranean (Peñuelas et al., 2004).

1.2 Challenges and Techniques in Plant DNA Extraction

The extraction of DNA from plants poses a number of challenges, mainly due to the rigid cellulose cell wall (Do & Adams, 1991; Pandey et al., 1996; Varma et al., 2007) and the presence specialized metabolites such as polyphenols, terpenes and polysaccharides. Specialized metabolites present in recalcitrant plants also increase the difficulty of the purification of DNA (Pratyusha, 2022; Wilson, 1997), as they co-precipitate with DNA (Scott & Playford, 1996; Sharma et al., 2002).

Many protocols have been developed for the purpose of plant genomic DNA extraction, however none of them has been found to be applicable to all plant species. This lack of standardisation is caused by the huge variability of concentration and type of contaminants present in different plant species. Moreover, the type and age of tissue, the storage and condition of the plant at the time of recollection also influence the presence of contaminants (Varma et al., 2007).

One of the most common DNA extraction methods from plant material is the CTAB (cetyltrimethylammonium bromide) protocol (Doyle & Doyle, 1987; Murray & Thompson, 1980; Saghai-Maroof et al., 1984). This protocol has been used to extract DNA from many plant species and has also been modified extensively to minimize contamination by species-specific compounds (Schenk et al., 2023).

The first step of this method is grinding plant tissue to a fine powder in liquid nitrogen and mixing it with pre-warmed CTAB extraction buffer containing 2-βmercaptoethanol (BME). CTAB is a cationic detergent that disrupts cell membranes and nuclear envelopes, thereby releasing cellular contents, including DNA. It also helps remove polyphenols and polysaccharides, which are very

common contaminants in DNA extractions of plants (Rogers & Bendich, 1989). BME is added as a reducing agent, which prevents oxidation and co-precipitation of polyphenols with DNA, and also reduces disulfide bonds in proteins, which helps to denature and solubilize them so they can be removed in the following steps (Heikrujam et al., 2020).

After the addition of CTAB and BME, the mixture is incubated at 65°C for about 30 minutes to lyse the cells. Then an equal volume of chloroform isoamyl alcohol (24:1) is added, mixed thoroughly, and centrifuged to separate phases. Chloroform denatures proteins, causing them to precipitate out of the aqueous phase. The aqueous phase containing the DNA is transferred to a new tube, mixed with cold isopropanol, and incubated to precipitate DNA, followed by centrifugation to pellet the DNA. The pellet is washed with 70% ethanol, air-dried, and resuspended in TE buffer for storage (Murray & Thompson, 1980). The many modifications that have been applied to this CTAB protocol are detailed in a recent article by Schenk et al (2023).

One of the problems of the CTAB protocol and other similar protocols, is that DNA must be precipitated after phase separation using some alcohol (typically isopropanol). As carbohydrates can co-precipitate with DNA (Do & Adams, 1991), this step can lead to a reduction in the quality of the DNA. A different approach to DNA extraction is the use of silica matrices, as silicates exhibit a strong binding affinity for DNA when exposed to alkaline conditions and elevated salt concentrations (Vogelstein & Gillespiet, 1979). The use of a silica membrane in DNA extractions is based on the principle of selective binding of negatively charged DNA to the matrix covered in positively charged ions. After the DNA is bound to the silica, the rest of the cellular components can be washed away and finally, the DNA can be eluted with any hypoosmotic solution such as distilled water or Tris-EDTA buffer. A simple protocol that involves the use of this type of matrices was described by Carter & Milton (1993).

With extraction methods based on silica columns, the DNA is not precipitated, but bound to a silica matrix and then eluted, which allows for the removal of carbohydrates.

Most commercial DNA extraction kits utilize silica column technology and examples of popular brands include Qiagen's, Invitrogen™, New England Biolabs or Macherey Nagal, among many others. Typically, silica matrices are used associated to columns and DNA is eluted by centrifugation, forcing the DNA to cross a membrane. While columns often help obtaining cleaner DNA, column elution can cause shearing of the DNA, which is inappropriate for next generation sequencing (NGS) platforms (Anderson et al., 2018; Xia et al., 2019).

The SILEX protocol (Vilanova et al., 2021) is a modification of the CTAB protocol where, instead of precipitating the DNA with isopropanol, it is absorbed by a silica matrix (same material used for the typical DNA extraction columns) and then eluted from it. In this way, this method avoids the precipitation step and the potential shearing due to the need of eluting the DNA from a membrane by centrifugation. It also keeps the price per sample low, in comparison with kits that utilize silica columns. Indeed, the authors claim that it can be applied to wild plants and that high molecular weight DNA is obtained (Vilanova et al., 2021).

There are many other methods of DNA extraction, including SDS-proteinase K protocols (EBELING et al., 1974), magnetic bead-based kits (Hawkins, 1999), filter paper- based methods (Shi

& Panthee, 2017) and the use of Chelex-100 resin (Hui et al., 2014), which will not be discussed in this work.

Another type of DNA extraction strategy is isolation of the nuclei and subsequent extraction of DNA. This method possesses significant advantages as nuclei isolation allows for the removal of many cellular contaminants and for the extraction of longer DNA fragments as the DNA remains in the nucleus and is more protected from shearing. It has also been established that nuclei isolation protocols generate more quantity of extracted DNA (Gong et al., 2019; Hanania et al., 2004; Kang et al., 2023; Mitchell et al., 2023; Xie et al., 2023).

1.3 Quantification and Evaluation of Nucleic Acid Purity and Integrity

Once DNA has been extracted its quantity and quality must be evaluated before proceeding with sequencing. The standard methods for the quantification and evaluation of the purity of extracted DNA are UV spectrophotometry (Gallagher, 1994) and fluorescence-based assays (Singer et al., 1997).

Spectrophotometric concentration measurements are based on the principles of the Lambert-Beer law. Double stranded DNA (dsDNA) shows an absorption peak at 260nm. Therefore, if DNA is pure, measurement at 260 nm is proportional to the concentration of DNA in a sample (Gallagher, 1998; Sambrook J & Russell D, 2006).

To evaluate sample purity, the ratios of absorbance values of 260 nm vs 280 nm (A260/A280) and the 260 nm vs 230 nm (A260/A230) are also determined. The A260/A280 ratio is an indicator of protein contamination as tyrosine and tryptophan (and to a very smaller extent Phe and disulfide bonds) absorb strongly at 280 nm (Aitken & Learmonth, 1996). So, the presence of proteins typically reduces the ratio. Additionally, the type of nucleic acid present influences the measurement, allowing RNA contamination to be detected by an increase in the ratio. Pure dsDNA typically displays A260/A280 of 1.85-1.88, while for RNA, the ratio is around 2.1 (Koetsier et al., 2019).

The A260/A230 ratio is an indicator of contaminants that absorb strongly at 230 nm, such as proteins (Liu et al., 2009), guanidine HCl, EDTA, carbohydrates, lipids, salts, phenol and other organic compounds (Stulnig & Amberger, 1994). The expected ratio when measuring pure dsDNA is between 2 and 2.2 (Lucena-Aguilar et al., 2016).

Additionally, fluorometric readings are usually taken to ensure more accurate estimations of DNA concentration. Fluorescence readings with the Qubit 3.0 Fluorometer (Thermo Fisher) are more accurate and sensitive than UV spectrophotometry, because the fluorescent reagent binds specifically to dsDNA, therefore the relation between the fluorescence emitted and DNA concentration is direct and there is less influence of contaminants such as proteins or other molecules (DeNovix, 2023; Downs & Wilfinger, 1983; Holden et al., 2009).

So, presently, the combination of Qubit fluorometric and Nanodrop spectrophotometric measures is routinely used to determine the concentration and quality (purity) of DNA preparations.

Finally, the standard method of separation of DNA for integrity and fragment size evaluation is an agarose gel electrophoresis, where the current is passed through an agarose gel containing the DNA samples in wells. When an electric current is applied, the negatively charged DNA fragments migrate through the porous agarose matrix towards the positive electrode. Smaller fragments move faster and travel further through the gel pores, while larger fragments move more slowly and travel shorter distances. This results in the separation of DNA fragments by size, which can be visualized using a DNA stain under UV light. The size of the fragments can be estimated by loading molecular markers that have bands of known molecular weight and comparing those to the bands of the sample. The intensity of the light emitted by the DNA bands can give an estimate idea of DNA concentration and if smears are present in the gel, it can be an indication of low DNA integrity (highly fragmented DNA). (Bjornsti & Megonigal, 1999; Lee et al., 2012; Vogelstein & Gillespiet, 1979; Wittmeier & Hummel, 2022).

In pulsed-field gel electrophoresis, the direction of the electric field changes periodically, allowing for the separation of large DNA fragments ranging from 10 kb to several megabases, providing high resolution for the better estimation of the size of the fragments (Schwartz et al., 1982; Schwartz & Cantor, 1984). This method enables the resolution of DNA fragments that are much larger than those typically separated by conventional gel electrophoresis.

OBJECTIVES

The objectives of this work are:

- Evaluate and compare the efficiency of various DNA extraction protocols in terms of DNA yield and purity across six different plant species (*Pistacia lentiscus, Limbarda crithmoides, Phillyrea angustifolia, Reseda alba, Reseda hookeri* and *Reseda barrelieri)*.
- Identify the protocol and modifications that produce the highest quantity and quality DNA suitable for long read sequencing for each species.
- Compare direct DNA extraction methods with nuclei isolation protocols to determine which would produce higher quality and quantity of extracted DNA
- Investigate the effectiveness of different modifications to the standard protocols in improving DNA yield and purity.

MATERIALS AND METHODS

3.1 Sample Collection and Processing

In the case of *Pistacia lentiscus, Limbarda crithmoides* and *Phillyrea angustifolia* plant material was collected from the wild (Parc Natural de La Dehesa del Saler, Valencia, Spain). Samples of *Reseda alba, Reseda hookeri, Reseda barrelieri* were collected from plants grown in the university premises from seeds collected in the wild, *Solanum lycopersicum (MoneyMaker)* was used in occasions as extraction control, and leaves were collected from plants grown in the greenhouses of COMAV (UPV). *Olea europaea* (Picual) was also used as control and the samples were collected at

the experimental fields of the University of Jaen. In all the cases, the required permits were obtained before collecting plant material from the wild.

The midribs of the leaves were removed with a clean scalpel, and the leaf lamina was frozen in a 50mL Falcon in liquid nitrogen.

The samples were ground in liquid nitrogen (-80ºC) with a pestle in a pre-cooled mortar for at least 30 minutes, until a fine powder resembling flour was observed. *Phillyrea angustifolia* leaves were very hard, therefore more grinding was required (~45 min). Ground samples were stored in 50 mL Falcon tubes in a -80ºC freezer.

3.2. Methods for DNA Extraction

3.2.1 CTAB Protocol

The CTAB protocol for DNA extraction and purification was developed in the 1980s and has been widely used to extract DNA from leaves and seeds, with various modifications for different plant species (Murray & Thompson, 1980).

For each sample of 50mg of ground leaf powder, 1 mL of pre-warmed CTAB extraction buffer containing 2-βmercaptoethanol (BME) at 1.4 % is added. Then, the mixture is incubated at 65°C for about 1 hour to lyse the cells and then an equal volume of chloroform: isoamyl alcohol (24:1) is added, mixed thoroughly, and centrifuged to separate the organic and aqueous phases. The upper aqueous phase containing the DNA is transferred to a new tube, mixed with equal volume of cold isopropanol, and incubated to precipitate DNA, followed by centrifugation to pellet the DNA. The pellet is washed with 70% ethanol, air-dried, and resuspended in TE buffer for storage.

3.2.2. SILEX Protocol

The SILEX method is derived from the standard CTAB method and incorporates DNA recovery using a silica matrix instead of alcohol precipitation (Vilanova et al., 2020). About 50 mg of sample (ground leaf powder) was needed for this procedure.

For this protocol, CTAB buffer with β-mercaptoethanol (reducing agent), are added to the frozen leaf powder and the mixture is incubated at 65ºC for 1 hour with occasional agitation to promote cell lysis. After this, chloroform: isoamyl alcohol (24:1) is added to separate the DNA (aqueous phase) from the organic phase. Then, RNase is added to the aqueous phase to remove RNA (37ªC, 15') and a binding buffer containing NaCl and PEG, and absolute ethanol are added. Next, silica matrix buffer is added, and the mixture is gently agitated for 5' to promote binding of the DNA to the silica matrix. Afterwards, the tubes are spun down, and the supernatant is decanted. The silica pellet is washed with ethanol twice and left to dry at room temperature. After the pellet is dry, it is resuspended in TE buffer (pH 8), incubated at 65ºC for 10 min and then centrifuged, to release the DNA. The supernatant is collected and transferred to a new 1.5 mL tube.

3.2.3 DNabsolute Kit (Idylle)

The DNabsolute Kit (Idylle) allows the isolation of DNA from different types of samples, including drosophila, coral and dried plant leaves. It is not designed as a specific kit for plant tissues, but the manufacturer recommends it for plant tissues too.

The first step of the protocol involves cell lysis with two lysis buffers. The first, containing proteinase K to remove protein contaminants and nucleases that can degrade DNA, is incubated at 65ºC for 1 hour. Then, to complete the lysis, RNase A and the second lysis buffer are added. For the precipitation of the DNA, absolute ethanol and DNAbsolute solution (proprietary formula) are added to the lysate, and then centrifuged at 10,000g at 4ºC for 5 min. After centrifugation, the supernatant is discarded, and the pellet is resuspended in 100µL of PBS buffer by vortexing. To purify the DNA, 600 µL of absolute ethanol are added and vortexed and then the samples are centrifuged at 10,000 g at 4ºC for 5 minutes, after which the supernatant is discarded. The pellet is washed 3 times with the addition of 600 µL of 70% ethanol and centrifugation at 10,000 g for 1 min at room temperature. Finally, the pellet is air dried and then resuspended in 50 µL of Tris Buffer.

3.2.4 CTAB and DNAbsolute Protocol Mix

A mix of the CTAB and DNAbsolute protocol was also tested. For this method, the CTAB protocol is followed until the addition of chloroform: isoamyl alcohol, and after the centrifugation, the supernatant was recovered and then the DNAbsolute protocol was followed with this supernatant as starting material.

3.2.5. Isolation of Nuclei from Plant Tissue using LN2 disruption (PacBio) followed by Extraction with Nanobind/PanDNA kits (PacBio) or SILEX Protocol

Ground plant material is resuspended in nuclei extraction buffer (NIB) (+ 35 mM β-mercaptoethanol added immediately before use) and incubated for 15' at room temperature in an orbital shaker. Next, the lysate is filtered using a Steriflip (20 µm pore) or filter cloth and centrifuged at 7000g for 20' at 4ºC. After centrifugation, the supernatant is discarded and 1mL of ice-cold NIB is added. Next, the pellet is gently resuspended using a paintbrush pre-soaked in NIB. Once the pellet is resuspended, we added 14mL of NIB to bring the final volume to 15 mL. Then, the tubes are centrifuged at 7000g at 4ºC for 10 min and the supernatant is decanted. These steps are repeated at least 3 times until the supernatant is colourless (in most experiments, this step was repeated 3 or 4 times). Finally, the last supernatant is decanted, 1 mL of cold 1X HB buffer is added and the pellets are resuspended with the paintbrush and a P1000 pipette. The nuclei suspension is transferred to a Protein LoBind tube and centrifuged at 7000g for 5 min at room temperature, after which the supernatant is discarded. The pellets can be snap frozen with liquid nitrogen or used immediately for DNA extraction.

Modifications to the PacBio Nuclei Isolation Protocol:

i) Increasing the % of β-mercaptoethanol and PVP360

The first 2 trials of the protocol were conducted with the recommended β-mercaptoethanol and PVP360 concentrations., which are 2.5 µL BME/mL NIB (0.25% BME) and 1% PVP360 respectively. However, in the following trials, we doubled the concentrations of both (0.5% BME and 2% PVP).

ii) Modifications for Resedaceae

For the Resedas, grinding on an ice-cooled mortar with the NIB before filtering was tested as they produce a highly viscous solution that is difficult to filter. Additionally, after the first centrifuge decantation and resuspension of the pellet in 1mL of NIB, the viscous, mucilaginous material produced by the Resedas and the green material that is mostly composed of chlorophyll is removed with the paintbrush and alternatively, with a P1000 pipette.

DNA extraction

After isolation of the nuclei, we proceeded to DNA extraction using the kit Nanobind/PanDNA from PacBio. This kit is a modification of the classical silica membranes, as in this case, instead of using a column, a magnetic silica disk is placed inside a tube for DNA binding. Therefore, instead of being forced through the membrane, the disk is captured using a magnetic rack, so the DNA can be washed without forcing it through a membrane and increasing the final size of the DNA fragments.

The DNA extraction using this kit involves a step for the lysis of the nuclei, with the addition of proteinase K and RNAse A, along with a lysis buffer and a 2-hour incubation period at 55ºC with intense agitation. After this, the tubes are centrifuged and the supernatant, which should contain the DNA that is released from the lysed nuclei, is transferred to a new 1.5 mL Protein LoBind tube, where a Nanobind disk and isopropanol are added. The tubes are left mixing in a HulaMixer for 20 minutes (9 rpm rotation, 70º tilting for 12 s and 2º vibration for 1 s). Theoretically, during the mixing, the DNA should bind to the Nanobind disk. The disks are later washed with a wash buffer, which contains ethanol, and the supernatants are decanted, using a magnetic rack to keep the Nanobind disk in place. To release the DNA from the disks, EB buffer is added, and the tubes are centrifuged, and the supernatant containing the DNA is transferred to a new tube. The DNA eluate is left to rest overnight at room temperature, to solubilize the DNA and is finally mixed with a P200 pipette and then stored in a -80ºC freezer.

Alternatively, we also tested the same protocol for nuclei isolation, followed by DNA extraction using the CTAB protocol, as described in section 3.2.1 and SILEX protocol, as described in section 3.2.2.

3.2.6. CellLytic™ PN Isolation/Extraction Kit (Sigma-Aldrich®) and DNA extraction using Nanobind PanDNA kit (PacBio)

The first step on this protocol is the preparation of 1X NIB from the 4X NIB in the kit with deionized water and DTT (1mM).

Three layers of filter mesh, (included in the kit), are formed into a conical shape and placed on an open 50mL Falcon tube; the layers are held by a cap with the centre cut out. Firstly, 3mL of 1X NIB buffer are added to 1g of frozen plant tissue and after mixing, the suspension is passed through the filter mesh into the 50mL tubes. The tubes are spun in the centrifuge at 1260 g for 10 min and the supernatant is discarded. Then, the pellet is resuspended in 0.5 mL on 1X NIB buffer with a paintbrush. To lyse the cell membrane, 10 % TRITON ™ X-100 solution is added at a specific concentration depending on the species of plant, that has to be determined experimentally by observing the lysis of the cells under an optical microscope. Manufacturers' instructions provided examples of some species like *S. lycopersicum* (1% of TRITON ™ X-100) or *Nicotiana benthamiana* (0.3% of TRITON ™ X-100).

For *Pistacia lentiscus*, the following final concentrations of TRITON ™ X-100 are tested: 0.3%, 0.6%, 0.8%, 1%, 1.2% and 2%, whereas for *Phillyrea angustifolia,* 0.6% TRITON ™ X-100 was used.

Manufacturers' instructions provide a protocol for crude, semi-pure and pure nuclei preparation. In our case, the protocol for the semi-pure preparation was followed. This procedure involved placing 0.8 µL of sucrose cushion (2.3M) in a 1.5 mL tube and then carefully applying the cell lysate on top. Then, the tubes are centrifuged at 12000g for 10 min and the upper green phase and sucrose cushion is discarded with a P1000 pipette. The pellets are washed twice, by resuspension in 1mL 1X NIB buffer and centrifugation at 12000g for 5 min. Finally, the supernatant is removed, the nuclei pellet is resuspended in 100 µL of Nuclei PURE Storage Buffer and stored in a -80ºC freezer.

After, nuclei isolation we proceeded to DNA extraction using the PanDNA kit protocol as described in section 3.2.5. Different amounts of nuclei pellets were used as starting material.

3.3. Methods for DNA purification

3.3.1. Precipitation of DNA with Ammonium Acetate and ethanol:

To further remove contaminants and purify the DNA, a DNA precipitation with ammonium acetate and ethanol was performed after the DNA extraction with the DNAbsolute kit. In this precipitation, 0.1 volumes of ammonium acetate 3M and 2.5 volumes of absolute ethanol are added to 50 µL of extracted DNA. After centrifugation and 2 washes with 70% ethanol, the pellets are left to dry and resuspended in 50 µL of TE buffer.

3.4. Methods of DNA Quality, Quantity and Integrity Evaluation

3.4.1. UV-Visible Spectrometry (Nanodrop) DNA Quantification

The concentration and quality of extracted DNA was measured spectrophotometrically using a NanoDrop™ ND-1000 (Thermo Scientific, Waltham, MA, USA). This is a standard method for the quantification and evaluation of the purity of extracted DNA (Gallagher, 1994).

For quantification, 2 µL of the DNA sample are placed in the Nanodrop, and absorbance at 230, 260 and 280 nm is recorded. Absorbance at 260 nm corresponds to the absorption peak of nucleic acids and, according to the Lambert-Beer law, it is proportional to the concentration in nucleic acid in the sample (Gallagher, 1998; Sambrook J & Russell D, 2006)

The ratios of absorbance values of 260 nm vs 280 nm (A260/A280) and the 260 nm vs 230 nm (A260/A230) are also determined, as an indicator of sample purity.

Following recommendations from the PacBio isolation protocol, triplicate Nanodrop UV/Vis measurements are taken from the top, middle and bottom of the tube to obtain accurate concentrations readings. This is necessary due to the viscosity and inhomogeneity of high molecular weight DNA (Smith & Cantor, 1987).

Manufacturer's specifications for nucleic acid detection set a lower detection limit of 2 ng/µL but warn that below 20 ng/µL the reliability of the purity ratios is compromised (Koetsier et al., 2019).

3.4.2. Fluorometric quantification (Qubit)

DNA quantification was also performed with a Qubit™ 2.0. Fluorometer (Thermo Scientific, Waltham, MA, USA). Between 1-10 µL of each sample were tested using the Qubit™ dsDNA BR Assay Kit (Thermo Scientific, Waltham, MA, USA) according to the instructions of the manufacturer.

To carry out the measurements, first, the Qubit was calibrated with the two standards in the kit and then, the sample DNA was mixed with 190 µL of the Quantitation solution, waiting for 2 minutes for the reaction to take place. This reaction is carried out in 500 µL thin-walled polypropylene tubes (Qubit™ Assay Tubes). Then, the tubes are placed in the apparatus for measurement of fluorescence. Similarly to the UV/Vis, measurements are taken from the top, middle and bottom of the tube to obtain accurate concentrations readings due to the viscosity and inhomogeneity of high molecular weight DNA.

3.4.3. Standard electrophoretic DNA analysis

To analyse the integrity and approximate size and concentration of the DNA fragments, standard agarose gel electrophoresis was performed. Agarose gels between 0.5-0.8% agarose in 1X TAE buffer were prepared, and 1 µL of GreenSafe dye (NZY tech) was added for the visualization of dsDNA per 100 mL of agarose gel. The amount of DNA placed in the gels was calculated and

adjusted for each sample based on the Nanodrop and/or Qubit concentration readings, so that approximately 100 ng of DNA was loaded in every well. Loading Buffer 6X (Thermo Fisher) was added to the samples in a final concentration of 1X, adjusting the volumes with milliQ water when necessary. Undigested lambda phage DNA (50 kb) and lambda phage DNA digested with HindIII (Thermo Fisher) were used as molecular markers to estimate the size and concentration of the DNA fragments. The gels were left running overnight at 10V and later visualized on a Gel Doc system (BioRad, CA).

This type of analysis allows the visualization and estimation of the size of the DNA fragments present, as well as identification of fragmented or degraded DNA and RNA, shown by the presence of smears in the gel (Bjornsti & Megonigal, 1999).

3.4.4. Pulsed-field DNA electrophoresis

Pulsed-field electrophoresis was carried out to resolve the very high molecular weight DNA present in some samples and have a more accurate estimation of the size, as the larger bands are not well resolved in standard agarose gels.

A 1% agarose gel was prepared with 1.3 g of agarose and 130 mL of 0.5X TBE buffer. The sample volume loaded was calculated using fluorometric estimates of DNA concentration so that approximately 100 ng of DNA was loaded in each well. Electrophoresis was carried out for 16 h with a pulse time of 30 s, at 100 volts and 80mA.

Similarly to the standard gel electrophoresis, undigested lambda phage DNA (50 kb) and lambda phage DNA digested with HindIII were used as molecular markers to estimate size and concentration of the DNA fragments. After electrophoresis, the gel is stained with a DNA-binding dye, GreenSafe Premium (NZY tech) in a 1:10000 dilution with 0.5X TBE buffer, and later visualized on a gel doc system (BioRad, CA).

For more detailed information on the materials used in this study, please refer to Annex 1.

RESULTS

The present work aims to obtain high-molecular weight DNA from wild plants for long-read sequencing with the objective of producing a reference genome for such species. Therefore, the DNA extracted must be, not only of high molecular weight, but also high quality (free from impurities). For this, we surveyed different extraction methods. As first approach, we tried direct extraction methods like CTAB or SILEX to evaluate whether these protocols can produce DNA of the required quality from the target plant species.

4.1. DNA extraction using SILEX protocol

In the first trial of DNA extraction with the SILEX protocol, we tested the protocol with *L. crithmoides*, *P. angustifolia* and *P. lentiscus*. After the extraction, quantification at the Nanodrop indicated that the extraction has failed for *L. crithmoide*s and *P. lentiscus,* as the concentration was close to zero for both species (Fig.1A), while in the case of *P. angustifolia,* we obtained a reasonable

amount of DNA. However, the A260/A230 ratio for this sample was <1, making the DNA inadequate for long-read sequencing (Fig.1A). The Qubit fluorometric readings indicated that we had extracted about 10 ng/µL of *L. crithmoides* DNA, 6 ng/µL of *P. angustifolia* and less than 1 ng/µL of *P. lentiscus* DNA. Loading the samples on an agarose gel, confirmed that the *P. lentiscus* extraction failed, but revealed that, although the concentration was low, we had some *L. crithmoides* DNA (Fig.1B). However, the inability of Nanodrop of providing a reliable measure for *L. crithmoides* indicates the presence of high amounts of contaminants making the DNA unsuitable for long-read sequencing.

A.

	Qubit	Nanodrop		
Sample	**[DNA] (ng/µL)**	**[DNA] (ng/µL)**	**A_{260}/A_{280}**	**A_{260}/A_{230}**
L.crithmoides	10.8	0	-0.32	-0.01
P.angustifolia	6.33	56.41	1.82	0.87
P.lentiscus	0.875	2.39	0.36	0.05

B.

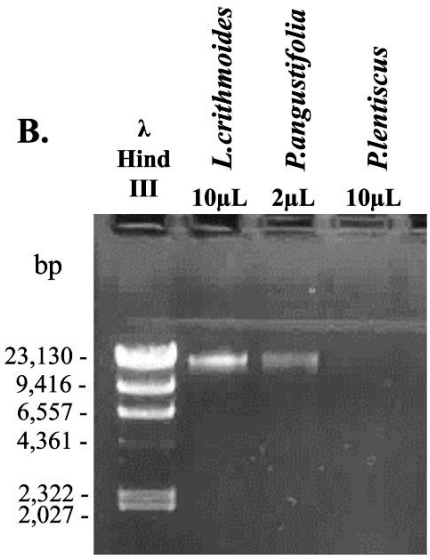

Fig1: Spectrophotometric (Nanodrop) and fluorometric (Qubit) quantifications and agarose gel electrophoresis analysis after DNA extraction of *L. crithmoides, P. angustifolia* and *P. lentiscus* with the SILEX protocol. A. Qubit and Nanodrop UV/Vis readings of DNA concentration and A260/A280 and A260/A230 absorbance ratios. **B**. Electrophoretic analysis of DNA (0.8% agarose gel in TAE1x) ran at 10V overnight. Lambda phage DNA digested with Hind III was loaded as a molecular weight marker in lane 1. In the case of *P. angustifolia*, the volume of DNA of the sample loaded was calculated with the Nanodrop concentrations so that approximately 100 ng of DNA are loaded. In the case of *L. crithmoide*s and *P. lentiscus,* 10 µL were loaded to ascertain whether the concentration measured by Nanodrop was reliable.

A second assay was performed with the SILEX to evaluate the performance of the protocol for Resedaceae plants and to repeat the test with *P. lentiscus* and *P. angustifolia.*

Compared to the first assay, the quality of the *Phillyrea* DNA improved overall, as the concentration according to the Qubit was much higher than in the first trial, but the A260/A230 ratio is close to the threshold value of 1.8 (Fig2A). Regarding the integrity of the DNA, although a light smear is present in the gel (Fig2B), the molecular weight is overall higher (around 50 kb) than in the previous experiment (Fig1B) and the smear stops around 20 kb (Fig2B). We observed an improvement for the *P. lentiscus* sample, as in this assay, some DNA was extracted and visualized in the agarose gel, even though both the concentration and the absorbance ratios are low (Fig2).

In the case of the Resedaceae, a band of high molecular weight DNA was observed for *R. alba* but not for the *R. hookeri* samples. Actually, for *Reseda alba*, a DNA that has not been resolved can be observed in the well indication that its weight is very high and/or that attached contaminants impair its migration.

In the case of *R. alba* and *R. hookeri,* the Nanodrop UV/Vis readings do not correspond with the Qubit fluorometric readings. While the Nanodrop measured a high concentration and A260/A280 and A260/A230 over 2, Qubit quantification and gel loading indicates that the Nanodrop overestimated the concentration. This might be due to the presence of contaminants that absorb light at 260 nm, like DNA, leading to the mistakenly high DNA concentration and ratios or to the low concentration of DNA present being close to the lower detection limit and compromising the reliability of the absorbance ratios. Qubit measures are more accurate due to the high specificity of the Qubit reagents for DNA. Therefore, even though at first glance, the absorbance ratios seem close to ideal ratios for pure DNA, they were not reliable.

A.

	Qubit	Nanodrop		
Sample	**[DNA] (ng/µL)**	**[DNA] (ng/µL)**	**A_{260}/A_{280}**	**A_{260}/A_{230}**
P.agustifolia	51.27	108.10	1.87	1.68
P. lentiscus	11.10	33.20	1.68	0.77
R.alba	20.57	497.20	2.11	2.04
R.hookeri	5.70	241.40	2.09	1.85
R.hookeri 2	4.25	216.80	2.13	1.90

B.

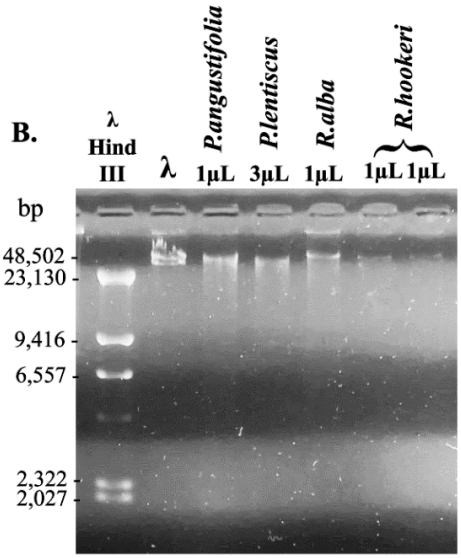

Fig2. Spectrophotometric (Nanodrop), fluorometric (Qubit) quantifications and agarose gel electrophoresis after DNA extraction of *P. angustifolia, P. lentiscus, R. alba* and *R. hookeri* with the SILEX protocol A. Qubit and Nanodrop UV/Vis readings of DNA concentration and A260/A280 and A260/A230 absorbance ratios. **B.** Electrophoretic analysis of DNA (0.8% agarose gel in TAE1x) ran at 10V overnight with lambda phage digested with Hind III as a molecular weight marker loaded in the first lane, and undigested lambda phage DNA in the second lane. The volume of DNA loaded was calculated with the Nanodrop concentrations so that approximately 100 ng of DNA are loaded.

4.2 Comparison of CTAB protocol against SILEX protocol

The CTAB protocol (Murray and Thomson, 1980) is a widely used method for plant DNA extraction. Therefore, after the low yields observed in the SILEX protocol trials, we decided to check whether the standard CTAB protocol could result in higher quantities DNA.

After the extraction, 1 and 10 µL were loaded in a gel to check the validity of the spectrophotometric results in Figure 3A, which seemed to indicate very high concentrations of DNA for both species tested. However, the Qubit fluorometric readings later indicated much lower concentrations than the Nanodrop, and according to these estimates, the concentration of the CTAB samples is lower than that of the SILEX samples. In the agarose gel, it is evident that DNA concentration is low and therefore this overestimation is likely due to contaminants absorbing at 260nm, skewing the absorbance curves. Overall, the SILEX protocol performed better than the CTAB protocol, however low quantities of DNA were obtained for both species.

A.

		Qubit	Nanodrop		
Sample	**DNA extraction**	**[DNA] (ng/µL)**	**[DNA] (ng/µL)**	**A_{260}/A_{280}**	**A_{260}/A_{230}**
L.crithmoides	SILEX	1.89	10.2	1.96	0.27
	CTAB	1.54	101.9	2.13	4.05
P.lentiscus	SILEX	1.77	4.9	1.15	0.26
	CTAB	0.224	75.1	1.91	1.52

B.

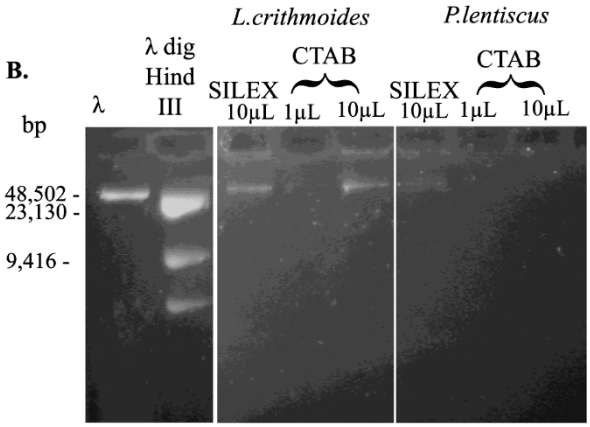

Fig3. Fluorometric (Qubit), spectrophotometric (Nanodrop) quantifications and agarose gel electrophoresis analysis after DNA extraction of *L. crithmoides* and *P. lentiscus* with the SILEX and CTAB protocols. A. Qubit and Nanodrop UV/Vis readings of DNA concentration and A260/A280 and A260/A230 absorbance ratios. **B.** Electrophoretic analysis of DNA (0.8% agarose gel in TAE1x) ran at 10V overnight with lambda phage as a molecular marker in the first lane, and lambda phage DNA digested with Hind III in the second lane. For the CTAB protocol samples, 1 and 10 µL were loaded to ascertain whether the concentration measured by Nanodrop was reliable.

4.3 DNA extraction using DNAbsolute kit (Idylle)

As the CTAB and SILEX protocols did not provide optimal results, we decided to try a kit for DNA extraction. The kit is not based on silica binding, but it contains a trademarked solution that allegedly helps to remove contaminants from the samples and has been used for the obtaining of HMW DNA from dried plant leaves.

Figures 4 and 5 show the 2 trials of the DNAbsolute protocol with the different plant species and *S. lycopersicum* as control, as it is a domesticated plant and is less problematic in DNA extractions. Different sample weights were tested as the protocol did not give any recommendations on the amount of plant material that should be used.

Overall, we were able to extract very high molecular weight DNA and ~20kbp bands in the cases of *S. lycopersicum, L. crithmoides* and *P. angustifolia (*Fig4B). The best results were obtained *S. lycopersicum*, as the bands in the gel are the brightest, indicating higher concentration of DNA. However, the A260/A280 Nanodrop ratio are too high in both samples. In the first samples, it is

somewhat closer to 1.8 (2.25), but in the second sample is abnormally high (10.95). For the other species, the A260/A230 ratios are not good, as they are all either above or below the ideal (2), except for the *L. crithmoides* 1 sample. However, this sample does not have a good A260/A280 ratio. For the A260/A280 ratios, the closest to 1.8 is the *L. crithmoides* 2 sample, but, the A260/A230 is <1, which indicates the presence of contaminants. For the rest of the samples, the absorbance ratios are all anomalous, therefore, the estimated concentrations (which would initially seem very high) are not accurate. Despite this, the presence of bands in the gel for all samples except *Pistacia*, even though only 1.5 µL of sample are loaded, do indicate high concentrations of DNA, however this DNA would have to be purified in order to be used for sequencing.

A.

Sample	Sample weight (mg)	[DNA] (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
S. lycopersicum1	47	175.95	2.25	1.29
S. lycopersicum2	87	593.88	10.95	3.31
L. crithmoides 1	55	365.14	5.54	1.97
L. crithmoides 2	22	80.16	1.96	0.99
P. lentiscus 1	45	207.86	22.73	4.34
P. lentiscus 2	15	29.90	3.11	0.78
P. angustifolia 1	20	123.62	3.48	0.98

B.

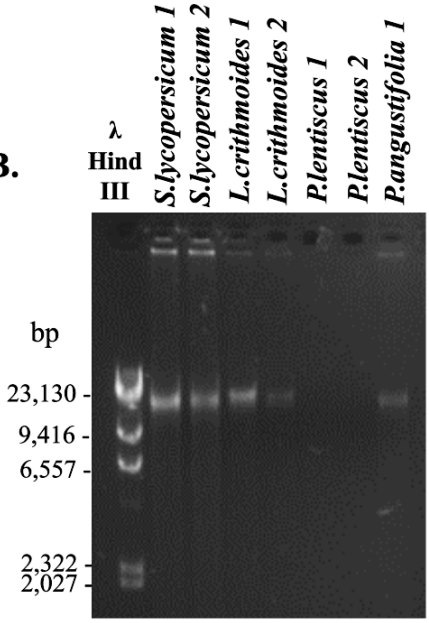

Fig4: Spectrophotometric (Nanodrop) quantifications and agarose gel electrophoresis analysis after DNA extraction of *S. lycopersicum, L. crithmoides, P. lentiscus* and *P. angustifolia* with the DNAbsolute kit (Idylle) protocol. Different sample weights were tested. **A**. Nanodrop readings of DNA concentration and A260/A280 and A260/A230 absorbance ratios. **B.** Electrophoretic analysis of DNA (0.8% agarose gel in TAE1x) ran at 10V overnight with lambda phage DNA digested with Hind III as a molecular marker in the first lane. 1.5 µL of DNA was loaded into each lane.

In the second trial with this protocol, we repeated the test with the *P. angustifolia* and *P. lentiscus* and also evaluated the extraction of *R. alba* and *R. hookeri*. The Qubit fluorometric readings do not correlate with the Nanodrop concentration estimates and the A260/A230 ratios are all below 1.5, indicating high levels of contamination (Fig5A). The A260/A280 ratio for *P. angustifolia* is close to 1.8, but the rest of the samples have ratios closer to 3. For *P. lentiscus*, the absorbance could not be determined by the Nanodrop because after resuspension of the pellet in TE, the DNA solution had a milky-white appearance, which is shown in Figure 6. Additionally, as seen by the Qubit quantification, the concentration of DNA is < 1 ng/µL. For this assay not enough quantity or quality of *P. lentiscus* DNA was extracted. As for the agarose gel in Fig5B, unresolved

bands of high molecular weight in the wells and also long smears can be seen for all samples except *Pistacia*. This suggests that even though high molecular weight DNA was extracted, the integrity was low.

The Qubit fluorometric concentration readings indicate low concentrations (between 10-20 ng/µL DNA) for the Resedas, which is reflected in the gel by the intensity of the bands. However, in the case of *P. angustifolia*, the estimated DNA concentration is quite high (90ng/µL), but in the gel, the bands have a similar intensity as the Resedas.

A.

	Qubit	Nanodrop		
Samples	**[DNA] (ng/µL)**	**[DNA] (ng/µL)**	**A_{260}/A_{280}**	**A_{260}/A_{230}**
P.angustifolia	90.7	403	1.70	0.95
P.lentiscus	0.316	-	-	-
R.alba	7.29	320	2.81	1.29
R.hookeri	17.8	242	5.82	1.32

B.

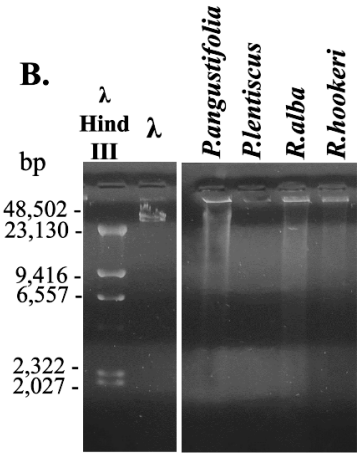

Fig5: Spectrophotometric (Nanodrop) and fluorometric (Qubit) quantifications and agarose gel electrophoresis analysis after DNA extraction of *P. angustifolia, P. lentiscus, R. alba* and *R. hookeri* with the DNAbsolute kit (Idylle). A. Qubit and Nanodrop readings of DNA concentration and A260/A280 and A260/A230 absorbance ratios. **B.** Standard electrophoresis gel (0.8% agarose in TAE) with lambda phage DNA digested with Hind III as a molecular marker in the first lane. The extracted DNA was diluted with ultra-pure water so that the final concentration of all samples, except *Pistacia lentiscus*, was 100 ng/µL. 1µL of the diluted DNA was loaded for these three samples and 5 µL of *P. lentiscus* DNA were loaded.

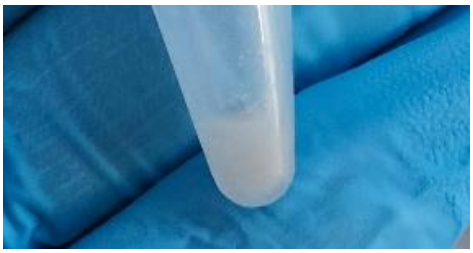

Fig6: Milky-white pellet of *P. lentiscus* DNA in TE buffer after DNA extraction with the second trial of the DNAbsolute protocol (Idylle).

4.4 DNA purification with ammonium acetate and ethanol after DNAbsolute extraction

Due to the anomalous absorbance ratios observed in the first trial of the DNAbsolute protocol (Fig 4B), we tried to precipitate the DNA with ammonium acetate and absolute ethanol to attempt to remove contaminants and obtain pure DNA, as acetate salts help to remove carbohydrates (Tel-Zur et al., 1999). Overall, improvement is seen in the absorbance ratios, mainly A260/A280 of *S. lycopersicum* and *L. crithmoides.* However, the A260/A230 ratios are all still < 2, the closest being *S. lycopersicum* and one of the *Pistacia* samples. Additionally, there is a considerable decrease in DNA concentrations, as is seen by the much lower intensity of the bands in the gel (Fig7B).The very high molecular bands that were in unresolved in the gels seem to have disappeared (with the exception of *S. lycopersicum* 2) suggesting that, indeed, they were caused by the presence of contaminants bound to DNA. *P. lentiscus* DNA is still not seen in the agarose gel in Figure, even though 12 and 15 µL are loaded in the respective lanes for samples 1 and 2.

These samples are not appropriate for long read sequencing, as the absorbance ratios are still not ideal and the high molecular weight bands are not present.

A.

Sample	[DNA] (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
S.lycopersicum1	88.88	1.86	1.56
S.lycopersicum 2	66.46	1.91	1.45
L.crithmoides 1	130.57	1.54	0.77
L.crithmoides 2	31.10	1.41	0.91
P.lentiscus 1	6.43	1.38	1.49
P.lentiscus 2	8.39	0.93	0.80
P.angustifolia	23.08	1.55	0.84

B.

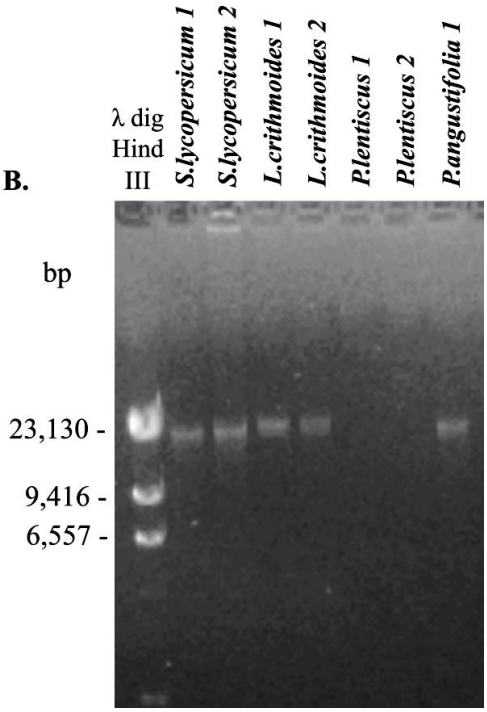

Fig7: Spectrophotometric quantifications and agarose gel after DNA extraction of *S. lycopersicum, L. crithmoides, P. lentiscus* and *P. angustifolia* with the DNAbsolute kit (Idylle) protocol and subsequent DNA precipitation with a standard ammonium acetate and absolute ethanol purification. A. Qubit and Nanodrop readings of DNA concentration and absorbance ratios. **B.** Electrophoretic analysis of DNA (0.8% agarose gel in TAE1x) ran at 10V overnight with lambda phage DNA digested with Hind III as a molecular marker in the first lane. The sample volume loaded in the agarose gel was decided based on the Nanodrop quantification values, aiming to load 100ng of DNA.

4.5 Comparison of CTAB and DNAbsolute protocol mix against SILEX protocol

Due to the unsuccessful trials of DNA extraction, a CTAB and DNAbsolute protocol mix was tested against a normal SILEX extraction for *P. angustifolia, P. lentiscus, R. alba* and *R. hookeri.*

In general, with the results of the modified CTAB and DNAbsolute protocol, in Figure 8B, it is apparent in the gel that the mixed protocol results in DNA with less integrity and more fragmentation than the normal SILEX protocol. Unresolved bands above 50 kbp are present for almost all samples, except *P. lentiscus,* and bands around 50kbp with some smears are also present for some samples.

An improvement is seen in the Nanodrop absorbance ratios compared to the previous assays, as the A260/A280 ratios are closer to 1.8 and the A260/A230 ratios are closer to 2 (Fig8A). It can be seen that the SILEX samples have better absorbance ratios, as *P. angustifolia* actually has acceptable A260/A280 and A260/A230 ratios, and *R. alba* and *R. hookeri* have better absorbance ratios than with the mixed protocol. However, the fluorescence quantification seems to indicate that overall, more DNA was present with the mixed protocol for all samples except *P. lentiscus*. *P. lentiscus* DNA however, is not visualized in the gel either way and Qubit quantification indicates that < 5 ng/µL of DNA is present.

So, in general, the CTAB and DNAbsolute protocol seems to be able to extract high molecular weight DNA in more quantity but less integrity and quality than the SILEX protocol.

A.

	Qubit	Nanodrop		
Sample	**[DNA] (ng/µL)**	**[DNA] (ng/µL)**	**A_{260}/A_{280}**	**A_{260}/A_{230}**
P.angustifolia S	41.80	86.15	1.88	1.93
P.angustifolia D	44.00	74.93	1.65	1.08
P.lentiscus S	3.40	14.00	1.64	0.61
P.lentiscus D	<0.01	95.53	1.01	0.71
R.Alba S	5.50	204.18	2.09	2.00
R.Alba D	32.20	104.50	1.93	1.32
R.Hookeri S	7.00	160.35	2.04	1.58
R.Hookeri D	13.10	82.23	2.04	0.82
R.Hookeri 2 S	5.50	138.98	2.07	1.58
R.Hookeri 2 D	12.20	71.33	1.87	0.68

B.

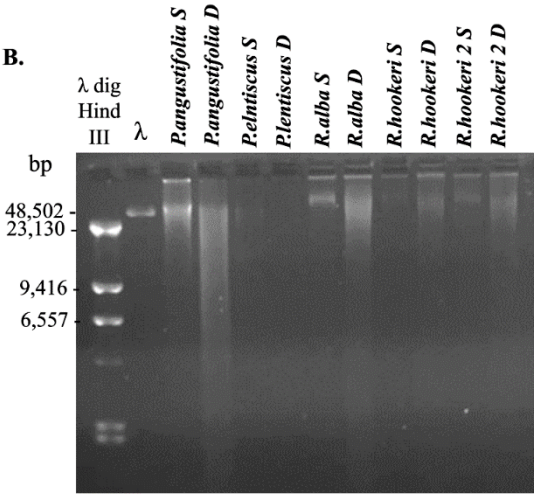

Fig8: Spectrophotometric quantifications and agarose gel after DNA extraction of *P. angustifolia, P. lentiscus, R. alba* and *R. hookeri* with the SILEX = S and modified CTAB protocol mix with DNAbsolute kit (Idylle) = D. A. Qubit and Nanodrop readings of DNA concentration and absorbance ratios. **B.** Electrophoretic analysis of DNA (0.8% agarose gel in TAE1x) ran at 10V overnight with lambda phage DNA digested with Hind III as a molecular marker in the first lane and not digested in the second lane. 5µL of DNA was loaded for each sample.

4.6 Nuclei isolation protocols

As the trials with direct extraction methods were mostly unsuccessful, we decided to try extraction methods based on the isolation of nuclei and a subsequent DNA extraction from the nuclei pellet. These methods have two advantages, first is that keeping the DNA inside the nuclei until the extraction steps helps to obtain longer DNA fragments. At the same time, isolating nuclei helps to get rid of contaminants interfering with the extraction process (i.e., chloroplasts containing carbohydrates or other specialized metabolites present in the cytoplasm) (Lutz et al., 2011). Two procedures for nuclei isolation are tested, a plant nuclei isolation protocol from PacBio and the CellLytic kit (Sigma-Aldrich), to see which would give the best results. As for DNA extraction from the nuclei pellets, two different kits that use magnetic Nanobind disks to specifically bind and isolate the DNA were used (Nanobind and PanDNA from PacBio) as one was discountinued (Nanobind) and substituted by the other (PanDNA), which is supposedly equivalent. The CTAB and SILEX protocols described previously were also used.

4.6.1 Nuclei isolation with PacBio and DNA extraction with Nanobind kit (PacBio)

This protocol is recommended by PacBio, one of the two providers of long-read sequencers. Nuclei were isolated following PacBio's recommendations regarding buffer composition, incubation and centrifugation times (see ANNEX 1.), and then we proceeded to extract the DNA using the Nanobind kit (PacBio).

For the first trial, we used olive leaves (Picual variety) as they had to be extracted for another ongoing project and optimization of the protocol was previously carried out by Dr. Manrique. We decided to try *P. angustifolia* in parallel as it is phylogenetically close to *O. europaea*, so the conditions used could be similar for both plants. After Dr. Manrique's optimization, for *Picual*, 5 samples containing 1g of ground leaves each were processed in parallel and merged after nuclei isolation. In the case of *P. angustifolia*, as a first trial, we decided to start from 1 aliquot containing 1g of plant material.

DNA extraction was done following manufacturers' instructions. As this protocol is potentially able to extract very high molecular weight DNA leading to inhomogeneous solutions, the protocol recommends measuring the concentration from the top, middle and bottom of the tube. Top, middle and bottom Nanodrop measurements show an important disparity with the measurements at the middle seeming to be the best ones. In the case of *P. angustifolia,* the ratios are slightly below of the desired 1.8, but close to it. In the case of *Picual*, the ratios are a bit lower but still, close enough to the threshold value of 1.8.

For all three samples in Figure 9, a high concentration of DNA was obtained (~260 ng/µL) according to Qubit measurements (pipetted from the centre of the tube). Although the concentrations obtained with the Nanodrop do not match with the Qubit concentrations, the difference is of around the 50% which is considered relatively acceptable (discrepancies of >50% in the measures of Nanodrop and Qubit are indicative of high level of contaminants). So, overall, in terms of purity these samples are not ready for long-read sequencing but they are on the right path.

However, in the gel, a smear from ~50kbp to 9kbp can be seen for each sample, which means that DNA integrity is low indicating that sample manipulation can be improved to reduce shearing. Nonetheless, bands of very high molecular weight DNA appearing much higher than 50kbp are seen for all three samples.

A.

	Qubit		Nanodrop		
	[DNA] (ng/µL)	position	[DNA] (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
P. angustifolia	262	top	-11.87	1.56	1.26
		middle	408.65	1.84	1.73
		bottom	-107.45	1.66	1.51
Picual 1	276	top	103.57	2.72	-16.3
		middle	570.72	1.81	1.59
		bottom	572.85	1.79	1.56
Picual 2	262	top	642.66	1.82	1.61
		middle	636.26	1.79	1.61
		bottom	683.56	1.77	1.49

B.

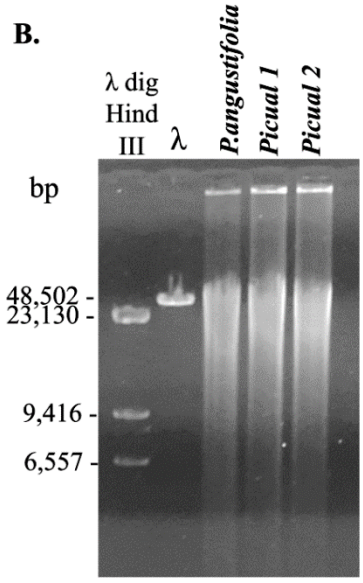

Fig9: Spectrophotometric and fluorometric quantifications and agarose gel after the nuclei isolation following the PacBio protocol and DNA extraction of *P. angustifolia* and *Picual* with the Nanobind kit (PacBio). A. Qubit and Nanodrop readings of DNA concentration and absorbance ratios. **B.** Electrophoretic analysis of DNA (0.8% agarose gel in TAE1x) ran at 10V overnight with lambda phage DNA digested with Hind III as a molecular marker in the first lane and not digested in the second lane. 1µL of DNA was loaded for each sample.

A second trial of nuclei isolation and DNA extraction with the Nanobind kit (PacBio) was performed, where *P. angustifolia, R. alba* and *R. hookeri* were evaluated and the DNA concentration obtained is reduced significantly (Figure 10). For *P. angustifolia*, which is evaluated twice, the concentration is more than halved in this trial (the only difference is the centrifugation speed is 7000g instead of 3000g). However, the UV/Vis absorbance ratios seem to have improved and are much closer to ideal values.

For the *R. alba* and *R. hookeri*, the concentration obtained is ~20 ng/µL, according to fluorometric measurements. The *Resedaceae* prove to be difficult in DNA extractions as they produce a mucilaginous material that is hard to remove, and in trying to remove this material, some DNA may also be discarded. The Nanodrop ratios for *R. alba* and *R. hookeri* are further from the ideal but still better than the previous trials. In the agarose gel, very high intensity high molecular weight bands above 50 kbp and around 50kbp are observed for all samples. This suggests that these samples are closer to the standard for long read sequencing, but some cleaning is required to remove contamination and improve the ratios.

A.

Sample	Qubit [DNA] (ng/µL)	Nanodrop position	Nanodrop [DNA] (ng/µL)	Nanodrop A_{260}/A_{280}	Nanodrop A_{260}/A_{230}
P. angustifolia	92.8	top	320.39	1.89	2.36
		middle	320.07	1.90	2.23
		bottom	326.21	1.84	2.22
R. alba	21.6	top	22.31	1.63	1.47
		middle	15.83	5.17	5.97
		bottom	17.51	1.15	-1.27
R. hookeri	24.8	top	5.55	1.39	0.64
		middle	53.80	1.42	0.80
		bottom	25.92	1.42	1.01
R. hookeri 2	8.07	top	9.46	2.14	2.27
		middle	12.07	1.63	1.33
		bottom	11.72	2.58	1.62

B.

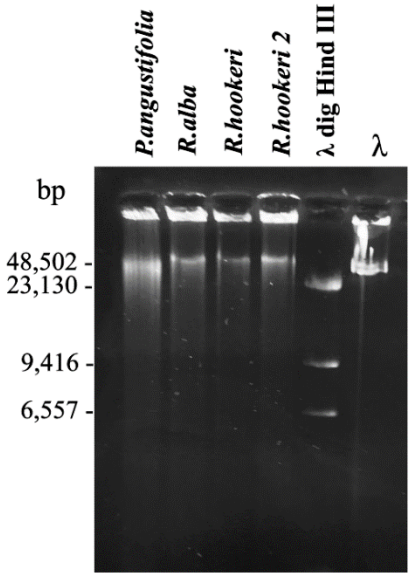

Fig10: Fluorometric (Qubit) and spectrophotometric (Nanodrop) quantifications and agarose gel after the nuclei isolation following the PacBio protocol and DNA extraction of *P. angustifolia, R. alba* and *R. hookeri* with the Nanobind kit (PacBio). A. Qubit and Nanodrop readings of DNA concentration and absorbance ratios. **B.** Electrophoretic analysis of DNA (0.8% agarose gel in TAE1x) ran at 10V overnight with lambda phage DNA digested with Hind III as a molecular marker in the fifth lane and not digested in the last lane. The volume of DNA loaded for each sample was calculated according to Qubit fluorometric readings.

4.6.2 Nuclei isolation with PacBio and HMWDNA extraction with PanDNA kit

In this trial, the *P. lentiscus* and *R. hookeri* DNA was evaluated after nuclei extraction (PacBio) and HMWDNA extracted from one nuclei pellet for each sample (ca. 1g of starting material). The PanDNA kit was used because the previous Nanobind kit was discontinued, however they are both supposedly interchangeable. For both samples, very low concentrations of DNA were obtained, as can be seen in Figure 11. For *P. lentiscus,* the Qubit quantification estimates a DNA concentration lower than 1 ng/µL and no bands are present in the agarose gel, suggesting very low concentration or none at all. For *R. hookeri,* the estimated concentration is about 6 ng/µL and a high molecular weight band is present in the gel at ~50 kbp. The Nanodrop A260/A230 ratios are all below 1 and the estimated concentrations do not match with the fluorometric DNA concentrations. The anomalous ratios may indicate high levels of contaminants or may be due to the very low concentrations of DNA, being close to the lower detection limit of the Nanodrop apparatus (Koetsier et al., 2019).

A.

		Qubit	Nanodrop		
Sample	**Position**	**[DNA] (ng/µL)**	**[DNA] (ng/µL)**	**A_{260}/A_{280}**	**A_{260}/A_{230}**
P.lentiscus	top	0.64	5.5	1.21	0.38
	middle	0.64	4.1	1.67	0.33
	bottom	0.525	3.8	2.89	0.22
R.hookeri	top	5.24	14.9	1.71	0.48
	middle	2.86	15.4	1.78	0.68
	bottom	6.61	15.1	1.59	0.53

B.

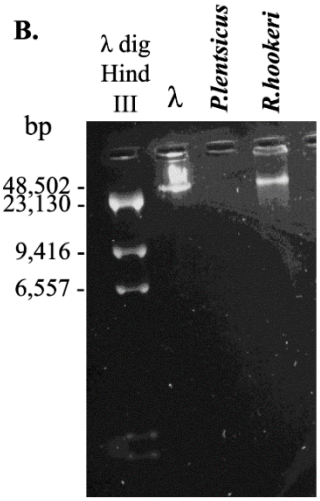

Fig11: Spectrophotometric quantifications and agarose gel after the nuclei isolation following the PacBio protocol and DNA extraction of *P. lentiscus* and *R. hookeri* with the PanDNA kit (PacBio). A. Qubit and Nanodrop readings of DNA concentration and A260/A280 and A260/A230 absorbance ratios. **B.** Electrophoretic analysis of DNA (0.8% agarose gel in TAE1x) ran at 10V overnight with lambda phage DNA digested with Hind III as a molecular marker in the first lane and not digested in the second lane. 15 µL of DNA was loaded for each sample.

For a second try with this protocol, to attempt to increase the DNA concentration, 3 nuclei pellets (from 0.5-1g of starting material) isolated from young leaves of *R. hookeri* were used for the DNA extraction with the PanDNA kit and the supernatants of the three samples were pooled after the lysis, to obtain a higher volume sample. As can be seen in Figure 12, the concentration of DNA according to fluorometric measurements increases by ~4-5X with respect to the previous trial where only 1 nuclei pellet was used. Moreover, there seems to be less contamination as the spectrophotometric and fluorometric DNA concentrations are similar. However, in the gel (Fig.12B), a smear is observed, and the band has low intensity, whereas in the previous test, a clean band was present. This suggests that by increasing the number of pellets, more DNA can be obtained but with less integrity.

A.

		Qubit	Nanodrop		
Sample	**position**	**[DNA] (ng/µL)**	**[DNA] (ng/µL)**	**A_{260}/A_{280}**	**A_{260}/A_{230}**
R.hookeri	top	20.2	30.9	1.62	0.91
	middle	26.4	35.8	1.63	1.06
	bottom	21.2	56.2	1.42	0.68

B.

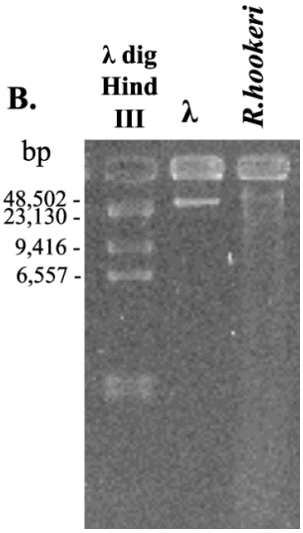

Fig12: Spectrophotometric quantifications and agarose gel after the nuclei isolation following the PacBio protocol and DNA extraction of *R. hookeri* with the PanDNA kit (PacBio). A. Qubit and Nanodrop readings of DNA concentration and A260/A280 and A260/A230 absorbance ratios. **B.** Electrophoretic analysis of DNA (0.8% agarose gel in TAE1x) ran at 10V overnight with lambda phage DNA digested with Hind III as a molecular marker in the first lane and not digested in the second lane. 15 µL of DNA were loaded.

Due to the low concentrations of DNA obtained from the previous tests, the following trial was done with different numbers of nuclei pellets to evaluate if the concentration of extracted DNA could be increased. In figure 13, it can be seen that overall, most of the samples have high molecular weight bands at around 48.5 kbp, and *P. angustifolia, L. crithmoides* and *R. alba*, have even higher molecular weight unresolved DNA.

For *P. angustifolia*, a concentration of around 80 ng/µL DNA was obtained with 4 nuclei pellets and the A260/A280 and A260/A230 absorbance ratios are close to the ideal. This sample may be ready for long-read sequencing. However, with the Nanobind kit, a similar quantity of DNA was obtained from only 1 nuclei pellet (Fig10). Even though the Nanobind and PanDNA kit are supposed to be interchangeable, these results may indicate that the Nanobind kit performs better in the species tested. Regarding the DNA integrity, a large smear can be seen in the gel for both *P. angustifolia* and *R. alba* DNA. For *P. angustifolia,* this could be due to the large volume of DNA loaded (10 µL), as when 1 µL was loaded, this smear is less visible, and the integrity is acceptable. On the other hand, for the *R. alba,* the smear could be due to shearing from sample manipulation or nucleases degrading the DNA.

For *P. lentiscus*, even though 5 nuclei pellets were used, the concentration of extracted DNA remained low (under 4 ng/µL) according to fluorometric readings. This was visualized in the gel as a high molecular weight band of around 48.5 kbp (Fig13B) and the absorbance ratios are anomalous, as is expected due to the low concentration of DNA (this is a trend that is being repeated throughout the experiments).

In the case of *L. crithmoides*, the DNA concentration estimated by Qubit was even lower than *P. lentiscus* and that is reflected in the gel as the DNA band has lower intensity, however it is of higher molecular weight than *P. lentiscus,* as it is unresolved in the well, above 50 kbp. The low DNA concentration may be caused by the fact that only one nuclei pellet was used, due to low sample availability. If more nuclei pellets were pooled for *L. crithmoides*, maybe a higher DNA concentration and better Nanodrop ratios could be obtained. For *R. alba* and *R. hookeri*, 2 and 4 nuclei pellets are used, respectively. However, even though less pellets are pooled for *R. alba,* the DNA concentration is greater than *R. hookeri.* The Nanodrop absorbance ratios for *R. alba* are far from the ideal except for the middle A260/A280 ratio, which is close to 1.8. This might be due to the low DNA concentration observed. If more pellets were used, then maybe these ratios could be improved upon. In the case of *R. hookeri,* a very low DNA concentration was observed, even though 4 nuclei pellets were used, additionally, in the gel only a faint band at 48.5kbp is observed, which is worse than the previous trials, where more DNA concentration was obtained with 1 or 3 nuclei pellets (Fig11 and 12).

A.

Sample	Nº of nuclei pellets	position	Qubit [DNA] (ng/µL)	Nanodrop [DNA] (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
P.lentiscus	5	top	2.18	5.1	2.82	0.48
		middle	3.13	5.7	2.24	0.68
		bottom	3.02	5.5	3.75	0.62
P.angustifolia	4	top	76.8	147.9	1.83	1.88
		middle	83.2	144.9	1.85	1.87
		bottom	89.2	151.8	1.80	1.90
L.crithmoides	1	top	1.51	8.6	1.69	0.26
		middle	0.774	9.6	2.01	0.32
		bottom	1.89	15.7	1.54	0.39
R.alba	2	top	2.88	5.6	6.58	0.46
		middle	14.2	30.0	1.89	1.07
		bottom	18.9	72.3	1.38	0.71
R.barrelieri	4	top	1.94	8.3	1.80	0.09
		middle	3.24	6.8	4.20	0.40
		bottom	3.50	7.7	2.19	0.45

B.

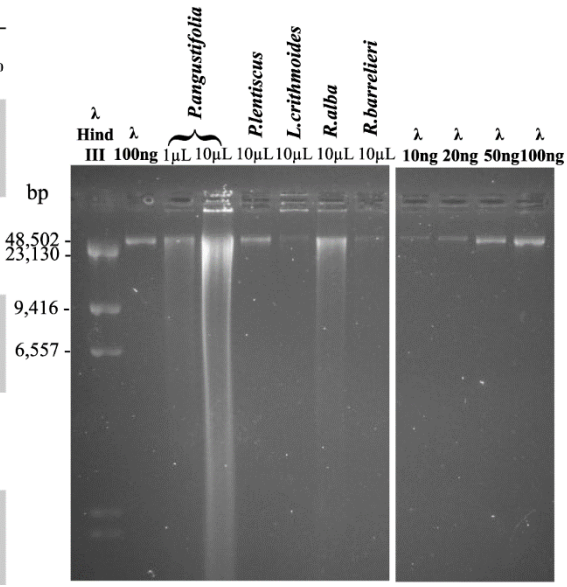

Fig13: Fluorometric (Qubit) and spectrophotometric (Nanodrop) quantifications and agarose gel after the nuclei isolation with the PacBio protocol and DNA extraction of *P. lentiscus, P.agustifolia, L. crithmoides, R. alba* and *R. hookeri* with the PanDNA kit (PacBio). A. Qubit and Nanodrop readings of DNA concentration and A260/A280 and A260/A230 absorbance ratios. B. Electrophoretic analysis of DNA (0.8% agarose gel in TAE1x) ran at 10V overnight with lambda phage DNA digested with Hind III as a molecular marker in the first lane and not digested in the last 4 lanes, with 10ng, 20ng, 50 ng and 100ng of DNA. 10 µL of DNA were loaded for each sample.

4.6.3 Nuclei isolation with PacBio and DNA extraction with SILEX and CTAB

To test whether the Nanobind/PanDNA kit are necessary for the procedure or, once nuclei are isolated, other simpler/more economic protocols can also be applied, we tried combining nuclei isolation with CTAB/SILEX protocols. In the following trial, the nuclei pellets of *P. lentiscus* and *R. hookeri* were isolated with the PacBio protocol and then, DNA extraction protocols tested were SILEX and CTAB.

Overall, the SILEX protocol for DNA extraction from the nuclei had better results than the CTAB protocol, in terms of DNA concentration and absorbance ratios, although the A260/A230 ratio is still not good (Fig 13A). In the gel (Fig13B), the SILEX samples have higher intensity bands of about 48.5 kbp, whereas for the CTAB samples, the bands are not present in *P. lentiscus* and are very subtle (have low intensity/concentration) in the case of *R. hookeri* also around 48.5kbp. The two species tested are the most challenging so far, so in comparison to the previous trials, similar concentrations were obtained.

A.

		Qubit	Nanodrop		
Sample	**DNA extraction**	**[DNA] (ng/µL)**	**[DNA] (ng/µL)**	**A_{260}/A_{280}**	**A_{260}/A_{230}**
P.lentiscus	SILEX	12.9	13.3	1.69	0.73
	CTAB	0.516	8.9	2.18	50.79
R.hookeri	SILEX	10.7	5.7	1.96	1.51
	CTAB	1.17	31.1	2.25	2.16

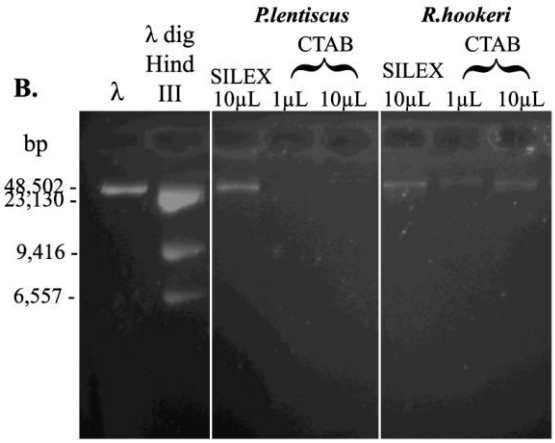

Fig14: Spectrophotometric (Qubit) and fluorometric (Nanodrop) quantifications and agarose gel after the nuclei isolation following the PacBio protocol and DNA extraction with the SILEX or CTAB protocol of *P. lentiscus* and *R. hookeri*. A. Qubit and Nanodrop readings of DNA concentration and absorbance ratios. **B.** 1 Dimensional electrophoresis gel (0.8% agarose in TAE) with lambda phage DNA digested with Hind III as a molecular marker in the first lane and not digested in the second lane. 10 µL of DNA were loaded for the SILEX samples, whereas 1 and 10 µL of DNA were loaded for the CTAB samples to ascertain whether the concentration measured by Nanodrop was reliable.

4.6.4 Nuclei isolation with CellLytic (Sigma-Aldrich) and DNA extraction with PanDNA kit (PacBio)

We also tested an alternative nuclei isolation protocol, using the CellLytic kit (Sigma-Aldrich). The kit was tested with *S. lycopersicum* as control and with the *P. angustifolia* and *P. lentiscus* samples. Different concentrations ranging from 0.3-2% of Triton X-100 were tested with the *P. lentiscus* samples. However, all of the pellets, except that of *S. lycopersicum*, were very green, indicating high level of contamination with chloroplasts. Ultimately, only two nuclei pellets were used for DNA extraction with the PanDNA kit, and 1 nuclei pellet was tested with a DNA precipitation with isopropanol.

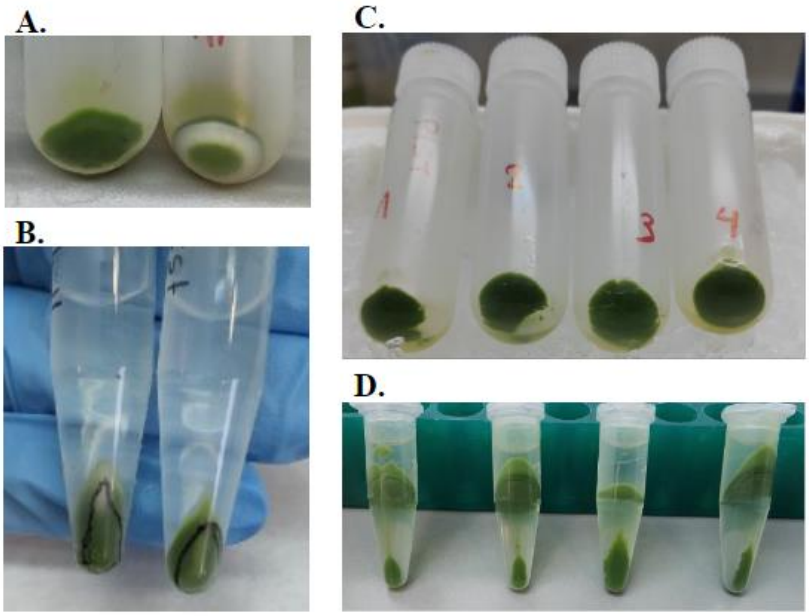

Fig15: Nuclei pellets *of P. lentiscus, S. lycopersicum* and *P. angustifolia* after nuclei isolation with CellLytic kit. A. Pellets after the cell lysis of *P. lentiscus* with 2% TRITON X-100 on the left and *S. lycopersicum* with 1 % TRITON X-100 on the right. **B**. Nuclei pellets of *P. angustifolia* (right) and *P. lentiscus* (left) after cell lysis with 0.6% and 0.3 % TRITON X-100 respectively and nuclei isolation with CellLytic kit. **C.** Pellets of *P. lentiscus* after cell lysis with 0.6%, 0.8%, 1% and 1.2% TRITON X-100 (in order from left to right). **D.** Nuclei pellets after nuclei isolation by centrifugation through sucrose cushion of *P. lentiscus* after lysis with 0.6%, 0.8%, 1% and 1.2% TRITON X-100, respectively.

The CellLytic kit (Sigma Aldrich) was used to isolate the nuclei of *P. lentiscus* and *P. angustifolia* along with a DNA extraction with the PanDNA kit. The spectrophotometric results indicated that a low concentration of DNA was present in both samples (~3-4ng/µL), however the Qubit was not able to detect DNA and no bands are present in the agarose gel (Figure 15). Therefore, the Nanodrop concentration readings are not reliable and could be due to contaminants or other factors. We can conclude that no DNA was present and this protocol is not suitable for DNA extraction of these species.

A.

Sample	position	Qubit [DNA] (ng/µl)	Nanodrop [DNA] (ng/µl)	Nanodrop A_{260}/A_{280}	Nanodrop A_{260}/A_{230}
P.lentiscus	top	<o.o1	4.0	1.42	0.09
	middle	<o.o1	4.0	3.68	0.18
	bottom	<o.o1	3.1	2.43	0.16
P.angustifolia	top	<o.o1	3.3	1.04	0.37
	middle	<o.o1	4.3	1.05	0.26
	bottom	<o.o1	3.0	1.62	0.21

B.

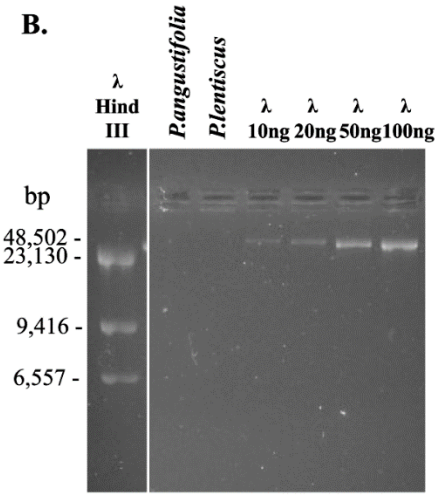

Fig16: Fluorometric (Qubit) and spectrophotometric (Nanodrop) quantifications and agarose gel after the nuclei isolation with the CellLytic kit (Idylle) and DNA extraction of *P. lentiscus* and *P. angustifolia* with the PanDNA kit (PacBio). A. Qubit and Nanodrop readings of DNA concentration and absorbance ratios. **B.** Electrophoresis gel (0.8% agarose in TAE) with lambda phage DNA digested with Hind III as a molecular marker in the first lane and not digested in the last 4 lanes, with 10ng, 20ng, 50 ng and 100ng of DNA. 10 µL of DNA were loaded for each sample.

4.6.5 Nuclei isolation with CellLytic (Sigma-Aldrich) and DNA precipitation with isopropanol

A nuclei isolation with CellLytic kit and a subsequent DNA precipitation with isopropanol for *P. lentiscus* was also tested, but no quality DNA was extracted, as seen with the anomalous absorbance ratios A260/A280 of 1.11 and A260/A230 of 0.37. The estimated DNA concentration by the Nanodrop was 23 ng/µL, however, we have previously observed that concentration estimates with such anomalous ratios are not reliable.

4.7 Evaluation of extracted DNA by PFGE

In order to better compare the most representative samples produced along this work, after the different trials, we analysed such samples by pulsed-field gel electrophoresis (PFGE) (Fig17).

The results indicate that in most cases, the nuclei isolation protocols seem to generate higher concentrations of DNA (except for *P. lentiscus*) as is seen by the higher intensity of bands in (Fig17). For most of the samples, high molecular weight bands are present above 48.5 kbp, however, smears are also seen for most. This could be due to DNA degradation during storage and defrosting or to sample contamination of nucleases or other compounds. In the case of, *L. crithmoides* and *R. barrelieri,* not enough DNA was extracted for visualization in the gel with any of the protocols (Fig17). With *P. angustifolia,* we can see that the original Nanobind kit seems to perform better than the PanDNA kit (PacBio), although they are supposed to be interchangeable. Even though 4 nuclei pellets were used for the PanDNA extraction, the extraction with the Nanobind kit with 1 nuclei pellet shows bands of higher intensity, indicating higher concentration.

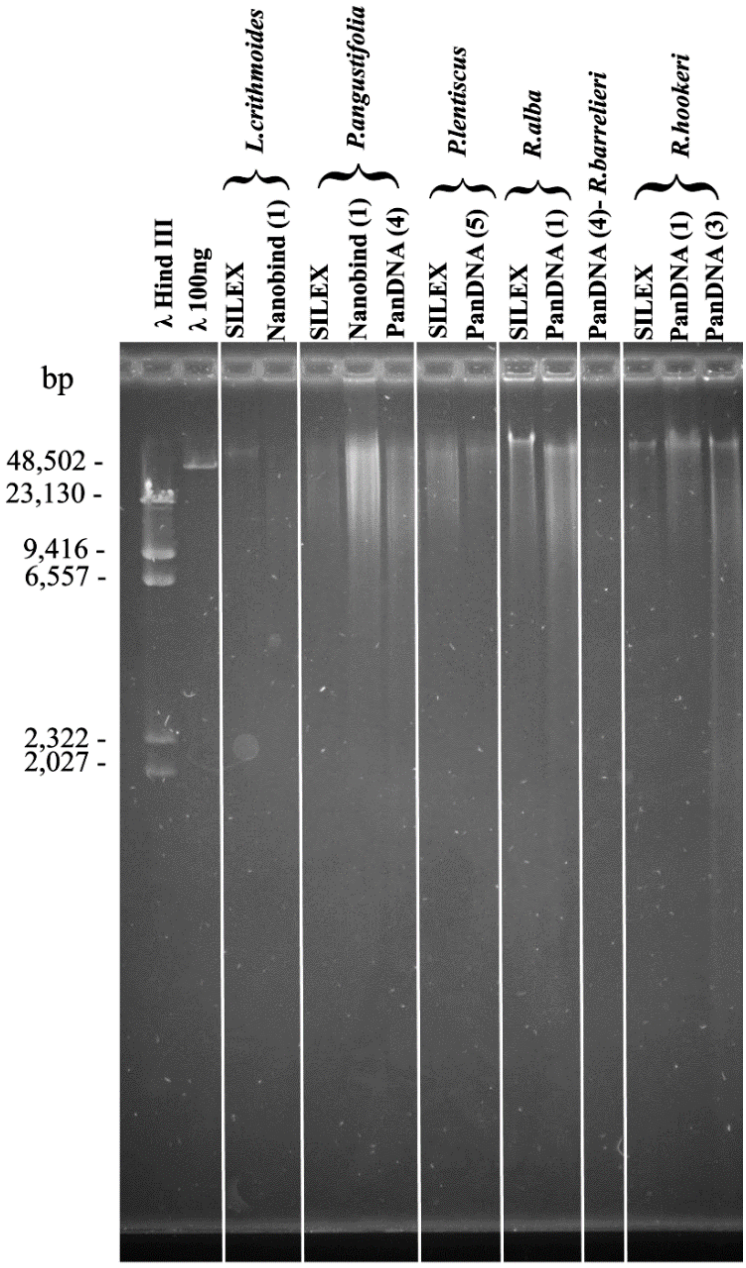

Fig17: Final pulsed-field agarose gel electrophoresis analysis after DNA extraction of *L. crithmoides*, *P. angustifolia, P. lentiscus, R. alba, R. barrelieri* and *R. hookeri* with the SILEX and PacBio nuclei isolation with either Nanobind or PanDNA kit (PacBio). Pulsed-field gel electrophoresis gel (1% agarose gel in 0.5X TBE) ran at 100V, 80mA with pulse intervals of 3 s for 16h. Lambda phage DNA digested with Hind III was loaded as a molecular weight marker in lane 1 and undigested in lane 2. 10 µL was loaded for every sample except the ones corresponding *P. angustifolia,* where only 1 µL was loaded per sample. The DNA extraction method is labelled for each sample and the number in brackets in the nuclei isolation protocols indicates the nº of nuclei pellets used as starting material for the DNA extraction.

DISCUSSION

The main objective of this work was to extract HMW DNA of high quality and enough quantity for long-read sequencing and generation of reference genomes.

The plant species tested were wild plants mainly present in the Mediterranean Basin that are adapted to the high temperatures and drought conditions of the Mediterranean summer (Álvarez et al., 2019; Gucci et al., 1997; LEPART & DOMMÉE, 1992; Peñuelas et al., 2004; Tattini et al., 2006; Zurayk & Baalbaki, 1996) and 2 of them, *L. crithmoides* and *P. angustifolia,* are halophytes (Gucci et al., 1997; Zurayk & Baalbaki, 1996). These are very recalcitrant plants that pose many problems in the process of DNA extraction.

We tested a total of six protocols for DNA extraction. These included four direct DNA extraction methods: CTAB, SILEX, DNAbsolute, and a modified CTAB protocol using DNAbsolute reagents and two nuclei isolation protocols: PacBio and CellLytic (Sigma Aldrich), followed by DNA extractions using SILEX, CTAB, or specific kits designed for DNA extraction from nuclei (Nanobind and PanDNA from PacBio), with various modifications.

BME (β-mercaptoethanol) is added as a reducing agent to nuclei extraction buffers to prevent oxidation and co-precipitation of polyphenols with DNA. It also reduces disulfide bonds in proteins, which helps to denature and solubilize them so they can be removed in the following steps (Heikrujam et al., 2020). Most protocols use between 0.2–0.5% of BME in DNA extraction buffers (Schenk et al., 2023) and according to the literature, increasing the % of BME can result in higher yields of cleaner DNA (Larridon et al., 2020; Schenk et al., 2023; Silva, 2010). Polyvinylpyrrolidone (PVP) is a high molecular weight polymer that forms hydrogen bonds with polyphenolic compounds and prevents their oxidation facilitating their removal (Loomis & Battaile, 1966; John, 1992). PVP is commonly used at 1–2.5% in the lysis or suspension buffer (Cullings et al.,1992; Sahu et al., 2012, Michiels et al., 2003; Arruda et al., 2017).

Therefore, for the first 2 trials with the PacBio nuclei isolation protocol (Figures 9 and 10), the recommended 0.25% of BME and 1% PVP360 was used, but later we decided to test doubling the concentrations to 0.5% BME and 2% PVP360 in the following assays, to evaluate if the purity of extracted DNA increased. However, ultimately, the effect of this change in concentrations cannot be reliably compared, as for the first 2 trials, the Nanobind kit was used, whereas for the rest of the trials, the PanDNA kit was used. These are supposed to be interchangeable, but the results of the first 2 trials show the highest concentration of extracted DNA, even with only 1 nuclei pellet per sample as starting material (Figures 9 and 10), whereas the rest of the extractions with the PanDNA obtained lower DNA concentrations (Figures 11, 12, 13 and 17) suggesting that the Nanobind kit provides higher yields than the PanDNA kit.

According to the literature, a darkness pre-treatment of the plants before DNA extraction results in less contaminants and higher purity of the DNA, as the photosynthetic products are reduced (Li et al., 2020). Additionally, it is hypothesized that young, rapidly expanding leaves have less accumulated secondary metabolites than older leaves, therefore resulting in higher quality extracted DNA (Lodhi et al., 1994; Mauro et al., 1992).

We attempted using young leaves from plants after 48-72 hours of dark treatment and old leaves without such treatment for nuclei extraction to test whether the age of the leaf and dark treatment would increase the yield or purity of extracted DNA. However, in the end, the low yields of DNA extraction with the PanDNA kit from 1 nuclei pellet (Fig11) forced us to ultimately combine pellets from young and old leaves to be able to obtain a significant concentrations of DNA in the following assays. These modifications should be further tested for these species.

In most cases, the A260/A280 and A260/A230 absorbance ratios provided by the Nanodrop were far from the ideal for pure DNA (1.8 and 2 respectively) for most of the samples and the concentrations estimated by the Nanodrop were often inconsistent with the fluorometric Qubit readings. A possible explanation for such discrepancies is the presence of contaminants absorbing at 260, 280 and 230 nm. Additionally, in most cases, DNA concentrations were close to the Nanodrop lower detection limit, rendering the absorbance ratios unreliable (Koetsier et al., 2019). In the agarose electrophoresis gels, unresolved DNA was observed in the wells. These bands could not be resolved in PFGE gels either, suggesting that DNA mobility might be impaired by the presence of contaminants rather than by DNA size. This is also supported by the fact that after precipitation of DNA with ammonium acetate, these bands disappeared (Fig7). In addition, bands at around 50kbp and 20kbp were also observed. For the purpose of long-read sequencing, the preferred molecular weight of DNA in this case was 50kbp or higher so protocols producing 20 kbp bands (DNAbsolute) are discarded for this application. Smears were also present in some of the gels, suggesting contamination or low integrity of DNA. This can be due to left over nucleases degrading the DNA or shearing by manipulation of the sample with pipettes.

The problems presented by these recalcitrant plants are often species or genus-specific, due to the differences in their secondary metabolites (Varma et al., 2007). This can be seen in the different performances of each protocol for each of the species tested.

For example, the *Resedas* (*R. alba, R. barrelieri* and *R. hookeri)* generated a mucilaginous material when the leaf powder was mixed with a solution, be it CTAB or nuclei extraction buffer. This viscous material is indicative of high levels of carbohydrates, which make pipetting and filtering more difficult (Fang et al., 1992; Porebski et al., 1997). In relation to this, we have observed that the DNA extraction from Resedas in general, was not very successful. In the case of *R. alba*, the maximum DNA concentration obtained was 32.30 ng/µL with the DNAbsolute-CTAB mixed protocol (Fig 8), although the Nanodrop A260/A230 ratio indicated contamination of the sample. Another sample for this species was the extraction with the nuclei isolation (PacBio) and DNA extraction with the Nanobind disk kit (PacBio), which also performed well with *R. hookeri*, yielding 21.6 ng/µL for *Reseda alba* and 24.6 ng/µL DNA for *R. hookeri* (Fig 10). Unresolved HMW DNA in the well and bands at 48.5kbp for both species were also present in the gel (Fig 10B). However, the Nanodrop ratios for both are still far from ideal, so a purification of some kind should be done for these samples to be used in long-read sequencing. For *R. hookeri,* the trial with the nuclei isolation (PacBio) and DNA extraction with the PanDNA kit (PacBio) with 3 pellets also performed well. This extraction also yielded about 20ng/µL of DNA. In the case of *R. barrelieri,* due to limitations with sample availability, only one protocol could be tested, and as the best-performing protocol seemed to be the nuclei isolation with PacBio and DNA extraction, this was tested. Even though this protocol

was performed with 4 nuclei pellets to increase the possible DNA concentration, the final yield of DNA was < 4 ng/µL and a very light band is seen in the gel (Fig13). For this species, more tests are needed for successful DNA extraction.

The most successful DNA extractions were produced from *P. angustifolia,* yielding high concentrations of DNA with the SILEX protocol of about 56 ng/µL (Fig2) and even higher with the nuclei isolation protocols, where the concentration of DNA ranged from 80-262ng/µL (Figures 9, 10 and 13).The best absorbance ratios are also seen with this species, specifically in the nuclei isolation and DNA extraction of 4 nuclei pellets (Fig 13), where ideal ratios are present. This DNA sample is appropriate for long read sequencing.

On the contrary, the least successful DNA extractions were produced from *P. lentiscus,* which usually yielded less than 5 ng/µL of DNA, with the exception of the SILEX protocol, where 11.10 ng/µL of DNA were obtained (Fig2A). *Pistacia lentiscus* presented distinct challenges due to its high concentration of polyphenolic compounds (Salhi et al., 2019; Saliha et al., 2013; Sehaki et al., 2023). Oxidized polyphenols bind to DNA irreversibly and cause enzymatic browning of the pellet, rendering it useless for downstream applications (Lodhi et al., 1994; Peterson et al., 1997; Porebski et al., 1997; Puchooa & Khoyratty, 2004). However, DNA has been successfully extracted from this species previously, with a modified CTAB protocol involving 5g of leaf tissue and 20mL of CTAB, where after the isopropanol precipitation, DNA was recovered with a glass hook and then cleaned (Hormaza et al., 1994). This option was not tested as the amount of material we had available was limited but considering the poor performance of all the protocols tested, testing this could be considered for future extractions of this species.

Finally, *L. crithmoides* also produced low yields of DNA in general and was not tested as thoroughly as *Pistacia* of *Phillyrea* because of low sample availability, as this species was collected from the wild. Similarly to *P. lentiscus*, *L. crithmoides* exhibits high levels of polyphenols (Bucchini et al., 2013; Ksouri et al., 2011), which could explain the low yields of contaminated DNA that was extracted. However, some DNA could be extracted with the SILEX protocol, about 10 ng/µL (Fig2). Although the nuclei isolation protocol seemed to work better for some of the other species, for *Limbarda crithmoides*, the maximum yield obtained with the PacBio nuclei extraction and DNA extraction from 1 nuclei pellet was 1.89ng/µL (Fig13A). As with the *R. barrelieri,* more testing needs to be done for DNA extraction of this species.

As for future work, for the Resedaceae, protocols specific for removing polysaccharides could be tested, due to their characteristic of producing viscous solutions. For example, washes with sorbitol before extraction may help remove cellular debris and contaminants before isolating the DNA without damaging the nuclei (Inglis et al., 2018; Jones et al., 2021). In the literature, DNA has been extracted from Reseda with the DNeasy Plant Mini Kit (Qiagen, California, USA) and the NucleoSpin Plant-Kit (Macherey-Nagel) (Al-Qurainy et al., 2021; Martín-Bravo et al., 2007).

In the case of *P. lentiscus,* as was mentioned before, the high levels of polyphenols in this species are one of the main challenges for the DNA extraction, therefore protocols that remove polyphenol contamination could be successful. Additionally, the modified CTAB with higher amounts of starting

material mentioned by Hormaza et al. (1994) could also be tested if enough material can be sampled.

In conclusion, the only species which yielded sufficient DNA of high quality and quantity for long-read sequencing was *P. angustifolia*, with the PacBio nuclei isolation and DNA extraction protocols. For the rest of the species, further testing and optimization is needed.

CONCLUSIONS

- Six protocols for DNA extraction were tested (4 direct DNA extraction protocols: CTAB, SILEX, DNAbsolute, a modified CTAB protocol using DNAbsolute and 2 nuclei isolation protocols: PacBio and CellLytic), with various combinations and modifications and the yield and purity of extracted DNA of each of the species was evaluated.
- The DNA samples with the highest quantity and purity was produced from *P. angustifolia* after nuclei isolation with PacBio and DNA extraction with Nanobind or PanDNA kits (PacBio) and are the only ones appropriate for long-read sequencing.
- In general, most DNA extractions had low quantity and quality, but nuclei isolation protocols performed better than direct DNA extraction methods, with few exceptions.

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ANNEX 1: Comprehensive List of Equipment, Disposables, Commercial Kits, Reagents, and Buffer Recipes for HMW DNA Extraction and Analysis

Index:

1. Equipment:

- Precision digital balance
- Thermoblock
- Vortex-Genie™ 2 (Scientific Industries SI™)
- Refrigerated tabletop micro-centrifuge
- Refrigerated centrifuge (with JA-14 rotor for 50 mL tubes)
- Orbital Shaker
- Thermomixer
- HulaMixer™ Sample Mixer
- Magnetic tube rack (DynaMag™-2 Magnet)
- NanoDrop 1000 Spectrophotometer (Thermo Fisher)
- Qubit 3.0 Fluorometer (Thermo Fisher)
- Optical microscope
- BioRad Gel electrophoresis system
- Pulsed-field gel electrophoresis (PFGE) system: Gene Navigator™ System (Amersham Biosciences, USA).

2. Disposables:

- Scalpel
- Cutting board
- 50 mL conical tubes (ThermoFisher)
- Liquid Nitrogen LN2
- Porcelain mortar and pestle
- P10, P200, P1000 pipettes

- Pipette tips, 10 µl, 200 µl, 1000 µl
- Filter cloth (100 µm)
- Steriflip (20 µm pore size, 50 mL process volume) (Merck)
- Small nylon or synthetic paintbrush
- 1.5 mL Protein LoBind micro-centrifuge tubes (Eppendorf)
- Qubit™ Assay Tubes

3. Commercial Kits:

- DNAbsolute (Idylle)
- Nanobind plant nuclei big DNA kit (PacBio®)
- NANOBIND® PANDNA KIT (PacBio®)
- CelLytic™ PN Isolation/Extraction Kit (Sigma-Aldrich)
- Qubit™ dsDNA Quantification Assay Kit, Broad Range

4. Reagents:

- RNase A (10mg/mL, VWR)
- 2-mercaptoethanol, 14 M Sigma-Aldrich (M3148)
- TRITON ™ X-100 Sigma-Aldrich (X100)
- Trizma base Sigma-Aldrich (T4661)
- Potassium chloride (KCl) Sigma-Aldrich (P9541)
- Ethylenediaminetetraacetic acid (EDTA), 0.5 M, pH 8.0 ThermoFisher (15575020)
- Spermidine trihydrochloride Sigma-Aldrich (S2501)
- Spermine tetrahydrochloride Sigma-Aldrich (S1141)
- Sucrose Sigma-Aldrich (S0389)
- Sodium hydroxide (NaOH), 10 M Sigma-Aldrich (72068)
- Polyvinylpyrrolidone (MW ~360 kD) (PVP360) Sigma-Aldrich (PVP360)
- Ultra-pure water
- Isopropanol (100%)
- Ethanol (96–100%)
- TE buffer
- DNA Gel Loading Dye (6X) Thermo Scientific™
- GreenSafe Premium DNA gel stain (NZY tech)
- Agarose

5. Buffer recipes:

5.1 SILEX protocol (Vilanova et al., 2021):

- Extraction buffer: 2% (w/v) CTAB, 2% (w/v) PVP-40, 20 mM EDTA, 100 mM Tris HCl (pH 8.0) and 1.40 M NaCl.
- Protein precipitation buffer: 24:1 chloroform: isoamyl alcohol.
- Binding buffer: 2.5 M NaCl and 20% PEG 8000.
- Silica matrix buffer: Mix 5 g of silicon dioxide (SiO_2) with 50 ml of MilliQ water and let stand for 24 h. Discard the supernatant and resuspend the pellet in 50 ml of MilliQ water and wait for another 5 h. Discard the supernatant and resuspend the pellet in 1:1 (v/v) MilliQ water. Finally, add 10 µl of HCl 36% per ml of silica matrix solution obtained.
- Washing buffer: Fresh prepared 70% ethanol.
- Elution buffer: 10 mM Tris HCl (pH 8.0) and 1 mM EDTA (pH 8.0).

5.2 Plant nuclei isolation (PacBio):

- 10X HB Buffer - homogenization buffer (500 mL): Add the following reagents to a clean beaker and stir until dissolved: Trizma 0.1M, KCl 0.8M, EDTA 0.1M, Spermidine 17mM, Spermine 17mM. Bring to the final volume with ultra-pure water and adjust pH to 9 with NaOH. Store at 4ºC for up to 1 year.
- 1X HB Buffer – homogenization buffer (1L): Buffer 10X HB (10%), sucrose 0.5M (171.2g/L). Bring to the final volume with ultra-pure water. Store at 4ºC for up to 3 months.
- TSB Buffer– triton-sucrose buffer (100 mL): Triton X-100 (20%), Buffer 10X HB (10%), sucrose 0.05M. Bring to the final volume with ultra-pure water. Store at 4ºC up to 1 year.
- NIB Buffer – nuclei isolation buffer (500 mL): 97.5% Buffer HB 1X, 2.5% TSB, 1% PVP360. Store at 4ºC for up to one week.

5.3 Electrophoresis buffers:

- 50X TAE Buffer: 242g of Tris-base (MW = 121.14 g/mol) and dissolve in approximately 700 mL of deionized water. Carefully add 57.1 mL of 100 % glacial acid (or acetic acid) and 100 mL of 0.5 M EDTA (pH 8.0). Adjust the solution to a final volume of 1 L and adjust the pH to 8.5.
- 1X TAE working solution: 20 ml 50 x TAE in 1000 ml distilled water.
- 10X TBE Buffer: 108 g tris base, 55 g boric acid, 900 ml double-distilled H2O, 40 ml 0.5 M EDTA solution (pH 8.0)
- 1X TBE: Dilute 100 mL of TBE Buffer, 10x stock solution into 900 mL deionised water to make 1L of TBE Buffer. (Final concentrations are: 89 mM Tris base, 89 mM Borate and 2 mM Na2EDTA.) On dilution to 1X, check pH and adjust as required.