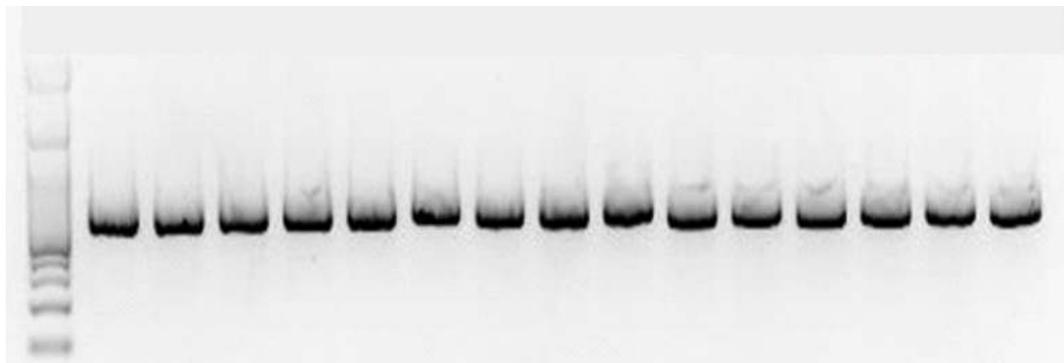




A DNA Extraction Protocol for Wood of *D. latifolia* in Java and West Nusa Tenggara, Indonesia



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Jakarta, Indonesia
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Contents

List of Figures.....	4
ACKNOWLEDGEMENTS	5
ACRONYMS AND ABBREVIATIONS	6
EXECUTIVE SUMMARY	7
1. Introduction	8
2. Sample preparation	8
3. DNA isolation	9
3.1. CTAB methods without RNase	9
3.2. CTAB methods with RNase.....	11
3.3. DNeasy® Plant Mini Kit	12
3.4. Genomic DNA Mini Kit (Plant) GeneAid.....	13
4. DNA quantification	14
5. Verification of the working protocol	15
6. Conclusion.....	16
References	16
Annex 1	17

List of Figures

Figure 1. Sample preparation for wood samples (a) taking the sample, and (b) weighing the sample.....	9
Figure 2. Grinding the wood sample to a fine powder using a mortar and pestle	9
Figure 3. Adding the extraction buffer	10
Figure 4. Homogenizing the sample with buffer extraction.....	10
Figure 5. Incubating the mixtures in a water bath	10
Figure 6. Centrifuging the mixtures	11
Figure 7. Transferring the supernatant to a new microtube.....	11
Figure 8. Chromatogram of DNA sequences from isolated DNA of wood samples	16

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On behalf of the project team,

Dr. Kusumadewi Sri Yulita

Project Team Leader

ACRONYMS AND ABBREVIATIONS

A	Adenin
bp	base pair
C	Cytosin
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CTAB	Cetyl Trimethyl Ammonium Bromide
CTSP	CITES Tree Species Programme
D.	<i>Dalbergia</i>
DNA	Deoxyribo Nucleic acid
G	Guanine
IGS	inter-genic spacer
mg	milligram
mL	millilitre
PCR	Polymerase Chain Reaction
PVP	polyvinylpyrrolidone
RNA	Ribo Nucleic acid
T	Thymine
μL	microliter
°C	degree Celsius

EXECUTIVE SUMMARY

The wood of *D. latifolia*, particularly the heartwood, is very dense, non-porous and very durable therefore suitable as a raw material for furniture, musical instruments, and other handicrafts. The local farmers considered the woodworking properties of *D. latifolia* to be difficult compared to the other timber species, such as teak and mahogany. The need to extract DNA from the wood and wood product become necessary in tracking the traceability of *D. latifolia* in the market chain, particularly when evidence of illegal logging is found.

The extraction of genetic material from wood is considered difficult because the wood contains fewer living cells, especially in dry wood and processed wood products. Hence, it is easier to extract genetic material from leaves. Generally, the wood in trade is not fresh wood and the wood products are from the heartwood, therefore the genetic material from wood is very small in quantity and of low quality. Various techniques to isolate DNA from the wood of timber species are available as reported in many scientific papers. In the case of *D. latifolia* which mainly uses the heartwood, a specific protocol of DNA extraction is required to be developed to obtain a better yield that is sufficient for further DNA analysis. The selection of the proper extraction method can provide better results and improve the extracted DNA quality of the *D. latifolia* wood samples, particularly those extracted from the heartwood.

The purpose of this activity was to develop a protocol for DNA extraction of *D. latifolia*. The selected method to isolate DNA was using the conventional method of CTAB and two commercial kits. The protocol was developed using simple equipment and basic reagents that are easy to find in a standard laboratory. Two commercial kits were selected as they are widely available in the market. It is expected that these minimum requirements of equipment and reagents are applicable and reproducible. The protocol to isolate DNA from the manufacturers was followed with minimum modification, particularly in the preparation steps. The results showed that the best method to isolate the wood and wood products was by using commercial kits. In the absence of commercial kits, the conventional method of modified CTAB can still be used. To improve the results from this protocol, it is important to carry out more experiments by performing PCR optimization and purification of the isolated DNA.

1. Introduction

Dalbergia latifolia (Fabaceae: Papilionaceae), locally known as sonokeling, is a commercial rosewood timber species distributed widely in tropical and subtropical regions in South America, Africa, Asia, and Madagascar. In Indonesia, the species is mainly distributed in Java and West Nusa Tenggara, and may be present in Timor Island, South Sumatra, Kalimantan, and Sulawesi. As a commercial tree species, *D. latifolia* is harvested for use as a raw material for furniture, musical instruments, and other handicrafts. The international trade in *D. latifolia* is regulated by CITES through Appendix II since 2017.

The heartwood of *D. latifolia* is very dense, non-porous and very durable. The local farmers considered the woodworking properties of *D. latifolia* very difficult compared to the other timber species, such as teak and mahogany. The feature of the wood of *D. latifolia* may be due to the production of secondary metabolites that formed complex compounds found in the wood content. The need to extract DNA from the wood and wood product become necessary in tracking the traceability of *D. latifolia* in the market chain, particularly when evidence of illegal logging is found. A DNA database based on DNA sequence has been developed for *D. latifolia* from Java and West Nusa Tenggara which can be used as a reference for forensic study. The development of a DNA extraction protocol for wood of *D. latifolia* in Java and West Nusa Tenggara, Indonesia, is needed to support the forensic tool.

The extraction of genetic material from wood is considered difficult because the wood contains fewer living cells, especially in dry wood and processed wood products. Hence, it is easier to extract genetic material from leaves. Generally, the wood in trade is not fresh wood and the wood products are from the heartwood, therefore, the genetic material from wood is very small in quantity and of low quality. Various techniques to isolate DNA from the wood of timber species are available as reported in many scientific papers. In the case of *D. latifolia* which mainly uses the heartwood, a specific protocol of DNA extraction is required to be developed to obtain a better yield that is sufficient for further DNA analysis. The selection of the proper extraction method can provide better results and improve the extracted DNA quality of the *D. latifolia* wood samples, particularly those extracted from the heartwood. The purpose of this activity was to develop a protocol for DNA extraction for *D. latifolia*. The selected method to isolate DNA was using the conventional method of CTAB and two commercial kits. The protocol was developed using simple equipment and basic reagents that are easy to find in a standard laboratory. Two commercial kits were selected as they are widely available in the market. It is expected that these minimum requirements of equipment and reagents are applicable and reproducible. The protocol to isolate DNA from the manufacturers was followed with minimum modification, particularly in the preparation steps.

2. Sample preparation

Samples of wood or wood products of *D. latifolia* collected directly from field surveys or other places such as the marketplace were cleaned by wiping them from dust/dirt. It is important to ensure that the part taken for the isolation of genetic material is clean. Approximately 60

mg of the heartwood is used for the conventional CTAB method and 20 mg for commercial kits. The wood is scraped into small pieces as the wood cannot be cut because it is very hard (**Figure 1**), before it is grounded to a fine powder. For wood that has been through industrial processes involving chemical treatments, the pieces need to be incubated in a 37°C water bath overnight in an extraction buffer before proceeding with the isolation process.



Figure 1. Sample preparation for wood samples (a) taking the sample, and (b) weighing the sample

3. DNA isolation

Wood samples were isolated for their total genomic DNA using the standard CTAB method (Doyle and Doyle 1990) and commercial kits. The standard CTAB methods comprised two techniques, with and without the addition of RNase. These two techniques are used to check the amount of contamination of RNA in the wood. The commercial kits used are the DNeasy® Plant Mini Kit from QIAGEN and the Genomic DNA Mini Kit (Plant) from GeneAid. A diagram of the workflow of DNA isolation of wood from *D. latifolia* is in **Annex 1**. The following is a detailed description of the procedure for the four methods.

3.1. CTAB methods without RNase

- a) Mix the prepared sample from section 2 with quartz sand (when liquid nitrogen is absent) and ground it to a fine powder using a pestle and mortar (**Figure 2**).



Figure 2. Grinding the wood sample to a fine powder using a mortar and pestle

- b) Pour the fine powder into a 1.5 mL microtube and add 700 μ L of extraction buffer and 14 μ L mercaptoethanol (**Figure 3**).
- c) Homogenize the sample with a vortex until the samples are mixed with the buffer (**Figure 4**), followed by incubation in a water bath for at least 3 hours at 65° C (**Figure 5**).
- d) Invert the microtubes every 30 minutes to ensure an evenly homogenized content.



Figure 3. Adding the extraction buffer

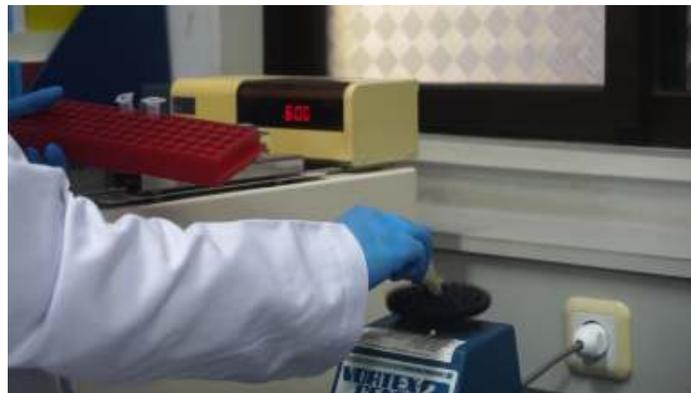


Figure 4. Homogenizing the sample with buffer extraction



Figure 5. Incubating the mixtures in a water bath

- e) Afterwards, add 600 μ L of chloroform-isoamyl alcohol (24:1) into the microtubes, followed by centrifugation for 5 minutes at 10,000 rpm (**Figure 6**).



Figure 6. Centrifuging the mixtures

- f) When the contents began to separate and formed layers of supernatant, organic materials, and chloroform, transfer the uppermost layer of the supernatant to a new microtube using a micropipette (**Figure 7**). This process is repeated twice.



Figure 7. Transferring the supernatant to a new microtube

- g) Afterwards, add 500 mL of cold isopropanol to the supernatant, then mix and store in a freezer overnight.
- h) Next, centrifuge the microtube for 5 minutes at 10,000 rpm to form precipitates. Remove the liquid that has been separated from the microtube and replaced it with 500 mL of 70% ethanol, followed by further centrifugation (**Figure 6**) for 2 minutes and discard the fluid. The process is carried out twice.
- i) Dry the pellets or DNA precipitates at room temperature for 30 minutes (with the tube cap opened) then add 20 μ L of nuclease-free water.
- j) Finally, the microtubes are flicked and the isolated DNA is subsequently used for PCR amplification.

3.2. CTAB methods with RNase

- a) Mix the prepared sample from section 2 with quartz sand (when liquid nitrogen is absent) and ground it to a fine powder using a pestle and mortar (**Figure 2**).

- b) Pour the fine powder into a 1.5 mL microtube and add 700 μ L of extraction buffer, 14 μ L mercaptoethanol, and 0.0008 g polyvinylpyrrolidone (PVP) (**Figure 3**).
- c) Homogenize the samples in a vortex until the samples are mixed with the buffer (**Figure 4**), followed by incubation in a water bath for at least 3 hours at 65° C (**Figure 5**).
- d) Invert the microtubes every 30 minutes to ensure an evenly homogenized content.
- e) When completed, add 600 μ L of chloroform-isoamyl alcohol (24:1) to the microtube. Next, centrifuge the mixture for 5 minutes at 10,000 rpm (**Figure 6**).
- f) When the contents began to separate and formed layers of supernatant, organic materials, and chloroform, transfer the uppermost layer of the supernatant to a new microtube using a micropipette (**Figure 7**). This process is repeated twice.
- g) Afterwards, add 500 mL of cold isopropanol to the supernatant, then mix and store in a freezer overnight.
- h) Next, centrifuge the microtube for 5 minutes at 10,000 rpm to form precipitates.
- i) Remove the separated fluid from the microtube and replaced it with 500 mL of 70% ethanol, followed by further centrifugation (**Figure 6**) for 2 minutes and discard the fluid. The process is carried out twice.
- j) Dry the pellets or DNA precipitates at room temperature for 30 minutes (with the tube cap opened), then add 20 μ L of nuclease-free water and 4 μ L RNase.
- k) At this stage of the process, the microtubes are flicked and the isolated DNA is subsequently used for PCR amplification.

3.3. DNeasy® Plant Mini Kit

The prepared sample from section 2 is mixed with quartz sand (when liquid nitrogen is absent) and ground to a fine powder using a pestle and mortar. The procedure for isolation should follow the manufacturer's instructions (HB-0542-003+1101205_PCard_DNY_Plant_Spi)¹.

The process of DNA isolation using DNeasy® Plant Mini Kit is as follows:

- a) Mix the prepared sample from section 2 with quartz sand (when liquid nitrogen is absent) and ground to a fine powder using a pestle and mortar and transfer it to a 1.5 mL microcentrifuge tube (**Figure 2**).
- b) Add 400 μ L buffer AP1 and 4 μ L RNase A. Vortex and incubate for 10 minutes at 65°C. Invert the tube 2-3 times during incubation.

Note: Do not mix buffer AP1 and RNase A before use.

- c) Add 130 μ L Buffer P3. Mix and incubate for 5 minutes on ice.
- d) Recommended: Centrifuge the lysate for 5 minutes at 14,000 rpm.
- e) Pipet the lysate into a QIAshredder spin column placed in a 2 mL collection tube. Centrifuge for 2 minutes at 14,000 rpm.
- f) Transfer the flow-through into a new tube without disturbing the pellet if present. Add 1.5 volumes of Buffer AW1, and mix by pipetting.

¹ <https://www.qiagen.com/no/resources/download.aspx?id=6b9bcd96-d7d4-48a1-9838-58dbfb0e57d0&lang=en>

- g) Transfer 650 μ L of the mixture into a DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge for 1 minute at 8,000 rpm. Discard the flow-through. Repeat this step with the remaining sample.
 - h) Place the spin column into a new 2 mL collection tube. Add 500 μ L Buffer AW2, and centrifuge for 1 minute at 8,000 rpm. Discard the flow through.
 - i) Add another 500 μ L Buffer AW2. Centrifuge for 2 minutes at 14,000 rpm.
- Note:** Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow trough.
- j) Transfer the spin column to a new 1.5 mL or 2 mL microcentrifuge tube.
 - k) Add 80 μ L Buffer AE for elution. Incubate for 5 minutes at room temperature (15-25°C). Centrifuge for 1 minute at 8,000 rpm.
 - l) Repeat step K.

3.4. Genomic DNA Mini Kit (Plant) GeneAid

The prepared sample from section 2 is mixed with quartz sand (when liquid nitrogen is absent) and ground to a fine powder using a pestle and mortar. The process of DNA isolation using the Genomic DNA Mini Kit (Plant) GeneAid mainly follows the manufacturer's instructions² with modification.

The process of DNA isolation using the Geneid kit is divided into 5 steps as follows:

Step 1: Tissue dissociation

- a) Mix the prepared sample from section 2 with quartz sand (when liquid nitrogen is absent) and ground to a fine powder using a pestle and mortar and transfer it to a 1.5 mL microcentrifuge tube (**Figure 2**).

Step 2: Lysis

Note: Mix GP1 Buffer or GPX1 Buffer and RNase A immediately prior to use.

- b) Add 400 μ L of GP1 Buffer or GPX1 Buffer and 5 μ L of RNase A into the sample tube and mix by the vortex.
- c) Incubate at 60°C for 30 minutes. During incubation, invert the tube every 5 minutes (at this time, pre-heat the required elution buffer (80 μ L per sample) to 60°C (for step 5 DNA elution)).
- d) Add 100 μ L of GP2 Buffer and mix by vortex then incubate on ice for 3 minutes.
- e) Centrifuge for 5 minutes at 13, 000 rpm (**modified from original protocol**).
- f) Place a filter column in a 2 mL collection tube then transfer the mixture to the filter column.
- g) Centrifuge for 1 minute at 3,500 rpm then discard the filter column.
- h) Carefully transfer the supernatant from the 2 mL collection tube to a new 1.5 mL microcentrifuge tube.

Step 3: DNA Binding

² <https://www.geneaid.com/data/download/attached/1602743693466329086.pdf>

- i) Add a 1.5 volume of GP3 Buffer (make sure isopropanol was added) then vortex immediately for 5 seconds.

Note: If precipitate appears, break it up as much as possible with a pipette.

- j) Place a GD column in a 2 mL collection tube.
- k) Transfer 700 μ L of the mixture (and any remaining precipitate) to the GD column.
- l) Centrifuge at 13,000 rpm for 2 minutes.
- m) Discard the flow-through then place the GD column back in the 2 mL collection tube.
- n) Add the remaining mixture to the GD column then centrifuge at 13,000 rpm for 2 minutes.
- o) Discard the flow-through then place the GD column back in the 2 mL collection tube.

Step 4: Wash

- p) Add 400 μ L of W1 Buffer to the GD column then centrifuge at 13,000 rpm for 30 seconds.
- q) Discard the flow-through then place the GD column back in the 2 mL collection tube.
- r) Add 600 μ L of wash buffer (make sure ethanol was added) to the GD column.
- s) Centrifuge at 13,000 rpm for 30 seconds.
- t) Discard the flow-through then place the GD column back in the 2 mL collection tube.
- u) Centrifuge for 3 minutes at 13,000 rpm to dry the column matrix.

Optional residual pigment removal step

If pigments remain on the column, perform this optional step

- v) Following wash buffer addition, add 400 μ L of absolute ethanol to the GD column.
- w) Centrifuge at 13,000 rpm for 30 seconds.
- x) Discard the flow-through then place the GD Column back in the 2 mL collection tube.
- y) Centrifuge for 3 minutes at 13,000 rpm to dry the column matrix.

Step 5: DNA Elution

- z) Transfer the dried GD Column to a clean 1.5 mL microcentrifuge tube.
- aa) Add 80 μ L of pre-heated elution buffer to the center of the column matrix.
- bb) Let stand for 3-5 minutes to ensure the elution buffer is completely absorbed.
- cc) Centrifuge at 13,000 rpm for 30 seconds to elute the purified DNA.

4. DNA quantification

The quality of the genomic DNA is tested by agarose electrophoresis 1% (w/v). As much as 2 μ L DNA was loaded to the gel and the electrophoresis is run at 100 volts for 30 minutes. The electrophoretic gel is stained with GelRed Biotium and then photographed using a gel documentation system to check the presence of DNA bands. In addition, the concentration of DNA extracts is measured using a nanodrop. The amount of extract used is 1 μ L per sample and measured at a wavelength of 260 nm. The DNA purity measurements are carried out to estimate the presence of contaminants in the DNA, where the purity was measured at wavelengths of 260/280 nm.

5. Verification of the working protocol

The isolated total genomic DNA should be further checked and verified by performing PCR analysis. Several non-coding chloroplast regions should be selected since the size of such regions is relatively short. This short fragment is suitable for use in wood as the DNA content may have been fragmented into small sizes due to the handling during wood processing. The chloroplast markers are chosen because chloroplast is only present in plants. The chloroplast is still present in wood tissue even though only in a small amount. The use of markers derived from nuclear genes may not be suitable because it is prone to contamination from other microorganisms and fungi that may infect or be present in the wood tissue.

Under the project, the PCR amplification test was performed using the isolated DNA from all samples using 6 non-coding chloroplast regions, i.e., CLP intron, IGS petD – rpoA, *trnL* intron, *trnG* intron, rpl16-rps3 and *psbA-trnH*. However, the result of PCR amplification only for *trnL* intron and *trnG* for template DNA of samples using the commercial kit (GeneAid). DNA sequencing was further performed to verify the results of the PCR products. The isolated DNA from the wood of *D. latifolia* was checked and verified by performing the DNA sequence analysis using the selected markers. The purified PCR products were bidirectionally sequenced and sequences generated assembled in Geneious (trial version) (**Figure 8**). The *trnG* intron showed a good performance as indicated by the length of the sequence (647 bp), although this size was shorter than the expected size (722 bp).



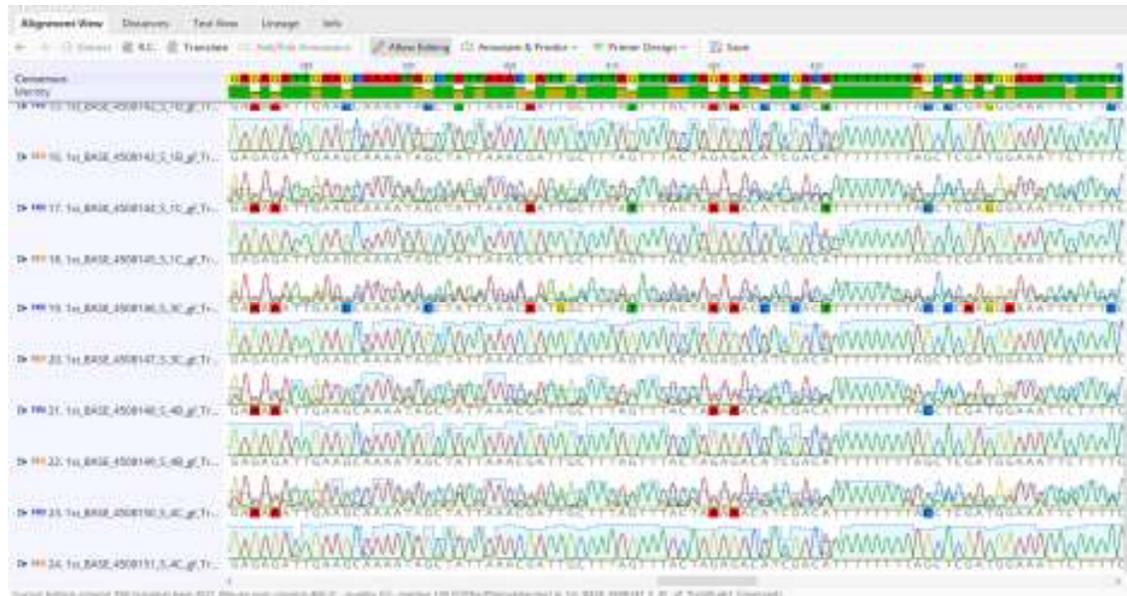


Figure 8. Chromatogram of DNA sequences from isolated DNA of wood samples

6. Conclusion

The developed protocol for DNA isolation from the wood of *D. latifolia* has successfully amplified the selected non-coding regions. The best method to isolate the wood is the use of commercial kits. In the absence of commercial kits, the conventional method of modified CTAB can still be used. To improve the results from this protocol, it is important to carry out more experiments by performing PCR optimization and purification of the isolated DNA.

References

Doyle, J.J. & Doyle, J.L. (1990). Isolation of Plant DNA from fresh tissue. *Focus* 12, 13-15.

Workflow of DNA isolation of wood from *D. latifolia*

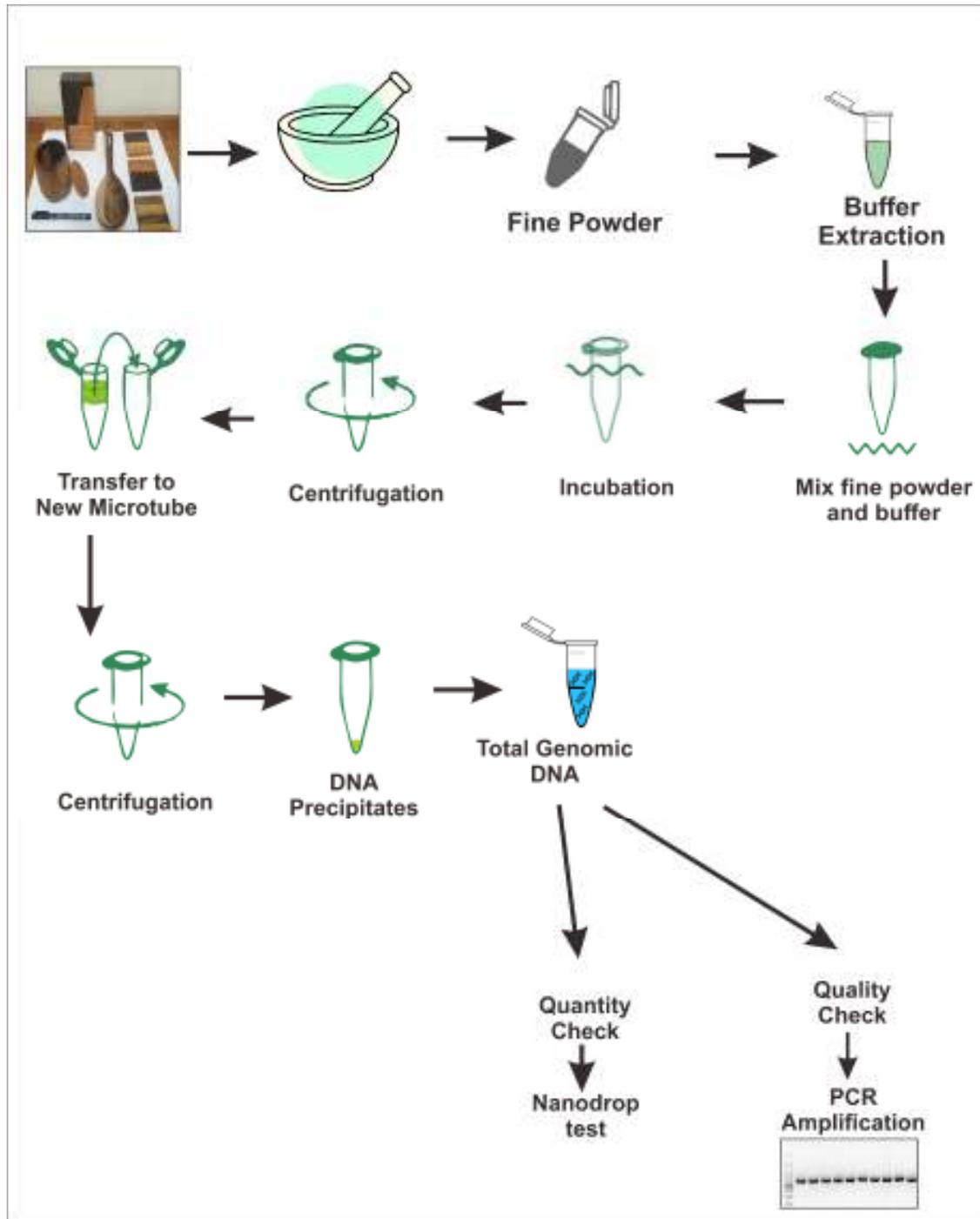


Diagram of principal work of DNA isolation of wood of *D. latifolia*