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DNA Extraction from Heartwood and Quick Species Authentication Using Real-Time PCR: A Case Study of the Rosewood (*Pterocarpus Indicus*)

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Abstract: Illegal logging, felling and timber trade have continued to increase over the past two decades, leading to a decline in forest biodiversity and the extinction of some wood species. *Pterocarpus indicus* Willd., listed in the National Standards of the People's Republic of China for Hongmu (GB/T 18107-2017), is widely used in production of high-end furniture, decorative flooring and musical instruments due to its high-quality timber. For molecular species identification, the quality and quantity of DNA extracted from wood samples should first be ensured. However, extracting DNA from dried, aged timber heartwood is difficult, as heartwood contains little fragmented DNA, along with lots of phenolic compounds known to impede sequence amplification. In order to protect *P. indicus* from over-exploitation and to achieve accurate species-level identification, we established a particular extraction method for obtaining amplifiable DNA from heartwood samples and the real-time PCR assay for species discrimination of *P. indicus* in this study. The quantity and quality of DNA extracted from dry heartwood samples using the modified CTAB method were 2.40-37.70 ng/μL and 1.55-2.12 demonstrated by $OD_{260/280}$, respectively. Primer set P9, targeting *P. indicus* specific microsatellite Pin2-20 sequence, was amplifiable in newly established real-time PCR. Through analysis, this real time PCR was shown to be specific and sensitive with a detection limit around 0.17 ng/μL. Hopefully, this study will contribute to heartwood DNA extraction and species identification of timber logs for forensic discrimination, law enforcement and natural resource conservation.

Keywords: DNA Extraction, Heartwood, Species Discrimination, Biodiversity

1. Introduction

Illegal logging, felling and timber trade have continued to increase over the past two decades, leading to a decline in forest biodiversity and the extinction of some wood species [1]. Both the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [2] and the List of National Key Protected Wild Plants in China [3] provide the legal basis for biodiversity conservation. More importantly, accurate species identification for forensics is the first step. It is believed that a deep combination of accurate species discrimination and law enforcement will prohibit or at least limit domestic and international trade in illegally sourced timber.

Pterocarpus indicus Willd. listed in the National Standards of the People's Republic of China for Hongmu (GB/T 18107-2017) [4], has a wide distribution range and considerable morphological variation throughout its range [5]. As a high-quality timber source, *P. indicus* is widely used in production of high-end furniture, decorative flooring, musical instruments, et al.. Due to the high profit in timber trade and

the relatively slow growth rate, the species resource itself is rapidly dwindling [6].

Traditional anatomical discrimination, which relies on macroscopic and microscopic observations of wood, has long been applied in timber industry and is still the most widely used. Molecular methods such as DNA barcoding [1] and mini-barcoding [7], have also been established, mostly to genus-level and requiring intensive professional data analysis. In order to use these molecular tools in actual identification, the quality and quantity of DNA extracted from wood samples should first be ensured. However, it has been reported that extracting DNA from dried, aged timber heartwood is difficult, as heartwood contains little fragmented DNA, along with lots of phenolic compounds known to impede sequence amplification [8, 9]. Overall, an effective DNA extraction method for wood samples should be developed, especially when heartwood is the only accessible botanical material.

In order to protect *P. indicus* from over-exploitation and to achieve accurate species-level identification, we established a particular extraction method for obtaining amplifiable DNA from heartwood samples and the real-time PCR assay for species discrimination of *P. indicus* in this study. The results are expected to facilitate heartwood DNA extraction and species identification of timber logs for forensic discrimination, law enforcement and natural resource conservation.

2. Materials and Methods

2.1. Plant Materials and Sources

In this study, 5 authentic leaf specimens of rosewood (4 species of *Pterocarpus* and 1 of *Dalbergia*) were donated by the Spice and Beverage Research Institute, Chinese Academy of Tropical Agriculture Sciences, while 16 authentic heartwood specimens of commercially important rosewood species were purchased from the State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China. Two dry wood samples claimed as *P. indicus* were donated by Nanjing Customs and Ningbo Customs, respectively. Details of the collection of all 23 samples are given in Table 1.

2.2. Genomic DNA Extraction

For leaf specimens used to establish real-time PCR method, total DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Wood samples were first cut into small pieces using an electric drill and sterile scalpel blades and then ground into fine powder using the Mixer Mill MM400 (Retsch, Germany). Method for DNA extraction from heartwood was modified from the conventional CTAB protocol [10]. A total of 8 g of fine powder was placed in a 50 ml centrifuge tube, mixed with 40 ml of CTAB solution and incubated at 65°C for 3 h with random shaking for 4-5 times. After incubation, the mixture was centrifuged at 5,000 rpm for 10 min. Clear supernatant was transferred to a new 50 ml centrifuge tube, mixed with an equal volume of chloroform:isoamyl alcohol (V/V: 24/1), and incubated at room temperature for 5 min. The mixture was then centrifuged at 8,000 rpm for 5 min. Clear supernatant was collected and mixed with an equal volume of isopropyl alcohol, and incubated at -20°C for at least 4 h. The mixture was centrifuged at 12,000 rpm for 15 min. The precipitation was collected, washed once with 75% ethanol, and centrifuged at 12,000 rpm for 5 min. Precipitation was dissolved in 100 μl of deionized water or TE after natural drying. Resulting solution was then purified using the TANBead Plant DNA Auto Kit (Taiwan TANBead Company) according to the kit instructions. Final DNA solution was stored at -20°C. The quality and quantity of extracted DNA were analyzed by a Nanodrop 2000c spectrophotometer (Thermo Scientific, USA).

2.3. Primer Design and Real-Time PCR Development

For target DNA region selection and primer design, 19 *P. indicus* sequences were downloaded from Genbank (https://www.ncbi.nlm.nih.gov/), and subject to specificity analysis via BLAST. The resulting 11 sequences with high specificity (Table 2) were then used for primer design by Primer Express v3.0.1 (Applied Biosystems, Thermo Fisher Scientific, UK). Primer synthesis and purification were performed by BGI Co., Ltd (Guangdong, China).

The 20 μL real-time PCR reaction mixture consisted of 10 μL TaqMan Universal PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, UK), 0.5 μM forward and reverse primers, 0.5 μM probe and 4 μL template DNA. Reaction was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, UK) with the following steps: heating at 50°C for 2 min, increasing to 95°C and incubating for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Method validation was performed basing on 5 authentic leaf specimens. Specificity and sensitivity analysis were carried out on 16 authentic heartwood specimens and two *P. indicus* dry wood samples. Sterile ddH_2O was adopted as negative control. Method validation and sensitivity testing were performed in two replicates.

3. Results

The quantity and quality of DNA extracted from dry heartwood samples are shown in Table 1, which demonstrates the feasibility of our modified CTAB extraction method with yields ranging from 2.40 ng/μL to 37.70 ng/μL. The purity of extracted DNA was ensured by $OD_{260/280}$ in the range of 1.55-2.12.

Of the 19 *P. indicus* sequences downloaded from Genbank, 11 candidates (57.89%) were selected for specificity and used as templates for primer design. A total of 5 primer sets (45.45%) were successfully obtained, leaving 6 out of 11 without primers. Unfortunately, only 1 primer set (20.00%) was amplifiable in tests using authentic leaf specimens (Table 2).

DNA extracts from 5 authentic leaf specimens were

employed for primer selection and method validation. Among the 5 primer sets, only P9 set targeting *P. indicus* microsatellite Pin2-20 sequence (Table 3) was amplifiable in real-time PCR resulting in a typical amplification curve for *P. indicus* leaf sample with Ct near 20 (Figure 1). However, 4 other leaf samples showed no amplification curves, suggesting the specificity of P9 primer set. Further specificity analysis of this real-time PCR was carried out basing on 16 authentic heartwood specimens and two *P. indicus* dry wood samples. According to the result, two *P. indicus* wood samples together with one authentic *P. indicus* heartwood sample had amplification curves (Ct ranging from 29 to 35), while other 15 heartwood samples had none (Figure 2).

In order to analyze the detection limit of this real-time PCR, DNA extraction at a concentration of 17.10 ng/μL was adopted as the parent solution which was serially diluted in a 10-fold gradient with sterile ddH_2O. A total of 4 concentrations (17.10 ng/μL, 1.71 ng/μL, 0.17 ng/μL and 0.02 ng/μL) were used in sensitivity test. The amplification result showed that all three concentrations except 0.02 ng/μL could achieve typical curves with Ct below 35 (Figure 3). The detection limit was therefore around 0.17 ng/μL.

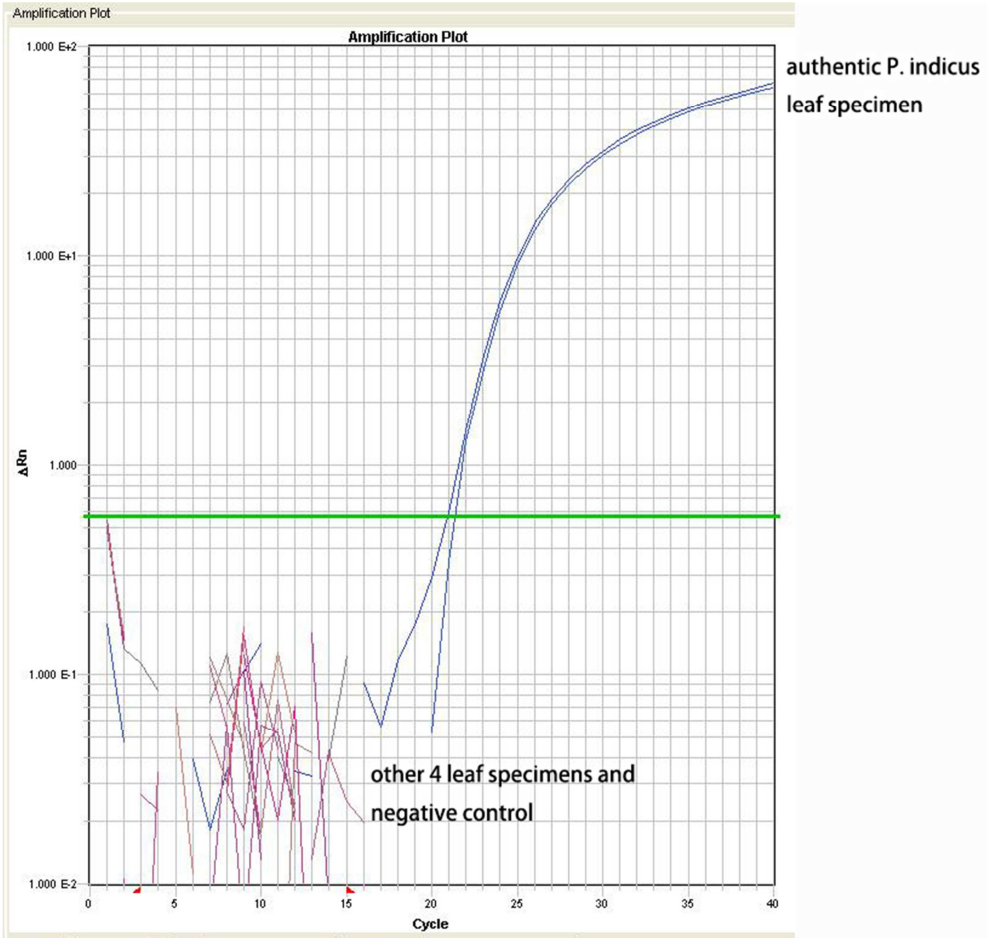

Figure 1. *Result of method validation using 5 authentic leaf specimens.*

Table 1. *Sample information and DNA extraction results.*

Sample code	Sources	Claimed taxonomic status	Type of sample	DNA concentration (ng/μL)	Purity ($OD_{260/280}$)
1	donated by Spice and Beverage Research Institute, Chinese Academy of Tropical Agriculture Sciences	*Pterocarpus erinaceus*	leaf	/	/
2	donated by Spice and Beverage Research Institute, Chinese Academy of Tropical Agriculture Sciences	*Pterocarpus macrocarpus*	leaf	/	/
3	donated by Spice and Beverage Research Institute, Chinese Academy of Tropical Agriculture Sciences	*Pterocarpus indicus*	leaf	/	/
4	donated by Spice and Beverage Research Institute, Chinese Academy of Tropical Agriculture Sciences	*Pterocarpus santalinus*	leaf	/	/
5	donated by Spice and Beverage Research Institute, Chinese Academy of Tropical Agriculture Sciences	*Dalbergia odorifera*	leaf	/	/
6	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Pterocarpus santalinus*	heartwood	10.00	1.60
7	bought from State Key Laboratory of Wood Identification	*Pterocarpus macrocarpus*	heartwood	3.10	1.75

Sample code	Sources	Claimed taxonomic status	Type of sample	DNA concentration (ng/μL)	Purity ($OD_{260/280}$)
	and Quarantine, Zhangjiagang Customs, China				
8	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Pterocarpus erinaceus*	heartwood	16.10	2.10
9	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Pterocarpus indicus*	heartwood	17.10	2.12
10	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Pterocarpus soyauxii*	heartwood	28.40	1.85
11	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Pterocarpus angolensis*	heartwood	37.70	1.83
12	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Dalbergia odorifera*	heartwood	13.80	1.66
13	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Dalbergia cochinchinensis*	heartwood	2.40	1.60
14	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Dalbergia bariensis*	heartwood	9.50	1.67
15	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Dalbergia oliveri*	heartwood	9.20	1.71
16	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Dalbergia retusa*	heartwood	9.20	1.62
17	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Dalbergia cultrate*	heartwood	10.70	1.55
18	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Dalbergia melanaoxylon*	heartwood	6.60	1.67
19	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Dalbergia stevensonii*	heartwood	7.10	1.58
20	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Dalbergia latifolia*	heartwood	3.50	2.02
21	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	*Dalbergia* sp.	heartwood	12.70	1.76
22	donated by Nanjing Customs	*Pterocarpus indicus*	dry wood	5.8	1.72
23	reserved test sample in our lab	*Pterocarpus indicus*	dry wood	6.1	1.81

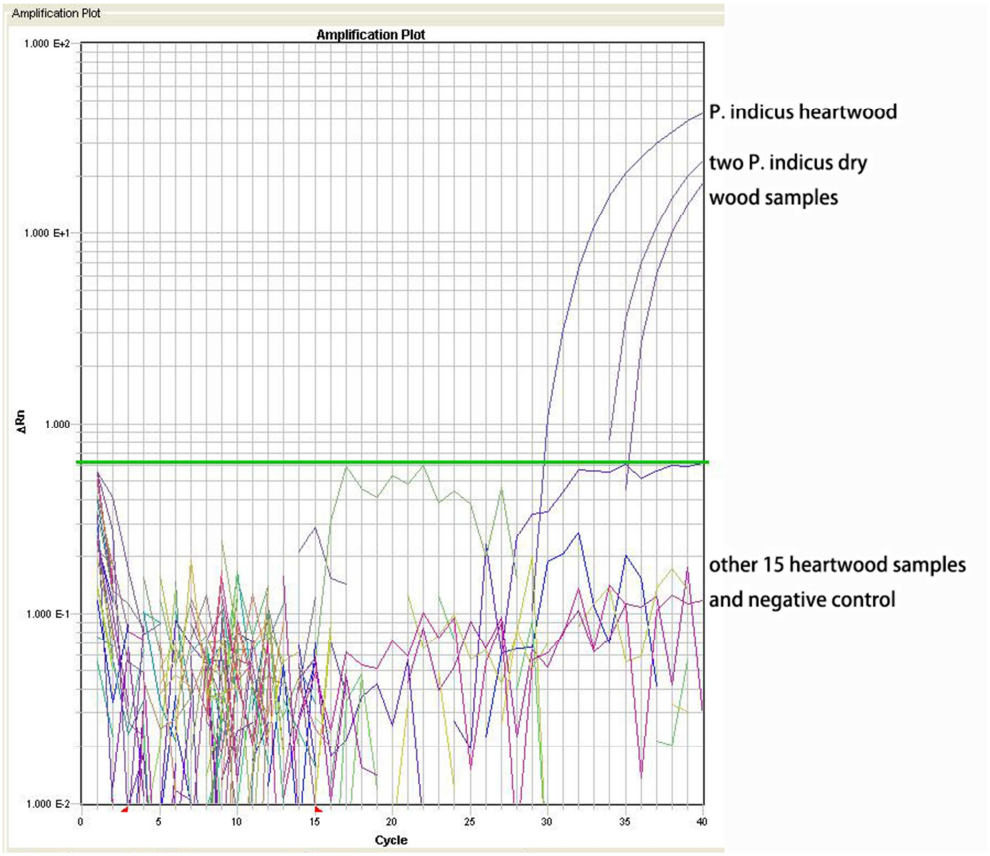

Figure 2. *Result of specificity test basing on 16 authentic heartwood specimens as well as two P. indicus dry wood samples.*

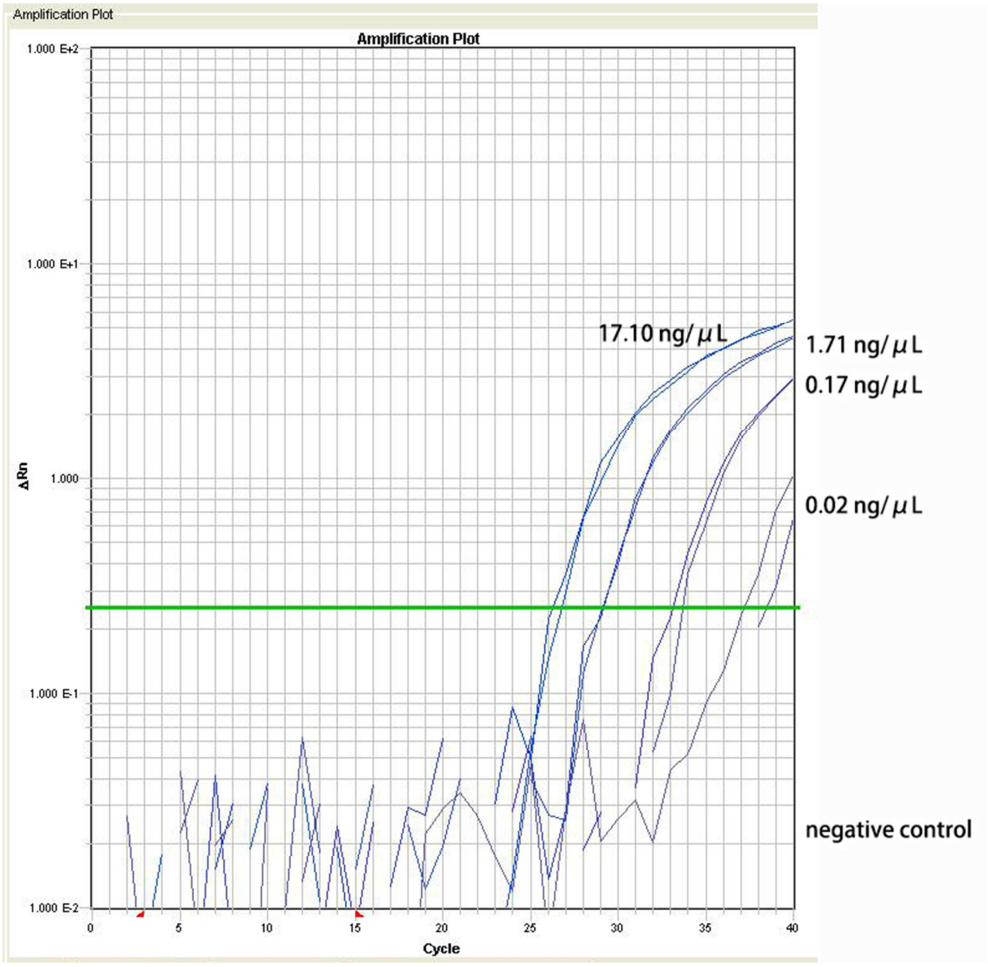

Figure 3. *Result of sensitivity analysis.*

Table 2. *Candidate sequences used for primer design.*

No.	Definition	Accession No.	Specificity	Primer design	Amplification
1	*Pterocarpus indicus* microsatellite Pin6-07 sequence	GU903087	low	/	/
2	*Pterocarpus indicus* microsatellite Pin5-44 sequence	GU903086	high	successful	failed
3	*Pterocarpus indicus* microsatellite Pin5-20 sequence	GU903085	low	/	/
4	*Pterocarpus indicus* microsatellite Pin2-41 sequence	GU903084	high	successful	failed
5	*Pterocarpus indicus* microsatellite Pin2-39 sequence	GU903083	low	/	/
6	*Pterocarpus indicus* microsatellite Pin2-36 sequence	GU903082	high	successful	failed
7	*Pterocarpus indicus* microsatellite Pin2-29 sequence	GU903081	high	failed	/
8	*Pterocarpus indicus* microsatellite Pin2-22 sequence	GU903080	low	/	/
9	*Pterocarpus indicus* microsatellite Pin2-20 sequence	GU903079	high	successful	successful
10	*Pterocarpus indicus* microsatellite Pin2-14 sequence	GU903078	high	failed	/
11	*Pterocarpus indicus* microsatellite Pin2-07 sequence	GU903077	high	failed	/
12	*Pterocarpus indicus* microsatellite Pin2-04 sequence	GU903076	high	failed	/
13	*Pterocarpus indicus* microsatellite Pin2-02 sequence	GU903075	low	/	/
14	*Pterocarpus indicus* microsatellite Pin1-34 sequence	GU903074	high	failed	/
15	*Pterocarpus indicus* microsatellite Pin1-23 sequence	GU903073	high	failed	/
16	*Pterocarpus indicus* microsatellite Pin1-07 sequence	GU903072	low	/	/
17	*Pterocarpus indicus* microsatellite Pin1-04 sequence	GU903071	low	/	/
18	*Pterocarpus indicus* microsatellite Pin1-01 sequence	GU903070	high	successful	failed
19	*Pterocarpus indicus* 1-deoxy-D-xylulose-5-phosphate synthase mRNA, complete cds	MN563181	low	/	/

Table 3. *Five primer sets generated from 5 candidate sequences.*

Primer Name	Sequence (5'-3')
P2-F	GCTAAGTCACCCCAACCCAAA
P2-R	ACCCTTCCCTTTCAGTTAGCACTA
P2-P	FAM-CTGCACCAAGCAGTATCGCAGTAATTCAAA-TAMRA

Primer Name	Sequence (5'-3')
P4-F	TCGTTGAGCTTGGTCATTTGTG
P4-R	AGAGACAAACAGAAAGAGGATAGGAGAA
P4-P	FAM-TGTATGAATACCCACCCCACGGCTCC-TAMRA
P6-F	CAAGGAGAGGATCTGCATTTGG
P6-R	AGCCTTTCACTTAACATTTCCAGAA
P6-P	FAM-CTAACCTGAATACCAAGAAAACTCCGCCCG-TAMRA
P9-F	CGGTCGGACCAGTCCAATT
P9-R	GGTTTTGGCCACATTCGATT
P9-P	FAM-CTGGACCCTAGGTCGATTCGGCCT-TAMRA
P18-F	TCACTTTATTCGCCGCACTGT
P18-R	AGAGTGTGCGTGTATGTGTGTTTG
P18-P	FAM-TCACCTCACCTCTGAGATCACTGTCCCA-TAMRA

F: forward primer; R: reverse primer; P: probe.

4. Discussion

Reliable and applicable discrimination methods depend on genetic information, as anatomical identification could fail to discriminate between look-alikes, especially for inexperienced examiners [11]. DNA recovery from long preserved dry heartwood is fundamental, while how to obtain sufficient high quality DNA from heartwood remains an unsolved problem [12]. Normally, DNA recovery rates have been higher for cambium and sapwood tissues than for heartwood. In heartwood, almost all cells are dead and empty, so the genomic DNA is under poor condition. It has been reported that preservation period has a strong influence on DNA recovery rate from dry wood, as genomic DNA would be degraded throughout the year [9]. In previous studies, DNA extracted from heartwood tissues often had problems such as amplification failure, allelic dropout or inconsistent genotyping, which largely limited applicability of heartwood for authenticity profiling [13]. A major reason for low amplification success rate of DNA from dried, aged wood samples is the co-existence of high content of polysaccharides and phenolic compounds which are difficult to remove. This was particularly true for *Pterocarpus* heartwood, which contains high content of pterocarpan [14]. In this study, a modified CTAB method was established for genomic DNA extraction from dry heartwood. To get rid of amplification inhibitors, primary DNA extraction was further purified using TANBead Plant DNA Auto Kit (Taiwan TANBead Company), which greatly increased amplification success rate.

Due to the low quality and quantity of DNA retrieved from heartwood, it is difficult to perform PCR amplification for large amplicon (>500 bp). It has been reported that amplicon length is negatively related to the success rate of PCR amplification [15]. Therefore, an ideal DNA target for species identification of heartwood samples is supposed to be both short and amplifiable, with sufficient information for each species. Microsatellite simple sequence repeats regions are widely used in genetic diversity and population structure analysis because of their high polymorphism and stability [16]. Here, *P. indicus* microsatellite Pin2-20 sequence was adopted for species specific primer design. Through BLAST on NCBI, microsatellite Pin2-20 sequence was found to be highly specific to *P. indicus*. In addition, the amplicon of P9 primer set is 77 bp.

Conventional PCR often has difficulty achieving sufficient amplification to be visible on an agarose gel when there is severe DNA degradation [17]. In contrast, real-time PCR is featured with high accuracy and sensitivity even with little degraded DNA as starting material [18]. Furthermore, real-time PCR amplification based on a species specific primer set has been shown with discrimination ability to the species level for *Aquilaria* samples under highly degraded and processed conditions [19]. In order to achieve a higher amplification success rate, we adopted real-time PCR technology combined with species specific primers designed from *P. indicus* microsatellite Pin2-20 sequence.

In this study, we have shown that real-time PCR can be utilized for species identification when long preserved dry heartwood is used as a source of DNA. Our work can contribute to international timber trade control. We also expect our DNA extraction protocol to serve as a model for DNA extraction from other heartwood timber and processed wood products.

5. Conclusion

Illegal logging, felling and timber trade have continued to increase over the past two decades, leading to a decline in forest biodiversity and the extinction of some wood species. *P. indicus*, listed in the National Standards of the People's Republic of China for Hongmu (GB/T 18107-2017), is widely used in production of high-end furniture, decorative flooring and musical instruments due to its high-quality timber. For molecular species identification, the quality and quantity of DNA extracted from wood samples should first be ensured. However, extracting DNA from dried, aged timber heartwood is difficult, as heartwood contains little fragmented DNA, along with lots of phenolic compounds known to impede sequence amplification. In order to protect *P. indicus* from over-exploitation and to achieve accurate species-level identification, we established a particular extraction method for obtaining amplifiable DNA from heartwood samples and the real-time PCR assay for species discrimination of *P. indicus* in this study. The quantity and quality of DNA extracted from dry heartwood samples using the modified

CTAB method were 2.40-37.70 ng/μL and 1.55-2.12 demonstrated by $OD_{260/280}$, respectively. Primer set P9, targeting *P. indicus* specific microsatellite Pin2-20 sequence, was amplifiable in newly established real-time PCR. Through analysis, this real time PCR was shown to be specific and sensitive with a detection limit around 0.17 ng/μL. Hopefully, this study will contribute to heartwood DNA extraction and species identification of timber logs for forensic discrimination, law enforcement and natural resource conservation.

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