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Rapid Species Discrimination of High Value Hardwood -- *Pterocarpus macrocarpus* via Real-Time PCR

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Abstract: *Pterocarpus macrocarpus*, a perennial, woody tree, is considered as a commercially valuable species with various applications in furniture making, building materials, drug development, and dyeing. The genus *Pterocarpus* comprises 66 species growing throughout the tropics, with *P. macrocarpus* and *P. indicus* being morphologically closely related, leading to difficulties in distinguishing them via anatomical features. The calcium-dependent protein kinase (CPK) gene is a Ser/Thr protein kinase existing in plants, whose Ca^{2+} sensing and kinase activities play a significant role in plant growth, development and response to various stresses. However, current molecular methods such as DNA barcoding and phylogenetic analysis are time-consuming and labour-intensive. In the present study, a rapid and reliable real-time PCR method for wood identification of this species was established. Through target regions selection, primer/probe design and testing, method validation, specificity and sensitivity analysis, the most efficient real-time PCR approach taking partial CPK gene region as the target, was finally built up. It has also been proven to be highly specific and sensitive with a detection limit near 1.8×10^{-2} ng/μL. This study provides a useful tool for wood species discrimination for the proper utilization of this valuable timber, which will certainly benefit the wood industry towards a better and reasonable circumstance.

Keywords: High-Value Hardwood, *Pterocarpus macrocarpus*, Species Discrimination, Real-Time PCR

1. Introduction

Pterocarpus macrocarpus Kurz (family: Fabaceae) is a perennial, woody tree, which can grow up to a height of 10-30 m. Native to Southeast Asia, including Myanmar, northern Thailand, Cambodia, Laos and Vietnam [1], it is often cultivated as an ornamental, for shade or for its economically important timber. Recently, over-exploitation and habitat loss have led to natural decline of *P. macrocarpus* [2] which has been listed as 'endangered' in the IUCN Red List [3].

P. macrocarpus is a commercially valuable species with various applications in furniture manufacturing, building materials, drug development, and dyeing, mainly because of its excellent heartwood properties. The hard, dense, relatively heavy and very durable wood can be made into furniture, construction lumber, cartwheels, tool handles and so on [4]. Famous for its resistance to termite attack [5], *P. macrocarpus* is considered a premium timber in both Laos and Vietnam. It is one of the main export timbers from Thailand and Myanmar due to the expensive reddish hardwood made from heartwood [4]. This tree is also used in reforestation schemes and on degraded sites to restore woodland [6, 7]. Crude extracts and homopterocarpin from heartwood of *P. macrocarpus* have been demonstrated to have anti-SARS-CoV-2, antiplasmodial, antioxidant, and antimicrobial activities [8]. The woody aroma from *P. macrocarpus* can benefit human blood circulation and immune function [9]. Many natural products isolated from heartwood of *P. macrocarpus* have anti-cancer [10], anti-Alzheimer's disease, antispasmodic and

neuro-protective effects [11].

The genus *Pterocarpus* contains 66 species growing throughout the tropics [12]. Among these, *P. macrocarpus* and *P. indicus* are morphologically closely related [13], leading to difficulties in distinguishing them with naked eyes. There are several molecular approaches for wood species identification, such as DNA barcoding and phylogenetic analysis.

The aim of this study was to establish a rapid species discrimination method for *P. macrocarpus* based on the fluorescence real-time PCR. This method has been shown to be highly sensitive, specific and simple, which would be a useful tool for wood species discrimination for the proper utilization of this valuable timber. It will certainly benefit the wood industry towards a better and reasonable circumstance.

2. Materials and Methods

2.1. Plant Materials and Sources

For method development, we used a leaf sample of *P. macrocarpus* donated by the Spice and Beverage Research Institute, Chinese Academy of Tropical Agriculture Sciences. For method specificity and sensitivity analysis, 17 heartwood specimens of commercially significant rosewood species taxonomically verified by the State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China, were selected. Two reserved samples (one of heartwood and one of sapwood) claimed as *P. macrocarpus* were analyzed simultaneously. Details of all 20 samples are given in Table 1.

Table 1. *Sample information.*

Sample code	Taxonomic status	Sources	Type of sample
1	*Pterocarpus macrocarpus*	donated by Spice and Beverage Research Institute, Chinese Academy of Tropical Agriculture Sciences	leaf
2	*Pterocarpus macrocarpus*	Reserved sample	heartwood
3	*Pterocarpus macrocarpus*	Reserved sample	sapwood
4	*Pterocarpus macrocarpus*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
5	*Dalbergia odorifera*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
6	*Dalbergia* sp.	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
7	*Dalbergia cochinchinensis*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
8	*Dalbergia bariensis*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
9	*Dalbergia oliveri*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
10	*Dalbergia retusa*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
11	*Dalbergia cultrate*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
12	*Dalbergia louvelii*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
13	*Dalbergia melanaoxylon*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
14	*Dalbergia stevensonii*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
15	*Dalbergia latifolia*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
16	*Pterocarpus santalinus*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
17	*Pterocarpus erinaceus*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
18	*Pterocarpus indicus*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
19	*Pterocarpus soyauxii*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood
20	*Pterocarpus angolensis*	bought from State Key Laboratory of Wood Identification and Quarantine, Zhangjiagang Customs, China	heartwood

2.2. Genomic DNA Extraction

Small pieces of dry wood samples were prepared using an electric drill or sterile scalpel blades. A mixer mill MM400 (Retsch, Germany) was used to grind wood pieces into fine powder which was suitable for DNA extraction. Method for DNA extraction from dry wood samples has been reported earlier [14]. Total DNA was extracted from the leaf sample via DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Resulted DNA solution was kept

at -20°C. Extraction success rate was confirmed via real-time PCR targeting 18S rRNA [15].

2.3. Target Region Selection and Primer Design

Two DNA regions, the calcium-dependent protein kinase (CPK) gene and the putative ornithine aminotransferase (OAT) gene, were selected as candidates [16] and subjected to sequence specificity analysis via BLAST. Primer design was performed by Primer Express v3.0.1 (Applied Biosystems, Thermo Fisher Scientific, UK). Primer synthesis and purification were carried out by BGI Co., Ltd (Guangdong, China).

2.4. Real-Time PCR Development

Reaction mixture (in a total volume of 20 µL) contained 10 µL TransStart Probe qPCR SuperMix (2×, TransGen Biotech Co., Ltd., China), 0.4 µL forward primer (10 µM), 0.4 µL reverse primer (10 µM), 0.8 µL probe (10 µM), 0.4 µL ROX, 4.0 µL sample DNA and 4.0 µL ddH_2O. Reaction was carried out on a Step-one Plus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, UK) in the following steps: incubating at 50°C for 2 min, increasing to 95°C and staying for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec.

2.5. Method Specificity and Sensitivity

Method validation was completed using the leaf sample of *P. macrocarpus*. Specificity test was demonstrated on 17 authentic specimens along with two *P. macrocarpus* wood samples. The concentration of DNA extraction solution of authentic *P. macrocarpus* heartwood specimen was analyzed with a Nanodrop 2000c spectrophotometer (Thermo Scientific, USA), which was used as the parent solution for serial dilution in method sensitivity determination. One leaf sample of *P. indicus* was used as the negative control with sterile ddH_2O as the blank control in method validation. In other analysis, sterile ddH_2O was exploited as the negative control. Method validation was performed in two replicates.

3. Results

3.1. DNA Extraction Confirmation

DNA extraction success rate was analysis via real-time PCR targeting 18S rRNA (Table 2). All 20 samples showed typical amplification curves with Ct ranging from 22 to 30 (Figure 1), representing a 100% success rate for DNA extraction. Thus these DNA solutions could be used in following analysis.

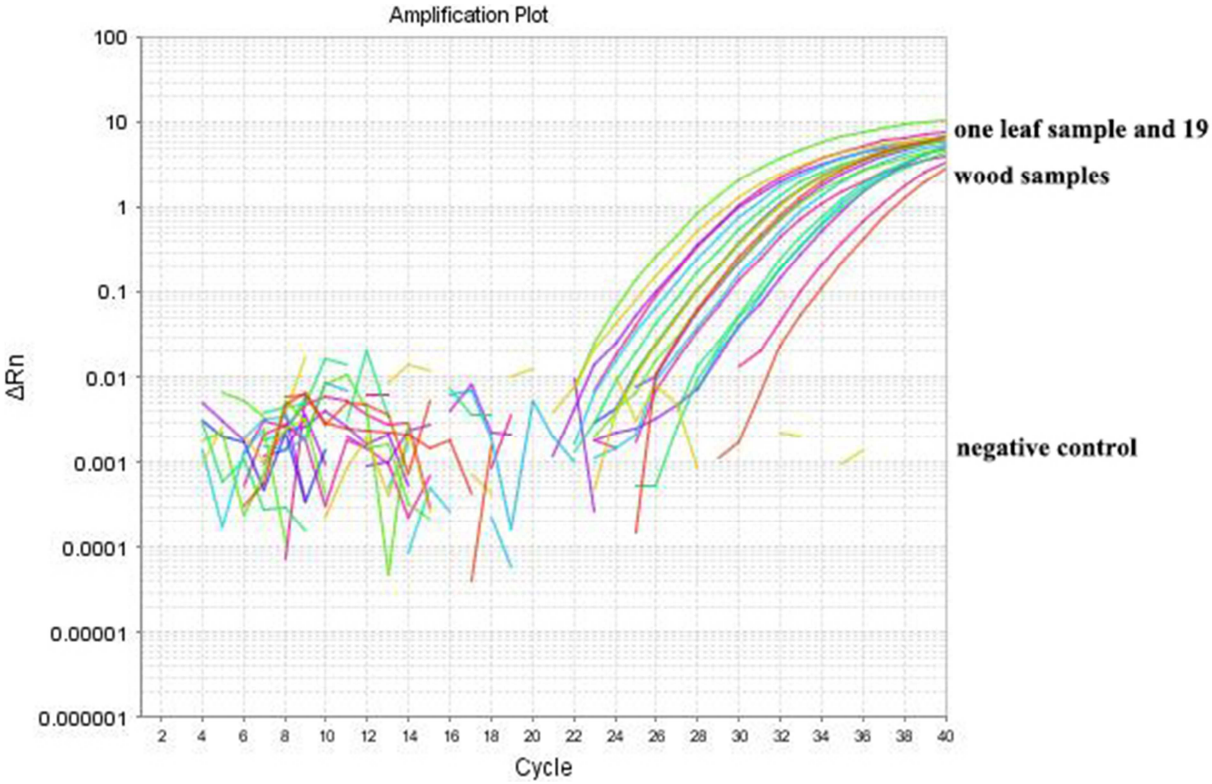

Figure 1. *DNA extraction confirmation.*

Table 2. *Primers used in this research.*

Target Region	Primer Name	Sequence (5'-3')	Amplicon (bp)
CPK gene	PM1-F	GCGTTTGCTTTCCCTACAATG	72
	PM1-R	CAGAACATGGGAAAATAATTCAACA	
	PM1-Probe	FAM- TCATCATTGTTCAACATTTG -MGB	

Target Region	Primer Name	Sequence (5'-3')	Amplicon (bp)
	PM2-F	ACTTGGAAGTCACGGGTTCAA	
CPK gene	PM2-R	GCAGCCTTACCCCCACAA	62
	PM2-Probe	FAM- AATGGGAAACAGCCTATC -MGB	
	PM3-F	GCAAATTTGATTGTACGCCTTTT	
OAT gene	PM3-R	AGCCCACAGTTCATTATTGCTGTA	71
	PM3-Probe	FAM- ATAATTAAGGTTCAGAGGTATC -MGB	
	PM4-F	TCTCTGTTGCGTGGTTAAGTTAAATAC	
OAT gene	PM4-R	TTGTGGACTTTGCTGATTAATTATCC	111
	PM4-Probe	FAM- CAATACATATCTAGAACGGGAAA -MGB	
	18SrRNA-F	CCTGAGAAACGGCTACCA	
18S rRNA	18SrRNA-R	CGTGTCAGGATTGGGTAAT	65
	18SrRNA-Probe	FAM- TGCGCGCCTGCTGCCTTCCT -Eclipse	

3.2. Method Validation

According to candidate genes, altogether four primer sets were generated, two for each region (Table 2). In order to rank these primer sets, work efficiency was tested with the *P. macrocarpus* leaf sample. Depending on Ct and amplification curves (Figure 2), only the leaf sample of *P. macrocarpus* showed positive results. Primer set PM2 with the best work efficiency targeting the CPK gene was selected finally.

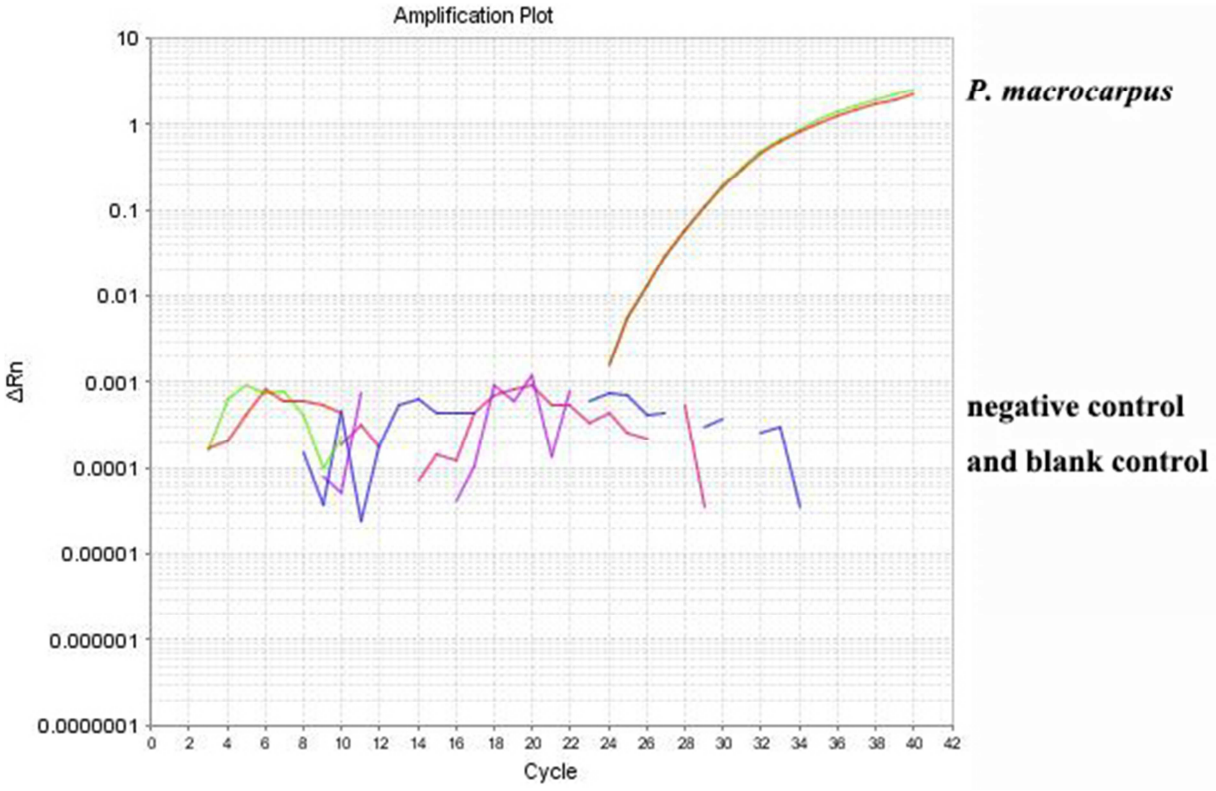

Figure 2. *Method validation with primer set PM2 and the leaf sample of P. macrocarpus.*

3.3. Specificity and Sensitivity Analysis

Method specificity was demonstrated basing on 17 authentic specimens and two *P. macrocarpus* samples. Only *P. macrocarpus* samples (one of sapwood, one of heartwood and one authentic specimen) showed typical amplification curves and their Ct(s) (Figure 3). Other samples had negative results. Thus, this real-time PCR method displays high specificity among common reddish wood species.

DNA solution of authentic *P. macrocarpus* heartwood specimen at the concentration of 1.80 ng/μL was used for serial dilution in a 10-fold gradient with sterile ddH_2O. Altogether 7 concentrations (1.80 ng/μL, 1.80×10^{-1} ng/μL, 1.80×10^{-2} ng/μL, 1.80×10^{-3} ng/μL, 1.80×10^{-4} ng/μL, 1.80×10^{-5} ng/μL and 1.80×10^{-6} ng/μL) were generated. Corresponding amplification result showed that former three concentrations (1.80 ng/μL, 1.80×10^{-1} ng/μL, and 1.80×10^{-2} ng/μL) had expected amplification curves with Ct below 35 (Figure 4). Therefore, the detection limit of this real-time PCR method was inferred to be near 1.8×10^{-2} ng/μL.

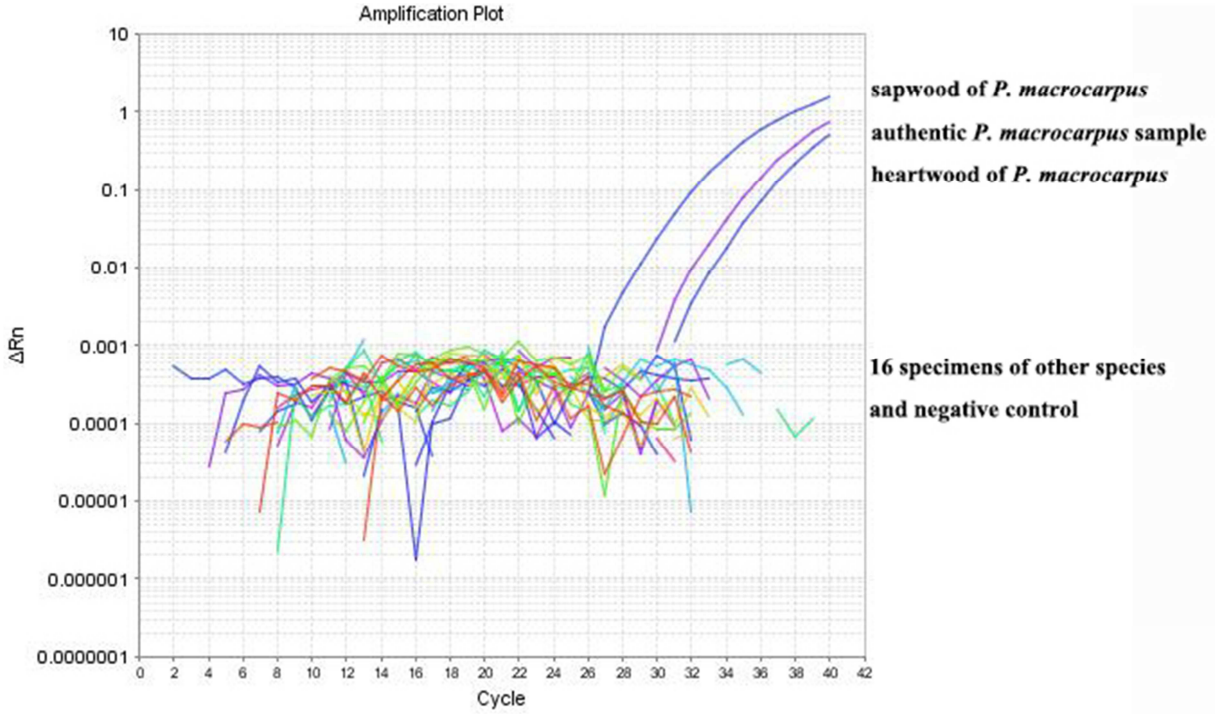

Figure 3. *Result of specificity analysis.*

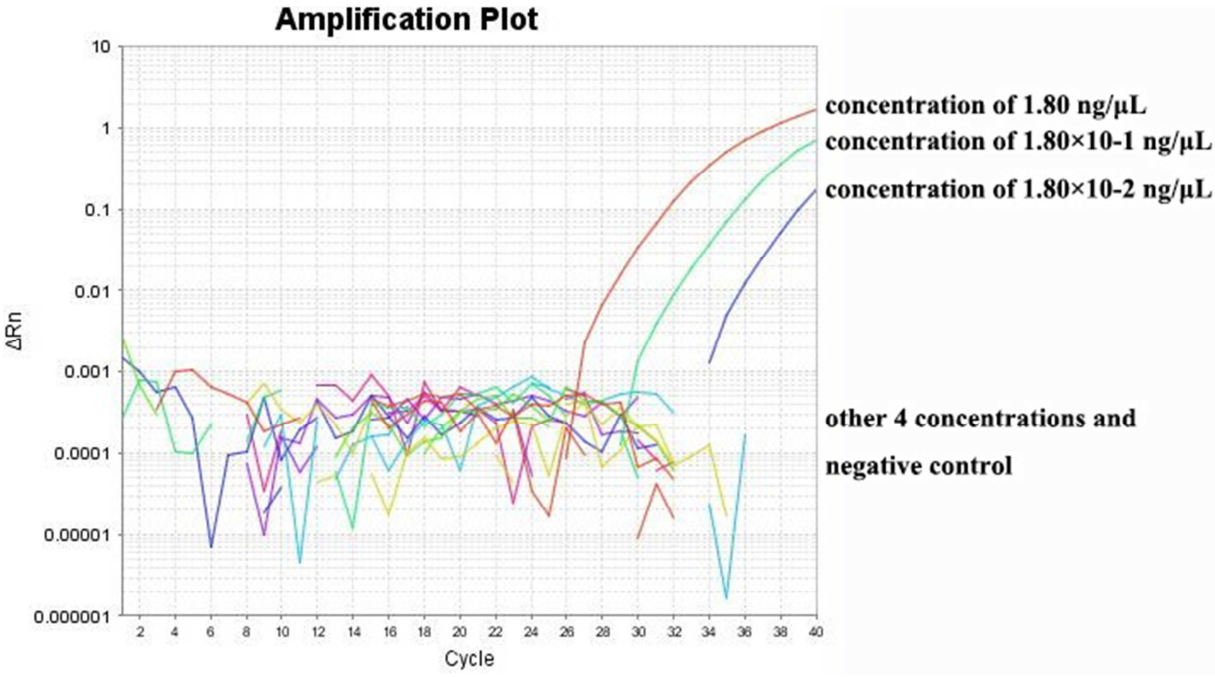

Figure 4. *Amplification curves in method sensitivity test.*

4. Discussion

Researches on species identification of commercially valuable wood are necessary for the timber industry. Traditionally, wood species discrimination is based mainly on anatomical characteristics including macroscopical and microscopic features which are classified by experienced experts. Lately, with machine vision and pattern recognition technology improving, wood recognition approaches have evolved from manual operation towards intelligent methods, such as machine learning. Automated wood identification system is so attractive that several scholars have attempted to do wood classification by combining anatomical analysis with various intelligent tools. Yang Xiaoxia et al. have developed a microstructure identification method based on vessel pore feature extraction of six hardwood species, with an accuracy rate of more than 98.9% [17]. New method basing on a non-destructive and non-invasive laboratory-scale tool, X-ray computed tomography (CT), and machine learning for image

recognition has been established for wood species identification [18]. Moreover, another rapid and non-destructive approach utilizing Attenuated total reflection Fourier transform infrared (ATR-FTIR) analytical technique with chemometric methods has also been applied in wood recognition [19].

There are several molecular approaches for wood species authentication, such as DNA barcoding and phylogenetic analysis. DNA barcoding is extensively used as a genetic tool for species identification based on a short region of DNA [20]. Recently, with advances in DNA extraction from wood specimens, many studies have applied DNA barcoding to wood species identification [21]. While DNA barcoding often exploits universal barcodes which lack sufficient resolution to distinguish between closely related species within the same genus [22]. More informative DNA barcodes have been identified basing on the complete chloroplast genomes [23]. For DNA barcoding, expanding reference libraries and high resolution barcodes are the two key points for accurate species identification. As for phylogenetic analysis, chloroplast and nuclear regions are constantly used for multi-level classification. Phylogenetic analysis requires more genetic information, and therefore provides the most accurate result so far. Obviously, shortcomings of the phylogenetic method is time consuming and labor intensive. Chloroplast genome of *P. macrocarpus* has been sequenced and ready for further use [13]. High-copy chloroplast genomes are inferred to be superior for genetic analysis compared to single-copy nuclear regions, particularly for degraded specimens.

Calcium-dependent protein kinase (CPK) is a family of Ser/Thr protein kinases existing in plants and some protozoa, whose Ca^{2+} sensing and kinase activities play a significant role in plant growth, development and response to various stresses [24]. In the present study, an efficient and reliable method for wood identification based on real-time PCR targeting the CPK gene was established. Through specificity and sensitivity analysis, this method was proved to be highly specific and sensitive with a detection limit near 1.8×10^{-2} ng/μL. Thus, our results provide a useful tool for species discrimination for a proper utilization of this valuable timber.

5. Conclusion

Pterocarpus macrocarpus, a perennial, woody tree, is considered as a commercially valuable species with various applications in furniture making, building materials, drug development, and dyeing. The genus *Pterocarpus* comprises 66 species growing throughout the tropics, with *P. macrocarpus* and *P. indicus* being morphologically closely related, leading to difficulties in distinguishing them via anatomical features. The calcium-dependent protein kinase (CPK) gene is a Ser/Thr protein kinase existing in plants, whose Ca^{2+} sensing and kinase activities play a significant role in plant growth, development and response to various stresses. However, current molecular methods such as DNA barcoding and phylogenetic analysis are time-consuming and labour-intensive. In the present study, a rapid and reliable real-time PCR method for wood identification of this species was established. Through target regions selection, primer/probe design and testing, method validation, specificity and sensitivity analysis, the most efficient real-time PCR approach taking partial CPK gene region as the target, was finally built up. It has also been proven to be highly specific and sensitive with a detection limit near 1.8×10^{-2} ng/μL. This study provides a useful tool for wood species discrimination for the proper utilization of this valuable timber, which will certainly benefit the wood industry towards a better and reasonable circumstance.

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