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Bar-HRM for Species Confirmation of Native Plants Used in Forest Restoration in Northern Thailand

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Abstract: Plant species confirmation is a crucial step in using native plant species for forest restoration. To enhance this, a hybrid method of DNA barcoding and high-resolution melting analysis (Bar-HRM) was investigated in this study. In total, 12 native plant species samples were collected from forest restoration sites in Nan, a province in Northern Thailand. Simulation HRM analysis was performed to find the most appropriate region for in vitro Bar-HRM analysis. After that, in vitro Bar-HRM was carried out to validate the performance of native plant species. Results from both simulation and in vitro analyses revealed that the nuclear ribosomal internal transcribed spacer (ITS) region can be used as a primer set that can clearly discriminate native plant species in this study. With our study, Bar-HRM was proved of use in native plant species confirmation, even if that species had no molecular data available. In this context, Bar-HRM would be useful for the identification of native plant species used in tropical forest restoration not only in Thailand but also in any areas with similar plant groups.

Keywords: native species; tropical forest; high-resolution melting analysis; species confirmation

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1. Introduction

Tropical forests are the habitat of approximately two-thirds of our world's flora and fauna, yet this type of forest covers less than 7% of the earth's land surface [1,2]. Southeast Asia was reported to be home to around 15% of the world's tropical forests [3]. There has been an unprecedented loss of tropical forests in the region because of population growth, infrastructure development, agricultural expansion, illegal logging, and uncontrolled forest fires [4–6]. The tropical forests of Southeast Asia are under immense pressure. This tropical forest region has lost a large proportion of its original forest cover and is now a deforestation hotspot [7]. Over the past two decades, Southeast Asia has lost 61 million hectares of forest; the annual loss was 4 million hectares a year on average from 2010 to 2019 [8]. Deforestation is a major problem in the region, with Indonesia in the lead, followed by other hotspots, such as Cambodia, Malaysia, Vietnam, and Thailand. The forest area in Thailand dramatically dropped from 70% in 1950 to 31% in 2018 [9]. Although all parts of Thailand are facing the same situation, the worst-affected area is the northern part of Thailand [10,11].

Over the past few decades, many countries have recognized the problem and put effort into forest conservation and restoration. One commonly used reforestation strategy is planting or seeding of native or introduced species [12,13]. Two main strategies of plantations have been established, which are monoculture and mixed-species plantations.

Monocultures (a single species with the same genotype with almost no variation) have been used extensively in the tropics to reforest and are well documented in forest research. However, monocultural plantations have also been reported to have several negative social and environmental impacts despite their economic benefits [14,15]. Monoculture forests may only provide short-term economic benefits, with lower ecosystem service and biodiversity conservation benefits [16,17]. In contrast, mixed-species plantations based on native trees have been found to be more productive and sustainable plantation systems over monocultures [18–20]. Thus, mixed-species plantations using indigenous species are increasingly being considered for sustainable reforestation and used worldwide to restore disturbed and degraded areas [18,21–24]. Many native species have proven ability to grow well in deforested sites and have higher growth rates than introduced species [25]. Perhaps the key to the success of mixed-species plantations using native plants is for a system to rely on accurate species identification and an efficient propagation approach [25–27].

Therefore, plant species identification is important for forest conservation and management [28]. However, morphological-based identification normally requires experts and is time consuming. Moreover, several parts of the plants are needed for species-level identification, such as flower, leave, and fruit. So, it is practically impossible to do this when plants are in a vegetative or seedling phase [29]. Having a reliable and fast method for species identification would benefit the mixed-species plantations. Several procedures, such as seed-handling, germination pretreatments, and storage, are involved in the mix-species, and wrong identification of native species stock could be a waste of time and money [30]. Various native plant species were selected to be included in the forest restoration project in Nan province, Thailand, launched by the Forest Restoration Research Unit, Chiang Mai University (FORRU-CMU). The majority of the plant materials generated in the project are seeds and plantlets, which are then used for restoration so that conventional plant species identification techniques are inefficient. Lack of appropriate identification and characterization of the plant materials could lead to failure. Thus, what is required is a method that can be used to identify plant species at an early stage (e.g., seed and plantlet).

The most popular molecular species identification system is the use of DNA barcoding. Two DNA regions, *rbc*L and *mat*K, were recommended by CBOL Plant Working Group as a universal barcode for plants [31]. In addition, the nuclear ribosomal internal transcribed spacer (ITS) is reported to be a good DNA barcode for species identification in several plant groups [32–34]. Recently, the combination of DNA barcoding and high-resolution melting analysis (HRM), called Bar-HRM, has been developed. Bar-HRM analysis has been used as a species authenticating method for herbal and agricultural products [35–41]. In addition, the method was used to identify a wide range of plant species [32,42–44]. Here, the Bar-HRM was evaluated for its performance in species identification of the native plants used for forest restoration in Nan province, Thailand. Results of this study will be useful for native plant species confirmation for tropical forest restoration and conservation.

2. Materials and Methods

2.1. Plant Samples and DNA Extraction

Plant tissue from 12 native species was collected from nine deforested sites in three districts (Pua, Phu Phiang, and Wiang Sa) of Nan province, Northern Thailand (Table 1 and Figure 1). Most of forest in Nan is mainly highland headwater mixed forest, which is an important watershed that feeds Thai people in many vital waterways. Chao Phraya River, the main river of Thailand, partly originates here. The nine selected sites were deforested due to agricultural in the past. Forest restoration is currently in progress here under the "From a bare mountain to a regenerated forest: comparing landscape planting design for forest restoration in NAN province" project, which was launched by the Forest Restoration Research Unit, Chiang Mai University (FORRU-CMU), in 2019.

Table 1. Twelve native plants samples used in this study.

Sample ID	Species	Sample ID	Species
N1	*Careya sphaerica* Roxb.	N7	*Phyllanthus emblica* L.
N2	*Hopea odorata* Roxb.	N8	*Albizia lebbeck* (L.) Benth.
N3	*Croton roxburghii* N.P. Balakr.	N9	*Chukrasia velutina* Wight & Arn.
N4	*Gmelina arborea* Roxb.	N10	*Spondias mombin* L.
N5	*Irvingia malayana* Oliv. ex A.W. Benn.	N11	*Oroxylum indicum* (L.) Benth. ex Kurz
N6	*Afzelia xylocarpa* (Kurz) Craib	N12	*Bauhinia purpurea* L.

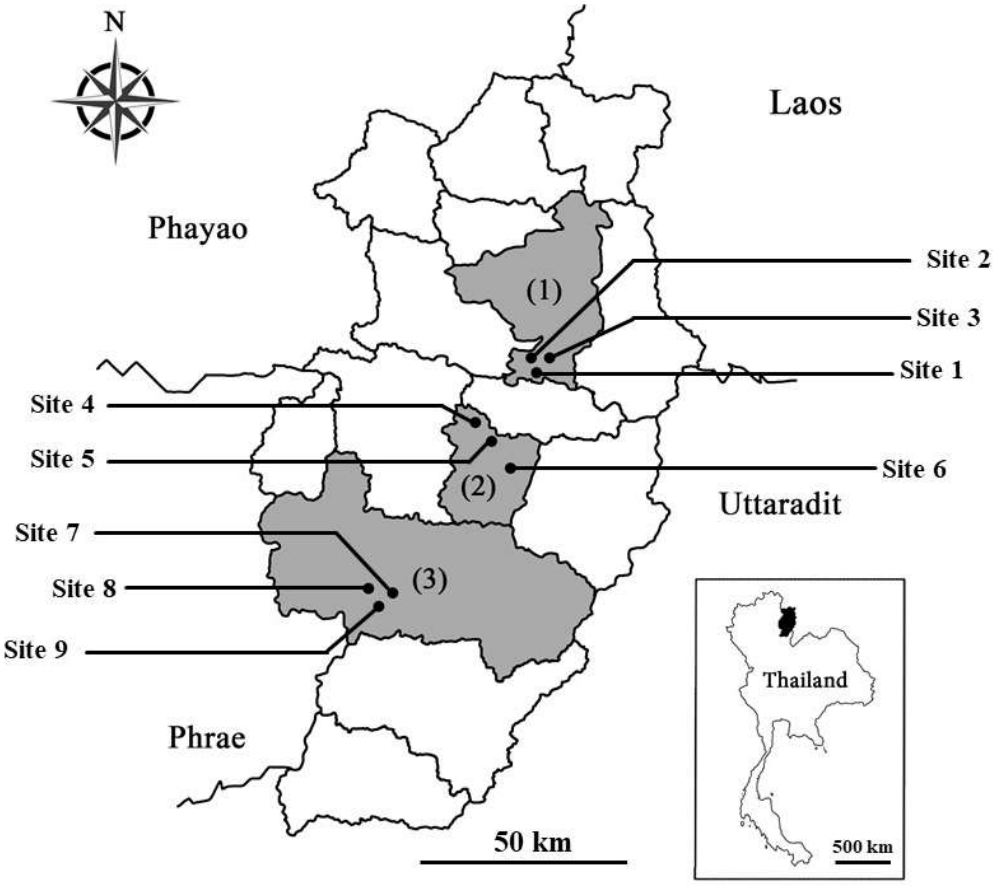


Figure 1. Locations of the nine sampling sites (black circle) in (1) Pua, (2) Phu Phiang, and (3) Wiang Sa districts in Nan, Northern Thailand.

The plant tissues were ground with liquid nitrogen. DNA from all samples was extracted using the CTAB-chloroform solution protocol [45]. DNA concentrations were determined using the Qubit dsDNA HS Assay (Invitrogen, CA, USA). Various DNA concentrations of the samples were obtained and are recorded in Table 2. Final concentrations of the DNA solutions were adjusted to 120 ng/μL. The DNA solutions were stored at −20 °C for further use.

Table 2. DNA concentration of each sample.

Sample ID	Included in In Vitro HRM	Average DNA Concentration (ng/uL)
N1	✓	327.52
N2	✓	578.21

N3	✓	1743.1
N4	✓	674.65
N5	x	16.18
N6	✓	827.69
N7	✓	306.78
N8	✓	382.46
N9	x	73.29
N10	x	18.17
N11	✓	170.58
N12	✓	2802.91

2.2. Data Mining

To address the most suitable markers for the identification of the tested species (Table 2) based on the Bar-HRM technique, a sequence dataset was constructed for sequence profile analysis. The sequences of the four selected regions (ITS, *mat*K, *rbc*L, and *trn*L) of the 12 native species were retrieved from GenBank. Multiple alignments of the obtaining sequences were performed using MEGA 11 [46], and sequence length (bp) and variable sites (%) were recorded.

2.3. Simulated High-Resolution Melting Analysis

The retrieved sequences were 5′ and 3′ trimmed. Six DNA sequences or fragments were selected based on their availability in all selected DNA regions, to test the feasibility of Bar-HRM. To determine the melting profile of each region, simulated HRM analyses were performed using uMELT Quartz (melting prediction software) following the user guide [47]. The species included in the assay were N2, N7-N8, and N10-N12 (Table 3). The melting curves of the four chosen regions of the tested species were compared for their efficiency in species discrimination. The region with the best performance was then used in the next experiment.

Table 3. Details of sequences retrieved from GenBank.

ID	Scientific Name	No. of Sequences in GenBank	DNA Region							
			ITS		*mat*K		*rbc*L		*trn*L	
			Accession Number	No. of Sequences	Accession Number	No. of Sequences	Accession Number	No. of Sequences	Accession Number	No. of Sequences
N1	*Careya sphaerica*	15	MN699345	2	MG742305	5	AF077655	3	DQ924313	2
N2	*Hopea odorata*	34	MZ782439	1	KY972951	7	DQ157306	6	KY972753	6
N3	*Croton roxburghii*	20	AY971244	6	AB428650	5	KP789671	2	AY971329	6
N4	*Gmelina arborea*	20,000	KR532195	5	JX495721	13	JF272486	16	JQ669310	3
N5	*Irvingia malayana*	107			EF135553	6	JX664054	7		
N6	*Afzelia xylocarpa*	22							KX690239	2
N7	*Phyllanthus emblica*	205	AY830087	47	AY936594	11	AY765269	13	FJ847837	1
N8	*Albizia lebbeck*	318	N181375	8	EU812047	8	KC417043	12	KX268143	4
N9	*Chukrasia velutina*	42	FJ518894	12	AB924836	8	AY128222	12	KU939115	1
N10	*Spondias mombin*	580	AF080064	2	AY594480	3	JQ590140	9	KC283103	45
N11	*Oroxylum indicum*	84	FJ606747	12	HQ384520	9	HQ384886	11	FJ870048	2
N12	*Bauhinia purpurea*	214	MH548397	15	JN881391	2	JX856647	10	FJ801069	4

2.4. In Vitro High-Resolution Melting Analysis

To distinguish the tested species, the melting profile of each species was generated in HRM. The extracted DNA was amplified using the Rotor-Gene Q 5plex HRM system (Qiagen, Hilden, Germany). The reaction mixture for the HRM analysis consisted of a total volume of 10 µL, containing 4 µL of Evagreen HRM Master Mix, 0.2 µL of 10 mM forward primer, 0.2 µL of 10 mM reverse primer, 1 µL of 120 ng DNA, and 3.6 µL of ddH_2O. The reaction conditions were as follows: an initial denaturing step at 95 °C for 5 min followed by 40 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 20 s. Melting curves were generated after the last extension step. Only the ITS primer pair was used in this experiment. The nucleotides of the forward primer (ITSF) are 5′-

GGTGAACCTGCGGAAGGATCATTG -3′ and the reverse primer (ITSR) are 5′- CCGAGATATCCATTGCCGAGAGTC -3′. The temperature for the HRM analysis was increased from 60 to 95 °C at 0.1 °C/s. The negative derivative of the fluorescence (F) over temperature (T) (dF/dT) curve displays the T_m, and the normalized raw curve depicts the decreasing fluorescence vs. the increasing temperature.

3. Results

To find the most suitable DNA region for the identification of the 12 target species, their sequences were retrieved from GenBank. Not all target species have sequences deposited in the database. There are 11 of 12 species of the *mat*K, *rbc*L, and *trn*L regions, whereas there are 10 for the ITS (Table 2). The *Afzelia xylocarpa* (N6) sequence of only the *trn*L region can be found in the database. However, 10 species (N1-N4 and N7-N12) contain sequences of all four selected regions (Table 3).

The sequence data of the four selected regions (ITS, *mat*K, *rbc*L, and *trn*L) were analyzed. ITS led the charts for nucleotide variation (61.56%), followed by *mat*K (41.99%), *trn*L (40.47%), and *rbc*L (21.56%), respectively (Table 4).

Table 4. Characteristics of the analyzed sequences.

DNA Region	Sample	Length (bp)	Nucleotide Variation
ITS	N1–N3, N7–N9, N10–N12	744	61.56% (458 bp)
*rbc*L	N1–N5, N7–N12	626	21.56% (135 bp)
*mat*K	N1–N2, N4–N5, N7–N9, N10–N12	1186	41.99% (498 bp)
*trn*L	N1–N4, N7–N12	677	40.47% (274 bp)

Before conducting an in vitro HRM, the DNA sequences were used in simulation HRM (uMELT Quartz) to predict the melting curves of each species. As can be seen in Figure 2, the melting curves of all the tested species were clearly distinguished only with the ITS primer set.

By containing the highest nucleotide variation (61.56%) and good performance in discriminating tested species in the simulation HRM, ITS is the most suitable for the task and thus only the ITS primer set was taken further to be used in an in vitro HRM assay. Although there were DNA samples of 12 native species (Table 2), only 9 were good enough for the HRM analysis: N1-N4, N6-N8, and N11-N12.

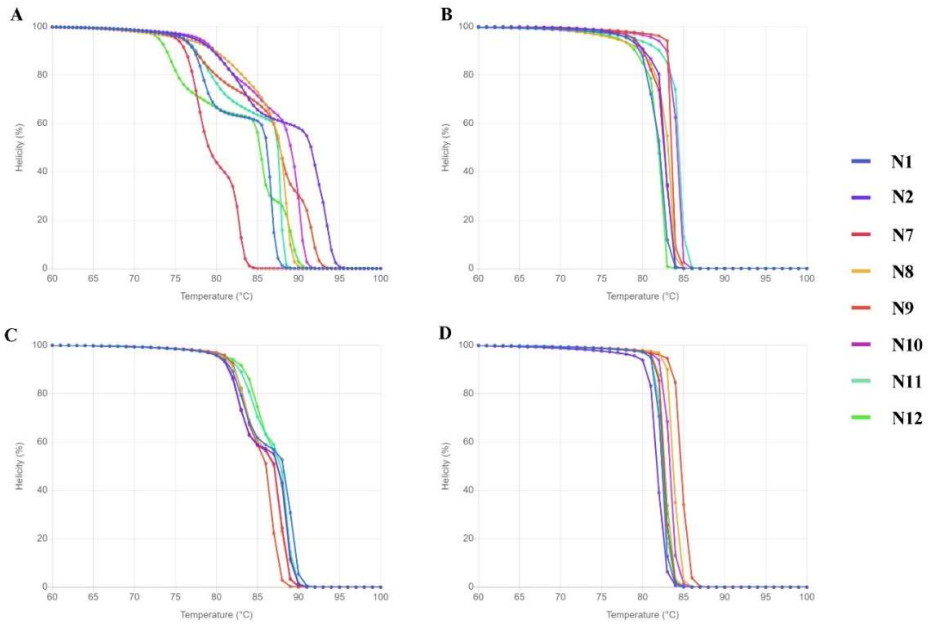
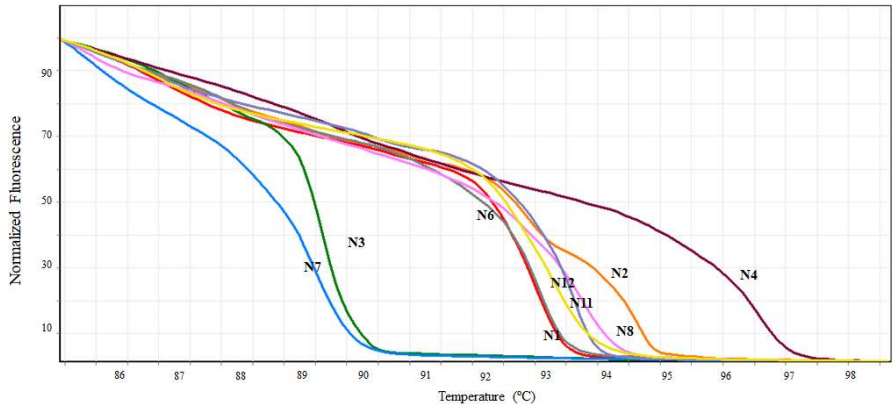


Figure 2. Melting curve prediction from uMELT Quartz based on DNA sequences from the four selected regions. (**A**) ITS, (**B**) *mat*K, (**C**) *rbc*L, and (**D**) *trn*L.

Results from the in vitro HRM analysis are similar to those obtained from the simulation. The ITS primer set can be used in HRM to separate the nine tested species as none of the melting curves of the tested species were similar (Figure 3). Melting temperatures of the tested species were also recorded, which range from 85.9 °C to 96.7 °C (Figure 3).



Color	Samples	Tm (°C)	Color	Samples	Tm (°C)	Color	Samples	Tm (°C)
	N1 : *Careya sphaerica*	93.2		N4 : *Gmelina arborea*	96.7		N8 : *Albizia lebbeck*	93.9
	N2 : *Hopea odorata*	94.7		N6 : *Afzelia xylocarpa*	93.2		N11 : *Oroxylum indicum*	93.8
	N3 : *Croton roxburghii*	89.7		N7 : *Phyllanthus emblica*	85.9		N12 : *Bauhinia purpurea*	93.4

Figure 3. Melting profiles of nine tested species generated from HRM analysis using ITS primers.

4. Discussion

Although DNA barcoding has been proven useful for the species-level identification of various plant groups, there are some limitations to the method. It is costly and time consuming and not easy to apply routinely in developing countries due to financial constraints for sequencing. The Bar-HRM is a free sequencing approach that is cost-effective

for a large-scale study. Bar-HRM analysis has proven to be one good molecular approach for plant identification. Several DNA regions were used with Bar-HRM to identify a wide range of plant groups in previous studies, such as *mat*K for flowering plants [42], *trn*L for bean crops [35] and nut products [36], *rbc*L for medicinal products from Acanthaceae species [37], ITS for Euphorbiaceae [38], and Dipterocarpaceae and Fagaceae [44]. It is therefore undoubtable that Bar-HRM would also be successful in identifying the tested species in this study.

Results from data mining and simulation HRM analysis indicate that the ITS is the most suitable region that can clearly discriminate the plant species in this study. As described in previous works, a key to success in HRM analysis is the variation in nucleotides within amplicon (more is better) [44,48]. The ITS as a non-coding region commonly exhibits higher nucleotide variations than coding regions, such as *mat*K and *rbc*L [32,49].

In the in vitro HRM analysis, only 9 of the 12 samples were good enough for Bar-HRM analysis. Due to both quantity and quality, three DNA samples (*Irvingia malayana*, *Chukrasia velutina*, and *Spondias mombin*) were omitted from the analysis. A main process in HRM analysis involves measuring the fluorescence dye bound to double-stranded DNA in a reaction. Thus, the initial DNA concentration of all samples needs to be the same [50]. In addition, DNA concentration correlates with melting temperature (T_m) [51]. Therefore, if there are any differences in the DNA concentration of the analyzed samples, results of HRM analysis could be wrong or uninterpretable [50].

5. Conclusions

Higher growth rate and greater ecosystem services are the main advantages of native plant species over other plant species for forest restoration. However, use of native plant species in forest restoration involves some time-consuming steps, including species identification. A rapid and reliable identification method is thus required. Bar-HRM has proved to be a rapid and reliable method for plant species identification in this study. The choice of the DNA region plays an important role in the success of Bar-HRM, as discriminated efficiency was found to be varied among the primer pairs selected (ITS, *mat*K, *rbc*L, and *trn*L). From the results shown here, a primer set based on the ITS region that exhibits the highest nucleotide variations is suitable for the identification of the tested native plant species. Thus, Bar-HRM with ITS primers could be used to confirm the species of plants used for reforestation.

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