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The Identification of Several Dipterocarpaceae and Fagaceae Trees by Barcode DNA Coupled with High-Resolution Melting Analysis

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Abstract: The loss of forests is a major environmental, social, and economic problem. The disappearance has been occurring to an extreme degree in many parts of Southeast Asia, including Thailand. Logging and clearing of forests for agriculture, cash crops, and food production has destroyed much of the tropical forests in Thailand. Floristic inventory could provide essential information for forest conservation but species identification as a part of inventory creating could be challenging in some cases. Barcode DNA coupled with High Resolution Melting analysis (Bar-HRM) was used here in aiding species identification of plant in Dipterocarpaceae (*Dipterocarpus alatus*, *D. costatu*, *D. intricatus*, *D. obtusifolius*, *Hopea ferrea*, *H. odorata*, *Shorea guiso*, *S. obtuse*, *S. roxburghii*, and *S. siamensis*) and Fagaceae (*Castanopsis echinocarpa*, *C. inermis*, *Lithocarpus wallichianus*, *Quercus aliena* and *Q. oidocarpa*) families. Two main experiments were conducted including: (1) a comparing method for primer design and (2) testing the robustness of the Bar-HRM by trying to identify tree samples that did not have sequences in the GenBank. In experiment 1, the manual design primer pair was found to be the best fit for the work. Of key importance is finding the primers which give the most nucleotide variations within the generated amplicon; this is a parameter that cannot be set in any web-based tools. Next, in experiment 2, Bar-HRM using primers of ITS1 and ITS2 regions were able to discriminate all 10 tested tree species without any problem, even when there were no sequences of the samples to be analysed before performing the HRM. Here, Bar-HRM poses potential to be a game-changer in tropical forest conservation, as it will be useful for species identification.

Keywords: tropical forests; High Resolution Melting analysis; species identification; primer design; Dipterocarpaceae; Fagaceae

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1. Introduction

Tropical forests host the most diverse plant communities on earth and play an important role in the global carbon cycle. Almost 15% of tropical forests are in Southeast Asia, which continues to be threatened or destroyed. According to recent research, Southeast Asia lost about 1.6 million hectares of forest per year, and it is estimated that such deforestation could lead to almost 50% of Southeast Asia's biodiversity loss by 2100 [1]. The effect of losing tropical forests is more devastating than previously thought, as also shown in a recent report [2]. Loss of forests is a major environmental, social, and economic problem. Thailand, Indonesia, and Malaysia are ranked top countries in Southeast Asia with high loss of forests.

Dipterocarpaceae and Fagaceae are two ecologically important plant families in tropical forests, often forming the backbone of the forest ecosystem. Both are main plant families for forest regeneration, conservation programmes and sustainable forest management [3–5]. Identification of plant species is also found to be important for conservation and management planning [6]. However, species identification of the plants in these families is still challenging. It is not only time-consuming but also requires experts. Most importantly, fruit and floral characteristics are needed to be identified [7], so it is almost impossible to identify them when they are seedlings or in a vegetative phase.

As stated previously, molecular technologies have recently paved the way in species identification. DNA barcoding is one of the biggest drivers to make identification easier and more reliable [8]. DNA barcoding involves sequencing a short standard region of DNA. Basically, DNA barcoding identification relies on genetic variability to establish the limits between species with lower levels of genetic divergence among individuals of the same species [9–11]. In animals, mitochondrial cytochrome c oxidase subunit I (COI) gene has been proposed as the standard barcode [8,12]. However, mutation rate of the COI is too low in plants so it cannot be used for plant species identification [13–16]. Although many researchers have made great contributions to the search for a core DNA barcode for plant identification, a consensus has not been reached [17–20]. Nevertheless, the CBOL Plant Working Group has suggested the use of two DNA regions (*rbcL* and *matK*) as a universal plant barcode [15]. Evaluation for candidate DNA barcodes in plants identified have also found other potential loci. The P6 loop of the *trnL* (UAA) intron is a good candidate as a supplemental barcoding locus as it can be routinely amplified across land plants with adequate species-level resolution, and can be used for highly degraded samples (e.g., [21,22]). Also, another commonly sequenced supplementary locus in plant identification is the nuclear internal transcribed spacer (ITS) (e.g., [23–25]).

Recently, DNA barcoding was used in combination with high-resolution melting analysis (called Bar-HRM). Bar-HRM analysis starts with PCR amplification of the selected barcode region in the presence of fluorescent dye. Amplification is then followed by a melting step under high-resolution conditions, which require a real-time PCR detection system with HRM analysis software. Bar-HRM requires no manual post-PCR processing as it is performed in a closed-tube system [26]. Species are discriminated according to base composition, length, and GC content [27,28]. Bar-HRM has been applied to authenticate commercial food, herbal, and agricultural products, e.g., [29–33]. Here, we show two different experiments for species identification of Dipterocarpaceae and Fagaceae using Bar-HRM with different DNA barcodes. In experiment 1, in order to have an effective Bar-HRM for species discrimination, two main approaches of primer design were tested. In experiment 2, Bar-HRM was evaluated to identify the tree samples. The developed method will be useful for forest health monitoring of the two ecologically important plant families.

2. Materials and Methods

2.1. Data Mining for Primer Design

To design primers for identification of Fagaceae plants based on the Bar-HRM technique, a dataset of a selected DNA region (Ribulose bisphosphate carboxylase large chain; *rbcL*) was constructed to conduct the sequence profile analysis. The *rbcL* sequences were retrieved from GenBank which included sequences from the entire family of Fagaceae (in total of 290 sequences from 92 species) (Supplementary Data A). Sequences obtained from public databases, including GenBank, are of low quality with no known associated herbarium vouchers. For this reason, all of the sequences were subjected to critical evaluation and any low-quality sequences were removed. Criteria used to filter the sequences were: (1) sequences are not 'unverified' without a species name, (2) contain <3% ambiguous base 'N', (3) maximum of 3 samples (sequences) are included from a species. After processing, multiple alignments were made from the selected sequences using MEGA10 and sequence

length (bp), conserved sites (%), variable sites (%), and GC content (%) of each dataset were recorded. Primers were designed manually by researcher and web-based programmes, including Primer3, Primer-BLAST, GenScript, and PrimerQuest IDT. All candidate primers were then compared and the best one selected for identification of the tested samples.

2.2. Plants Samples and DNA Extraction

Plant tissues of five Fagaceae species and ten Dipterocaparceae species used in experiment 1 and 2, respectively (Table 1), were collected from a forest in Doi Suthep-Pui and provided by the Queen Sirikit Botanical Garden. The plant tissues were ground with liquid nitrogen. DNA from all samples was extracted using the Nucleospin Plant® II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA concentrations were adjusted to a final concentration of 20 ng/µL. The DNA was stored at −20 °C for further use.

Table 1. Details of the plant samples.

Used in	Species	Voucher Number
Experiment 1	*Castanopsis echinocarpa* A.DC	25819
	Castanopsis inermis (Lindl. ex Wall.) Benth. & Hook.f.	7316
	Lithocarpus wallichianus (Lindl. ex Hance) Rehder	25181
	Quercus aliena Blume	34038
	Quercus oidocarpa DC.	90636
Experiment 2	*Dipterocarpus alatus* Roxb. ex G.Don	46253
	Dipterocarpus intricatus Dyer	10895
	Dipterocarpus costatus C.F. Gaertn.	10567
	Dipterocarpus obtusifolius Teijsm. ex Miq.	8889
	Hopea odorata Roxb.	28852
	Hopea ferrea Laness.	27258
	Shorea roxburghii G.Don	32238
	Shorea obtusa Wall. ex Blume	28848
	Shorea siamensis Miq.	33182
	Shorea guiso Blume	20500

2.3. In Silico Analysis of the Most Suitable Region for HRM Analysis

To address the most suitable DNA region for identification of Dipterocarpaceae and Fagaceae species based on Bar-HRM technique, a dataset was constructed to conduct the sequence profile analysis. Sequence profile of maturase K (*matK*), Ribulose bisphosphate carboxylase large chain (*rbcL*), *trnL* (UAA) intron (*trnL*), and Internal transcribed spacer (ITS) were retrieved from GenBank, which included sequences from the Dipterocarpaceae. There are in total 469 sequences from 188 species of *matK*, 425 sequences from 152 species of *rbcL*, 1112 sequences from 246 species of *trnL*, and 161 sequences from 75 species of ITS (Supplementary Data A). All the retrieved sequences were processed, as described in the data mining for primer design section.

2.4. Simulation High-resolution Melting (HRM)

uMelt Quartz was used for simulation of high-resolution melting assays (https://dna-utah.org/umelt/quartz/ (accessed on 27 February 2021). For experiment 1, sequences of five Fagaceae species (Table 1) were trimmed to leave only the region that would be generated by each pair of the candidate primers (one pair form a manual design and four pairs from each web-based tool). The amplicons were then put into the uMelt Quartz to get the melting profiles of each species. The primer pair that gives the best result in uMelt Quartz was then selected for HRM analysis. All melting profiles are shown in Supplementary Data B.

2.5. High-resolution Melting (HRM) Analysis

In order to determine the characteristic melting profile for each sample that could be used to distinguish the two different species, DNA amplification using real-time PCR was performed using the Rotor-Gene Q 5plex HRM system (Qiagen, Hilden, Germany). The reaction mixture for the real-time PCR and 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 20 ng DNA, and 3.6 μL of ddH2O. The nucleotides of forward and reverse primers were detailed in Table 2. Fluorescence dye was used to monitor both the accumulation of the amplified product and the high-resolution melting process in order to derive the Tm value during PCR. The reaction conditions were as follows: an initial denaturing step at 95 °C for 5 min followed by 40 cycles of 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. 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 Tm, and the normalised raw curve depicts the decreasing fluorescence vs. increasing temperature. To generate normalised melting curves and differential melting curves, pre- and post-melt normalisation regions were set to define the temperature boundaries of the normalised and difference plots.

Table 2. Primers used in HRM assays.

Primer	Length	Sequence (5′—3′)	Region	Amplicon Size (bp)
M1F	22	GGTACATGGACAACTGTGTGGA	*rbcL*	149
M1R	25	ACAGAACCTTCTTCAAAAAGGTCTA		
ITS1F	24	GGTGAACCTGCGGAAGGATCATTG	ITS1	200–230
ITS1R	24	CCGAGATATCCATTGCCGAGAGTC		
ITS2F	21	CGCCTGCTTGGGCGTCATGGC	ITS2	200–230
ITS2R	22	GGGCCTCGCCTGACTTGGGGCC		

3. Results

3.1. Experiment 1: Comparing Primer Design Methods

Sequences of plants in Fagaceae (*Castanopsis*, *Lithocarpus* and *Quercus*) retrieved from GenBank were used to design primers. Two strategies (manual design and programme-based design) were performed to find the best primer pair for identification of the tested samples. Basically, primers should have the following properties: (i) length of 18–24 bases, (ii) 40–60% GC content, (iii) melting temperature (T_m) of 50–60 °C, (iv) primer pairs (forward and reverse) should not have complementary regions. However, for the primers for species identification/discrimination here, variable nucleotides within the product generated from the primer pair (amplicon) are important.

For manual design, a region with high variable sites was targeted. First, *rcbL* sequence (1,428 bp) was divided into seven segments (A–G), 200 bp each, except the last segment, which is 228 bp in length (Figure 1). The retrieved *rbcL* sequences from GenBank were analysed to find the most suitable *rbcL* segment for HRM analysis. The variable sites within the segment were found to be as follows: segment D > segment C > segment E > segment F > segment G > segment B > segment A. There are two primer pairs obtained using the manual design, the sequence of which was located within segment B and C (Table 3). Primer M1 contains more variable sites (6.0%) than M2 (2.0%); thus, the latter was chosen as a candidate of a manual design primer and taken for further analysis.

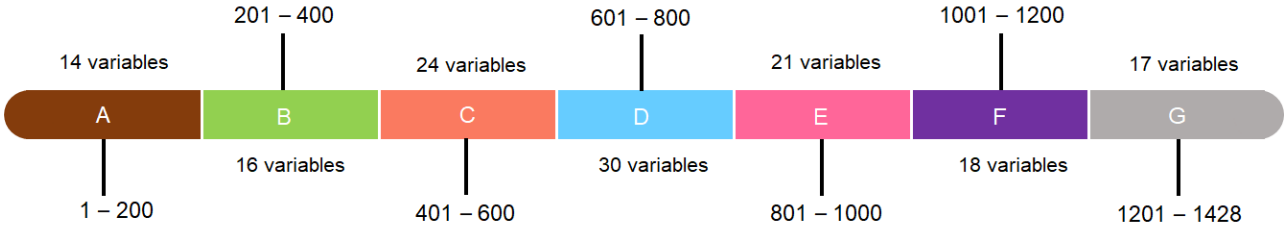

Figure 1. Seven analysed segments of the *rbcL* sequence for manual design of a primer.

For the programme-based primer design, a consensus sequence of *rbcL* sequences was built from all the retrieved sequences. Four web-based primer design tools, namely GenScript, Primer3, Primer-BLAST, and PrimerQuest IDT, were selected. All the selected programmes are similar in term of ease of use, quantity of time taken to get the results, and all are free to use. Different numbers of primers were obtained from each tool: three primer pairs were generated from GenScript, four from Primer3, ten from Primer-BLAST, and five from PrimerQuest IDT. All generated pairs of primers were then analysed. Most of the primers designed by web-based tools were located in segment B and C. Variable nucleotides within the amplicon of each primer pairs were calculated and shown in Table 3. The primer pair of each programme with the highest variable nucleotides within amplicon (G3, P3, PB4, and PQ1) were chosen for further analysis (Table 4).

The length, GC content, and annealing temperature (T_m) of the five chosen primer pairs were recorded. All five pairs were also evaluated in simulation HRM (uMelt Quartz) to find the best one that can discriminate five selected Fagaceae sequences (Table 1). Nucleotide variable and GC content of analysed amplicons are important factors to be considered for HRM analysis, so the two values were calculated and focused. Amplicons generated from a manual designed primer pair (M1) was found to have the highest nucleotide variable (6.0%) in comparison to all five chosen pairs. Among four programme-based designed primers, G3 contain the highest %variation site, which is 2.3%, followed by P3 (2.0%), PB1 (0.6%), and PQ1 (0.5%). GC content of amplicon generated from primer M1 had the highest GC content (46.9). Furthermore, the GC content of others is still at an acceptably value (Table 4). Simulation HRM (uMelt Quartz) was carried out, revealing that none of the designed primers were able to discriminate between all five tested species. None of the designed primers can discriminate between all the tested species from each other based on their melting profile obtained from Simulation HRM. M1 and P3 enable separation of the five tested species into three groups; PB4 and PQ1 can only divide the five species into two groups and G3 cannot separate any species so they are showed in one group (Supplementary Data B).

Table 3. Details of primer pairs generated by manual and programs.

Programme	Primer	Variable Nucleotide	Segment
Manual	M1	6.0	B
	M2	2.0	C
GenScript	G1	3.9	E/F
	G2	7.5	B
	G3	8.7	D/E
Primer3	P1	5.5	B
	P2	5.9	B
	P3	6.0	B
	P4	5.9	B/C
Primer-BLAST	PB1	11.7	C/D
	PB2	11.9	C/D
	PB3	4.0	F
	PB4	12.3	C/D

	PB5	7.6	B/C
	PB6	8.2	F
	PB7	8.0	F
	PB8	9.6	F/G
	PB9	10.4	C/D
	PB10	7.4	B/C
PrimerQuest IDT	PQ1	10.5	C/D
	PQ2	9.8	C
	PQ3	8.0	D/E
	PQ4	7.7	D/E
	PQ5	5.2	E/F

As the M1 seems to be the best candidate for the task here (highest variable sites), it was then used in HRM analysis in the lab. The DNA of five Fagaceae species (same as used in the simulation HRM) were used as a template for in vitro HRM. The melting profiles of all amplicons are illustrated in Figure 2. The M1 primer enabled division of the five tested species into four groups. Only *Castanopsis inermis* and *Quercus oidocarpa* cannot be discriminated from each other, which is consistent with the simulation HRM result.

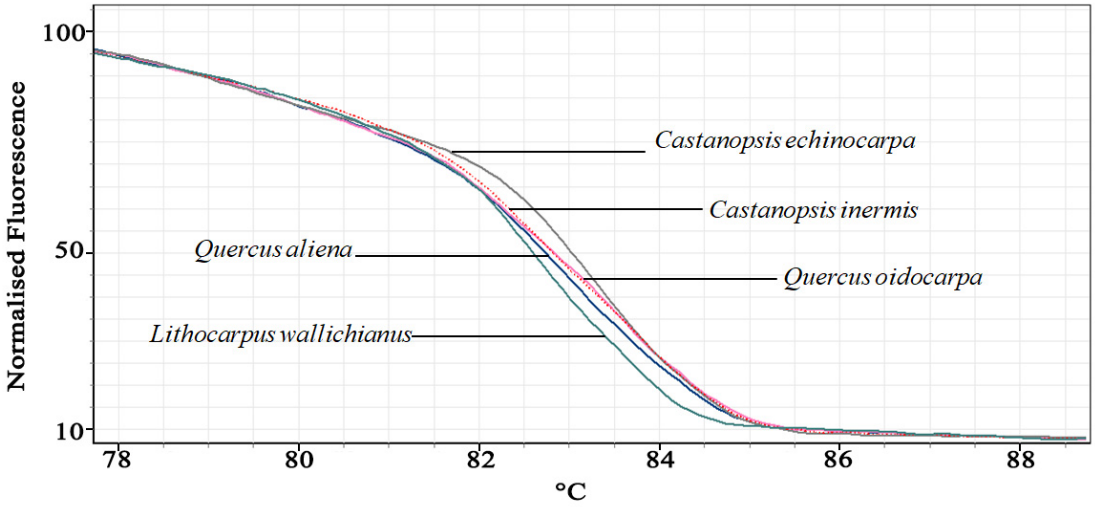

Figure 2. Melting profiles of five Fagaceae species generated from HRM analysis using M1 primers.

Table 4. Details of the candidate primers from manual design and web-based tools.

Primer	Length	T_m (°C)	GC Content	Sequence (5′—3′)	Variable Site (%)	Group in Simulation HRM	Amplicon Size (bp)
M1F	22	55.93	46.8	GGTACATGGACAACTGTGTGGA	6.0	3	149
M1R	25	54.25		ACAGAACCTTCTTCAAAAAGGTCTA			
G3F	20	59.04	44.8	CAACCATTTATGCGTTGGAG	2.3	1	266
G3R	20	59.24		GCACGATGGATGTGAAGAAG			
P3F	20	59.85	44.2	GGACATGGACAACTGTGTGG	2.0	3	200
P3R	20	59.89		CAGGGCCTTGAATCCAAATA			
PB4F	20	60.25	43.6	AACTTTCCAAGGTCCGCCTC	0.6	2	163
PB4R	20	59.83		GGTAAAATCAAGCCCACCGC			
PQ1F	22	62.00	42.1	GCATCCAAGTTGAGAGGGATAA	0.5	2	190
PQ1R	20	62.00		CGGTCTCTCCAACGCATAAA			

3.2. Experiment 2: Using Bar-HRM to Identify Unknown Species (Dipterocarpaceae)

In a similar situation, species identification of both Fagaceae and Dipterocarpaceae plants is difficult. Several molecular approaches have been introduced in aiding the identification. One of the most promising molecular techniques is DNA barcoding. However, the main limitation of the barcoding is that sequences are needed. Results from our search of four main DNA barcode regions (*matK*, *rbcL*, *trnL*, and ITS) in GenBank found that in a total of 695 species in Dipterocapaceae family, there are 205, 165, 268, and 77 species having *matK*, *rbcL*, *trnL*, and ITS sequences deposited in the database, respectively (Supplementary Data A). All of the selected barcodes of Dipterocarpaceae species having nucleotide sequences deposited in the GenBank less than 50% of the total species number. Bar-HRM is the molecular method that could eliminate the limitation of DNA barcoding in which sequences of the studies plants are not required. According to in silico test of all five DNA regions (*matK*, *rbcL*, *trnL*, ITS1, and ITS2), only three DNA regions (*rbcL*, ITS1, and ITS2) contain variable sites that can be used to distinguish the tested plants species (10 Dipterocarpaceae species) here. Thus, three primer pairs from the three DNA regions were used. First, the melting profiles of all amplicons generated from primer M1 (*rbcL*) are illustrated in Figure 3A-D. The ten selected species were divided into seven groups in which *D. obtusifolius*, *D. tuberculatus* and *S. obtuse* are in one group, *D. intricatus* and *S. siamensis* are also grouped together; the rest are well separated (Figure 3A). When looking closely at each genus (*Dipterocarpus*, *Hopea*, and *Shorea*), the M1 primers can only discriminate four *Dipterocarpus* into three group (Figure 3B), whereas all *Hopea* and *Shorea* species were all well separated (Figure 3C, 3D).

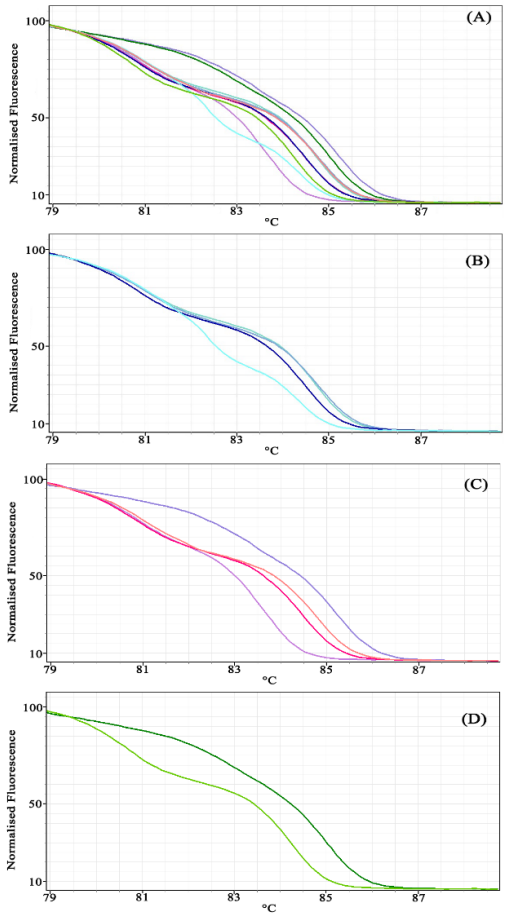

Figure 3. Melting profiles of 10 Dipterocarpaceae species generating from HRM analysis using M1 primers (*rbcL*). Normalised curves of (**A**) all 10 species; (**B**) species from genus *Dipterocarpus*; (**C**) species from genus *Shorea*; (**D**) species from genus *Hopea*.

The ITS1 and ITS2 primer sets were also used for the amplification of DNA fragments from all 10 samples, and the amplicons were analysed using HRM to define melting profile of each species and found that none of the melting curves of the tested species were shared or close to one another (Figures 4A–D and 5A–D). Thus, both ITS1 and ITS2 primers were equally good to discriminate the 10 tested species here.

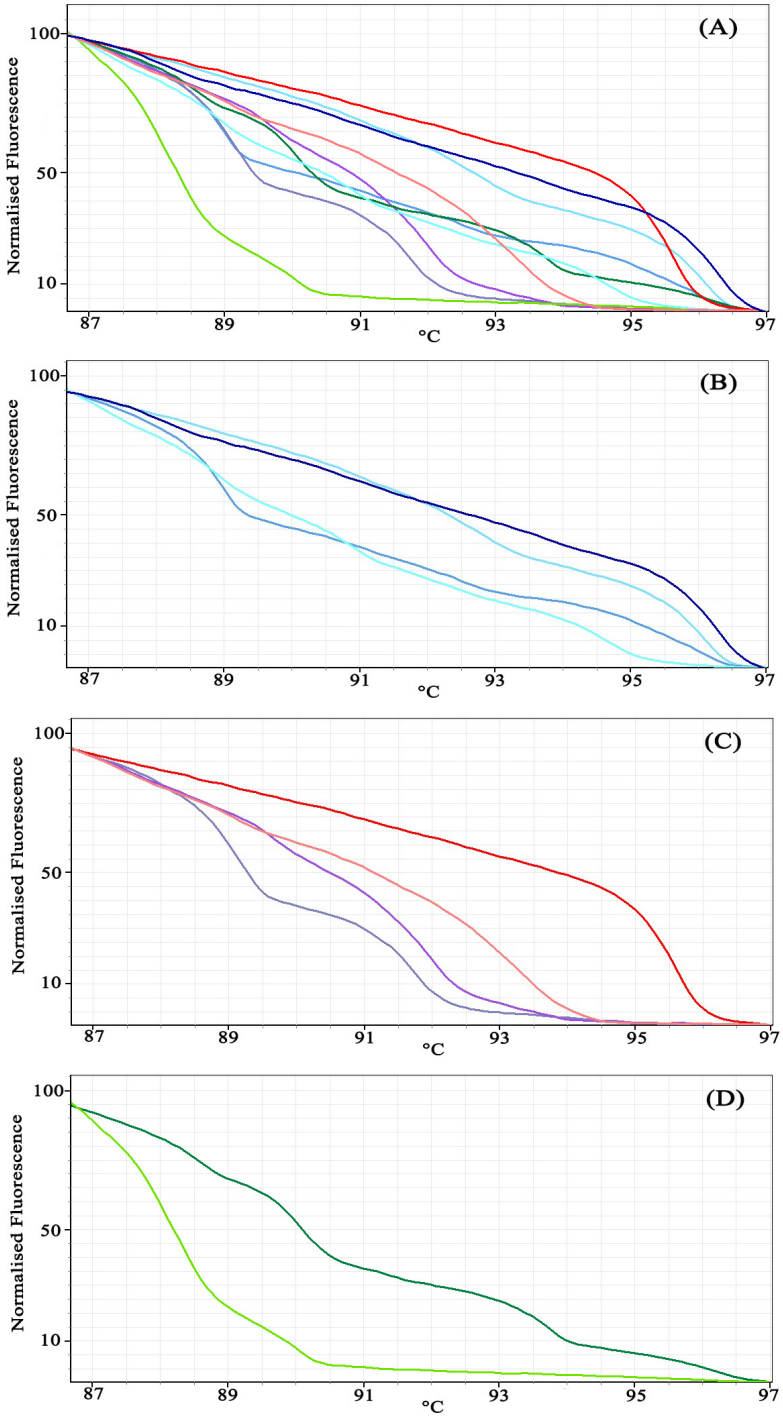

Figure 4. Melting profiles of ten Dipterocarpaceae species generated from HRM analysis using ITS1 primers. Normalised curves of (**A**) all 10 species; (**B**) species from genus *Dipterocarpus*; (**C**) species from genus *Shorea*; (**D**) species from genus *Hopea*.

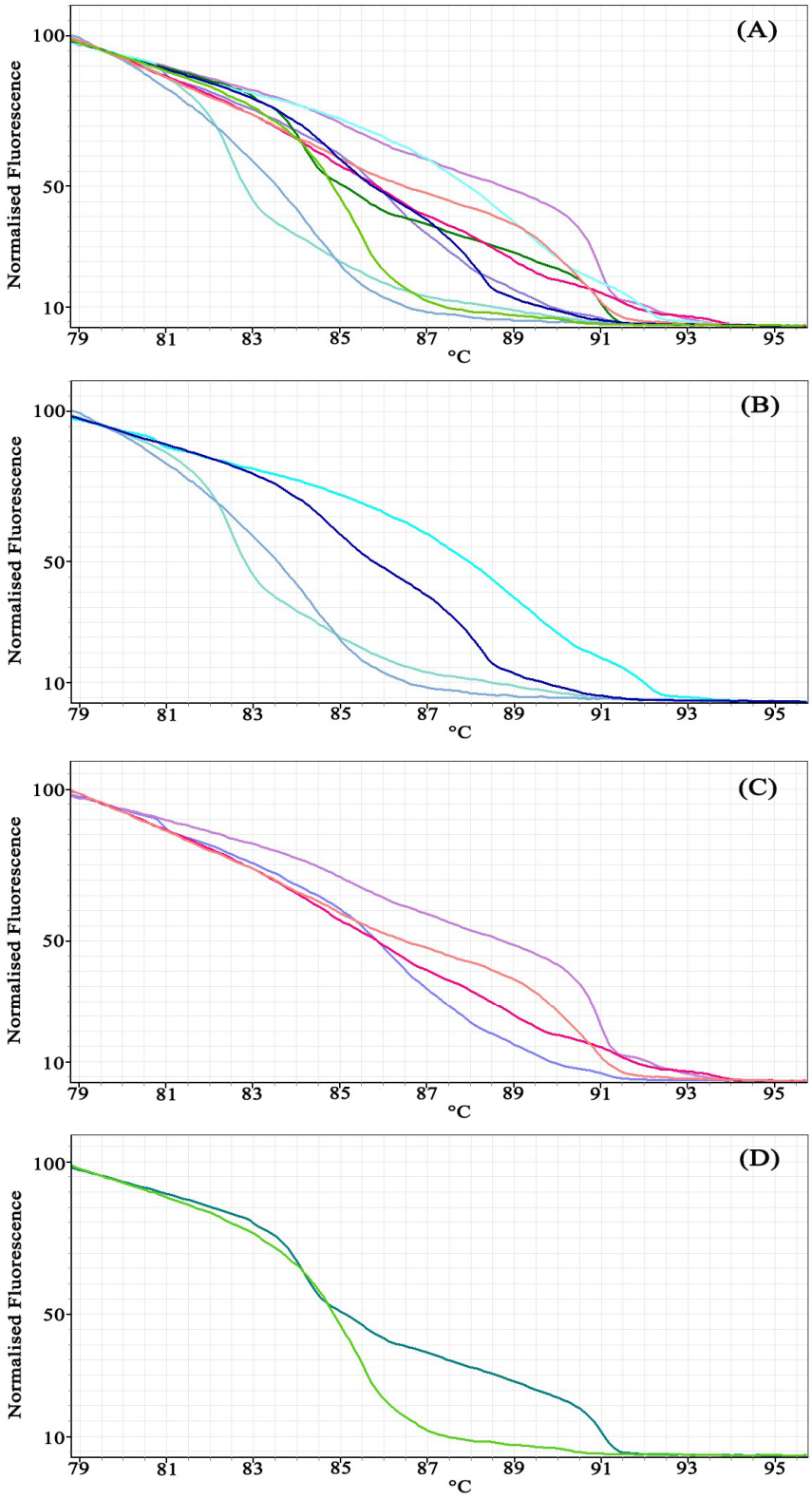

Figure 5. Melting profiles of 10 Dipterocarpaceae species generated from HRM analysis using ITS2 primers. Normalised curves of (**A**) all 10 species; (**B**) species from genus *Dipterocarpus*; (**C**) species from genus *Shorea*; (**D**) species from genus *Hopea*.

4. Conclusions

From the results showing here, manually designed primers were most suitable for Bar-HRM technique, as there were several factors affecting HRM analysis. One of the most important factors is variable nucleotides within the product generated from the primer pair (amplicon). Choice of DNA barcodes also has an impact on the success of Bar-HRM, as can be seen when comparing primer pairs generated from different DNA regions.

5. Discussion

5.1. Experiment 1: Comparing Primer Design Methods

Fagaceae is a big plant family comprising of nearly a thousand species, but less than 30% (260 species) of them have *rbcL* sequences deposited in GenBank. As mentioned earlier, morphological identification of the Fagaceae species is known to be difficult; thus, molecular data is necessary. Lack of sequences in public domain can hinder species identification via DNA barcoding, which relies heavily on the sequences. High-resolution melting (HRM) analysis has been proven to be alternative molecular tool in several cases, which sequence is not essential. Two main factors that contribute to the success of HRM analysis are GC content (40–60%) and variation nucleotide (more is better) within amplicon [34]. Therefore, primers generate amplicon with high variable sites and GC content within the range are desirable. Several programmes for primer design are now available; some with license some are free of charge. Many of the web-based primer design tools permit the user to set parameters as per their preference. Generally, criteria for primer design are all similar, such as length of primers (18–24 bp), melting temperature (56–62 °C), hairpin (try to avoid), and terminal nucleotide at 3′ (not complementary between forward and reverse primers) [35]. In the case of HRM for species discrimination, primers need to generate amplicon around 150–300 bp in length because a larger DNA size could reduce the distinction between variants [27,28]. Also, the generated amplicons should contain as many as nucleotide variations as possible and this is not a parameter that can be normally set in web-based primer design tools. In contrast, the manual design allows researchers to look for the most variable region and then design primers in that region. However, manual design takes longer time than web-based primer design tools.

5.2. Experiment 2: Using Bar-HRM to Identify Unknown Species (Dipterocarpaceae)

HRM coupled with DNA barcode (called Bar-HRM) has proved promising in species identification. However, none of the previous applications of Bar-HRM were used with Dipterocarpaceae before. Also, although there are several choices of DNA regions, *rbcL* and ITS were chosen in this study, which is based on several works on plant species identification, e.g., [15,17–20,23–25]. Several molecular works on Dipterocarpaceae species identification have been done, but there is still insufficient sequence data to cover a greater range of Dipterocarpaceae species [36–39] Interestingly, as mentioned earlier, not even 50% of Dipterocarpaceae species have their DNA sequences deposited in GenBank, but that is not a problem when using Bar-HRM, as proven here.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/f12111466/s1.

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References

1. Estoque, R.C., Ooba, M., Avitabile, V., Hijioka, Y., DasGupta, R., Togawa, T., Murayama, Y. The future of Southeast Asia's forests. *Nat. Commun*. **2019**, *10*, 1829.
2. Maxwell, S.L., Evans, T., Watson, J., Morel, A., Grantham, H., Duncan, A., Harris, N., Potapov, P., Runting, R. K., Venter, O., Wang, S., Malhi, Y. Degradation and forgone removals increase the carbon impact of intact forest loss by 626%. *Sci. Adv*. **2019**, *5*(10), eaax2546.
3. Sasaki S. Physiological characteristics of tropical rain forest tree species: a basis for the development of silvicultural technology. *Proc Jpn Acad Ser B Phys Biol Sci*. **2008**, *84*(2), 31-57.
4. Singh, P.P., Chakraborty, T., Dermann, A., Dermann, F., Adhikari, D., Gurung, P.B., Barik, S.K., Bauhus, J., Fassnacht, F.E., Dey, D.C., Rösch, C., Saha, S. Assessing Restoration Potential of Fragmented and Degraded Fagaceae Forests in Meghalaya, North-East India. *Forests* **2020**, 11, 1008.
5. Widiyatno, Fanny, H., Suryo, H., Sapto I., Susilo P. J., Naoki, T., Mohammad, N. Selection of dipterocarp species for enrichment planting in a secondary tropical rainforest. *Forest Science and Technology.* **2020**, *16*(4), 206-215.
6. Margules, C. R., and Pressey, R. L. Systematic conservation planning. *Nature*. **2000**, *405*, 243.
7. Forman, L.L. *Trigonobalanus* a new genus of Fagaceae with notes on the classification of the family. *Kew. Bull*. **1964**, *17*, 381-396.
8. Hebert, P. & Gregory, T. R. The promise of DNA barcoding for taxonomy. *Syst. Bot*. **200**5, *54*, 852–859.
9. Hajibabaei, M.; Singer, G.A.; Hebert, P.D.; Hickey, D.A. DNA barcoding: How it complements taxonomy, molecular phylogenetics and population genetics. *Trends Genet.* **2007**, *23*, 167–172.
10. Joly, S.; Davies, T.J.; Archambault, A.; Bruneau, A.; Derry, A.; Kembel, S.W.; Peres-Neto, P.; Vamosi, J.; Wheeler, T.A. Ecology in the age of DNA barcoding: The resource, the promise and the challenges ahead. *Mol. Ecol. Resour*. **2013**, *14*, 221–232.
11. Pečnikar, F.Z.; Buzan, E.V. 20 years since the introduction of DNA barcoding: From theory to application. *J. Appl. Genet.* **2014**, *55*, 43–52.
12. Hebert, P.D.; Ratnasingham, S.; de Waard, J.R. Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proc. R. Soc. Lond. B Biol. Sci.* **2003**, *270* (Suppl. 1), S96–S99.
13. Chase, M.W.; Cowan, R.S.; Hollingsworth, P.M.; Van Den Berg, C.; Madriñán, S.; Petersen, G.; Seberg, O.; Jørgsensen, T.; Cameron, K.M.; Carine, M.; et al. A proposal for a standardised protocol to barcode all land plants. *Taxon* **2007**, *56*, 295–299.
14. Kress, W.J. Use of DNA barcodes to identify flowering plants. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 8369–8374.
15. CBOL Plant Working Group. A DNA barcode for land plants. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 12794–12797.
16. Pennisi, E. Wanted: A barcode for plants. *Science* **2007**, *318*, 190–191.
17. Erickson, D.L.; Spouge, J.; Resch, A.; Weigt, L.A.; Kress, J.W. DNA barcoding in land plants: Developing standards to quantify and maximize success. *Taxon* **2008**, *57*, 1304–1316.
18. Lahaye, R.; Van der Bank, M.; Bogarin, D.; Warner, J.; Pupulin, F. DNA barcoding the floras of biodiversity hotspots. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 2923–2928.
19. Ledford, H. Botanical identities: DNA barcoding for plants comes a step closer. *Nature* **2008**, *451*, 616.
20. Farrington, L.; MacGillivray, P.; Faast, R.; Austin, A. Investigating DNA barcoding options for the identification of *Caladenia* (Orchidaceae) species. *Aust. J. Bot*. **2009**, *57*, 276–286.
21. Taberlet, P.; Coissac, E.; Pompanon, F.; Gielly, L.; Miquel, C.; Valentini, A.; Vermat, T.; Corthier, G.; Brochmann, C.; Willerslev, E. Power and limitations of the chloroplast *trnL* (UAA) intron for plant DNA barcoding. *Nucleic Acids Res*. **2006**, *35*, e1.
22. Fahner, N.A.; Shokralla, S.; Baird, D.J.; Hajibabaei, M. Large-scale monitoring of plants through environmental DNA metabarcoding of soil: Recovery, resolution, and annotation of four DNA markers. *PLoS ONE* **2016**, *11*, e0157505.
23. Chen, S.; Yao, H.; Han, J.; Liu, C.; Song, J.; Shi, L.; Zhu, Y.; Ma, X.; Gao, T.; Pang, X.; et al. Validation of the ITS2 Region as a Novel DNA Barcode for Identifying Medicinal Plant Species. *PLoS ONE* **2010**, *5*, e8613.
24. Li, D.Z.; Gao, L.M.; Li, H.T.; Wang, H.; Ge, X.J.; Liu, J.Q.; Chen, Z.D.; Zhou, S.L.; Chen, S.L.; Yang, J.B. Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 19641–19646.
25. Feliner, G.N.; Rosselló, J.A. Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants. *Mol. Phylogenetics Evol.* **2007**, *44*, 911–919.
26. Ganopoulos, I.; Sakaridis, I.; Argiriou, A.; Madesis, P.; Tsaftaris, A. A novel closed-tube method based on high resolution melting (HRM) analysis for authenticity testing and quantitative detection in Greek PDO Feta cheese. *Food Chem.* **2013**, *141*, 835–840.
27. Ririe, K.M.; Rasmussen, R.P.; Wittwer, C.T. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem*. **1997**, *245*, 154–160.

28. Reed, G.H.; Wittwer, C.T. Sensitivity and Specificity of Single-Nucleotide Polymorphism Scanning by High-Resolution Melting Analysis. *Clin. Chem*. **2004**, *50*, 1748–1754.
29. Madesis, P.; Ganopoulos, I.; Anagnostis, A.; Tsaftaris, A. The application of Bar-HRM (Barcode DNA-High Resolution Melting) analysis for authenticity testing and quantitative detection of bean crops (Leguminosae) without prior DNA purification. *Food Control* **2012**, *25*, 576–582.
30. Madesis, P.; Ganopoulos, I.; Bosmali, I.; Tsaftaris, A. Barcode High Resolution Melting analysis for forensic uses in nuts: A case study on allergenic hazelnuts (*Corylus avellana*). *Food Res. Int.* **2013**, *50*, 351–360.
31. Osathanunkul, M.; Madesis, P.; de Boer, H. Bar-HRM for Authentication of Plant-Based Medicines: Evaluation of Three Medicinal Products Derived from Acanthaceae Species. *PLoS ONE* **2015**, *10*, e0128476.
32. Singtonat, S.; Osathanunkul, M. Fast and reliable detection of toxic *Crotalaria spectabilis* Roth. in *Thunbergia laurifolia* Lindl. herbal products using DNA barcoding coupled with HRM analysis. *BMC. Complementary Altern. Med.* **2015**, *15*, 162.
33. Osathanunkul, M.; Suwannapoom, C.; Khamyong, N.; Pintakum, D.; Lamphun, S.; Triwitayakorn, K.; Osathanunkul, K.; Madesis, P. Hybrid analysis (barcode-high resolution melting) for authentication of Thai herbal products, *Andrographis paniculata* (Burm.f.) Wall. ex Nees. *Pharmacogn. Mag.* **2016**, *12*, 71–75.
34. Wittwer, C.T.; Reed, G.H.; Gundry, C.N.; Vandersteen, J.G.; Pryor, R.J. High-Resolution Genotyping by Amplicon Melting Analysis Using LCGreen. *Clin. Chem*. **2003**, *49*, 853–860.
35. Dieffenbach, C.W.; Lowe, T.M.; Dveksler, G.S. General concepts for PCR primer design. *PCR Methods Appl*. **1993**, *3*, S30–S37.
36. Kajita, T.; Kamiya, K.; Nakamura, K.; Tachida, H.; Wickneswari, R.; Tsumura, Y.; Yoshimaru, H.; Yamazaki, T. Molecular phylogeny of Dipterocarpaceae in Southeast Asia based on nucleotide sequences of *matK, trnL* intron, and *trnL-trnF* intergenic spacer region in chloroplast DNA. *Theor. Appl. Genet.* **1998**, *10*, 202–209.
37. Kamiya, K.; Harada, K.; Kajita, T.; Yamazaki, T.; Lee, H.S.; Ogino, K.; Ashton, P.S. Molecular phylogeny of dipterocarp species using nucleotide sequences of two noncoding regions in chloroplast DNA. *Tropics* **1998**, *7*, 195–207.
38. Dayanandan, S.; Ashton, P.S.; Williams, S.M.; Primack, R.B. Phylogeny of the tropical tree family Dipterocarpaceae based on nucleotide sequences of the chloroplast rbcL gene. *Am. J. Bot*. **1999**, *86*, 1182–1190.
39. Yulita, K.S.; Bayer, R.J.; West, J.G. Molecular phylogenetic study of *Hopea* and *Shorea* (Dipterocarpaceae): Evidence from the *trnL-trnF* and internal transcribed spacer regions. *Plant Species Biol.* **2005**, *20*, 167–182.