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Botanical Biometrics: Exploring Morphological, Palynological, and DNA Barcoding Variations in White Kwao Krua (*Pueraria candollei* Grah. ex Benth. and *P. mirifica* Airy Shaw & Suvat.)

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Abstract: White Kwao Krua, a crucial Thai medicinal plant, contains various phytoestrogen substances used to alleviate menopausal symptoms in estrogen-deficient women. It originates from two species, namely *Pueraria candollei* Wall. ex Benth. and *P. mirifica* Airy Shaw & Suvat. However, there exists morphological variation, and the taxonomic status between both species is ambiguous, making discrimination challenging. In this study, we aim to clarify and differentiate the morphological characteristics, palynology, and DNA barcoding of both species. The morphological results showed the stipule size is a phenotypic marker for the differentiation of both species during the vegetative stage. The palynological results, however, exhibited similarity. Through an examination of nucleotide sequences and neighbor-joining tree analysis, it was determined that the DNA barcoding of the *mat*K region has the capability to distinguish between *P. candollei* and *P. mirifica* at nucleotide position 702. Specifically, *P. candollei* manifested a G base, contrasting with the C base observed in *P. mirifica*. This study concludes that stipule size and the *mat*K gene in DNA barcoding serve as a distinctive characteristic for distinguishing between *P. candollei* and *P. mirifica*. These methodologies prove valuable for ensuring the accurate identification of white Kwao Krua for horticulturists.

Keywords: DNA barcoding; herbal medicine; *mat*K; morphological characteristics; palynological characters; phylogentic analysis; pollen grain; *Pueraria candollei*; *Pueraria mirifica*; white Kwao Krua

1. Introduction

White Kwao Krua (various spellings) is an important Thai medicinal plant constrained to a select few deciduous montane woodlands situated in the northern and western regions of Thailand [1,2]. The botanical origin of white Kwao Krua comes from two species, namely *Pueraria candollei* Wall. ex Benth. [3–5] and *P. mirifica* Airy Shaw & Suvat. [6–8], according to the world flora online database [9,10]. However, both species have been categorized to the variety level of *P. candollei*: *P. candollei* var. *candollei* [11,12] and *P. candollei* var. *mirifica* (Airy Shaw & Suvat.) Niyomdham [2,13,14] in much of the literature, respectively. The tuberous root of white Kwao Krua has found extensive utilization within the traditional Thai medicinal system, primarily for the purposes of promoting skin health, improving cognitive function, and rejuvenating properties, particularly among the elderly [15]. Nowadays, white Kwao Krua is utilized to alleviate

menopausal symptoms experienced by estrogen-deficient women. These symptoms include vasomotor manifestations such as hot flashes and night sweats, reproductive issues like decreased libido and urogenital disorders, as well as psychological aspects, including depression and musculoskeletal discomfort [2]. White Kwao Krua contains chromene compounds, such as deoxymiroestrol, miroestrol, and isomiroestrol, exhibiting a distinctive estrogen-like molecular structure, and isoflavonoids, namely genistein, daidzein, and puerarin [3,16]. Additionally, it is employed for breast augmentation and esthetic enhancement purposes [17]. Consequently, it is distributed through herbal markets, drugstores, and online retail platforms in various forms, including tablets, capsules, cookies, creams, and gels. Its usage is predominantly directed towards mitigating symptoms associated with estrogen deficiency, such as hair loss, wrinkles, and breast sagging [2,3,15]. However, white Kwao Krua faces a critical conservation status due to extensive exploitation because of high demand, limited resources, and soil erosion [3]. Consequently, it is extensively cultivated for its utilization in medicinal and dietary supplement applications for domestic and international consumption. Nonetheless, white Kwao Krua encounters challenges related to the quality of its raw materials [4,18]. An underlying factor contributing to this issue is the lack of precise identification of the botanical origin [3,4].

In plant populations, variations manifest among species that are spatially separated, encompassing both genotype and phenotype [19]. Different environmental conditions generate distinct selection forces to occur in various plant populations of the same species, resulting in genetic and phenotypic divergence between populations [19,20]. The large number of varieties within crop plants poses challenges in identifying and characterizing them [21]. Numerous plants reported the variation with the horticultural species, such as *Bixa orellana* L. [22], *Cucumis melo* L. [23], *Solunum lycopersicum* L. [24], and *S. melongena* L. [25]. Our preliminary investigation showed the variance of floral size, color, marking, and morphogenetic characteristics of white Kwao Krua (Figure 1). Furthermore, the conspicuous similarity in morphological characteristics between *P. candollei* and *P. mirifica* is evident, with precise details remaining elusive. The botanical characteristics and ISSR-Touchdown PCR technique were used to analyze and classify the variation of *P. mirifica* [26]. Moreover, DNA barcoding, a modern advancement in biological identification technique, was employed to identify and analyze the variation of *P. candollei* and *P. mirifica* [3,4,18]. According to our previous study, there were genetic variations of *P. candollei* in Internal Transcribed Spacer and *trn*H-*psb*A regions; however, the study lacked a connection between genetic traits and morphological features [3]. In order to render the morphological diversity characteristics of white Kwao Krua clear and easily identifiable for its botanical origin, which is beneficial to farmers in cultivation, this research aims to clarify the taxonomic status and description using morphological characteristics, palynology, and DNA barcoding between *P. candollei* and *P. mirifica*.

Figure 1. Flora variance of white Kwao Krua.

2. Materials and Methods

2.1. Plant Materials Collection

In total, thirty-eight accessions of white Kwao Krua seeds were collected from various locations in Thailand and then planted in open field at Phichit Agricultural Research and Development Center in Phichit, Thailand (Supplementary Table S1). In this study, the white Kwao Krua sample was gathered for comprehensive examination encompassing morphological, palynological, and molecular analyses. Voucher samples for reference were duly deposited in the official herbarium of the Faculty of Pharmacy, Chiang Mai University (bearing the Index Herbarium acronym: CMU). Morphological and palynological assessments were conducted on fresh stems, leaves, flowers, and fruits. Meanwhile, for DNA barcoding analysis, fresh leaves were meticulously stored in polyethylene bags containing silica gel for subsequent analysis.

2.2. Morphological Analyses

Morphological features of vegetative components, including stem and leaves (specifically lamina and stipule), as well as reproductive structures such as inflorescence, flower, and fruits, were meticulously examined. The attributes encompassing size, shape, and color of the white Kwao Krua floral samples were thoroughly investigated and dissected in triplicate using stereo microscopes. Hierarchical cluster analysis (HCA) was employed to conduct a qualitative analysis to elucidate the relationship among white Kwao Krua samples and distinguish between *P. candollei* and *P. mirifica*. Various characteristic data, encompassing stipule shape, stipule length (cm), indumentum of young leaves, inflorescence length, flower length (cm), calyx length (cm), calyx tube shape, vexillum blotch patterns, apex vexillum blotch patterns, wing-to-keel ratio, and pod indumentum, were utilized to generate row and column data matrices. The dataset included sample codes as rows and their corresponding characteristic data as columns (Supplementary Table S2). Using the complete method in hierarchical cluster analysis with RStudio version 1.2.5033 software [27], these data were clustered into a dendrogram.

2.3. Palynological Analyses

To analyze the morphological characteristics of pollen grains from the white Kwao Krua in the two species, representative samples from *P. candollei* (D1) and *P. mirifica* (C4), including some *P. candollei* samples such as A3, A5, AA7, B3, D4, E3, K4-6, and L were selected for examination. The fresh pollen grains were mounted on an aluminum stub using double-sided adhesive carbon tape and then coated with a layer of gold through sputter-coating. Subsequently, the micromorphological attributes of the pollen grains were observed utilizing a scanning electron microscope (JSM-590LV, JEOL, Tokyo, Japan) at central science laboratory, Faculty of Sciences, Chiang Mai University. The pollen terminology adopted in this study adheres to that defined by Halbritter et al. [28].

2.4. Molecular Analyses Using DNA Barcoding Technique

2.4.1. DNA Isolation

The dried leaves of white Kwao Krua were cryogenically pulverized into a fine powder using liquid nitrogen. Total genomic DNA was extracted employing the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's guidelines, with slight modifications. The extracted DNA was quantified using a NanoDrop One UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.4.2. DNA Amplification of Barcoding Regions and Sequencing

Polymerase Chain Reaction (PCR) was conducted to amplify four DNA barcode regions, specifically the Internal Transcribed Spacer (ITS), maturase K gene (*mat*K), ribulose-bisphosphate carboxylase gene (*rbc*L), and *trn*H-*psb*A intergenic spacer region. Each reaction mixture comprised approximately 100 ng of genomic DNA, 12.5 µL of KOD One™ PCR Master Mix (TOYOBO Life Science, Osaka, Japan), 0.75 µL of each upstream and

downstream primer at 10 pmol/µL concentration, and PCR-grade water to reach a final volume of 25 µL. Detailed information regarding the primers used can be found in Supplementary Table S3. The amplification process involved 40 cycles at 98 °C for 10 s, 52 °C (for *mat*K and *rbc*L) or 55 °C (for ITS and *trn*H-*psb*A) for 5 s, and 68 °C for 3 s. Subsequently, all PCR amplicons underwent electrophoresis on a 1.8% agarose gel and were visualized by staining with RedSafe™ nucleic acid staining solution (iNtRON Biotechnology, Seongnam, Republic of Korea) under UV light radiation utilizing a Gel Doc™ EZ Imager (Bio-Rad, Hercules, CA, USA). Successful PCR products were subjected to bidirectional sequencing using an ABI PRISM 3730XL sequencer (Applied Biosystems, Waltham, MA, USA).

2.4.3. Bioinformation Analysis

The raw sequence data underwent meticulous manual examination and quality-based trimming. BioEdit Software version 7.2.6 [29] was employed with default parameters to excise primer regions and segments exhibiting low quality. The refined nucleotide sequences were aligned using MUSCLE Software 3.8.31 [30] and MEGA software version 11.0.10 [31]. Alignment parameters were judiciously selected, encompassing clustering methods such as UPGMB, a minimum diagonal length of 24, a gap open penalty of −400, and a gap extend value of 0. To comprehend and visually represent the relationship among white Kwao Krua samples and the differentiation of its two species, *P. candollei* and *P. mirifica*, genetic distances based on single nucleotide polymorphisms (SNPs) and insertion/deletion in three DNA barcoding regions—namely ITS, *mat*K, and *trn*H-*psb*A—among white Kwao Krua samples were calculated. The resulting genetic distance matrix was then subjected to Principal Coordinate Analysis (PCoA) using GenAlEx 6.5 [32,33] to generate a graphical representation. Phylogenetic analysis involving a neighbor-joining tree based on a combination of all DNA barcoding regions was conducted using MEGA software version 11.0.10, employing the K2P model of evolution and executing 10,000 bootstrap replications. *Pueraria* species were utilized as the outgroup for this analysis. The intraspecific value was assessed using the Kimura-2 parameter (K2P) model of evolution with 10,000 bootstrap replications [34].

3. Results

3.1. Morphological Analysis

3.1.1. Comparison of Morphological Characteristics of *Pueraria candollei* and *P. mirifica*

A meticulous morphological analysis of both species of white Kwao Krua, namely *P. candollei* and *P. mirifica*, was undertaken. Essential morphological characteristics, as indicated by prior publications, were employed to distinguish between the two species. These characteristics encompassed traits such as hairiness, inflorescence length, flower length, calyx length, and the presence of indumentum on pods (Table 1) [1,35]. The distinctiveness between both species was apparent in various features. For instance, the inflorescence length of *P. candollei* exceeded that of *P. mirifica* (Table 1; Figure 2a–d). Additionally, the flower and calyx sizes of *P. mirifica* were relatively smaller compared to *P. candollei* (Table 1; Figure 2e–h). Furthermore, the pod of *P. candollei* was glabrous (Figure 2i), whereas *P. mirifica* exhibited dense pubescence (Figure 2j). In this study, specimens B1, C1, and D1-D3 exhibited consistent features with *P. candollei*, while C3-C5, AA5, and AA6 demonstrated the morphological characteristics of *P. mirifica*. Nevertheless, other specimens exhibited ambiguous characteristics, displaying features common to both species. Moreover, various plant structures of *P. candollei* and *P. mirifica* were evaluated, as illustrated in Figure 3 for *P. candollei* and Figure 4 for *P. mirifica*. A distinct difference in stipule length was observed between the two species; the stipule of *P. candollei* (0.9–1.8 cm) (Figure 3f) was longer than that of *P. mirifica* (0.3–0.4 mm) (Figure 4f). Regarding seeds, *P. candollei* displayed a marble dark red seed coat, with seed dimensions measuring 4.1–4.4 × 3.3–3.6 mm and an ellipsoid-shaped hilum 1.5 mm in diameter (Figure 3h). Conversely, *P. mirifica* exhibited a marble pinkish white seed coat, with seed dimensions measuring 4.82–4.9 × 3.5–3.6 mm and an oval-shaped hilum of 1.0 mm in diameter (Figure 4h).

Table 1. Key morphological features of *Pueraria candollei* and *P. mirifica* based on our study and the published data.

Morphological Characters	Our Research		Published Data [1,35]	
	P. candollei	*P. mirifica*	*P. candollei*	*P. mirifica*
Hairiness	glabrous	densely pubescence	sparsely hairy or glabrous	Densely pubescence
Inflorescence length (cm)	72.0–77.0	25.0–35.0	30-up to 80	Up to 30
Flower length (mm)	17.6–19.8	17.9–18.2	18–20	13–15
Calyx length (mm)	8.1–9.0	6.4–7.1	8–12	5–8
Pod	glabrous	densely pubescence	rather glabrous	-

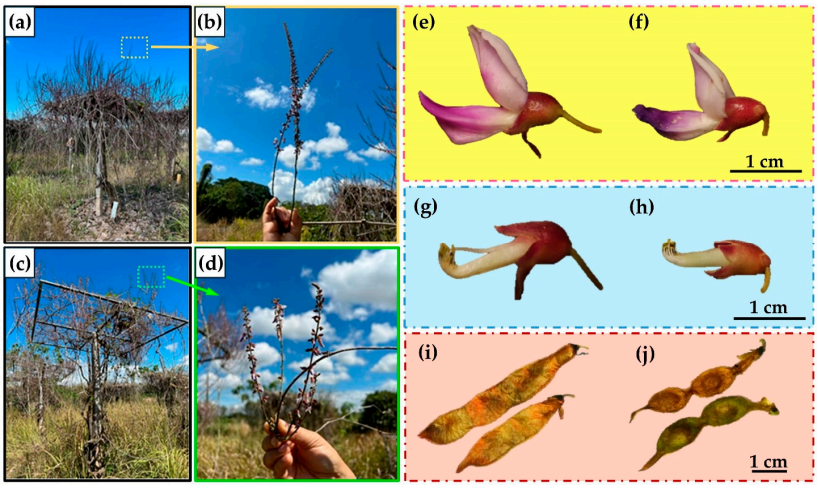

Figure 2. Key morphological features of white Kwao Krua for distinguishing its two species: *P. candollei* and *P. mirifica*. Habit, illustrating inflorescence during the flowering stage of *P. candollei* (**a**) and *P. mirifica* (**c**). Inflorescence structures of *P. candollei* (**b**) and *P. mirifica* (**d**). Flower characteristics of *P. candollei* (**e**) and P. mirifica (**f**). Calyx and *androecium* of P. candollei (**g**) and *P. mirifica* (**h**). Pod characteristics of *P. candollei* (**i**) and *P. mirifica* (**j**).

Based on the analysis of morphological characteristics between *P. candollei* and *P. mirifica*, we delineated the morphological classification using a narrative description and a dichotomous key for distinguishing between both species.

Deciduous lianas, tuberous root large. Shoots and young branches tomentose. Leaves tri-foliolate pinnate, alternate. Stipule peltate, lanceolate, persistent. Stipel filiform. Petiole slender, 8–12 cm. Petiolule short, 7–9 mm. Leaflets broad ovate, 12–15 × 7.5–15 cm, apex acute to acuminate, terminal leaflets base round to obtuse, lateral leaflets base round to truncate and oblique, chartaceous–coriaceous, entire to slightly sinuate. Lateral vein 5–7 pairs. Deciduous when flowering. Inflorescences axillary, pseudoraceme, branched. Flowers 3 per node, minute, 1.75–2.00 cm long, purple, bracteate and caducous, bracteole minute; pedicel scanty. Calyx united, 6.5–9 mm long, maroon, appressed pubescent; calyx tube shorter than calyx lobes; upper lobe completely united, obtuse and shorter; lower lobes 3, deltoid, middle lobes longer than lateral lobe, recurved at anthesis. Corolla clawed, blueish purple fades out to the base; vexillum thickened on the inner surface; alae spurred. Vexillum stamen united to others. Stigma penicillate below. Pod flat with tan walls, allowing the outlines of the seeds to be seen. Seed shagreened and carunculate. Hilum ellipsoid to oval.

1a Inflorescence often greater than 30 cm long; stipule often not less than 9 mm; pod glabrous; seed abundant shagreen; hilum ellipsoid. (*P. candollei*);

1b Inflorescence often less than 30 cm long; stipule not more than 5 mm; pod hairy; seed sparsely shagreen; hilum oval. (*P. mirifica*).

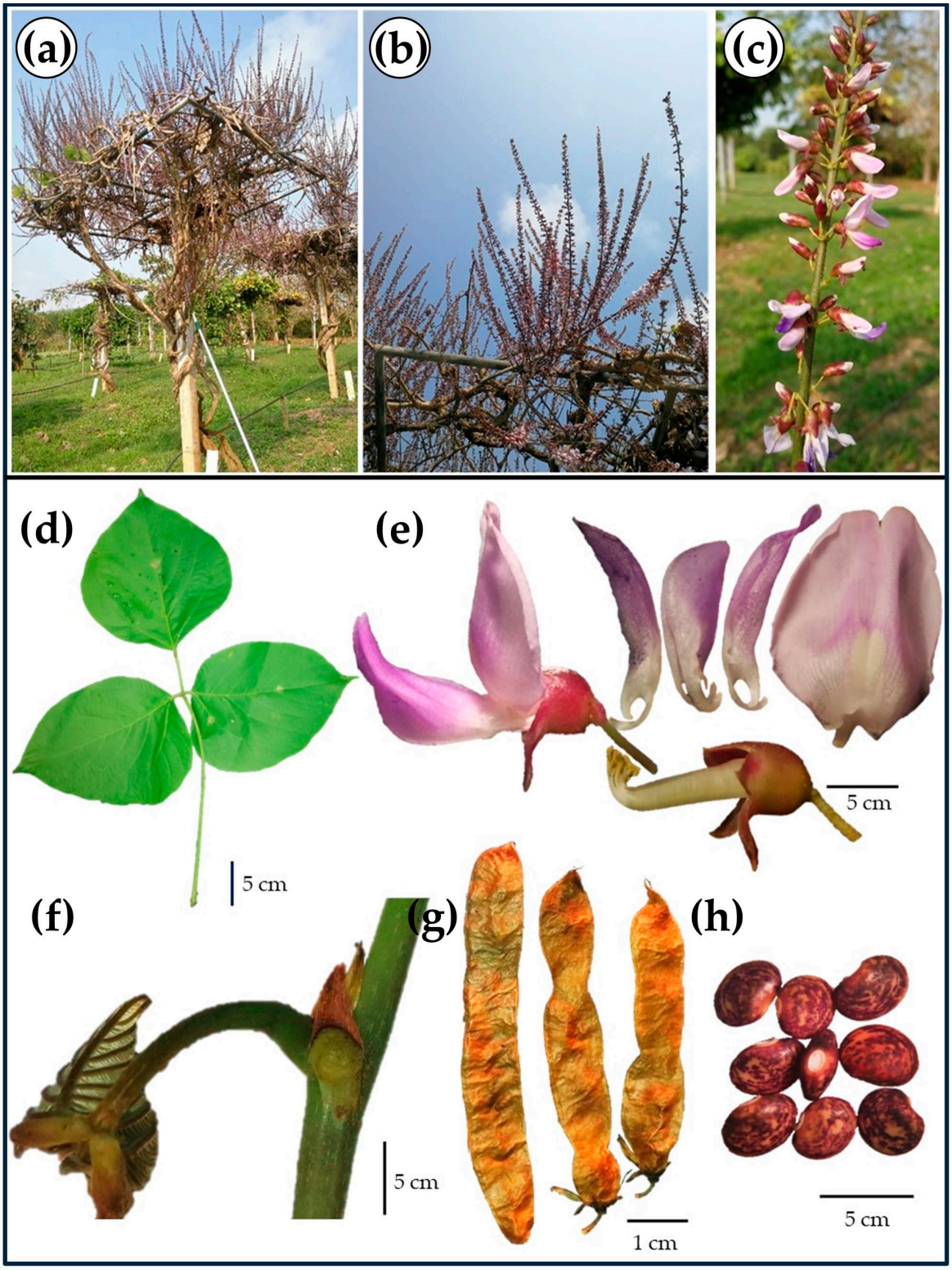

Figure 3. Morphological characteristics of *P. candollei* (specimens B1, C1, and D1–D3). Habit during flowering stage (**a**); inflorescence (**b**); floral structures (**c**); ternate leaves (**d**); complete flower and its components: standard, wings, keels, and calyx with stamens (**e**); stem displaying young leaves and stipule (**f**); pods (**g**); and seeds (**h**).

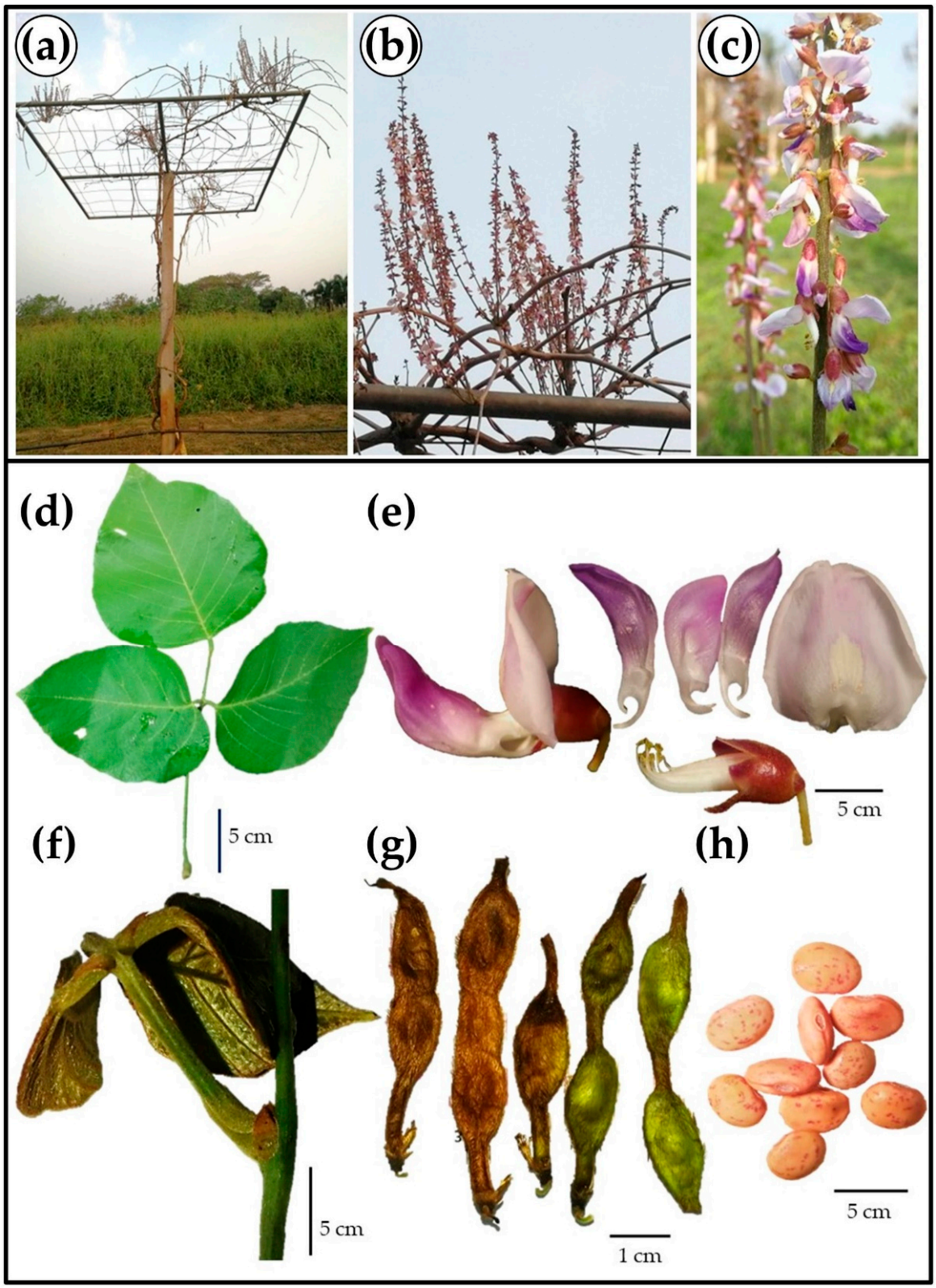

Figure 4. Morphological characteristics of *P. mirifica* (specimens C3–C5, AA5, and AA6). Habit during flowering stage (**a**); inflorescence (**b**); floral structures (**c**); ternate leaves (**d**); complete flower and its components: standard, wings, keels, and calyx with stamens (**e**); stem displaying young leaves and stipule (**f**); pods (**g**); seeds (**h**).

3.1.2. Hierarchical Cluster Analysis (HCA) of Morphological Features of Two Botanical Origins of White Kwao Krua: *P. candollei* and *P. mirifica*

In accordance with the morphological features observed in specimens of white Kwao Krua, hierarchical cluster analysis (HCA) was conducted using RStudio version 1.2.5033 software to elucidate the relationship between white Kwao Krua samples and distinguish between the two species. In addition to the primary morphological characteristics outlined in previous studies [1,35], other features were also examined, such as stipule shape and size, the indumentum of young leaves, calyx tube shape, vexillum blotch patterns on the standard, and the ratio of wing and keel length (Supplementary Table S2). The dendrogram resulting from HCA depicted the division of white Kwao Krua samples into five clusters (Figure 5). Five specimens exhibited discernible morphological traits characteristic of *P. candollei* (B1, C1, and D1–D3), placing them within cluster 5 with A2, E3, D4, and D5 samples. Conversely, C3–C5, AA5, and AA6 displayed precise morphological characteristics of *P. mirifica*, positioning them in the clade 2, along with the K4-6 sample. Within clade 1, there were eight specimens of white Kwao Krua, specifically B3–B7, C6, C7, and L. All exhibited distinctive characteristics, including a large flower size (2.4–2.5 cm) with white petals, a stipule size of 0.6 cm, and glabrous pods. While both clade 3 and clade 4 exhibited shared characteristics of both species, specimens in clade 3, namely A4–A7, D6, and D7, had stipule sizes approximately 0.2–0.3 cm, resembling those of *P. mirifica*. In contrast, clade 4, consisting of A1, B2, and AA1-AA4, featured large stipules (0.9–1.8 cm) resembling those of *P. candollei*.

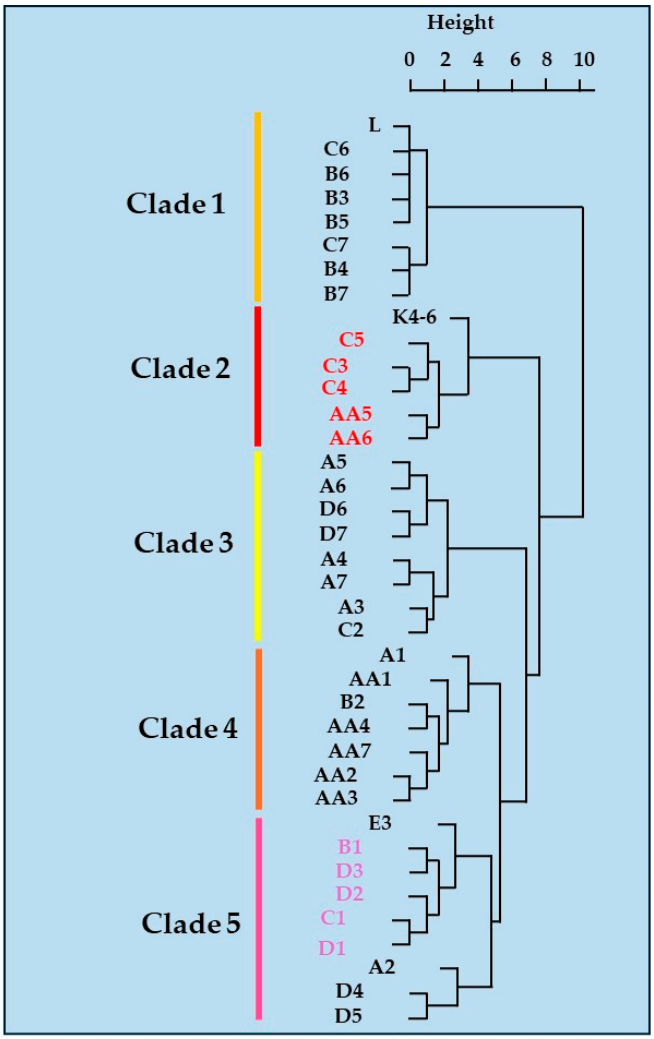

Figure 5. Hierarchical cluster analysis based on morphological characters depicting the relationship of white Kwao Krua samples (*P. candollei* and *P. mirifica*).

3.2. Palynological Characters of Pollen Grains of P. candollei and P. mirifica

Palynological analysis was performed to investigate the difference in both species: *P. candollei* (D1) and *P. mirifica* (C4). The scanning electron microscopy (SEM) images of both species are shown in Figure 6. The SEM photographs showed that both species demonstrated similar pollen micro-morphological characteristics. Both species contain tricolporate pollen grains with a spheroidal shape. The surface ornamentation of the pollen grains of all samples is the microreticulate type.

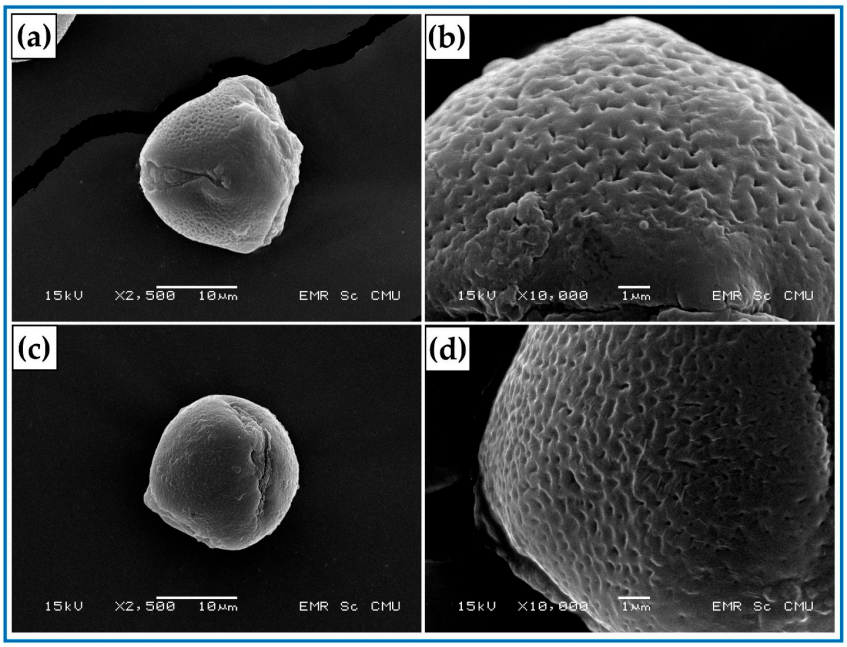

Figure 6. Scanning Electron Micrographs (SEM) depicting the shape and surface characteristics of pollen grains in *P. candollei* (D1) (**a**,**b**), *P. mirifica* (C4) (**c**,**d**).

3.3. DNA Barcoding Analysis

3.3.1. DNA Sequencing Analysis of White Kwao Krua in ITS, *mat*K, *rbc*L, and *trn*H-*psb*A

In this study, we successfully amplified and sequenced the DNA from all thirty-eight white Kwao Krua samples across four core DNA barcoding regions, namely the Internal Transcribed Spacer (ITS) region of the nuclear genome and three regions from the chloroplast genome: *mat*K, *rbc*L, and *trn*H-*psb*A. All nucleotide sequences were submitted in the DDBJ/EMBL/GenBank database (Supplementary Table S1).

1. Internal Transcribed Spacer sequencing

The total length of nucleotide sequences within the ITS regions of all white Kwao Krua samples ranged from 683 to 689 base pairs. The nucleotide sequences of the ITS regions (ITS1-5.8S-ITS2) exhibited intraspecific DNA polymorphism among all white Kwao Krua samples (Supplementary Figure S1). Eight base substitutions were identified in the ITS1 region at nucleotide positions 40, 41, 45, 86, 87, 106, 170, and 230. In the ITS2 region, six base substitutions were observed at positions 548, 580, 667–669, and 675. Additionally, we investigated the insertion/deletion of nucleotide bases at two locations: five nucleotides at position 62–66 and one nucleotide base at position 106 of the ITS1 region. Upon analyzing the DNA sequencing electropherogram, we detected three locations with overlapping peaks caused by nucleotide insertion. In the DNA sequencing electropherogram of sample L3, an insertion of five nucleotides (CGGGC) was noted at position 76 of the ITS1 region. In ITS2, the insertion of the base TA was identified at position 661 in samples A3–A7, C2, D6, and D7. Moreover, samples AA1–AA4, AA7, B2, D4, and D5 revealed an insertion of the base CTC, resulting in overlapping peaks at nucleotide position 671. The pairwise genetic distance based on the ITS region was calculated to be 0.00249 ± 0.00087.

2. Maturase K (*mat*K) sequencing

In this study, the total length of nucleotide sequences within the *mat*K region was 858 base pairs (Supplementary Figure S2). The analysis of the nucleotide sequences revealed a single polymorphic site at aligned nucleotide position 702, displaying either base C or G. The white Kwao Krua samples were categorized into two groups based on this polymorphism. Specifically, members of *P. candollei*, including B1, C1, D1–D3, A1–A2, AA1–AA4, AA7, B2, D4, D5, and E3, exhibited base G at nucleotide position 702. Conversely, *P. mirifica*, encompassing AA5, AA6, C3–C5, A3–A7, B3–B7, C2, C6, C7, K4-6, and L, demonstrated base C at the same nucleotide position. Utilizing the *mat*K nucleotide sequences, the pairwise genetic distance among white Kwao Krua specimens was determined to be 0.00058 ± 0.00059.

3. Ribulose-bisphosphate carboxylase gene (*rbc*L) sequencing

Based on the *rbc*L region, the total length of nucleotide sequences of white Kwao Krua samples was 721 bp (Supplementary Figure S3). The *rbc*L nucleotide sequences exhibited no intraspecific variation among white Kwao Krua samples, encompassing both of its varieties. The overall mean interspecific distance was calculated to be 0.00000 ± 0.00000.

4. *trn*H-*psb*A intergenic spacer sequencing

The aligned nucleotide sequences of white Kwao Krua specimens, based on the *trn*H-*psb*A intergenic spacer, were 232 base pairs in length (Supplementary Figure S4). These sequences displayed significant genetic variability, resulting in a high overall mean interspecific distance of 0.01318 ± 0.00511. Upon closer examination of the aligned nucleotide sequences, two polymorphic sites were identified among white Kwao Krua specimens. Specifically, A1, A2, AA1–AA4, AA7, B1–B7, C1, C6, C7, D1-D5, E3, and L exhibited the base AAAAG, whereas A3–A7, AA5, AA6, C2–C5, D6, D7, and K4-6 showed the base CTTTT at nucleotide positions 153–157. Furthermore, A5, A6, AA5, AA6, C3, and C4 revealed the base AA at nucleotide positions 218–219, while the remaining samples demonstrated TG at these positions.

3.3.2. Principal Coordinate Analysis (PCoA) and Phylogenetic Analysis of White Kwao Krua

Principal Coordinates Analysis (PCoA) was conducted based on SNPs and insertion/deletion within the ITS, *mat*K, and *trn*H-*psb*A regions to differentiate between the two species of white Kwao Krua and elucidate the relationship within the white Kwao Krua germplasm. Because there was no different base nucleotide in the *rbc*L region among all white Kwao Krua, this region was excluded from this analysis. The resultant PCoA unveiled a notable diversity among white Kwao Krua samples, representing a cumulative percentage variation of 1-axis, 2-axis, and 3-axis = 44.07%, 67.61%, and 76.22%. As illustrated in Figure 7, white Kwao Krua samples were categorized into three major groups. The first group, which is the largest, comprised 17 samples: A1, A2, AA1–AA4, AA7, B1, B2, C1, D1–D5, E3, and L. These samples exhibited distinct characteristics of *P. candollei*. The second group included 14 members: A3–A7, AA5, AA6, C2–C5, D6, D7, K4-6, and showcasing the predominant attributes of *P. mirifica*. The third group encompassed B3–B7, C6, and C7.

The nucleotide sequences obtained from core DNA barcoding regions (ITS, *mat*K, *rbc*L, and *trn*H-*psb*A) were utilized both individually and in combination to construct phylogenetic trees employing the neighbor-joining (NJ) method. The resulting phylogenetic trees, constructed using individual markers, revealed distinct structures. Specifically, the NJ trees exhibited 3, 2, 1, and 2 major clades for ITS, *mat*K, *rbc*L, and *trn*H-*psb*A, respectively (Figure 8). Considering the sample locations of *P. candollei* (B1, C1, and D1–D3) and *P. mirifica* (C3–C5, AA5, and AA6) on the NJ tree, the results indicated that the ITS and *rbc*L gene loci were unable to distinctly separate these two plants. Fortunately, *mat*K and *trn*H-*psb*A loci could discriminate between these species. Furthermore, the analysis results from both of these DNA barcoding loci yielded identical outcomes. Nevertheless, samples B3-B7, C6, and C7 exhibited divergent outcomes in the analysis of two genes: *P. mirifica* in the *mat*K gene and *P. candollei* in the *trn*H-*psb*A gene. Upon integrating all DNA barcoding regions, the NJ phylogeny unveiled four major clades (Figure 9). The first clade comprised A1, A2, AA1–AA4, AA7, B1, B2, C1,

D1–D5, and E3. The second clade included A3–A7, AA5, AA6, C2–C5, D6–D7, and K4-6. The third clade encompassed seven members, namely B3–B7, C6, and C7. Lastly, the fourth clade consisted of a single member (L).

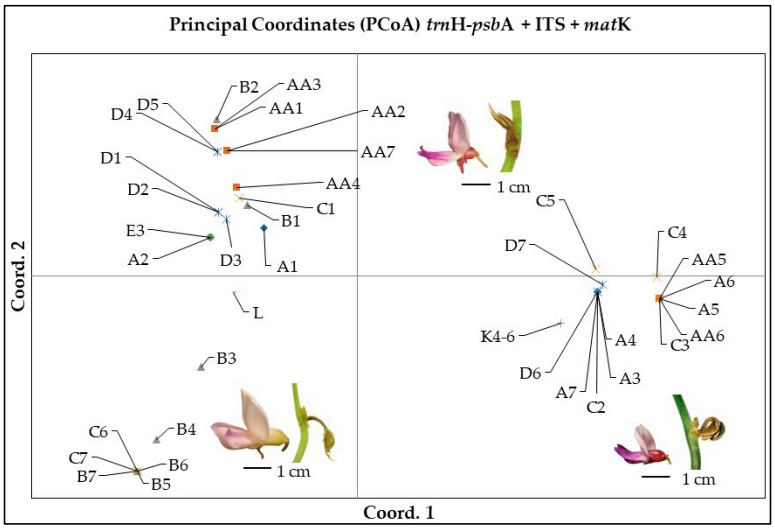

Figure 7. Principal Coordinate Analysis (PCoA) of 38 white Kwao Krua specimens based on SNPs in three DNA barcoding loci: ITS, *mat*K, and *trn*H-*psb*A.

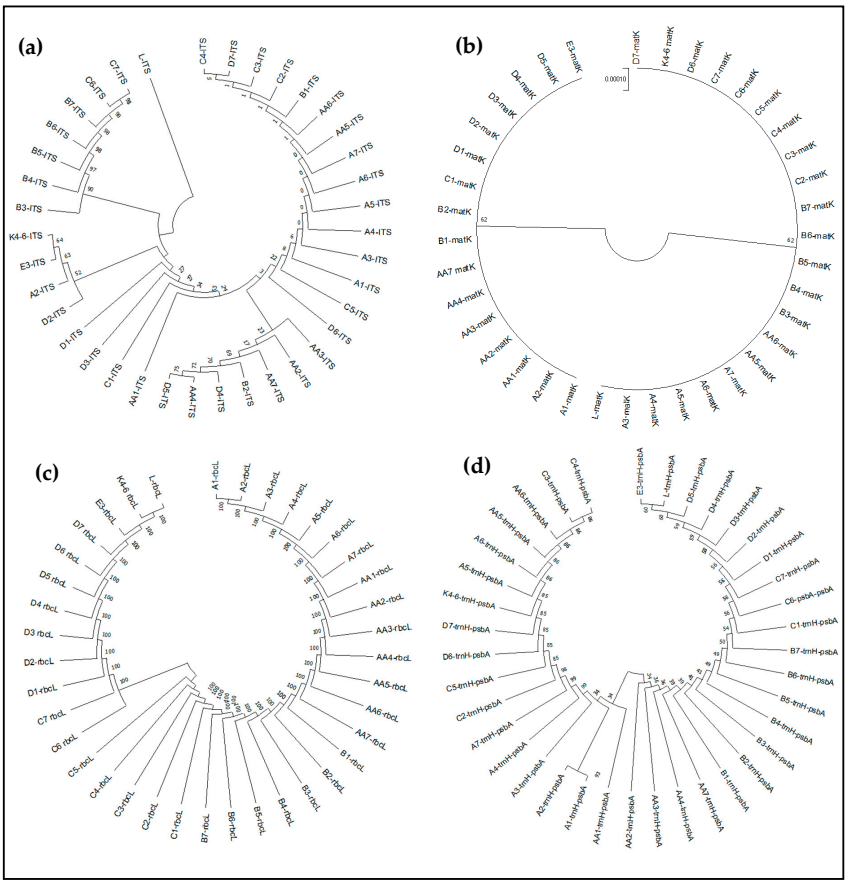

Figure 8. Phylogenetic trees illustrating the relationships among white Kwao Krua samples, reconstructed using the neighbor-joining method with MEGA software version 11.0.10. Trees are based on ITS (**a**), *mat*K (**b**), *rbc*L (**c**), and *trn*H-*psb*A (**d**) regions.

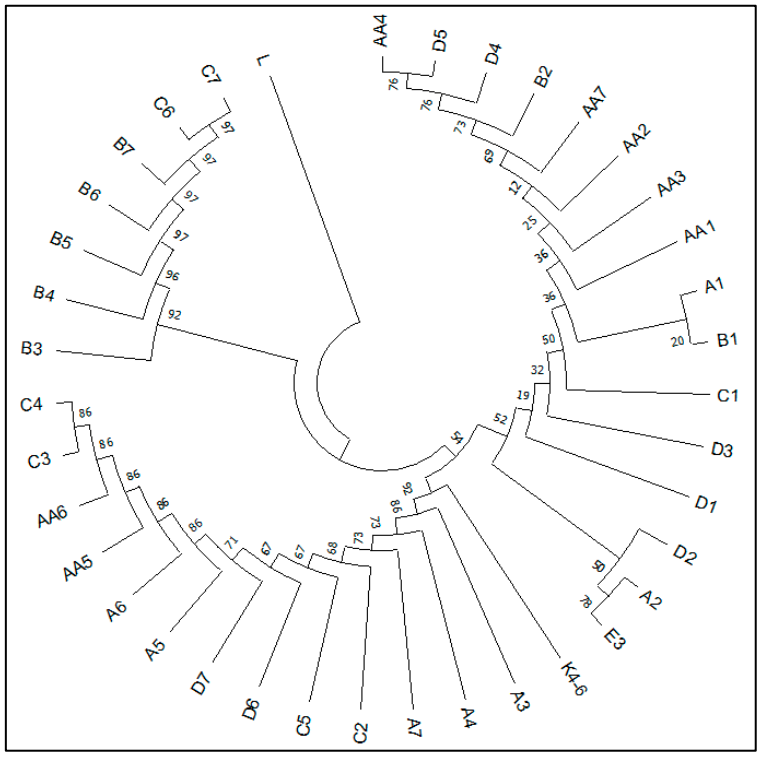

Figure 9. Phylogenetic trees illustrating the relationships among white Kwao Krua samples, reconstructed using the neighbor-joining method with MEGA software version 11.0.10. Trees are based on a combined analysis of all DNA barcoding regions.

4. Discussion

White Kwao Krua was endorsed as a "Thailand herbal champion product" by the Ministry of Public Health, emphasizing its role in the development of herbal products intended for both domestic consumption and export [36]. The accurate identification of plant species and the selection of high-quality strains are essential initial steps in cultivating medicinal plants for the development of high-value products. From our preliminary study, we observed a diversity of white Kwao Krua in morphological characteristics indicative of both *P. candollei* and *P. mirifica.* However, the morphological characteristics distinguishing *P. candollei* from *P. mirifica*, pertaining to the botanical origin of white Kwao Krua, remain unclear. Therefore, the morphological analysis, incorporating palynological and DNA barcoding analyses, was employed.

Being a leguminous plant akin to beans, white Kwao Krua is capable of undergoing cross-pollination, which is likely a contributing factor to the observed high genetic variation among these plants [26]. Genetic diversity leads to variations in the morphological expression of plants, yielding distinct characteristics that may contribute to confusion in cultivation or utilization. Therefore, in this study, morphological analysis was used to clarify the variation of white Kwao Krua and distinguish between *P. candollei* and *P. mirifica*. Thirty-eight samples of white Kwao Krua were collected from different locations in Thailand. Based on the essential morphological features [1,35], certain samples exhibit characteristics consistent with both *P. candollei* and *P. mirifica*, while some specimens display ambiguous features that make differentiation between the two species challenging due to the pronounced plasticity in leaf morphology, which undergoes changes under environmental influences [37,38]. Suwanvijitr et al. reported that the leaf size of *P. mirifica* may be influenced by the climate and humidity of its habitat [38]. Therefore, the morphological characters of leaves are not considered in this analysis. Other morphological traits, such as petiole morphology, seed shape, and patterns, were subsequently utilized for further morphological analysis. Utilizing the morphological features of white Kwao Krua samples, hierarchical cluster analysis (HCA) was conducted

using RStudio version 1.2.5033 software. The resulting dendrogram from HCA illustrated the segregation of white Kwao Krua samples into five distinct clusters (Figure 5). Although the prior research found that the seven characteristics, encompassing the leaf upper color, leaf blade, hairs at stem parts, hair at pod, overall flower color, size of leaf, and inflorescence length, the UPGMA-generated dendrogram revealed a division within white Kwao Krua samples, classifying them into two groups [26]. However, it is noteworthy that this study does not specify the plant type serving as the botanical source of white Kwao Krua. In the present study, a phenotypic characteristic emerged as a distinctive feature between the two plant species. Specifically, the stipule size of *P. candollei* (clade 5) was observed to be greater in length compared to those of *P. mirifica* (clade 2). However, three clades shared characteristics between these two species. Regarding stipule size, samples in clades 1 and 3 exhibited small stipule sizes resembling those of *P. mirifica*, whereas members of clade 4 had larger stipules, correlating with *P. candollei*. This finding can be employed for species identification on living plants for farmers, horticulturists and plant breeders, either independently or as a complementary method to genetic analyses.

Palynology is the scientific study of palynomorphs, a broad term encompassing all entities present in palynological preparations, such as pollen, spores, cysts, and diatoms [28]. Palynomorphological characters were acknowledged as a valuable tool for the identification of species and genera [39] in various applications such as environment [40], forensic [41], and herbal medicine [42]. There are many studies that reported pollen micromorphology can be used to discriminate plant species such as *Malva neglecta* Wallr. and *M. parviflora* L. (*Malvaceae*) [43], *Nelumbo nucifera* Gaertn. and *Nymphaea lotus* L. (*Nelumbonaceae*) [44], and the spineless *Astragalus* plants (*Fabaceae*) [45]. In this study, a comparative analysis of palynological morphology between *P. candollei* and *P. mirifica* was conducted. The results revealed that both species exhibit tricolporate pollen grains with a spheroidal shape, and the surface ornamentation is of the microreticulate type. This finding indicates that the pollen phenology between these two plant species cannot be differentiated. This observation aligns with previous research suggesting that genetically closely related species cannot be distinguished through palynology. For example, *Musschia aurea* Dumort. and *M. wallastonii* Lowe (*Campanulaceae*) [46], *Xanthium strumarium* L. and *X. brasilicum* Vell. (*Asteraceae*) [47], and *Ampelocissus artemisiifolia* Planch. and *A. indica* Planch. (*Vitaceae*) [48].

DNA barcoding is a methodology employed to identify biological specimens through the utilization of short DNA sequences extracted from either nuclear or organelle genomes, applicable to plants, animals, and microbes [49]. The principal barcoding regions encompass brief segments derived from chloroplast and nuclear genomes, ITS, *mat*K, *rbc*L, and *trn*H-*psb*A [49]. In addition to utilizing DNA barcodes for species identification, the study of genetic diversity in interspecific and intraspecific plant variation is also conducted, for example, *Phellodendron* species (*Rutaceae*) [50], *Panax* species (*Araliaceae*) [51], and *Pueraria* species (*Fabaceae*) [52]. In the analysis of genetic variation in white Kwao Krua, previous studies employed ISSR [26] and RAPD [38] techniques to assess genetic diversity. However, these methods are insufficient for verifying the botanical origin of white Kwao Krua. In this study, an assessment of genetic variation was conducted on 38 white Kwao Krua samples, focusing on DNA barcoding regions, namely ITS, *mat*K, *rbc*L, and *trn*H-*psb*A. The results indicated that, based on pairwise genetic distance, the highest genetic variation was observed in the *trn*H-*psb*A region, followed by ITS and *mat*K. However, no genetic variation was observed in the *rbc*L region. According to Principal Coordinate Analysis (PCoA) constructed by using a combination of all DNA barcoding regions, it was observed that the white Kwao Krua samples were categorized into more than two groups, consistent with findings from the previous studies employing ISSR [26] and RAPD [38] techniques. However, the PCoA result could discriminate *P. mirifica* from *P. candollei*. Focusing on the correlation between DNA barcoding and stipule size, the *P. mirifica* group exhibits small stipules, whereas the *P. candollei* group displays larger stipules (Figure 7). Additionally, the small group of B3–B7, C6, and C7 shows the different flower and stipule sizes in the range between *P. mirifica* and *P. candollei*. Based on the NJ tree, each DNA barcoding region

(Figure 8) and the integration of all loci (Figure 9) were reconstructed. From four DNA barcoding regions, *mat*K and *trn*H-*psb*A regions could separate *P. mirifica* from *P. candollei*. The analysis of the nucleotide sequence in the *mat*K gene locus unveiled a singular polymorphic site at the aligned nucleotide position 702, indicating the presence of the base G for *P. candollei* or C for *P. mirifica*, enabling the differentiation of the two plant species. The nucleotide position 702 serves as a distinctive nucleotide signature that separates the two species. This finding aligns with the research conducted by Wiriyakarun et al. [4]. In case of *trn*H-*psb*A, there were many polymorphic sites but the NJ tree was differentiable white Kwao Krua sample to two species. However, the samples of B3-B7, C6, and C7 were placed in *P. mirifica* clade for *mat*K gene, while they were positioned in *P. candollei* clade for *trn*H-*psb*A gene. Considering the morphological characters, it was observed that they predominantly exhibited features typical of *P. mirifica*. Consequently, based on the results, they should be identified as *P. mirifica*. This suggests that differentiating between plant species that are the botanical source of white Kwao Krua can be achieved by the nucleotide base sequence at the *mat*K region. This information can be leveraged to develop a more rapid, accurate, and convenient differentiation method, such as Bar-HRM, LAMP, immunochromatographic assays, or DNA chromatographic detection strips, which can play a pivotal role in advancing applications related to horticulture.

5. Conclusions

The botanical origin of white Kwao Krua is attributed to *Pueraria candollei* and *P. mirifica*; nevertheless, both the taxonomic status and description remain ambiguous. Both species can be easily distinguished based on morphological characteristics during flowering and fruiting; however, these stages are relatively short. Therefore, during the vegetative stage, the distinction is made by observing differences in the size of the stipule. In terms of palynology, the pollen micromorphology of both species is identical. However, nucleotide sequencing analysis of three DNA barcoding regions, namely ITS, *mat*K, and *trn*H-*psb*A, showed genetic variation among white Kwao Krua samples. Only the *mat*K gene, as revealed by nucleotide sequencing and neighbor-joining tree analysis, allows for the differentiation between the two species. Therefore, the development of future tools characterized by simplicity, speed, and accuracy, such as Bar-HRM, LAMP, immunochromatographic assays, or DNA chromatographic detection strips, aims to distinguish between these two species. This discovery provides valuable information for accurate identification methods applicable to farmers, horticulturists, and plant breeders. For future research, an analysis of the chemical constituents and metabolomic profile of both species of white Kwao Krua will be undertaken to select high-quality variants for the production of high-value herbal products in the pharmaceutical industry.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10020162/s1, Figure S1: Alignment of ITS barcode region of white Kwao Krua samples; Figure S2: Alignment of *mat*K barcode region of white Kwao Krua samples; Figure S3: Alignment of *rbc*L barcode region of white Kwao Krua samples; Figure S4: Alignment of *trn*H-*psb*A barcode region of white Kwao Krua samples; Table S1: Lists of white Kwao Krua samples collected in Thailand and accession numbers submitted in GenBank database; Table S2: Morphological characteristics of leaf, inflorescence, flower, and pod from white Kwao Krua samples; Table S3: Primers used in this study. References [53–57] are cited in the supplementary materials.

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