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Full Length Research Paper

Using DNA markers and barcoding to solve the common problem of identifying dried medicinal plants with the examples of *Smilax* and *Cissus* in Thailand

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Medicinal herbs called *Khao-Yen Nuer* and *Khao-Yen Tai* in Thai traditional medicines have been used as common ingredients in several preparations. They were claimed to be *Smilax china* or *Smilax glabra*. In order to identify the dried medicines, fresh samples of *S. china* and *S. glabra* were collected. Another two fresh samples with the same local names were also collected from Chiang Mai province, and were later morphologically identified as *Cissus repens*. These samples served as reference standards for the other dried samples. Nine dried samples of the two herbs were randomly collected from pharmacies in the provinces of Thailand. DNA barcodes by *trn*H-*psb*A spacer region show great variations in banding sizes as DNA markers and genetic distances (D) from sequences. The D values in same, and different species ranged from 0 to 12.9%, and 23.2 to 32.3%, respectively. Based on the D values, four dried samples were identified as *S. china* and *S. glabra* while the other five dried samples are of the same unknown species and are used as *Khao-Yen Nuer* and *Khao-Yen Tai* in different provinces and parts of Thailand. The *trn*H-*psb*A spacer region is an efficient barcode and marker identifying the studied plant group. Aside from reaching success in the research aims, the results have led us to an accidental discovery. One of the more important practices of traditional healers is to use *C. repens* and other unknown plant alternatives with *S. china* or *S. glabra* to create the traditional medicine called *Khao-Yen Nuer* and *Khao-Yen Tai*.

Key words: *Cissus repens*, DNA barcode, medicinal plant discovery, *Smilax china*, *Smilax glabra*, *trn*H-*psb*A

INTRODUCTION

Medicinal herbs are used in alternative medicine worldwide. *Smilax* (Smilacaceae; about 350 species) is distributed widely in the tropical and temperate regions of the world, especially in East Asia and North America. Many species of *Smilax* have long been used as medicinal herbs. They are known to be rich in steroidal saponins (Jia and Ju, 1992; Ju and Jia, 1992, 1993; Kubo et al., 1992; Sashida et al., 1992; Ju et al., 1994; Bernardo et al., 1996; Sautour et al., 2005; Shao et al., 2007). For example, the root of *Smilax china* L., known as *Ba Qia* (or *Jin Gang Teng*) in China, is used in traditional Chinese medicine as a diuretic and detoxifying agent. It is also used in the treatment of rheumatoid arthritis, lumbago, gout, tumors and inflammatory symptoms (Shao et al., 2007). It has also demonstrated

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free radical scavenging activity (Lee et al., 2001). Some pharmacological investigations show that *S. china* and other species of *Smilax* have anti-inflammatory properties (Shu et al., 2006; Shao et al., 2007) and some extracts including steroidal saponins or flavonoid glycoside when isolated from these plants exhibit a significant cytotoxicity against several tumor cell lines (Hu and Yao, 2002; Thabrew et al., 2005; Li et al., 2007). In addition, *S. glabra* Roxb. found in traditional Chinese medicine is commonly used for the treatment of liver disease (Sa et al., 2008) and has been reported to have a hypoglycemic effect (Fukunaga et al., 1997) and immunomodulatory activity (Jiang and Xu, 2003).

In Thai traditional medicines, two medicinal herbs named *Khao-Yen Nuer* and *Khao-Yen Tai* have been used as common ingredients in several preparations, including those used in the treatment of lymphopathy, dermopathy, venereal diseases, leprosy, and cancers. Interestingly, despite their close resemblance, the drugs available in traditional drug stores throughout the country are, in fact, rhizomes from different plant species from at least three genera, *Dioscorea* (Dioscoreaceae), *Smilax* (Smilacaceae), and *Pygmaeopremna* (Verbenaceae) (Itharat et al., 2003). *Khao-Yen* is as useful as the species of *Smilax* from China. It is sold in the form of sliced dried rhizomes in many pharmacies throughout Thailand. *Khao-Yen Nuer* and *Khao-Yen Tai* are used in combination with other medicinal herbs, but the true species of these plants have not been identified.

Traditionally, morphological characters have been used to characterize levels and patterns of diversity (genetic, environmental, and species diversity). Attempting to identify dried medicinal plant parts solely based on morphology can lead to deadly results as well. In 1997 there was the case of a woman who was hospitalized after ingesting medicinal herbs labeled as plantain leaves (*Plantago major*), but, they were, in fact, toxic foxglove (*Digitalis purpurea*) (Tewfik, 2008). When medicinal plants that contain pyrrolizidine alkaloids are commonly misidentified as less toxic plants (Huxtable, 1989; Neuman et al., 2007), these ingestions can cause severe health problems. In one case, it has lead to the death of a 2-month old infant, who was administered a Mexican herb *Gordoloba yerba* which can be comprised of several species in diverse families such as *Senecio longilobus* (Asteraceae), *Gnaphalium macounii* (Asteraceae) and *Verbascum thapsus* (Scrophulariaceae) (Croom, 1983). Because of these potentially drastic consequences of medicinal plant misidentification, better methods of identification of dried plant specimens and plant parts are called for. Additionally, there has been a recent push in the field of ethnobotany to embrace molecular techniques and to use genetic identification to distinguish plant material, or to merely differentiate between several species possibilities derived from the common name (Newmaster and Ragupathy, 2010).

Various molecular approaches have been devised to overcome identification problems. DNA fingerprints are used effectively to support the morphological data (Mokkamul et al., 2007; Chaveerach et al., 2008). Alternatively, sequence divergence of parts of genes or spacer regions is an option for DNA barcoding. It is a relatively new concept aiming to provide rapid, accurate and automatic species identification by using a standardized DNA region as a tag (Hebert and Gregory, 2005). Actually, the DNA barcoding system should meet many criteria as described by Taberlet et al. (2007), but, a high level of variation with sufficient phylogenetic information will be most important for taxonomists (Hebert and Gregory, 2005).

Even though there are many advantages in all three plastid regions, *rpo*C1, *mat*K, and *trn*H-*psb*A intergenic spacer, these three regions combined are proposed to be one option to use as a standard protocol for barcoding all land plants. This option substitutes for the relatively conserved coding region, *rpo*B, *mat*K, and highly length-variable, non-coding intergenic spacer, *trn*H-*psb*A (Chase et al., 2007). There are already at least 276 plant species completely sequenced listed in GenBank, and numerous others that have the sufficient two or three regions sequenced for barcoding (Benson et al., 2005). The most recent proposal by Hollingsworth et al. (2009) approved and recommended that *rbc*L and *mat*K are the core DNA barcodes for land plants.

Therefore our study aims to identify the two dried rhizomes of the medicinal herbs called *Khao-Yen* by comparing them with molecular markers such as the DNA barcoding of known species that have been identified by morphological characters.

MATERIALS AND METHODS

Plant materials

The numbers of specimen collected and details are listed in Table 1 including dried samples from four parts of Thailand and fresh samples.

Dried samples

Nine dried rhizome samples (numbers K1, K2, K4, K5, K6, K7, K8, K9, K10) of *Khao-Yen Nuer* and *Khao-Yen Tai* were randomly collected from pharmacies in randomly selected provinces of Thailand namely Bangkok and Nakhon Pathom (Central Thailand), Chon Buri (Eastern Thailand), Chiang Mai (Northern Thailand), and Nong Khai (Northeastern Thailand). The dried rhizome samples are shown in Figure 1.

Fresh samples

Fresh samples were collected for reference. These are *S. china* L. (called *Khao-Yen Nuer*, K12) from Khon Kaen province, and *S. glabra* (called *Khao-Yen Tai*, K17) from Nong Khai province. Additionally, two samples from Chiang Mai province locally called *Khao-Yen Nuer* (K15) and *Khao-Yen Tai* (K16) were collected. They have different rhizome colors as shown in Figure 2. Voucher specimens are A. Chaveerach 675 for *S. china* (K12), A. Chaveerach 676 for *S. glabra* (K17), and A. Chaveerach 677, 678 for *C. repens* (K15, K16), and these are kept at The Bangkok

Table 1. List of fresh and dried samples including local names, scientific names, collection sites (pharmacy collected sites for dried samples), colors of fresh or dried rhizomes and GenBank accession numbers of *trn*H-*psb*A region.

Specimen no.	Local name	Scientific name	Dried / Fresh	Collection site	Rhizome color	Accession no.
K1	*Khao-Yen Nuer*	*Smilax china*	Dried	Nong Khai	Reddish	GU372808
K2	*Khao-Yen Tai*	unknown	Dried	Nong Khai	Whitish	GU372809
K4	*Khao-Yen Nuer*	unknown	Dried	Chiang Mai	Whitish	GU372810
K5	*Khao-Yen Nuer*	unknown	Dried	Nakhon Pathom	Whitish	GU372811
K6	*Khao-Yen Tai*	*S. glabra*	Dried	Nakhon Pathom	Reddish	GU372812
K7	*Khao-Yen Nuer*	unknown	Dried	Bangkok	Whitish	GU372813
K8	*Khao-Yen Tai*	*S. china*	Dried	Bangkok	Reddish	GU372814
K9	*Khao-Yen Nuer*	*S. glabra*	Dried	Chon Buri	Reddish	GU372815
K10	*Khao-Yen Tai*	unknown	Dried	Chon Buri	Whitish	GU372816
K12	*Khao-Yen Nuer*	*S. china*	Fresh	Khon Kaen	Not seen	GU372817
K15	*Khao-Yen Nuer*	*C. repens*	Fresh	Chiang Mai	Bright red	GU372818
K16	*Khao-Yen Tai*	*C. repens*	Fresh	Chiang Mai	Dark red	GU372819
K17	*Khao-Yen Tai*	*S. glabra*	Fresh	Nong Khai	Not seen	GU372820

Figure 1. Dried rhizome samples from a pharmacy which are always sold and used together and have been alternatively called *Khao-Yen Nuer* and *Khao-Yen Tai*.

Herbarium (BK).

DNA extraction

Genomic DNA was extracted from the leaves of freshly collected samples and from the vascular cambium area of dried rhizome using a Plant Genomic DNA Extraction kit (RBC Bioscience). Extracted DNA was examined by agarose gel electrophoresis and ethidium bromide staining. The quality and quantity of DNA were determined by a gel documenting instrument. Then, DNA samples were diluted to a final concentration of 20 ng/µl, and these dilutions were used as DNA templates in the Polymerase Chain Reaction (PCR).

Amplification and sequencing

DNA amplifications were done for the standard barcoding regions. Primer sequences obtained from Kew Barcoding Project (http://www.kew.org/barcoding/update.html; 28 January 2009) are 5′-GTT ATG CAT GAA CGT AAT GCT C-3′ and 5′-CGC GCA TGG TGG ATT CAC AAT CC-3′ for *trn*H-*psb*A spacer regions. The 25 µl reaction mixture consisted of GoTaq Green Master mix (Promega), 0.25 µM of each primer and 10 ng DNA template. The reaction mixture was incubated at 94°C for 1 min and amplification was performed with 35 cycles of 94°C for 30 s, 53°C for 40 s, 72°C for 40 s, followed with 5 min final extension at 72°C. The amplified products were detected by 1.2% agarose gel electrophoresis in TAE buffer and were visualized by ethidium bromide staining.

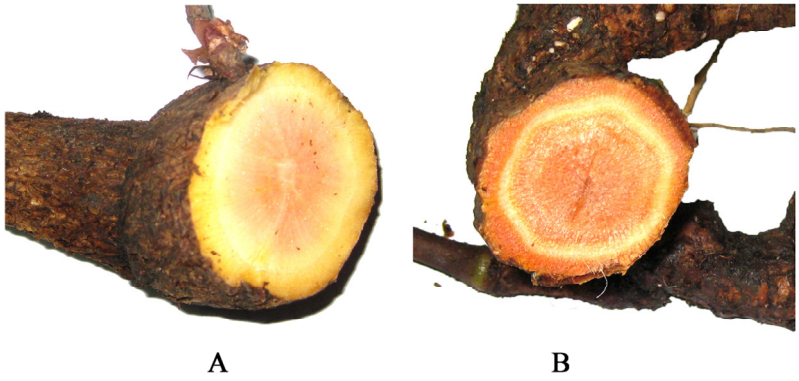

Figure 2. Differences in fresh rhizome colors of *Khao-Yen Nuer* (A) and *Khao-Yen Tai* (B) from Chiang Mai province which were verified to be the same species, *Cissus repens*. The durability begets different colors in the younger (samples) used as *Khao-Yen Nuer* instead of *Smilax china* and the older (samples) used as *Khao-Yen Tai* instead of *Smilax glabra*.

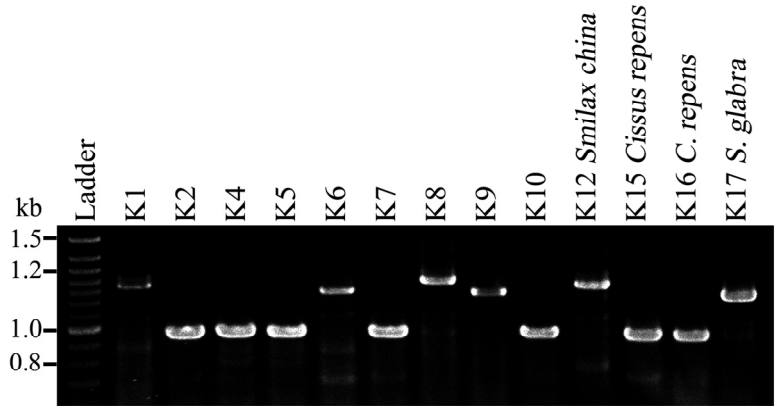

Figure 3. DNA bands of *trn*H-*psb*A for collected samples locally called *Khao-Yen Nuer* and *Khao-Yen Tai* including dried (K1-K10) and fresh (K12-K17) samples.

The amplified specific fragments of *trn*H-*psb*A spacer region were sequenced and the sequences were tested for genetic distances using MEGA software version 4 (Tamura et al., 2007). The sequences were submitted to the GenBank database.

RESULTS

Dried rhizome colors

Plants with different rhizome colors have alternative local names. The reddish rhizomes are called *Khao-Yen Nuer* and the whitish rhizomes are called *Khao-Yen Tai* in Nong Khai and Chon Buri. On the contrary, in Nakhon Pathom and Bangkok, the reddish rhizomes are called *Khao-Yen Tai* and the whitish rhizomes are called *Khao-Yen Nuer*. A group of reddish rhizomes included both *Smilax china* and *S. glabra*. The other group of white rhizomes in this study is an unknown (Table 1).

Morphological detection

Two of the fresh samples from Chiang Mai province called *Khao-Yen Nuer* and *Khao-Yen Tai* were morphologically identified as *C. repens* Lam., in the family Vitaceae using the key and description from the flora of China (Chen et al., 2007).

Obtaining the efficient standardized DNA barcode region showing different banding sizes

The successful amplifications of DNA barcoding regions are shown in Figure 3. Different DNA banding sizes of

Table 2. Genetic distances (D) of all studied samples based on *trn*H-*psb*A sequence analysis by MEGA4.

Species	*S. china* (K1)	Unknown (K2)	Unknown (K4)	Unknown (K5)	*S. glabra* (K6)	Unknown (K7)	*S. china* (K8)	*S. glabra* (K9)	Unknown (K10)	*S. china* (K12)	*Cissus repens* (K15)	*C. repens* (K16)	*S. glabra* (K17)
Smilax china (K1)	0.000												
Unknown (K2)	0.419	0.000											
Unknown (K4)	0.403	0.016	0.000										
Unknown (K5)	0.419	0.016	0.016	0.000									
S. glabra (K6)	0.265	0.468	0.452	0.468	0.000								
Unknown (K7)	0.403	0.016	0.000	0.016	0.452	0.000							
S. china (K8)	0.065	0.419	0.403	0.419	0.297	0.403	0.000						
S. glabra (K9)	0.281	0.435	0.419	0.435	0.097	0.419	0.248	0.000					
Unknown (K10)	0.403	0.016	0.000	0.016	0.452	0.000	0.403	0.419	0.000				
S. china (K12)	0.016	0.435	0.419	0.435	0.265	0.419	0.048	0.297	0.419	0.000			
Cissus repens (K15)	0.339	0.371	0.355	0.371	0.371	0.355	0.323	0.339	0.355	0.323	0.000		
C. repens (K16)	0.339	0.371	0.355	0.371	0.371	0.355	0.323	0.339	0.355	0.323	0.000	0.000	
S. glabra (K17)	0.248	0.435	0.419	0.435	0.097	0.419	0.281	0.129	0.419	0.232	0.323	0.323	0.000

*trn*H-*psb*A spacer regions vary according to different families and species that are Smilacaceae (*S. china* and *S. glabra*) and Vitaceae (*C. repens*). There are three DNA banding patterns: 800-900 bp of *S. china*, 700-800 bp of *S. glabra*, and 300-400 bp of *C. repens*.

Sequencing

Sequencing was done and sequences were recorded in the GenBank database with the accession numbers shown in Table 1.

Lengths of the DNA sequences of *trn*H-*psb*A bands are in three range groups following the three groups of DNA banding patterns of the fresh known species: 882 bp for K12 (*S. china*), 770 bp for K17 (*S. glabra*), and 369 bp and 372 bp for K15 and K16 (*C. repens*). When comparing these groups to the known species sequences, dried rhizome sequence groups K1 (867 bp) and K8 (878 bp); K6 (771 bp) and K9 (739 bp); and K10 (365 bp), K5 (366 bp), K7 (372 bp), K2 (385 bp) and K4 (398 bp) are nearly same sizes as K12, K17, and K15 and K16, respectively.

Sequence alignment for obtaining genetic distance of same and different species

Sequence alignment takes the following genetic distances as shown in Table 2. The genetic distance ranges are accorded with same and different species group in the three identified plants and DNA banding sizes: *S. china*, *S. glabra*, and *C. repens*.

The same species level posses the genetic distance (D) values of K1, K8 and K12 (*S. china*) at 1.6 to 6.5%, and K6, K9 and K17 (*S. glabra*) at 9.7 to 12.9%. There is only one base variation (Figure 4) in two individuals, K15 and K16 showing the D value of 0% (very little to zero) supporting a species of *C. repens*. At the *C. repens* banding site, there are two D levels namely an identity and a different D levels. The D level of identity of dried samples including K2, K4, K5, K7 and K10 are 0% (K4-K7, K4-K10, K10-K7) to 1.6% (K2-K4, K2-K5, K2-K7, K2-K10, K4-K5 and K5-K10) (Table 2). A different D level appears in the fresh samples (K15, K16) and the dried samples (K2, K4, K5, K7, K10). They show a D value of 35.5% to 37.1%, supporting different species groups.

The difference in the species level is 23.2% in *S china* and *S. glabra*, 32.3% in *S. china* and *C. repens*, and in *S. glabra* and *C. repens*.

DISCUSSION

Attempting to identify any species of indistinguishable dried medicinal plant parts solely by the plant common names is an all-too-frequent problem in ethnobotany. The problem of dried medicinal plant parts locally called *Khao-Yen Nuer* and *Khao-Yen Tai* can be readily solved using molecular markers as different banding sizes on the barcoding with *trn*H-*psb*A spacer region and sequence variations.

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Cissus repens (K15)--------------TGAAGCTCCATCTATAAATGGATAAGATTTACGTCTTAGTGTATAC 46
C. repens (K16)    TCAACTACTGCTGTTGAAGCTCCATCTATAAATGGATAAGATTTACGTCTTAGTGTATAC 60
                              *******************************************
C. repens (K15)    GAGTTTTTGAATGTAAAGGAGCAATGACCAATTTCCTCTTCTATCAAGGAGGTGGTATTG 106
C. repens (K16)    GAGTTTTTGAATGTAAAGGAGCAATGACCAATTTCCTCTTCTATCAAGGAGGTGGTATTG 120
                   ************************************************************
C. repens (K15)    CTCCTTTATTTAGTAGTCTTTTACTTTTCTAAGTTTTTTTTATTTCAACTAAAACTAAAA 166
C. repens (K16)    CTCCTTTATTTAGTAGTCTTTTACTTTTCTAAGTTTTTTTTATTTCAACTAAAACTAAAA 180
                   ************************************************************
C. repens (K15)    TATTAATAATCGTAAAAAGCATTCTCAGAGGTTGGTTTATGATTGAGTATCATACTTTGG 226
C. repens (K16)    TATTAATAATCGTAAAAAGCATTCTCAGAGGTTGGTTTATGATTGAGTATCATACTTTGG 240
                   ************************************************************
C. repens (K15)    TTTTGTTGTTTAATACTTTAATCTTAATTTAATCTTAAAATAAGATAATAAAAAACATGA 286
C. repens (K16)    TTTTGTTGTTTAATACTTTAATCTTAATTTAATCTTAAAATAAGATAATAAAAAACATGA 300
                   ************************************************************
C. repens (K15)    TGAATGGTGGAAATTTAATCTTTTTCAATTTATTATTTCTTGTAATGTGAACAGTAAAAG 346
C. repens (K16)    TGAATGGTGGAAATTTAATCTTTTTCAATTTATTATTTCTTGTAATGTGAACAGTAAA-G 359
                   ************************************************************ *
C. repens (K15)    GGCGGATGTACCCAGTGGATCCCAGG 372
C. repens (K16)    GGCGGATGT----------------- 369
                   *********
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Figure 4. DNA sequence alignment of two studied samples of *Cissus repens*. The alignment shows that they are the same species which varied in only one base (boxed) according to morphological characters.

Performing DNA extractions from dried samples is more difficult than with fresh samples. Because the dried samples were rhizomes filled with polysaccharides, the vascular cambium connecting bark, which is meristematic tissue, was scraped and used in the DNA extraction. When the extracted DNA of the vascular cambium was compared to other parts of the plant tissue, it showed that the existing DNA can only be found in the scraped vascular cambium.

There are many advantages to the plastid regions, such as conserved gene order and high copy number in each cell enabling the easy retrieval of DNA for PCR and sequencing. Therefore, these DNA samples had PCR performed for barcoding with specific primers of *trn*H-*psb*A. In the studied sample group, the *trn*H-*psb*A spacer region is distinct and efficient indicating a three banding size group as displayed in Figure 3 following the known species as *S. china*, *S. glabra* and *C. repens*. DNA banding sizes differ in different families accordingly. The Smilacaceae are *Smilax china* including K1, K8 and K12 with 867-882 bp, and *S. glabra* including K6, K9 and K17 with 739-771 bp. The Vitaceae is *C. repens* including K15 and K16 with 365-398 bp.

The sequence alignment of the *trn*H-*psb*A spacer region indicates genetic distances in accordance with three groups of identified plants and DNA banding sizes. The first group consisted of dried samples, K1 and K8, and the fresh K12 are *S. china* and showed the same banding sizes and the same genetic distance (D) range of 1.6 to 6.5%. The second group consisted of dried samples, K6 and K9 and the fresh K17 is *S. glabra* which showed the same banding sizes and D values of 9.7% to 12.9%. The third group consisted of fresh samples, K15 and K16, with a D value of 0%. The species identification of the third group supported the morphological identification. They are both actually *C. repens*.

The D values between species, *S. china* and *S. glabra*, are 23.2 to 29.7%, while the values between the families, Smilacaceae (*S. china* and *S. glabra*) and Vitaceae (*C. repens*), are as high as 32.3 to 37.1%. There is no standard level between genera of the same family.

Accordingly, the D values of the dried sample group (K2, K4, K5, K7, K10), Smilacaceae are 0.00% to 1.60%. So, these dried samples should be same species, they cannot be *C. repens* with genetic distances of 35.5% to 37.1% which ranges on the limits of a different species even though there is an equivalent banding size. These dried samples were randomly collected from different provinces and parts of Thailand (K2 from Nong Khai (Northeast), K4 from Chiang Mai (North), K5 from Nakhon Pathom (Central), K7 from Bangkok (Central), and K10 from Chon Buri (East). This shows that they are all locally called and used as *Khao-Yen Nuer* and *Khao-Yen Tai* in the above-mentioned parts of Thailand. Additionally, they are neither *S. china* nor *S. glabra* showing both different banding sizes and D values. The values between dried samples K2, K4, K5, K7 and K10 with *S. china* and *S. glabra* are 40.3% to 43.5% and 41.9 to 46.8% which shows that the samples belong to a different family as earlier mentioned.

It is possible that the five unknown samples could be *Dioscorea* and/or *Pygmaeopremma* as Itharat et al. (2003) proposed. However, we have known the samples for comparison, because of a lack of pharmacists', traditional healers' or local peoples' guidelines for using them. They have introduced only *S. china*, *S. glabra* and *C. repens*, which means that they are not popularly used leading to rare collection. The reason we have are including *C. repens* in the research is because we do not have the two samples earlier mentioned.

In Chiang Mai province, we collected dried rhizomes of *Khao-Yen Nuer* and *Khao-Yen Tai* from a pharmacy in the Amphur Maerim region, about 20 km from Chiang Mai city. We collected fresh samples from the Doi Chiangdown Forest about 100 km away from Chiang Mai city. Agreeing with the herbalists there, the two fresh samples of *Khao-Yen Nuer* and *Khao-Yen Tai* showed morphological characteristics that identified them as *C. repens*, with the defining character being the rhizome color.

The one called *Khao-Yen Tai* is older than the one called *Khao-Yen Nuer*, and very much darker due to substance accumulation such as nutritional elements or secondary metabolites. Another characteristic of the older *Khao-Yen Tai* is that it shows clarified wood and annual rings as shown in Figure 2. Accordingly, the medicinal plantation of a university in Central Thailand labels *C. repens* by the local name of *Khao-Yen Nuer*. The authors still have not seen a dried rhizome of *C. repens* at the collection times as expected. Perhaps, the harvesting has not yet started its yearly cycle.

Formerly, traditional medicine usages have led us to speculate that combinations of *Khao-Yen Nuer* and *Khao-Yen Tai* that have been used for disease treatment in Thailand could have possibly been *S. china* and *S. glabra* which the traditional literature calls *Khao-Yen Nuer* and *Khao-Yen Tai*, respectively. In Thailand they are considered to have the same medicinal properties. Later in this research, we discovered that the four possible species corresponding to these common names are *C. repens*, *S. china*, *S. glabra*, and an unknown species. The names *Khao-Yen Nuer* and *Khao-Yen Tai* are used interchangeably to describe the reddish and whitish rhizome colors. These interchangeable names have lead to the common notion that these species have identical medicinal properties and usages. This historical shift is thought to have happened because *S. glabra* and *S. china* populations have diminished and have became rare. As a result, *C. repens* and/or an unknown species was discovered or traditional healers already knew that these two later species had the same properties as the two *Smilax* species. However, there are no publications or evidence to show that these have identical medicinal properties.

Hollingsworth et al. (2009) suggested that the *trn*H-*psb*A region is a strong candidate for plant barcoding aside from core barcodes such as *rbc*L and *mat*K.

A suitable region should ideally show enough variation within it to discriminate among species. Therefore, all of the genetic distance values taken from the *trn*H-*psb*A spacer region are standardized enough in the studied plant group to distinguish species between fresh-fresh samples and dried-fresh samples at standard levels in the same species and in different species, as well as, in the same family and in different families. The *trn*H-*psb*A region in the studied plant group can be called DNA markers by providing different banding sizes in three plant group with four species. However, it can be called barcodes with the following causes. Firstly, the primer used is designed from the region of it. The second, the genetic distance values which are considered for different and same species, genera and families are the base variation in the nucleotide sequences.

Thailand has high level of biodiversity and, therefore, diverse medicinal plants are used. Only a few collection areas, pharmacies and plant samples were studied in this research, however, we covered the areas of Thailand where *Khao-Yen Nuer* and *Khao-Yen Tai* are widely used.

Additionally, because of a high level of skill and knowledge of traditional medicine plants among Thai traditional healers, we believe that there may still be other unidentified plant species other than the plants studied being called *Khao-Yen Nuer* and *Khao-Yen Tai*, but being used for the same purposes as *S. china* and *S. glabra*. Moreover, it may be worthwhile to make *C. repens* available worldwide because of its great value. Consequently, the unknown species must be identified. There should be further studies to acquire new knowledge which confirms and leads to new discoveries of biodiversity and sustainable use of medicinal plants.

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