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Full Length Research Paper

Ethnobotany and species specific molecular markers of some medicinal *sakhan* (*Piper*, Piperaceae)

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Sakhan, _jakhan_ and _takhan_ are some of Thai common names for _Piper_ species especially for unidentified forest species. They are widely used as vegetables, spices, decoration, traditional medicines and ceremonially. Seven of these species were collected and identified of which the sliced stems are often used as medicinal plants and spices, and are easily preserved as dried powder or slices: _Piper betle_ L. (_phlu_), _Piper colubrinum_ Link. (_prik thai tua phu_), _Piper nigrum_ L. (_prik thai_), _Piper pendulispicum_ C. DC. (_jakhan jin_), _Piper retrofractum_ Vahl. (_dee plee_), _Piper ribesioides_ Wall. (_takhan lek_), and _Piper sarmentosum_ Roxb. (ชะพลู: _cha phlu_). Stem slices and a powder of what was called _sakhan_ were brought from pharmacies for analysis. The ethnobotany of these species was studied by literature reviews, traditional healers and interviews with locals. DNA barcoding of the main stem from all wild species and slices and powder from pharmacies was performed to provide specific markers which can be standardized for further identification of medicinal _sakhan_ samples. The barcode marker accession numbers are GQ500612-GQ500619 for the _rpo_C1 gene, GU372746-GU372751 for _mat_K gene, and GQ891994-GQ892001 for _trn_H-_psb_A.

Key words: DNA barcodes, ethnobotany, molecular markers, *Piper*, Thai medicinal *sakhan.*

INTRODUCTION

Sakhan, *jakhan* and *takhan* generic names are supplemented with specific suffixes, such as *sakhan daeng*, *jakhan jin*, and *takhan lek* are the names for *Piper* species growing in the forest. Also, *sakhan* is the name for powders or slices of medicinal *Piper* species sold at drugstores. *Piper* species used in traditional Thai medicine for a long time include well known species such as *Piper betle*, *Piper nigrum*, *Piper sarmentosum*, *Piper retrofractum* and unidentified species just called *sakhan*.

Piper species are commonly used by Thai people as vegetables, spices, decoration, in ceremonies and as traditional medicines. There are presently forty species documented in Thailand (Chaveerach et al., 2008) and some are commonly found cultivated in home gardens, which suggests that Thais have had a long relationship with them and are familiar with their use. *Piper* leaves contain distinctively aromatic and acrid volatile oils such as cadinene, carvacrol, caryophyllene, chavibetol, chavicol, eugenol, terpinyl and acetate (Dyer et al., 2004), piperine, piperlongumine, pyridine aikaloids, sesamin, tannins, oxalic acid and iron (De Waard and Anunciado, 1999; Teo and Banka, 2000).

Several *Piper* species have great economic and cultural importance among Thais and are used in complex spice mixtures and as medicines, stimulants, antiseptics and antioxidants (Chaveerach et al., 2006, 2008). Since *Piper* species are always used for medicinal plants, traditional healers will often take unidentified *sakhan* species as from the nearest forest. Some *Piper* species are made into powders or slices for pharmacies or used as cultivated species in Thailand as spices or

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traditional medicines namely *P. betle*, *P. retrofractum* and *P. nigrum*. Therefore, the species of what is called *sakhan* will depend on geographic collection area, the seller, the traditional healer, the illness treated and the form of the final medicine (that is, only common species such as the four mentioned above are abundant enough to be turned into powder, while rarer species are usually just sold as dried slices). This leads us to assert that a better taxonomic understanding of the *Piper* genus and how common names relate to species leads to the benefit of knowing which species most effectively treats each illness.

DNA barcoding is a popular method for species identification and can be used in samples which have only a short region of reliable DNA or even highly degraded DNA found in processed food, fossil remains, and herbarium specimens. However, Taberlet et al. (2007) claimed that this approach only allows the identification to the level of families, but not genera or species in most cases. Conversely, there has been much more research of this approach in plants starting from 2003 by Hebert et al. (2003). Subsequently, there have been many studies testing standard barcoding regions in different plant taxa aiming to provide rapid, accurate and automatable species identification (Hebert and Gregory, 2005). The land plant chloroplast genome has been suitably proposed as a standard region for plant barcoding (Palmer, 1985, 1991; Downie and Palmer, 1991; Katayama and Ogihara, 1993; Downie et al., 1996; Thiede et al., 2007). There is still a lot of DNA barcoding research in progress such as Kress et al. (2005) who identified some flowering plants using certain regions and suggested that the sequences in the pair of loci, the nuclear internal transcribed spacer region and the plastid *trn*H-*psb*A intergenic spacer are potentially usable DNA regions for barcoding flowering plants.

As a standard protocol for barcoding all land plants, Chase et al. (2007) proposed to use two options of three regions each: *rpo*C1, *mat*K, and *trn*H-*psb*A intergenic spacer or *rpo*B, *mat*K and *trn*H-*psb*A. Newmaster et al. (2008) proposed using *mat*K and *trn*H-*psb*A to identify plants solely in Myristicaceae. Finally, Hollingsworth et al. (2009) at the Consortium for the Barcode of Life (CBOL) plant working group, recommended *rbc*L and *mat*K as the core DNA barcode for land plants. Our research aims to study ethnobotany of *sakhan* and make standard barcode markers for automatable identification of many species of the popular Thai medicinal plants named *sakhan*. Powder and slices of unknown species of *Piper* were also barcoded and compared against the known species in order to determine the species of the dried pharmaceutical samples.

MATERIALS AND METHODS

Plant materials

Fresh plant materials were collected from sites described in Chaveerach et al. (2008). Powder and slices of *sakhan* were bought from pharmacies in Khon Kaen province, northeastern Thailand in 2008.

Procedures

Plant usage information was recorded from observations, market surveys, literature review and interviews with traditional healers and local people. Samples were collected and identified by morphological characters from Chaveerach et al. (2008). All collected samples from fresh plants and pharmacies were analyzed with DNA extraction, DNA barcoding amplification and DNA barcoding sequencing, as described below. DNA barcoding was done following Chase et al. (2007) suggestion of using the *rpo*C1, *trn*H-*psb*A and *mat*K regions. Standard sequence regions of seven known species were aligned and compared with stem slices and powder from pharmacy samples based on DNA barcoding sequence analysis.

DNA extraction

Genomic DNA was extracted in all collected samples using the Plant Genomic DNA Extraction Kit (RBC Bioscience). Extracted DNA was examined by subjecting it to 0.8% agarose gel electrophoresis stained with ethidium bromide and observed. The quality and quantity of DNA were determined by a gel documenting instrument. DNA samples were then diluted to a final concentration of 20 ng/µl, and these dilutions were used as DNA templates in the PCR reaction.

DNA barcoding amplification

Amplifications were performed for DNA barcoding development using forward and reverse primers, 5′-GTGGATACACTTCTTGATAATGG-3′ and 5′-TGAGAAAACATAAGTAAACGGGC-3′ for *rpoC*1 gene, 5′-TAATTTACGATCAATTCATTC-3′ and 5′-GTTCTAGCACAAGAAAGTCG-3′ for *mat*K gene, and 5′-GTTATGCATGAACGTAATGCTC-3′ and 5′-CGCGCATGGTGGATTCACAATCC-3′ for *trn*H-*psb*A (http://www.kew.org/barcoding/update.html; 28 January 2009). The reaction mixture was done in 25 µl consisting of GoTaq Green Master mix (Promega), 0.25 µM each primer, and 10 ng DNA template. The reaction mixture was incubated at 94°C for 1 min and the amplification was performed with the following thermal cycles: 35 cycles of denaturation for 30 s at 94°C, 40 s annealing at 53°C, 40 s extension at 72°C, and 5 min final extension at 72°C. Amplification products were detected by 1.2% agarose gel electrophoresis in TAE buffer and visualized using ethidium bromide staining.

DNA barcode sequencing

The amplified specific fragments of the studied samples were sequenced and the sequences were tested for genetic distances using MEGA software version 4 (Tamura et al., 2007). The sequences were submitted to GenBank database.

RESULTS

Morphological treatment

Collected fresh samples were identified using

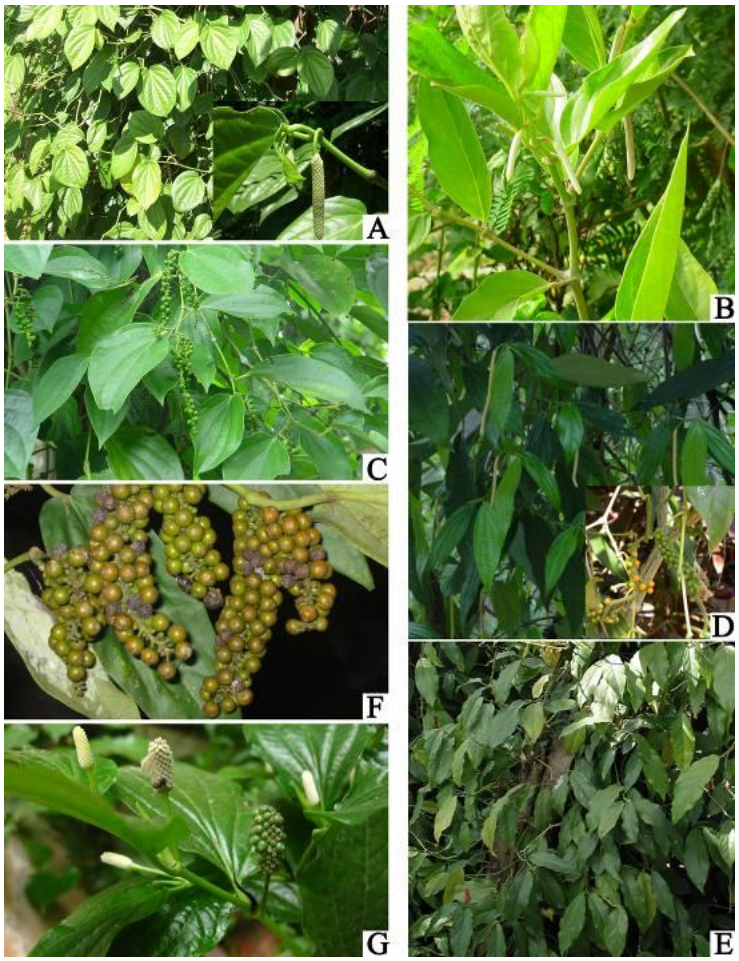

Figure 1. Collected plants, *P. betle* (A), *P. colubrinum* (B), *P. nigrum* (C), *P. pendulispicum* (D), *P. retrofractum* (E), *P. ribesioides* (F), and *P. sarmentosum* (G).

morphological characters from Chaveerach et al. (2008) for which the major distinguishing characters are stamen and stigma numbers and characters, floral bract morphology, leaf shape, and leaf venation. The collected fresh samples were identified as *P. betle* L. (*phlu*), *Piper colubrinum* Link. (*prik thai tua phu*), *P. nigrum* L. (*prik thai*), *P. pendulispicum* C. DC. (*jakhan jin*), *P. retrofractum* Vahl. (*dee plee*), *P. ribesioides* Wall. (*takhan lek*), and *P. sarmentosum* Roxb. (*cha phlu*) as shown in Figure 1. Powder and slices of *sakhan* from pharmacies in Khon Kaen province are shown in Figure 2.

Ethnobotany treatment

The five species including *P. betle*, *P. nigrum*, *P. pendulispicum*, *P. retrofractum* and *P. sarmentosum* have long been used in traditional Thai medicine as mentioned in Chaveerach et al. (2006). The two most commonly used species are *P. colubrinum* and *P. ribesioides* and their uses are described subsequently.

P. colubrinum is cultivated in lowland gardens usually used as a rootstock for grafting *P. nigrum*, which is the most important economic plant in Thailand. *P. nigrum* are cultivated largely in eastern Thailand especially Chanthaburi province, but bacterial sensitivity has usually caused root rot. Therefore, agriculturalists currently use *P. colubrinum* as the rootstocks for *P. nigrum* in plantations (Figure 3). *P. colubrinum* is locally called "*prik thai tua phu*" which derives from its usage as a rootstock and the fact that it has only a male flower. *Prik thai* is the local name of *P. nigrum* and *tua phu* means "male plant". Stems of *P. ribesioides* have long been used to flavor food giving its spicy, peppery taste, as well as being a traditional medicine in Thailand. For food, *P. ribesioides* greatly improves the flavor and smell of *kaeng* (a Northeastern Thai food resembling a curry), such as in Loei province (a province at the Northeast Thai border with Laos) where it is used in *kaeng naw mai* (curry with bamboo shoot), *kaeng om* (curry with many kinds of

Figure 2. Sliced *sakhan* (A) and powdered *sakhan* (B) bought from pharmacies in Khon Kaen province, northeastern Thailand.

Figure 3. *Piper colubrinum* prepared as rootstocks (A) and the grafted plants of *P. nigrum* (B).

vegetables and a kind of meat such as fish, pork or beef), among other dishes, *P. ribesioides* is always added with pieces of *jakhan jin* (*P. pendulispicum*; Chaveerach et al., 2006). As a traditional medicine, all parts of *P. ribesioides* have been used to treat many different symptoms. The root is used to treat an illness caused from asthma, secrete sweat and treat abnormalities in the body's wind element. The stem helps secrete sweat, increases appetite and supports body elements activities and treats abnormalities in the body's wind element, excess phlegm, and disability of four body elements (earth, water, wind and fire), diarrhea, and abdominal pain. The leaves treat body wind element abnormality incurred in phlegm and blood, alleviate chest congestion, and excrete phlegm. The flowers treat urticaria. The fruits treat body air element abnormality. All parts are use for countering disabilities with and support of body element activities, and to treat diarrhea, abdominal pain, flatulence, and colic.

Molecular treatment

DNA barcoding amplifications for *rpo*C1, *mat*K, and *trn*H-*psb*A showed DNA fragment sizes of about 700, 900 and 500 bp, respectively (Figure 4).

Sequence alignment

DNA barcodes of the seven known *Piper* samples acted as standards for the powdered *sakhan* samples from pharmacies via sequence analysis and alignment (an example shown in Figure 5). The comparison of the genetic distance of the two sequences, *rpo*C1 and *trn*H-*psb*A, of an unknown powder and the seven studied species are shown in Tables 1 and 2. Genetic distances between species range from 0.036 (*P. pendulispicum* and *P. betle*) to 0.205 (*P. colubrinum* and *P. sarmentosum*) in the *rpo*C1 sequence. For the *trn*H-*psb*A sequence, the distances range from 0.004 (*P. betle* and *P. nigrum*) to 0.031 (*P. colubrinum* and *P. pendulispicum*). All studied sample sequences were submitted to the GenBank database with accession numbers shown in Table 3.

DISCUSSION

The *Piper* species sampled in the research were based on popularity of use among Thais, abundance, and ease

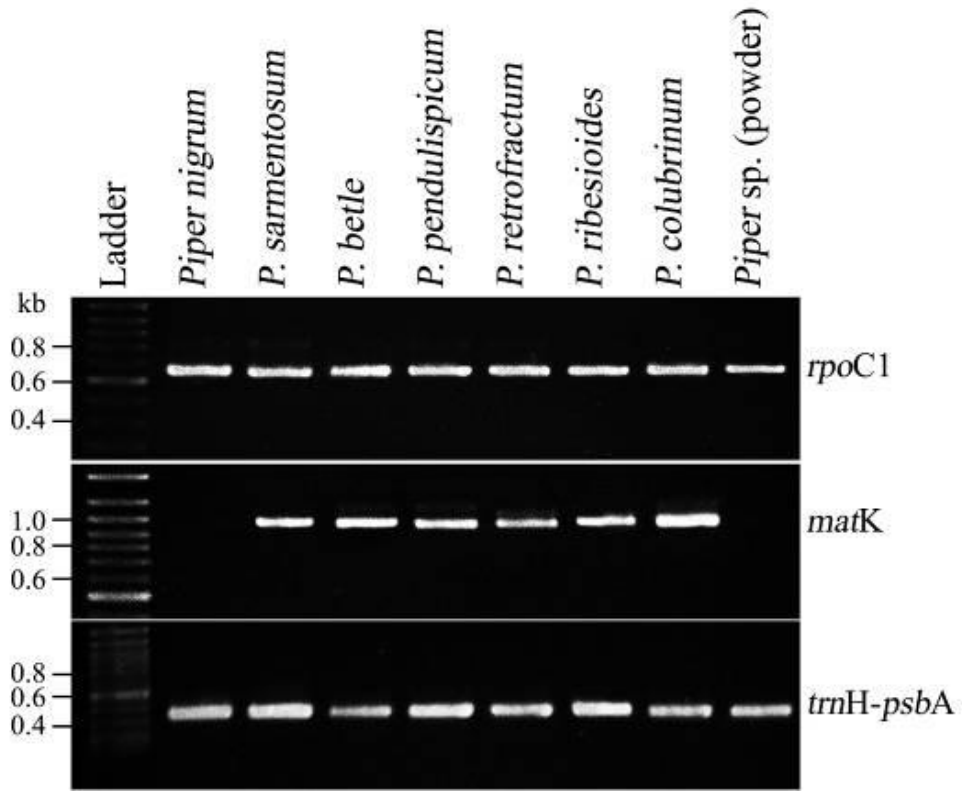

Figure 4. DNA barcode fragments of the seven fresh samples and powdered *sakhan* of the standard *rpo*C1, *mat*K, and *trn*H-*psb*A regions.

of collection for trade of powder and dried stem slices: *P. betle*, *P. colubrinum*, *P. nigrum*, *P. pendulispicum*, *P. retrofractum*, *P. ribesioides*, and *P. sarmentosum*. The other *Piper* species mentioned in Chaveerach et al. (2008) that grow in Thailand are not usually found ground or sliced because they grow mainly in evergreen forests, are rare, and are difficult to collect as these forests are protected areas and access is restricted by national law.

DNA extraction was successful for fresh samples and powdered *sakhan*. Powdered *sakhan* found in pharmacies should be made from a mixture of young and mature plant parts, but only the young plant parts yield viable DNA. While stem slices are usually made from mature plants because the plant's medicinal substances are mostly secondary metabolites and gradually accumulate as the plant ages, and therefore the more mature specimens are more powerful medicines. This meant that stems harvested for medicinal use are mature which lead to our unsuccessful DNA extraction attempts, even after trying other *sakhan* powders from other pharmacies. The factor should be considered before attempting DNA barcoding in other dried plants.

Chase et al. (2007) give two useful region combinations, *rpo*C1, *trn*H-*psb*A and *mat*K, and *rpo*B, *trn*H-*psb*A and *mat*K for barcoding all land plants. We tried the first option, but were unsuccessful as the amplification of *mat*K region failed with *prik thai* and powdered *sakhan*. In our second attempt to amplify *matK*, we were successful only with powdered *sakhan*. *Prik thai* may have base variations at the priming site caused by human activities. Since *prik thai* is a very important economic plant and widely planted in Thailand for medicine, food and spices. Human breeding of *prik thai*, fertilizers, soil, or weather may affect the genetic variation in the chloroplast DNA.

For *rpo*C1 sequence analysis, the distance values of *Piper* sp. and the other studied species are 0.113 (*Piper* sp. and *P. betle*) to 0.205 (*Piper* sp. and *P. retrofractum*) agreeing with the distance ranges of the other studied species (Table 1). The *trn*H-*psb*A distance values of *Piper* sp. and the other studied species are rather high, ranging from 0.602 (*Piper* sp. and *P. retrofractum*) to 0.614 (*Piper* sp. and *P. pendulispicum*, and *Piper* sp. and *P. colubrinum*) which are much higher values than the range found among the known species (Table 2). The sequence similarity had good enough resolution to determine fresh samples, but the dissimilarity of the powdered *sakhan* sequence from any studied sequence did not allow us to decide whether the unknown powdered *sakhan* was one of the studied species or

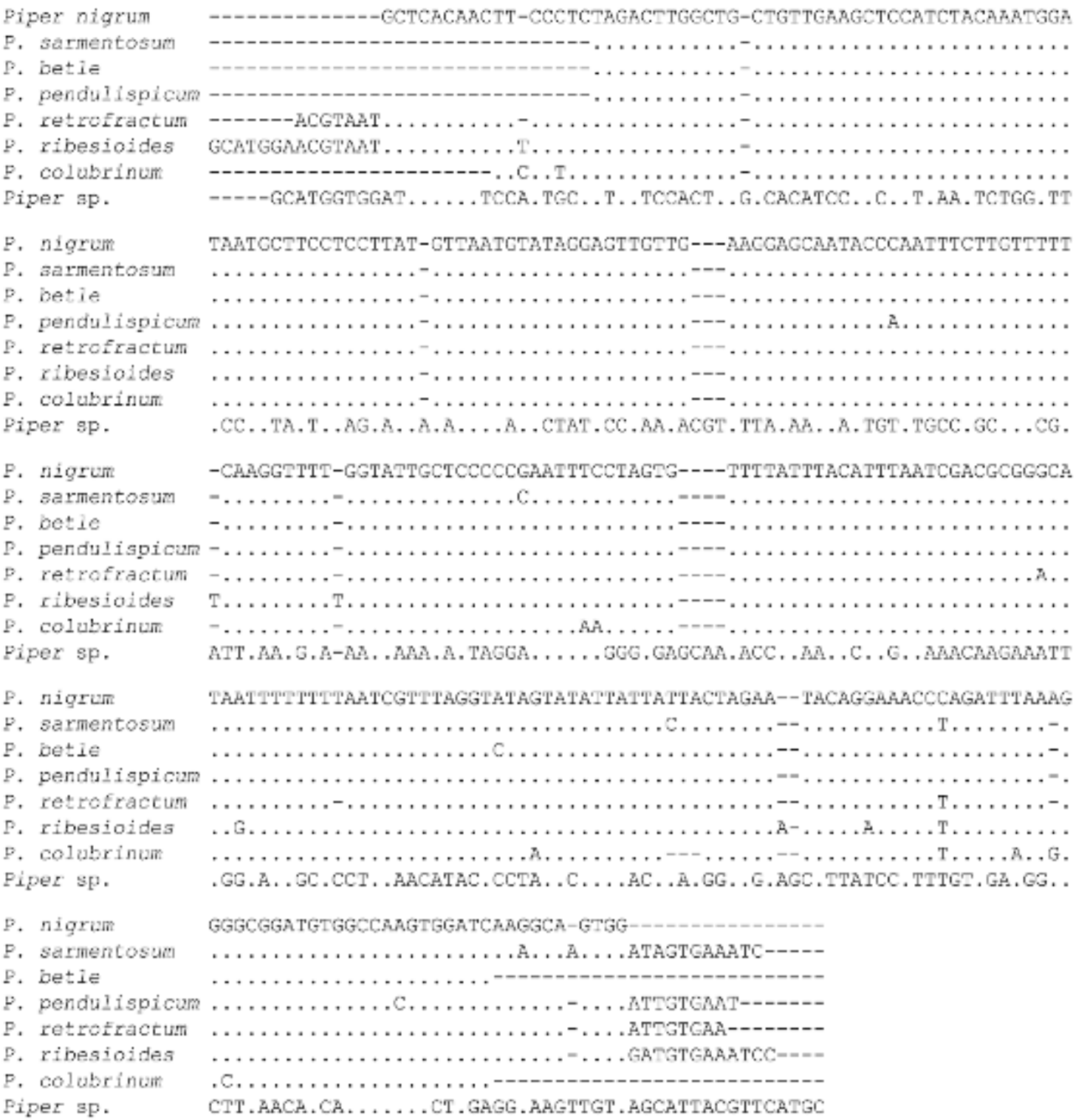

Figure 5. Sequence analysis of the *trn*H-*psb*A spacer region of the seven studied samples and an unknown powdered *sakhan* (*Piper* sp.) from a pharmacy in Khon Kaen province using MEGA4.

different species. To better make this determination, we would need sequences for all *Piper* in Thailand and perhaps worldwide. We are gradually accumulating barcodes for all the *Piper* species in Thailand, as shown

Table 1. Genetic distance (D)* matrix of *Piper* species from sequence analysis of the *rpo*C1 region.

Variable	*P. nigrum*	*P. sarmentosum*	*P. betle*	*P. pendulispicum*	*P. retrofractum*	*P. ribesioides*	*P. colubrinum*	*Piper* sp.
Piper nigrum	0.000							
P. sarmentosum	0.169	0.000						
P. betle	0.108	0.187	0.000					
P. pendulispicum	0.095	0.174	0.036	0.000				
P. retrofractum	0.113	0.172	0.086	0.079	0.000			
P. ribesioides	0.099	0.176	0.086	0.072	0.113	0.000		
P. colubrinum	0.133	0.205	0.041	0.065	0.108	0.113	0.000	
Piper sp.	0.149	0.205	0.113	0.122	0.133	0.142	0.129	0.000

*Overall D = 0.136.

Table 2. Genetic distance (D)* matrix of *Piper* species from sequence analysis of the *trn*H-*psb*A region.

	P. nigrum	*P. sarmentosum*	*P. betle*	*P. pendulispicum*	*P. retrofractum*	*P. ribesioides*	*P. colubrinum*	*Piper* sp.
Piper nigrum	0.000							
P. sarmentosum	0.008	0.000						
P. betle	0.004	0.012	0.000					
P. pendulispicum	0.008	0.016	0.012	0.000				
P. retrofractum	0.008	0.008	0.012	0.016	0.000			
P. ribesioides	0.012	0.012	0.016	0.020	0.012	0.000		
P. colubrinum	0.024	0.024	0.028	0.031	0.024	0.028	0.000	
Piper sp.	0.610	0.606	0.606	0.614	0.602	0.602	0.614	0.000

*Overall D = 0.360

by the sequences in GenBank database. This research shows advantages and disadvantages of barcoding for *Piper* species. However, one must consider the possibility of sample adulteration when attempting species determination as it is difficult to test adulterated powder or dried slice samples.

There are four Thai traditional medicine formulas used to treat four kinds of cancer (liver, lung, breast cervical and skin cancer), and *sakhan* is important because it is an ingredient in one of these, called “the Five Spices Formula” (*Tumrub benja kun*) which comprises *dee plee* (*P. retrofractum*), *cha phlu* (*P. sarmentosum*), *sakhan* (*Piper* sp.), and other two plant species, *Plumbago indica* Linn. and *Zingiber officinale* Roscoe (Juengprasert, 2010). In addition to the *Piper* species used in this formula, traditional healers in different regions of Thailand may often add other unidentified plants which may or may not be the same *Piper* species as powder or slices. The benefits of this research is to use species specific barcode markers to better determine the species of *Piper* for the medicinally important plants called *sakhan* in Thailand. This will lead to taking the right plant for the right disease.

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Table 3. Scientific names and GenBank accession numbers of the three barcoding regions of the *Piper* species studied.

Lab designation	Scientific name	Accession number		
		***rpo*C1**	***mat*K**	***trn*H-*psb*A**
Pi-1	*P. nigrum*	GQ500612	-	GQ891994
Pi-4	*P. sarmentosum*	GQ500613	GU372746	GQ891995
Pi-5	*P. betle*	GQ500614	GU372747	GQ891996
Pi-6	*P. pendulispicum*	GQ500615	GU372748	GQ891997
Pi-7	*P. retrofractum*	GQ500616	GU372749	GQ891998
Pi-14	*P. ribesioides*	GQ500617	GU372750	GQ891999
Pi-15	*P. colubrinum*	GQ500618	GU372751	GQ892000
Pi-B	*Piper* sp., unknown powder	GQ500619	-	GQ892001

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