SNRU Journal of Science and Technology

Journal home page: snrujst.snru.ac.th

Molecular analysis of selected *Strychnos* species from Thailand

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Received: 5 May 2020; **Revised:** 14 August 2020; **Accepted:** 17 August 2020; **Available online:** 1 September 2020

Abstract

Strychnos species are well known for use as medicinal plants in both the Western and Eastern part of the world including Thailand. Many *Strychnos* species have their vernacular names and similar morphological appearances resulting in unintentional substitution. The aims of the current research were to investigate the full-length of nucleotide sequencing ITS region, *rbc*L gene and *mat*K gene among four *Strychnos* species including *S. lucida, S. thorelii, S. nux-blanda* and *S.nux-vomica*. The results showed that the amplified PCR products in ITS region, *rbc*Lgene and *mat*K gene were approximately 700, 1500 and 1800 base pairs, respectively. The intra-species of three sequences showed 96 – 99% similarity and the inter-species showed 87 – 99% similarity. PCR-RFLP analysis based on the *mat*K gene was developed and digested with two specific restriction enzymes using *Xba*I and *Dra*I. *S. thorelii* can be distinguished from *S. lucida, S. nux-blanda* and *S. nux-vomica* when digested with *Xba*I whereas *S. lucida, S. nux-blanda* and *S. nux-vomica* can be distinguished to each other when digested with *Dra*I. Four *Strychnos* species were easily differentiated based on the different sizes of the digested fragments. In conclusion, the use of DNA molecular techniques was successful in the identification of four *Strychnos* species in Thailand.

Keywords: *Strychnos* species; DNA sequencing; PCR-RFLP

1. Introduction

The genus *Strychnos* is the largest genus of family Strychnaceae, comprising about 200 species range from forest lianas to shrubs and trees, and can be subdivided into three groups. There are at least 73 species originating from South and Central America, 75 species from Africa and 44 species from Asia including Australia [1, 2]. Smitinand recorded the presence of 11 species in Thailand. Among these, four species were revealed as important medicinal plants in folk medicine namely *Strychnos lucida* R. Br., *Strychnos thorelii* Pierre ex Dop, *Strychnos nux-blanda* A.W. Hill and *Strychnos nux-vomica* L. [3]. *Strychnos* species have their ethnomedicinal uses. A few species are well known as arrow poison and ordeal poison [4]. In ethnomedicine, the genus *Strychnos* has long been used for rheumatism, as a fever reducer, analgesic, and antidote for snake poisoning [5]. The major constituents in the *Strychnos* plant are reported to contain alkaloids like strychnine and brucine, which are responsible for pharmacological and toxicological activities.

There was a cause of unintentional substitution of herbals medicinal from *Strychnos* species due to several species have the same vernacular names and similar morphological appearances [6]. There are many methods used for examination of medicinal plants such macroscopic and microscopic examinations, chemical compound identification and genetic information. DNA-based molecular techniques have been proved to be the powerful way to discriminate species with high accuracy

because DNA characteristics are the unique heredity of each species and are not affected from environmental factors. Plant genomes are more complex than other eukaryotic organisms due to present of multiple chromosomes; nuclear genome, chloroplast genome and mitochondrial genome. A specific region of DNA sequence in plant genome has been used as a modern genomics tool for herbal plant identification [7]. The most commonly used marker for plant identification is an internal transcribed spacer (ITS) region of nuclear ribosomal DNA [8]. Many chloroplast, mitochondrial and nuclear genes have been utilized for studying sequence variation at genus or species level [9].

Misidentification might cause inconsistent results because of the different therapeutic effects of these species and may be poisonous if taken in excess [10]. There are limited studies about *Strychnos* species existing in Thailand. As a result, it is essential for studying the genetic information of *Strychnos* species. The information obtain from this study can be useful for species identification and development of a better polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for rapid identification.

2. Materials and methods

Plant materials

The fresh young leaves of *S. lucida*, *S. thorelii, S. nux-blanda*, and *S. nux-vomica* were collected from various geographical areas from Thailand as showed in Table 1. Plant materials were authenticated by Associate Professor Nijsiri Ruangrangsi, Ph.D. College of Public Health Sciences, Chulalongkorn University. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

Table1 1 List of four *Strychnos* species

Scientific name	Locality
S. lucida	Bangkok
	Pathum Thani
	Nonthaburi
S. thorelii	Chachoengsao
	Phitsanulok
	Chonburi
S. nux-blanda	Chiang Mai
	Phitsanulok
	KhonKaen
S. nux-vomica	Pathum Thani
	KhonKaen
	Songkla

DNA Extraction and PCR-RFLP method

Genomic DNA was extracted from fresh young leaves of each *Strychnos* species using modified CTAB method [11]. The genomic DNA was examined on 1.50% agarose gel electrophoresis after straining with ethidium bromide as showed in Fig. 1. The genomic DNA samples were stored at –20 ºC for further use as DNA templates in PCR amplification.

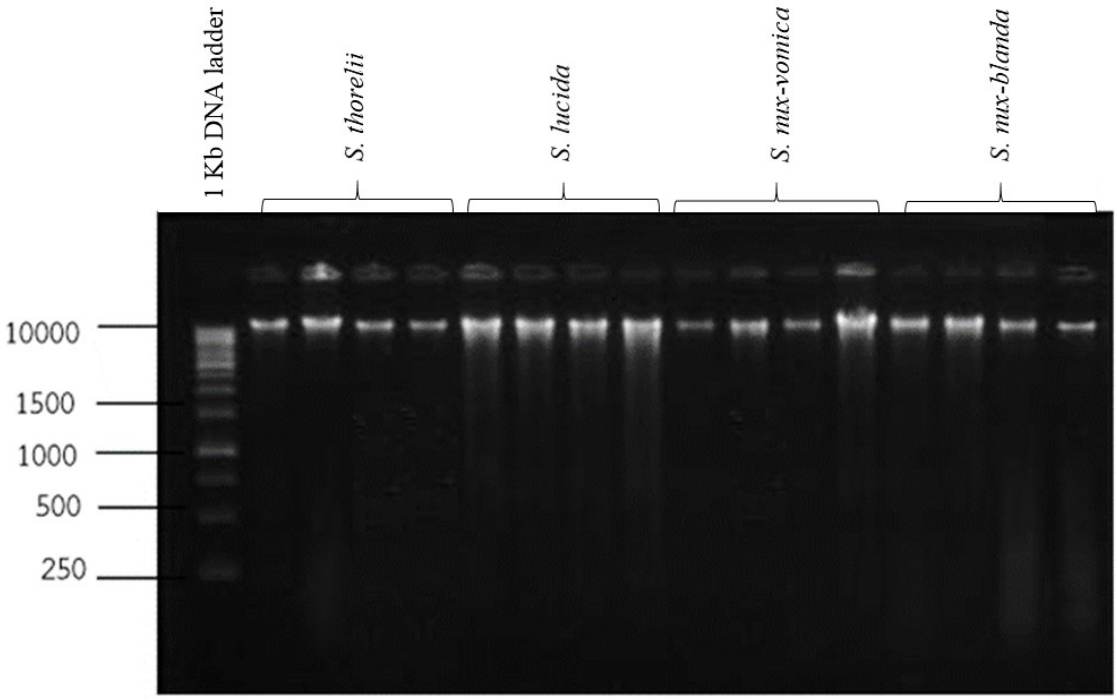

Fig. 1 Genomic DNA of four *Strychnos* species on 1.50% agarose gel electrophoresis

Amplification of the ITS region, the ITS including ITS1-5.8S rDNA - ITS2 region was amplified using a pair of universal primers [12], the ITS5 primer (5'-GAAGTAAAAGTCGTAACAAGG-3') and the ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') (Operon Biotechnologies, Germany). PCR amplification was performed in a 25 µL reaction volume, containing of 1X PCR buffer (100 mM KCl, 20 mM Tris-HCl pH 8), 2.50 mM $MgCl_2$, 0.20 mM dNTPs, 0.20 µM of each primer, 0.50 unit of *Taq*DNA polymerase (Fermentas, USA) and 1 µL of DNA template. PCR amplification was performed in Thermal cycler (Thermo Electron Corporation, USA) under the following condition: initial denaturation step at 95 ºC for 5 min, 30 cycles of 95 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s, a final extension at 72 ºC for 5 min, and then hold at 4ºC. Five microliters of PCR product were separated by 1.50% agarose gels electrophoresis in 1XTBE buffer (Fluka, Germany) and stained with ethidium bromide.

The ribulose-bisphosphate carboxylase (*rbc*L) gene was amplified using a pair of primer, forward *rbc*L primer (5'-TGTCACCACAAACAGAGACTAAAGCAAGT-3') and reverse *rbc*L primer (5'AGTCTTTTAGTAAAGATTGGGCCGAG-3'**)** (Operon Biotechnologies, Germany). PCR amplification was performed in a 25 µL reaction volume, containing of 1X PCR buffer (100 mM KCl, 20 mM Tris-HCl pH 8), 2.50 mM $MgCl_2$, 0.20 mM dNTPs, 0.20 µM of each primer, 0.50 unit of *Taq* DNA polymerase (Fermentas, USA) and 1 µl of DNA template. PCR amplification was performed in Thermal cycler (Thermo Electron Corporation, USA**)** under the following condition: "initial denaturation step at 95 ºC for 5 min, 30 cycles of 95 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 30 s, a final extension at 72 ºC for 5 min, and then hold at 4 ºC. Five microliter of PCR product were separated by 1.50% agarose gels electrophoresis in 1X TBE buffer (Fluka, Germany) and stained with ethidium bromide.

The maturase K (*mat*K**)** gene was amplification using a pair of primer, *trn*K forward primer (5'CTGTTGATAAGTTTACCTGCCTCCG-3') and *trn*K reverse primer (5'-ATTGCACACGGC TTTCCCTATG-3') (Operon Biotechnologies, Germany). PCR amplification was performed in a 25 µL

reaction volume, containing of 1X PCR buffer (100 mM KCl, 20 mM Tris-HCl pH 8), 2.50 mM $MgCl_2$, 0.20 mM dNTPs, 0.20 µM of each primer, 0.50 unit of *Taq* DNA polymerase **(**Fermentas, USA) and 1 µL of DNA template. PCR amplification was performed in Thermal cycler (Thermo Electron Corporation, USA) under the following condition: initial denaturation step at 95 ºC for 5 min, 30 cycles of 95 ºC for 30 s, 54ºC for 30 s, 72 ºC for 1 min, a final extension at 72 ºC for 5 min and then hold at 4 ºC. Five microliter of PCR product were separated by 1.50% agarose gels electrophoresis in 1X TBE buffer (Fluka, Germany) and stained with ethidium bromide.

The PCR products were purified by PCR purification kit (QIAGEN) according to the manufacturer's protocol prior sequencing. The DNA sequencing the ITS region, *rbc*L and *mat*K gene from both sense and antisense strands were analyzed using BioEdit sequence alignment version 7.0.9 for Windows.

Development of PCR-RFLP method for *mat*K gene, the *mat*K sequences data were analyzed and the PCR products were digested with restriction enzymes (*Dra*I, *Xba*I) (Eurofons MWG operon, Germany) according to manufacturer's instructions. The reaction mixture was carried out in 20 µL which consisting of 10 µL of *mat*K gene PCR amplification product, 2 µL of restriction buffer, 10 U/µL of restriction enzyme. The reaction was incubated at temperature 37 ºC for 30 min in shaking incubator. Ten microliters of the restriction reaction were separated through their length by 1% agarose gel electrophorese along with 1 kbDNA ladder (Promega, USA). Electrophoresis was performed in 1X TBE buffer (Fluka, Germany) at constant voltage of 80 V until the faster migration dye (bromophenol blue) has traveled to two-third of gel and then stained with ethidium bromide. Agarose gels were photographed using InGenius 3 with GeneSis Software **(**Syngene, UK) and fragment pattern were estimated using GeneTools Software **(**Syngene, UK).

3. Results and Discussion

In order to characterize an herbal plant, various analytical methods were employed ranging from simple morphological to physicochemical analytical and DNA molecular techniques. This study aimed to identify the four *Strychnos* species using molecular analysis.

DNA sequencing

The length of the ITS, *rbcL* and *matK* sequence obtained from various geographical areas of four *Strychnos* species were approximately 700, 1,500 and 1,800 base pair (bp), respectively. The similarity of the ITS, *rbcL* and *matK* sequence among the intra-species ranges from 98 – 99%, 95 – 99%, and 96 – 99% while the inter-species ranges from 87 – 99%, 94 – 97% and 90 – 95%, respectively.

DNA identification provided the effective and reliable pharmacognostic tool resolving the confusion in case of similar morphological identification. DNA sequencing can serve as information of nucleotide order in comparison to their closely related species [9]. The ITS regions of ribosomal DNA gene sequences have been employed as genetic markers for various medicinal plant identification. The ITS sequences of *S. lucida, S. nux-blanda and S. nux-vomica* were relatively similar [10]. This region of ribosomal DNA gene is believed to be fast evolving and measurable variations between species [11]. PCR primers designed from highly conserved flanking the ITS regions which is relatively small size about 600 – 700 bp and high copy number more than 100 per cell, enable easy amplification of the ITS region. As a result, ITS region of ribosomal DNA gene has been preferred choice for DNA fingerprint. However, the sequences of ITS region and *rbcL* gene from this study could not apply for PCR-RFLP technique due to no restriction enzyme recognition site for discrimination of 4 *Strychnos* species which is the limitation of this technique. Although, important advantages of the PCR-RFLP technique include inexpensiveness and lack of requirement for advanced instruments, but the disadvantages of this technique include the requirement for specific endonucleases. Apart from ITS sequence of nuclear DNA, the sequencing of chloroplast DNA has

been used for plant species discrimination and identification [12]. PCR-RFLP analysis based on the sequence of *mat*K gene was developed in this study for discrimination of 4 *Strychnos* species.

PCR-RFLP method for species differentiation using matK gene

The PCR products were digested with two restriction enzymes, *Dra*I and *Xba*I. When the PCR product was digested with *Xba*I, the PCR-RFLP restriction pattern can be distinguished *S. thorelii* from the other three *Strychnos* species by observing the single uncut 1800 bp band while the other three *Strychnos* species shown the 1,400 and 400 bp bands as showed in Table 2 and Fig. 2.

Table 2 The restriction fragment size of four *Strychnos* species digested with *Xba*I and *Dra*I

***Strychnos* Species**	**Restriction fragment (base pair)**	
	***Xba*I**	***Dra*I**
S. thorelii	1,800	1,210, 380, 210
S. lucida	1,400, 400	1,150, 360, 290
S. nux-vomica	1,400, 400	1,620, 180
S. nux-blanda	1,400, 400	1,210, 380, 210

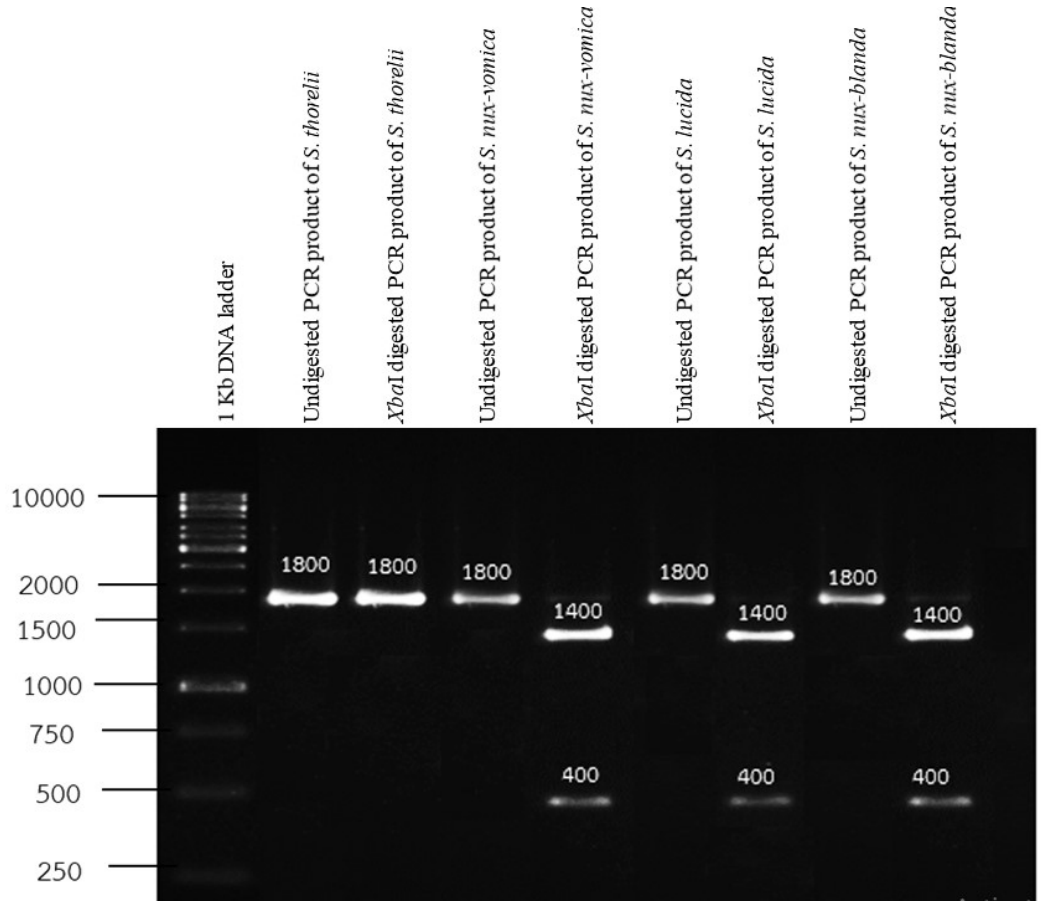

Fig. 2 PCR-RFLP pattern of four *Strychnos* species digested with *Xba*I

To distinguish the three *Strychnos* species, the PCR products were digested with *Dra*I restriction enzyme. The PCR-RFLP restriction pattern can be distinguished *S. nux-vomica, S. lucida and S. nux-blanda.* In *S. nux-vomica*, there are two bands of 1,620 and 180 fragments in size were observed after digestion with *Dra*I. For *S. lucida*, there are three bands of 1,150, 360 and 290 fragments in size while *S. nux-blanda* showed the three bands of 1,210, 380 and 210 fragments in size after digestion with *Dra*I restriction enzyme as showed in Table 2 and Fig. 3.

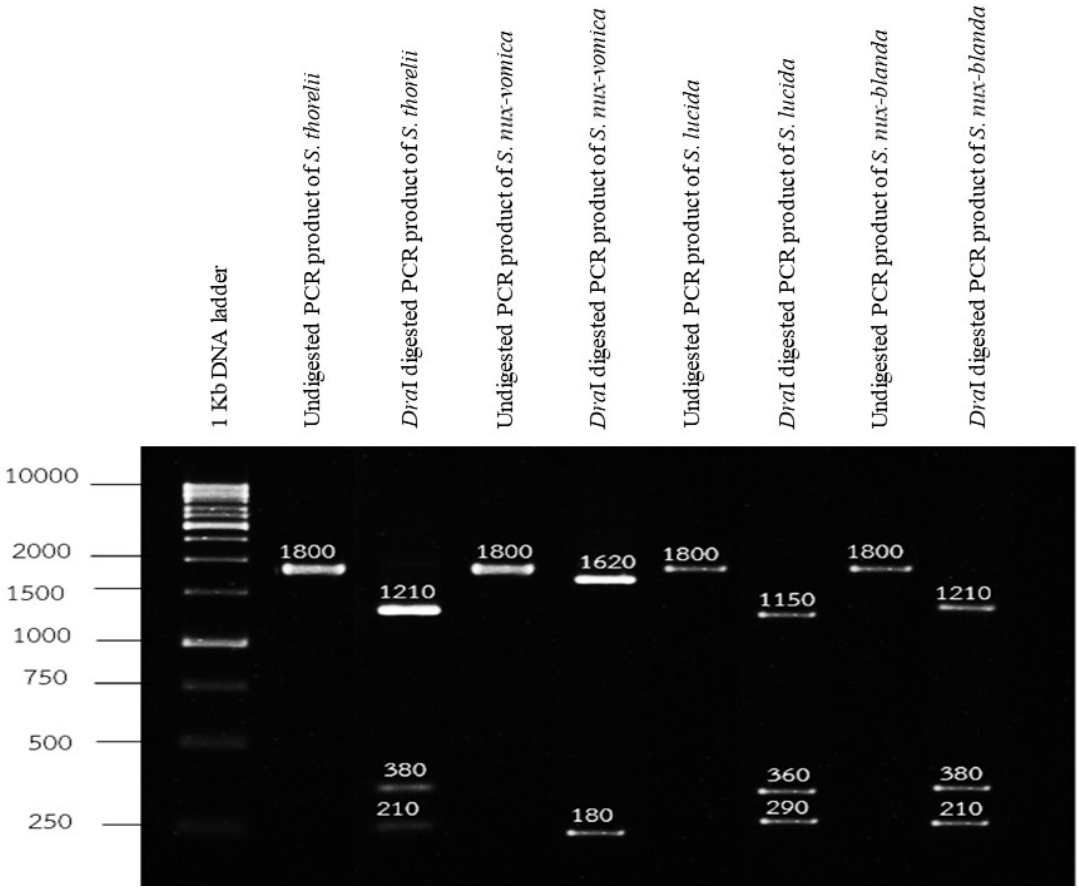

Fig. 3 PCR-RFLP pattern of four *Strychnos*species digested with *Dra*I

*rbc*L and *mat*K gene has been widely used for identification of medicinal plant [17]. The ITS sequence, *rbc*L and *mat*K sequences in this study were supported the identification of four *Strychnos* species. Currently, PCR-RFLR of nuclear DNA and chloroplast DNA (cpDNA) has been widely used in species identification and genetic diversity in several medicinal plant species [18]. PCR-RFLP analysis of cpDNA was applied for intra-specific variation of Tea cultivars (*Camellia sinensis* L.) [19]. PCR-RFLP technique showed potential for identifying species present in *Strychnos* crude drugs. The results showed that after digestion of the *mat*K gene with specific restriction enzyme using *Dra*I and *Xba*I, four *Strychnos* species including *S. lucida, S. thorelii,S. nux-blanda* and *S. nux-vomica* were easily distinguished based on the different sizes of the digested fragments. Herein, a convenient PCR-RFLP technique based on the *mat*K gene that would enable rapid and accurate identification was developed to distinguish four *Strychnos* species from Thailand. As indicated in some previous reports, the sequence of the chloroplast *mat*K gene provides useful information to assist in the taxonomic classification and identification of herbal medicine [20].

4. Conclusion

The ITS region, *rbc*L and *mat*K gene sequences provide valuable information for identification of four *Strychnos* species. The development of PCR-RFLP based on *mat*K sequence analysis provides the simple, rapid, and identification of four *Strychnos* species from Thailand. For future research, combination of various techniques should be applied for comparison such as morphological characterization, chemical composition and other molecular identification.

5. Acknowledgement

The authors appreciate all staff member of College of Public Health Sciences, Chulalongkorn University, Thailand for assistance and instrument supports.

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