CHAPTER IV

AUTHENTICATION OF "KRAI-KRUE" DERIVED FROM THREE ARISTOLOCHIA SPECIES USING MULTIPLEX PCR

4.1 Introduction

"Krai-Krue" is a name of a crude drug used in many Thai folk medicines. The drug is derived from dried roots of three *Aristolochia* plants, *A. pothieri* (Athikomkulchai and Ruangrungri 2001), *A. pierrei* and *A. tagala* (Sathornviriyapong, Picheansoonthon et al. 2007). It can also be derived from dried roots of *Raphistemma pulchellum* (Apocynaceae) (Vuthithammavech 1997), *Jasminum* spp. (Oleaceae) (Picheansoonthon, Chawalit et al. 2001), and *Gymnopetalum integrifolium* (Cucurbitaceae) (Vuthithammavech 1997). Crude drug "Krai-Krue" is available in the local dispensaries in the forms of powders and dried root slices, which no longer bear the original morphological characters, making them difficult to identify.

Recently, many types of DNA-based molecular technique have been developed for rapid and reliable herbal materials identification tool (Zhao, Hu et al. 2006), and the ITS region is the most frequently used (Li, Cao et al. 2011). Multiplex PCR based on polymorphic sites of nucleotide sequence is a suitable technique for the authentication of herbal medicines by amplification of more than two different loci simultaneously (Lee, Kim et al. 2012).

In this study, a multiplex PCR method using diagnostic primers via interspecific variation analysis of the ITSS2 region was developed for the convenient and rapid identification of the three *Aristolochia* species used as Krai-Krue herbs, *A. pothieri*, *A. pierrei* and *A. tagala*. To the best of our knowledge, this is the first study on the discrimination of the herb using the multiplex PCR technique.

4.2 Materials and methods

4.2.1 Plant materials

Forty-two *Aristolochia* samples as shown in Table 2 were studied. Other Krai-Krue herbs comprising *Raphistemma pulchellum* Wall, *Jasminum sp*, *J. sombac* (L.) Aiton, *J. adenophyllum* Wall. Ex C.B. Clarke and *Gymnopetalum integrifolium* Kurz. collected from various locations were also included in this study (Table 10).

Table 10 Plant samples used as sources of Krai-Krue.

Sample	Place of collections (Thailand, Province)	Voucher number
Raphistemma pulchellum Wall	Bangkok	MUS-5414
Gymnopetalum integrifolium Kurz.	Sakaeo	MUS-5415
	Phetchaburi	MUS-5416
Jasminum sambac (L.) Aiton	Bangkok	MUS-5417
Jasminum adenophyllum Wall. Ex C.B. Clarke	Bangkok	MUS-5418
Jasminum sp	Bangkok	MUS-5419

4.2.2 Genomic DNA extraction

Total genomic DNA was extracted from 100 mg of leaves from each individual plant specimen and was frozen using liquid nitrogen and ground with a mortar and pestle to obtain a fine powder. The isolation of the total DNA from the powder was performed using genomic DNA extraction kit as described previously.

4.2.3 Multiplex PCR of the ITS2

4.2.3.1 ITS2 multiple sequence alignment

The DNA sequences of ITS region from eleven *Aristolochia* plants were aligned using ClustalX Version 2.1 software. According to the multiple sequence alignment, polymorphic sites were detected on the ITS region. The diagnostic forward

primer was designed to be complementary to a region of the PCR product where interspecific variation of interest occurs (Figure 7). The primer pairs that would produce different sizes of PCR products were designed as shown in Table 11. Each primer was designed to anneal to a specific region of each interested *Aristolochia* plants. The two common primers, ITS-Aris-371F and ITS4, were also designed to amplify DNA sequence as an internal amplification control.

Table 11 Species-specific primers used in multiplex PCR in this study.

Primer	Specificity	Sequence (5'⟶3')	Tm (°C)
ITS2-poF	*A. pothieri*	GCC GCG AGG ACC C*AA TG*	60.0
ITS2-piF	*A. pierrei*	GAC TAC TGG TGG CTC CAC GC*A*	63.7
ITS2-taF	*A. tagala*	GGC GGG GGC GAG CAG *GC*	67.2
ITS-Aris-371F	Internal control	AAT TGC AGA ATC CCG CGA AC	57.3
ITS4	Internal control	TCC TCC GCT TAT TGA TAT GC	55.3

(The underlined nucleotide(s) were specifically designed for target sequence(s))

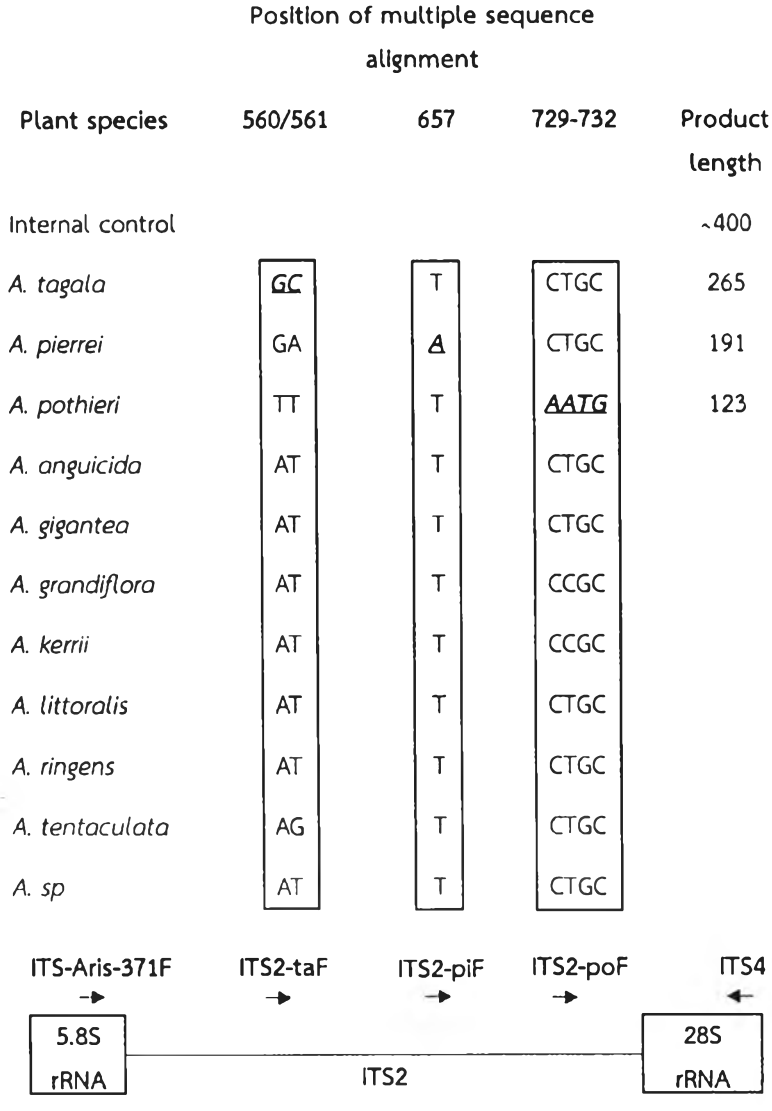

Figure 7 Positions of diagnostic primers for multiplex PCR for discrimination of *Aristolochia* plants used as Krai-Krue herbs.

4.2.3.2 Multiplex PCR

Nucleotide polymorphisms-based multiplex PCR was performed to authenticate three *Aristolochia* plants using species-specific primers. The multiplex PCR should be first examined the specificity of diagnostic primers by singleplex PCR (Sint, Raso et al. 2012). Each diagnostic primer pair was conducted separately using genomic DNA of each species as template. With the multiplex PCR system, three diagnostic

primers and two common primers were included in the multiplex PCR reaction using genomic DNA of each species as template. The amplification reaction was performed in 25 µL of GoTaq® Flexi DNA Polymerase reaction mixture, consisting of 5X PCR buffer, 25 mM $MgCl_2$, 2.5 mM of each dNTP, 10 mM of each primer, 5U *Taq* polymerase (Promega, USA), and 10–100 ng of total genomic DNA as a template.

Annealing temperatures were determined by gradient PCR with temperatures increasing from 52 to 58°C. The optimal PCR condition obtained was at 96°C for 3 min, followed by 30 cycles of 96°C for 60 s, 55°C for 1 min and 72°C for 30 sec, and final extension at 72°C for 10 min. The amplified products were detected by 1.7% agarose gel electrophoresis in 1X TAE buffer at 80 V for 40 min. Separated PCR products were then visualized by ethidium bromide staining under UV light. Fragment sizes were estimated by comparison with DNA marker. This experiment was repeated in all samples in the same species for three times to verify the stability and reproducibility of banding patterns.

4.3 Results

4.3.1 ITS2 sequences analysis and species-specific primers for multiplex PCR

According to the multiple sequence alignments of ITS regions of eleven *Aristolochia* plants, three interspecific variation sites specific to each of *A. pothieri*, *A. pierrei* and *A. tagala* were detected. These sites were chosen to design three diagnostic primers. The nucleotide at positions 560-561 was GC which specific in *A. tagala*. The nucleotide at positions 657 was T in all *Aristolochia* plants, whereas in *A. pierrei* was A. The positions 729-732 in *A. pothieri* was AATG, but not in the other species. These diagnostic forward primers were designed for specific amplification using the available variation sites on the ITS2 region of eleven *Aristolochia* plants. The forward primers, ITS2-poF, ITS2-piF and ITS2-taF, were used to amplify specific fragments of 123, 191 and 265 bp for *A. pothieri*, *A. pierrei* and *A. tagala*, respectively.

The common forward primer, ITS-Aris-371F, and universal primer, ITS4, were used as internal amplification control to all *Aristolochia* plants with ~ 400 bp fragment (Figure 7).

4.3.2 Multiplex PCR analysis

The specificity of each primer was done using singleplex PCR. DNA template of each species was amplified individually with three pairs of diagnostic primers. The results revealed that only species-specific product was amplified. Each primer pairs generated specific fragments with different size. For example, only specific fragment of 123 bp was generated in *A. pothieri*. Likewise, *A. pierrei* and *A. tagala* generated their specific fragments of 191 and 265 bp of PCR products, respectively. These results indicated that each primer was specific to each species (data not shown).

In multiplex PCR reaction, each specific PCR product was amplified specifically from its target species with the combination of the three diagnostic forward primer and common forward primer. The PCR result was observed by gel electrophoresis. Three individual species-specific fragments were shown with different sizes. The fragments of 123, 191 and 265 bp was amplified by the primer pairs ITS2-poF/ITS4, ITS2-piF/ITS4 and ITS2-taF/ITS4 specifically for *A. pothieri*, *A. pierrei* and *A. tagala*, respectively, while the 400 bp fragment generated from internal control primer pair of ITS-Aris-371F/ITS4 in all target species. The PCR product was determined by visualization under UV light after gel electrophoresis. Therefore, two different size of PCR products were simultaneously amplified in all of individual target species. The result analysis was based on the condition of one fragment used as an internal control and one fragment used as specific fragment of each species (Figure 8). To confirm the reproducibility of this method, the experiment was repeated in all samples in the same species for three times.

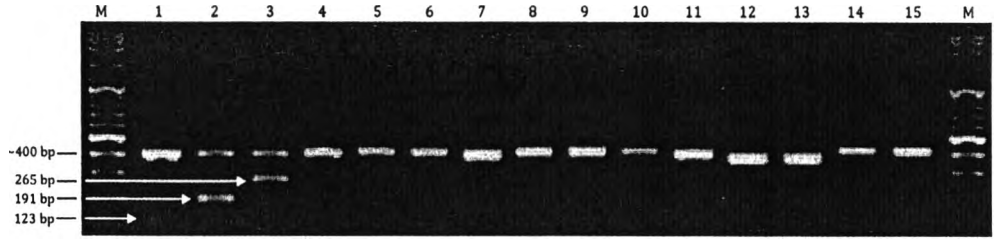

Figure 8 1.7% agarose gel electrophoresis image of PCR products generated with a set of species-specific PCR primers on ITS2 region of Krai-Krue herb. Lane;

1: *Aristolochia pothieri*
2: *A. pierrei*
3: *A. tagala*
4: *A. ringens*
5: *A. kerrii*
6: *A. littoralis*
7: *A. grandiflora*
8: *A. gigantea*
9: *A. tentaculata*
10: *A. anguicida*
11: *Gymnopetalum integrifolium*
12: *Raphistemma pulchellum*
13: *Jasminum* sp
14: *J. sambac*
15: *J. adenophyllum*
M: VC 100-bp plus DNA ladder

4.4 Discussion

Dried roots of three *Aristolochia* plants, *A. pothieri* (Athikomkulchai and Ruangrungri 2001), *A. pierrei* and *A. tagala* (Sathornviriyapong, Picheansoonthon et al. 2007) and other plants, *Raphistemma pulchellum* (Apocynaceae) (Vuthithammavech 1997), *Jasminum* spp. (Oleaceae) (Picheansoonthon, Chawalit et al. 2001), and *Gymnopetalum integrifolium* (Cucurbitaceae) (Vuthithammavech 1997) have been recognized as sources of Krai-Krue. Such misidentification of herbs is particularly common for species that share a similar name or similar features but significantly vary in their medicinal or toxic properties. Therefore, the proper identification of Krai-Krue herbs is needed, especially when the herbs appear in the form of a powder, shredded material, or a formulated mixture. To ensure the safety of using these herbs, species-specific method for the discrimination of *A. pothieri*, *A. pierrei* and *A. tagala* is neccessary.

Morphological characteristics of plants have been widely used for plant classification. Regarding the botanical origins of Krai-Krue, generally, *A. pothieri* has been clearly differentiated by leaf morphology from *A. pierrei* and *A. tagala* (Phuphathanapong 1987). But *A. pierrei* and *A. tagala* are difficult to differentiate by morphology. The discrimination of these *Aristolochia* plants is difficult especially when they are ground into small pieces or powder. To ensure the safety of consumers from Krai-Krue containing herbal products, the development of a simple and reliable method for discrimination of these *Aristolochia* plants is necessary. In this study, based on the obtained nucleotide sequences of four DNA regions (*rbc*L, *mat*K, ITS and *trn*H-*psb*A) from eleven *Aristolochia* species as described in the previous chapter, the ITS2 region was used as a tool for discrimination of the three *Aristolochia* plants because it is a high variable region and has been successfully used in several herbal identifications (Li, Cao et al. 2011). Based on the diagnosed polymorphic sites from sequencing technique as in Figure 7, the three *Aristolochia* Krai-Krue can be differentiated by multiplex PCR.

Multiplex PCR was developed for identification of the three *Aristolochia* plants using a species-specific primer set. The three forward species-specific primers located on ITS2 region, ITS2-poF, ITS2-piF and ITS2-taF, were specifically designed for *A. pothieri*, *A. pierrei* and *A. tagala*, respectively to generate PCR products of 123, 191 and 265 bp in length. The generated amplicon size of each specific fragment should be different when detected by gel electrophoresis (Sint, Raso et al. 2012). The internal control of amplification was included in the experiment design to ensure that the reaction was successful because the PCR product of internal control should always be amplified even though there is no target DNA sequence. The PCR products of ITS region from all samples were amplified with the internal control primers ITS-Aris-371F and ITS4 located on conserved region of 5.8s rDNA and 28s rDNA to generate 400 bp fragments in all samples. The test of specificity of each diagnostic primer before starting

the multiplex PCR was performed by singleplex PCR. The conditions of PCR for both singleplex and multiplex PCR were optimized by gradient PCR. The optimal annealing temperature was at 55 °C for all primers (data not shown). The result indicated that multiplex PCR simultaneously amplified two fragments, one corresponding to target sequence for each species and the other to an endogenous sequence as an internal control for the PCR. Interestingly, only one specific fragment of each species was amplified by its specific primer. This result indicated that the each species-specific primer designed was highly specific to its target sequence. The three *Aristolochia* plants used as sources of Krai-Krue herbs could be discriminated individually between species with their specific primers. The results from this study are consistent with previous studies showing that the multiplex PCR could be applied as an effectively authenticate medicinal plants tool (Lee, Doh et al. 2008, Jigden, Wang et al. 2010).

4.5 Conclusion

The multiplex PCR based on ITS2 region was examined and successfully applied for the discrimination of three *Aristolochia* plants used as Krai-Krue herbs from other sources. The results indicated that this method is a convenient and specific tool for raw material identification. This is the first report of the authentication of three *Aristolochia* species used as Krai-Krue herbs by molecular approach, and this could be adapted for identification of other medicinal plants in a simple, accurate, time-saving and cost-effective method.