CHAPTER VII
DISCUSSION AND CONCLUSIONS

Recently, natural products have become a popular supplement for maintaining good health. The misidentification of material derived from toxic herbs such as *Aristolochia* herbs have been reported as one of the global concerns (Debelle, Vanherweghem et al. 2008). To assure the correct authentication of natural materials, many identification tools have been developed (Heubl 2013). Currently, the DNA barcodes of many herbal *Aristolochia* plants have been studied. Chemical profiling has been often combined with DNA fingerprinting to investigate the botanical sources of suspected herbal materials (But, Shaw et al. 2007, Li, Au et al. 2012, Chan 2014, Li, Au et al. 2014, Liu, Chuang et al. 2015).

The present studies provide genetic and phytochemical tools for identification of *Aristolochia* plants. They were applied for identification of the crude drug called "Krai-Krue" which was derived from different plants including dried roots of *A. pothieri*, *A. pierrei*, *A. tagala*, *Raphistemma pulchellum*, *Jasminum* spp and *Gymnopetalum integrifolium*. Genetic assessment by DNA barcoding technique of four standardized DNA regions (*rbc*L, *mat*K, ITS and *trn*H-*psb*A) were conducted on eleven *Aristolochia* plants, *A. anguicida*, *A. gigantea*, *A. grandiflora*, *A. kerrii*, *A. littoralis*, *A. pierrei*, *A. pothieri*, *A. ringens*, *A. tagala*, *A. tentaculata* and *A.* sp. The nucleotide variations of these species were found in the order of ITS (28.96%) > *trn*H-*psb*A (13.35%) > *mat*K (11.22%) > *rbc*L (3.29%). Although intraspecific variations of samples were detected, the information could still be served as an effective approach to discriminate different or confusing plant species of the same genus. The obtained nucleotide sequences are also useful as the centralized nucleotide database for global uses. The combined data of many DNA regions could be used for further study such as development of molecular markers, phylogenetic analysis and forensic science.

The systematic of *Aristolochia* has been based on morphological characters such as perianth tubes, leaves, number of styles and anthers on gynostemium and fruit and chromosome number (González 1999, Murata, Ohi et al. 2001, Ohi-Toma, Sugawara et al. 2006). In this study, the phylogenetic tree constructed from nucleotide sequences of complete *mat*K gene of *Aristolochia* were analyzed. The phylogenetic analysis supported the morphological criteria, habitat and chromosome number. This result agrees well with the previously published *mat*K phylogenetic tree (Murata, Ohi et al. 2001, Ohi-Toma, Sugawara et al. 2006) and *trn*L-*trn*F phylogenetic tree (Neinhuis, Wanke et al. 2004). In addition, this is the first study of *A. pothieri*.

The high level of ITS2 sequence variations in eleven *Aristolochia* plants, was used for detection of Krai-Krue sources. Unfortunately, direct DNA amplification and sequencing process by universal primer of this region failed to discriminate crude drugs bought from local dispensaries. The results indicated that crude drugs were contaminated with the microorganism during post-harvesting processes and storage conditions (data unpublished). However, the sequences were analyzed for the development of other molecular markers. Multiplex PCR based on the ITS2 region were then employed. The method successfully differentiated the three *Aristolochia* Krai-Krue from the other botanical origins by different sizes of PCR products on agarose gel electrophoresis. In addition, HPTLC using AAI as standard reference was helpful in aiding the multiplex PCR to identify the botanical sources of Krai-Krue herbs. The results indicated that crude drugs "Krai-Krue" from various local dispensaries were derived from *A. pierrei* and other species. These studies confirmed that the combined identification tools, multiplex PCR and HPTLC, are suitable, convenient and specific technique for the discrimination of Krai-Krue herbs used in Thailand. The combined techniques can be modified for the identification of other several pharmaceutical herbs or individual species in herbal drug formulations.

Nevertheless, this PCR based method was not suitable for Krai-Krue containing formulas because there are very low amount of Krai-Krue in those formulas. Therefore, the twenty-three Krai-Krue containing formulas manufactured before and during the Krai-Krue removal regulations were assessed by HPTLC. According to the result, 13 formulas probably contained AAI. This method could be used as AAI-screening test by the herbal industries and regulatory authorities. As recommendations, the more sensitivity of molecular markers is needed in this case for example real-time PCR analysis for further study.

The results from our studies indicated that the combination of genetic and phytochemical assessments is useful and could be used as suitable tools for both genus level and species-level identification of *Aristolochia* plants. Moreover, natural products should be subjected to the same stringent scrutiny and controls as modern drugs before their release onto the market.