DNA Barcoding of Aristolochia Plants

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Abstract

The anecdotal evidence is outstanding on the uses of *Aristolochia* plants as traditional medicines and dietary supplements in many regions of the world. However, herbal materials derived from *Aristolochia* species have been identified as potent human carcinogens since the first case of severe renal disease after ingesting these herbal preparations. Any products containing *Aristolochia* species have thus been banned on many continents, including Europe, America and Asia. Therefore, the development of a method to identify these herbs is critically needed for customer safety. The present study evaluated DNA barcoding of the *rbc*L, *mat*K, ITS2 and *trn*H-*psb*A regions among eleven *Aristolochia* species collected in Thailand. Polymorphic sites were observed in all four DNA loci. Among those eleven *Aristolochia* species, three species (*A. pierrei*, *A. tagala* and *A. pothieri*) are used as herbal materials in Thai folk medicine, namely, in Thai “Krai-Krue”. “Krai-Krue” herbs are interchangeably used as an admixture in Thai traditional remedies without specific knowledge of their identities. A species-specific multiplex PCR based on nucleotide polymorphisms in the ITS2 region was developed as an identification tool to differentiate these three *Aristolochia* species and to supplement the HPTLC pattern in clarifying the origins of herbal materials. The combination of multiplex PCR and HPTLC profiling achieves accurate herbal identification with the goal of protecting consumers from the health risks associated with product substitution and contamination.

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Protocol

Plant materials

Step 1.

Thirty-eight specimens of eleven *Aristolochia* taxa were collected from various regions of Thailand. All specimens were identified by Associate Professor Thatree Phadungcharoen at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. Herbarium specimens were prepared and kept at the Museum of Natural Medicines, Faculty of Pharmaceutical Science, Chulalongkorn University.

Genomic DNA extraction

Step 2.

Total genomic DNA was extracted from 80 to 100 mg of fresh leaves of the plants or from 15 to 25 mg of dried commercially available crude drug samples. DNA was extracted by a DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA quality and quantity were determined by agarose gel electrophoresis; the gels were stained with ethidium bromide and visualized under UV light.

PCR amplification and electrophoresis

Step 3.
The *rbc*L, ITS2, and *trn*H-*psb*A of *Aristolochia* were amplified by amplification primers. Nucleotide sequences of the *trn*K-*mat*K regions of *A. pierrei* (accession number DQ296649), *A. grandiflora* (accession number DQ532052), and *A. gigantea* (accession numbers JX485569 and DQ882187) were retrieved from GenBank for primer design. New primers, matK-Aris-F1, matK-Aris-F2 and matK-Aris-R1, were used to amplify the complete *mat*K sequence of the genus *Aristolochia*.

DNA locus	Sequencing primer	Sequence (5'→3')
***rbc*L**	rbcL_aF*	ATG TCA CCA CAA ACA GAG ACT AAA GC
	rbcL-Aris-327R	TTC AAA AAG GTC TAA AGG GTA AGC
	rbcL_636F	GCG TTG GAG AGA TCG TTT CT
	rbcL_R23*	TTT TAG TAA AAG ATT GGG CCG
***mat*K**	matK-Aris-F1*	ATC CCC TAT TCC TTC AGT TCA A
	matK-Aris-F2*	CCT TGT TTT GAC TGT ATC GCA C
	matK-Aris-F458	ATA CCC CAC CCC ATC CAT CTG
	matK-Aris-F967	CAC TTG TGG TCT CAA CCG GG
	matK-Aris-R1*	GCA CAC GGC TTT CCC TAT G
***trn*H-*psb*A**	psbAF*	GTT ATG CAT GAA CGT AAT GCT C
	trnHR*	CGC GCA TGG TGG ATT CAC AAA TC
ITS2	ITS1*	TCC GTA GGT GAA CCT GCG G
	ITS3*	GCA TCG ATG AAG AAC GCA GC
	ITS4*	TCC TCC GCT TAT TGA TAT GC
	ITS-Aris-390F	AAT TGC AGA ATC CCG CGA AC

The PCR amplification was performed in 50 µL of reaction mixture consisting of 5X PCR buffer, 25 mM $MgCl_2$, 2.5 mM each dNTP, 10 mM each primer, 5U GoTaq® Flexi DNA polymerase (Promega, USA), and 10–100 ng of total DNA as a template. PCR amplifications were carried out in a C1000™ Thermal Cycler (Bio-Rad, USA) using cycling conditions of 96°C for 3 min; followed by 30 cycles of 96°C for 1 min, 55°C for 1 min and 72°C for 2 min (for *rbc*L and *mat*K) and 45 sec (for ITS2 and *trn*H-*psb*A); and a final extension at 72°C for 10 min. The amplified products were detected by 1.2% agarose gel electrophoresis in 1X TAE buffer and were visualized by ethidium bromide staining.

Sequence analysis

Step 4.
The sequencing process was performed by capillary sequencing (AIT Biotech, Singapore) with sequencing primers for each region. The sequences were aligned, edited and analysed using BioEdit Sequence Alignment Editor Version 7.2.5. The obtained sequences were assembled to create consensus sequences using the DNASTAR® (Version 8.0.2) program (USA). The sequences were then submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases, and their accession numbers.