A DNA Microarray for the Authentication of Toxic Traditional Chinese Medicinal Plants

Maria Carles[1, 2], Matthew Kin Lok Cheung[2, 3], Shanti Moganti[1, 2], Tina T. X. Dong[3], Karl W. Tsim[2, 3], Nancy Y. Ip[1, 2], Nikolaus J. Sucher[2, 3]

Abstract

A silicon-based DNA microarray was designed and fabricated for the identification of toxic traditional Chinese medicinal plants. Species-specific oligonucleotide probes were derived from the 5S ribosomal RNA gene of *Aconitum carmichaeli, A. kusnezoffi, Alocasia macrorrhiza, Croton tiglium, Datura inoxia, D. metel, D. tatula, Dysosma pleiantha, Dy. versipellis, Euphorbia kansui, Hyoscyamus niger, Pinellia cordata, P. pedatisecta, P. ternata, Rhododendron molle, Strychnos nux-vomica, Typhonium divaricatum* and *T. giganteum* and the leucine transfer RNA gene of *Aconitum pendulum* and *Stellera chamaejasme.* The probes were immobilized via dithiol linkage on a silicon chip. Genomic target sequences were amplified and fluorescently labeled by asymmetric polymerase chain reaction. Multiple toxic plant species were identified by parallel genotyping. Chip-based authentication of medicinal plants may be useful as inexpensive and rapid tool for quality control and safety monitoring of herbal pharmaceuticals and neutraceuticals.

Traditional Chinese Medicine (TCM) is an integral part of Chinese diet and culture [1]. It has been used to meet medical needs for several millennia. TCM drugs are derived from natural sources (plant, animal and mineral). To date, more than 8,000 medicinal species are used in China in TCM prescriptions. About 500 species have been categorized as "toxic TCMs" [2]. Nonetheless, some are still commonly used in certain prescriptions due to their unique therapeutic effects [3]. Even though these herbs are classified as "toxic or regulated", they can often be acquired in TCM dispensaries and are contained in various "patent medicines", the TCM version of over-the-counter drugs [4].

Although, in China, medicinal herbs are usually prescribed and sold by experienced TCM practitioners, unintended poisonings occur due to the presence of contaminants or inclusion of toxic herbs that were mistaken for a similar looking non-toxic species

Affiliation: [1] Department of Biochemistry, Hong Kong University of Science & Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China · [2] Biotechnology Research Institute, Hong Kong University of Science & Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China · [3] Department of Biology, Hong Kong University of Science & Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China

Correspondence: Dr. Nikolaus J. Sucher · Department of Neurology/Division of Neuroscience · Children's Hospital and Harvard Medical School · Enders 353 · 300 Longwood Avenue · Boston · MA 02115 · U.S.A · Phone: +1-617-355-3515 · Fax: +1-617-730-0243 · E-mail: nikolaus.sucher@childrens.harvard.edu

Received: August 23, 2004 · **Accepted:** March 12, 2005

Bibliography: Planta Med 2005; 71: 580–584 · © Georg Thieme Verlag KG Stuttgart · New York · DOI 10.1055/s-2005-864166 · ISSN 0032-0943

Downloaded by: Hong Kong University of Science & Technology. Copyrighted material.

[5], [6], [7]. In many Western countries, herbal products are considered as dietary supplements and therefore not subject to the same stringent safety controls as drugs [8]. Furthermore, herbal products are most often self-administered by patients without the proper guidance by a TCM practitioner and the knowledge of a physician, who may be treating the patient in parallel with Western medicines [9].

Traditionally, medicinal plants are identified and authenticated by experienced collectors, botanists or trained TCM experts. Initially, these experts identify the samples by their characteristic morphology, appearance, smell, taste, texture, size and color. The inspection may be followed by anatomic dissection, microscopic analysis of the structure and shape of the cells as well as specific physical and/or chemical assays. Morphological methods of identification/authentication of TCMs are only applicable to unprocessed raw materials and require expertise from well-trained experts. Detailed chemical analyses require expensive equipment and the chemical composition of herbs often varies with environmental conditions, time of harvest, and post-harvest processing. Moreover, TCMs used for the preparation of prescriptions are usually dried and highly processed, making their identification in some cases very difficult.

Recently, DNA polymorphism-based assays have been developed for the identification of herbal medicines [10], [11]. In this approach, small amounts of DNA are amplified by the polymerase chain reaction (PCR) and the reaction products are analyzed by gel electrophoresis, sequencing, or hybridization with species-specific probes. In particular, the 5S ribosomal RNA (5S-rRNA) gene has been used for species identification [12], [13], [14], [15], [16], [17].

In an effort to develop methods for the reliable, rapid and inexpensive authentication of medicinal herbs [18], we report the development of a DNA microarray for the identification of common toxic traditional Chinese medicinal plants. We determined the nucleotide sequences of the 5S-rRNA from multiple specimens each of the toxic TCM herbs *Aconitum carmichaeli*, *A. kusnezoffi, Alocasia macrorrhiza*, *Croton tiglium*, *Datura inoxia*, *D. metel*, *D. tatula*, *Dysosma pleiantha, Dy. versipellis*, *Euphorbia kansui*, *Hyoscyamus niger*, *Pinellia cordata, P. pedatisecta, P. ternata*, *Strychnos nux-vomica*, *Typhonium divaricatum* and *T. giganteum*. Comparative sequence alignment revealed species-specific nucleotide sequences, which we used to design and synthesize probes for the construction of a DNA microarray for the authentication of these herbs. Sequence-specific oligonucleotides consisted of a set of 3 primers per species: a primer pair used in the asymmetric PCR reaction (reverse primer-fluorescently labeled) and the immobilization primer or "probe" (Table **1**).

Each silicon chip for the identification of these herbs contained probes corresponding to 20 plant species. Oligonucleotides serving as molecular probes for each species were spotted in columns containing five rows. An additional row was added to each column consisting of a fluorescently labeled control oligonucleotide indicating the quality of probe immobilization. The target sequences were labeled with a fluorescent dye by asymmetric PCR using genomic plant DNA as templates. After hybridization and stringent washing of the microarray, the presence of hybridized target sequence was detected with a confocal laser scanner. Quantification of the fluorescence images indicated that the molecular probes used on the chip unequivocally distinguished between the different plants with a high signal to noise ratio. Some cross-hybridization was observed under the hybridization conditions used between *Datura innoxia* and *D. metel* of which the target and probe sequences differed by only two nucleotides. Nonetheless, the sensitivity of the assay allowed the unequivocal identification of both species (Fig. **1**).

The use of DNA microarrays should make it relatively easy and cost-effective to incorporate the DNA-based authentication of medicinal herbs into a series of measures aimed at the quality assurance and quality control of medicinal herbs [8]. The unequivocal identification of the precise nature of medicinal herbs can constitute a first step before more elaborate and expensive testing methods are applied. In cases of poisoning, this method may be useful for the *post hoc* positive identification of toxic herbal or animal material.

Materials and Methods

All chemicals were purchased from Fluka and Sigma-Aldrich (Milwaukee, WI). 5′-Thiol-modified oligonucleotides and fluorescently labeled probes (Texas Red™) were purchased from Synthetic Genetics (San Diego, CA). All other oligonucleotides were synthesized using an ABI Nucleic Acid synthesis system (Expedite 8909).

Experienced botanists collected plant specimens from China and Hong Kong as follows: *Aconitum carmichaeli* Debeaux (Ranunculaceae) from Jiangyou (Sichuan), *A. kusnezoffi* Reichb. (Ranunculaceae) from Badaling (Beijing), *Alocasia macrorrhiza* Schott (Araceae) from Clear Water Bay (Hong Kong), *Croton tiglium* L. (Euphorbiaceae) from Xinglong (Hainan), *Datura metel* L. (Solanaceae) from Fengtai (Beijing), *D. inoxia* Mill. (Solanaceae) and *D. tatula* L. (Solanaceae) from Xibeiwang (Beijing), *Dysosma pleiantha* R. E. Woodson (Berberidaceae) and *Dy. versipellis* M. Cheng (Berberidaceae) from Yulin (Guangxi), *Euphorbia kansui* S. B. Ho (Euphorbiaceae) from Guangzhou (Guangdong), *Hyoscyamus niger* L. (Solanaceae) from Anguo (Hebei), *Pinellia cordata* N. E. Br. (Araceae) from Conghua (Guangdong), *P. pedatisecta* Schott (Araceae) from Xibeiwang (Beijing), *P. ternata* Ten. ex Breitenb. (Araceae) from Badaling (Beijing), *Strychnos nux-vomica* L. (Loganiaceae) from Xinglong (Hainan), *Typhonium divaricatum* Decne. (Araceae) from Clear Water Bay (Hong Kong), and *T. giganteum* Engl. (Araceae) from Conghua (Guangdong). At least five individuals of each species were authenticated again by one of us (T. T. X. Dong) and used for the experiments. Voucher specimens have been deposited in the Department of Biology at the Hong Kong University of Science & Technology. The sequences of the 5S-rRNA gene of *Dysosma versipellis, Dy. pleiantha*, and *Rhododendron molle* G. Don. (Ericaceae), and the leucine tRNA gene of *Aconitum pendulum* Busch (Ranunculaceae) and *Stellera chamaejasme* L. (Thymelaeaceae) were obtained from GenBank. Plant DNA was extracted from the leaves of fresh plants as described [12]. The 5S-rRNA gene intergenic spacer domain was amplified by PCR (amplicons ranged from 200 – 360 bp). The PCR primers had the following sequence: 5S-rRNA-forward: 5′GGA TCC GTG CTT GGG CGA GAG TAG TA 3′

Downloaded by: Hong Kong University of Science & Technology. Copyrighted material.

Table **1** Sequences of the immobilization, forward and reverse primers of the TCM species

Sequence	*Primer*	*% GC*
Aconitum carmichaeli		
5′-SH-CGTCGCACCCCGTCAACCAC-3′	immobilization	70
5′-Texas Red-GTGGTTGACGGGGTGCGACG-3′	reverse primer	70
Aconitum pendulum		
5′-SH-GTTGACTGCGTTGATCGAGG-3′	immobilization	55
5′-Texas Red-CCTCGATCAACGCAGTCAAC-3′	reverse primer	55
Alocasia macrorrhiza		
5′-SH-TGCGCAATTTTTTTTGAAAAG-3′	immobilization	30
5′-TGCGCAATTTTTTTTGAAAAG-3′	forward primer	30
5′-Texas Red-GGACAACTTACTCCGTTTGC-3′	reverse primer	50
Croton tiglium		
5′-SH-GACCTGCGCGCTCGGCGCGG-3′	immobilization	85
5′-GACCTGCGCGCTCGGCGCGG -3′	forward primer	85
5′-Texas Red-GGATCCTTAGTGCTGGTATGATCGCA-3′	reverse primer	50
Datura inoxia		
5′-SH-AGCTAGCTGGGTCTTCGTGTTGCATCCCGCT-3′	immobilization	58
5′-AGCTAGCTGGGTCTTCGTGTTGCATCCCGCT-3′	forward primer	58
5′-Texas Red-TAACGACACCCCGCCCCGAA-3′	reverse primer	65
Datura metel		
5′-SH-GGGGCCCCGGGTCCTCGTGTTGCATCCCGCT-3′	immobilization	74
5′-GGGGCCCCGGGTCCTCGTGTTGCATCCCGCT-3′	forward	74
5′-Texas Red-TAACGACACCCCGCCCCGAA-3′	reverse	65
Datura tatula		
5′-SH-GCCGGAACTCCGCTTAATTATG-3′	immobilization	50
5′-GCCGGAACTCCGCTTAATTATG-3′	forward primer	50
5′-Texas Red-TAACGACACCCCGCCCCGAA-3′	reverse primer	65
Dysosma pleiantha		
5′-SH-CTTACAGGTGTGGTCGGCTC-3′	immobilization	60
5′-Texas Red GAGCCGACCACACCTGTAAG-3′	reverse primer	60
Dysosma versipellis		
5′-SH-GGTAATACGGTGGGGTGCAA-3′	immobilization	55
5′-Texas Red TTGCACCCCACCGTATTACC-3′	reverse primer	55
Euphorbia kansui		
5′-GTCATCGTATTAAGGCCTCG-3′	immobilization	50
5′-GTCATCGTATTAAGGCCTCG-3′	forward primer	50
5′-Texas Red-GGATCCTTAGTGCTGGTATGATCGCA-3′	reverse primer	50
Hyoscyamus niger		
5′-SH-GCTATCGCTCGGCGAATCTC-3′	immobilization	53
5′-GCTATCGCTCGGCGAATCTC-3′	forward primer	53
5′-Texas Red-GGATCCTTAGTGCTGGTATGATCGCA-3′	reverse primer	50
Pinellia cordata		
5′-SH-TTTTTGCAGCGCACCAC-3′	immobilization	50
5′-TTTTTTGCAGCGCACCAC-3′	forward primer	50
5′-Texas Red-CTGAGAGGTGTCGAAAAG-3′	reverse primer	50
Pinellia pedatisecta		
5′-SH-TTGCCGCATGTCCCATTTTT-3′0	immobilization	45
5′-TTGCCGCATGTCCCATTTTT-3′	forward primer	45
5′-Texas Red-CCGAGTCTGCTTTCC-3′	reverse primer	60
Pinellia ternata		
5′-SH-ACAGTGCACCATCGTC-3′	immobilization	56
5′-ACAGTGCACCATCGTC-3′	forward primer	56
5′-Texas Red-CTGAGAGGTGTCGAAAAG-3′	reverse primer	50
Rhododendron molle		
5′-SH-CGACTCTCGGCAACGGATAT-3′	immobilization	55
5′-Texas Red-ATATCCGTTGCCGAGAGTCG-3′	reverse primer	55
Stellera chamaejasme		
5′-SH-GCCGACCCAAACCCGTATTT-3′	immobilization	55
5′-Texas Red-AAATACGGGTTTGGGTCGGC-3′	reverse primer	55

Downloaded by: Hong Kong University of Science & Technology. Copyrighted material.

Table **1** Cont.

Sequence	*Primer*	*% GC*
Strychnos nux-vomica		
5′-SH-GCTATCGCTCGGCGAATCTC-3′	Immobilization	60
5′-GCTATCGCTCGGCGAATCTC-3′	forward primer	60
5′-Texas Red-GGATCCTTAGTGCTGGTATGATCGCA-3′	reverse primer	50
Typhonium divaricatum		
5′-SH-ACACCCCGTGGACACACGCC-3′	immobilization	70
5′-ACACCCCGTGGACACACGCC-3′	forward primer	70
5′-Texas Red-GGATCCTTAGTGCTGGTATGATCGCA-3′	reverse primer	50
Typhonium giganteum		
5′-SH-ATCCCATCACCTGCATGGAC-3′	immobilization	55
5′-ATCCCATCACCTGCATGGAC-3′	forward primer	55
5′-Texas Red-CTACGTCGACGCAGGTCTTC-3′	reverse primer	60

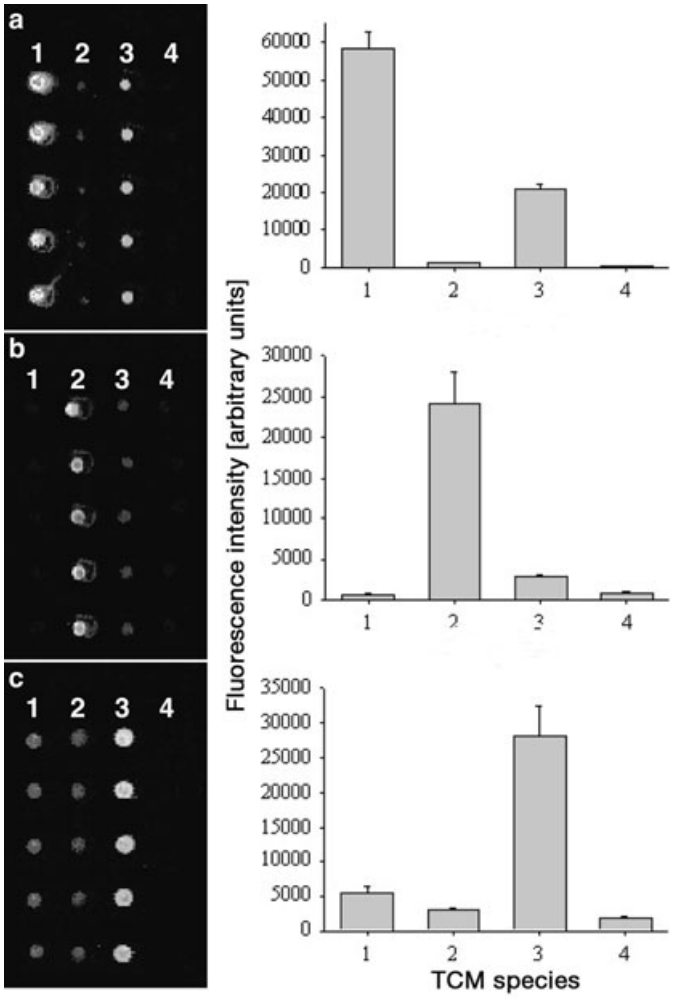

Fig. **1** Authentication of *Datura* species with a DNA microarray. The left panel shows fluorescent micrographs of microarrays containing probes for 1) *Datura metel*, 2) *Datura tatula*, 3) *Datura inoxia*, 4) *Rhododendron molle*. DNA from *Datura metel* (**a**), *Datura tatula* (**b**) or *Datura inoxia* (**c**) was hybridized to the microarrays. The right panel shows the histograms of the measured fluorescent intensities of the corresponding microarray in the left panel.

and 5S-rRNA-reverse: 5′ GGA TCC TTA GTG CTG GTA TGA TCG CA 3′. PCR products were purified, sub-cloned and electrophoresed into *E. coli*. Plasmid DNA from positive clones was subjected to DNA sequencing. The DNA sequences were deposited in GenBank with accession numbers: AB020374, AY334493, D8449, AY334501, AY334496, AY334494), AY334495, L75878, AF328968, AY334497, AY334499, AY334500, AY334498, AF072489, AJ308657, AY334503, and AY334502.

The species-specific 5S-rRNA sequences of the toxic TCM species were amplified and labeled by asymmetric PCR [19]. Microarrays were fabricated, hybridized and analyzed as described previously [19], [20].

Acknowledgements

This work was supported by grants from the Hong Kong Jockey Club and the Innovation and Technology Commission (AF/150/99) and the Areas of Excellence Scheme under the University Grants Committee (AoE/B-15/01) of the Hong Kong Special Administrative Region, China.

References

[1] Yin H, Shuai X. Fundamentals of Traditional Chinese Medicine. Foreign Language Press, Beijing: 1995
[2] Guo XH, La WY, Zhang SF, Wu AH, Wang ZW. A dictionary of poisonous Chinese herbal medicines; Vol 1 Tianjin ke ji fan yi chu ban she: Tianjin: 1992
[3] Pharmacopoeia Commission of the Ministry of Public Health. Pharmacopoeia of the People's Republic of China (English Edition); Vol 1 Chemical Industry Press, Beijing: 1997
[4] The Hong Kong Special Administrative Region. (1999) Ordinance No. 47. Hong Kong: 1999
[5] Ko RJ. A U.S. perspective on the adverse reactions from traditional Chinese medicines. J Chin Med Assoc 2004; 67: 109 – 16
[6] But PP. Herbal poisoning caused by adulterants or erroneous substitutes. J Trop Med Hyg 1994; 97: 371 – 4
[7] Chan TY, Critchley JA. Usage and adverse effects of Chinese herbal medicines. Hum Exp Toxicol 1996; 15: 5 – 12
[8] Calixto JB. Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). Braz J Med Biol Res 2000; 33: 179 – 89
[9] Boullata JI, Nace AM. Safety issues with herbal medicine. Pharmacotherapy 2000; 20: 257 – 69

Downloaded by: Hong Kong University of Science & Technology. Copyrighted material.

[10] Shaw P, Wang I, Butt P. Authentication of Chinese Medicinal Materials by DNA Technology. World Scientific Publishing Co, Singapore: 2002
[11] Hon CC, Chow YC, Zeng FY, Leung FC. Genetic authentication of ginseng and other traditional Chinese medicine. Acta Pharmacol Sin 2003; 24: 841 – 6
[12] Cai ZH, Li P, Dong TTX, Tsim KWK. Molecular diversity of 5S-rRNA spacer domain in *Fritillaria* species revealed by PCR analysis. Planta Med 1999; 65: 360 – 4
[13] Cui XM, Lo CK, Yip KL, Dong TT, Tsim KW. Authentication of *Panax notoginseng* by 5S-rRNA spacer domain and random amplified polymorphic DNA (RAPD) analysis. Planta Med 2003; 69: 584 – 6
[14] Ma XQ, Duan JA, Zhu DY, Dong TT, Tsim KW. Species identification of Radix Astragali (Huangqi) by DNA sequence of its 5S-rRNA spacer domain. Phytochemistry 2000; 54: 363 – 8
[15] Ma XQ, Zhu DY, Li SP, Dong TT, Tsim KW. Authentic identification of stigma Croci (stigma of *Crocus sativus*) from its adulterants by molecular genetic analysis. Planta Med 2001; 67: 183 – 6
[16] Wang Y, Zhou K, Xu L, Dong TT. Tsim KW. Authentication of an animal crude drug, *Zaocys*, by diagnostic PCR. Biol Pharm Bull 2000; 23: 585 – 8
[17] Zhao KJ, Dong TT, Tu PF, Song ZH, Lo CK, Tsim KW. Molecular genetic and chemical assessment of radix Angelica (Danggui) in China. J Agric Food Chem 2003; 51: 2576 – 83
[18] Carles M, Lee T, Moganti S, Lenigk R, Tsim KWK, Ip NY et al. Chips and Qi: microcomponent-based analysis in traditional Chinese medicine. Fresenius J Anal Chem 2001; 371: 190 – 4
[19] Trau D, Lee TMH, Lao AIK, Lenigk R, Hsing IM, Ip NY et al. Genotyping on a complementary metal oxide semiconductor silicon polymerase chain reaction chip with integrated DNA microarray. Anal Chem 2002; 74: 3168 – 73
[20] Lenigk R, Carles M, Ip NY, Sucher NJ. Surface characterization of a silicon-chip based DNA microarray. Langmuir 2001; 17: 2497 – 500

Downloaded by: Hong Kong University of Science & Technology. Copyrighted material.