Identification and Quantification of the Traditional Chinese Medicinal Plant *Gentiana macrophylla* using Taqman Real-Time PCR

Author
Chun-Ying Xue[1], **De-Zhu Li**[1], **Qing-Zhong Wang**[2]

Affiliation
[1] Laboratory of Plant Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, P. R. China
[2] School of Chemical Science and Technology, Yunnan University, Kunming, Yunnan, P. R. China

Key words
- DNA
- *Gentiana macrophylla*
- Gentianaceae
- real-time PCR
- species identification

Abstract

Gentiana macrophylla Pall. is a commonly used antirheumatic herb. There are four species of *Gentiana* recorded as herbal drugs in the Chinese Pharmacopoeia. The other species are often marketed as *G. macrophylla*, and thus the therapeutic effects of *G. macrophylla* are not achieved. A novel one-step methodology based on real-time polymerase chain reaction (PCR) technology has been developed for the identification of *G. macrophylla*. This relative quantification methodology does not require a known amount of standard, allowing the analysis of many more samples together. The utilization of real-time PCR does not require sample handling, preventing contamination and resulting in much faster and higher throughput results.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

received January 12, 2008
revised August 11, 2008
accepted August 28, 2008

Bibliography
DOI 10.1055/s-0028-1088329
Planta Med 2008; 74: 1842–1845

Published online November 17, 2008
ISSN 0032-0943

Correspondence
Dr. Chun-Ying Xue
Kunming Institute of Botany
Chinese Academy of Sciences
Kunming 650204
Yunnan
People's Republic of China
Tel.: +86-871-522-3508
Fax: +86-871-521-7791
chyxue@mail.kib.ac.cn

Dr. Qing-Zhong Wang
School of Chemical Science and Technology
Yunnan University
Kunming
Yunnan 610091
People's Republic of China
Tel.: +86-871-503-3722
Fax: +86-871-503-2180
qzhwang@ynu.edu.cn

Introduction

The plant *Gentiana macrophylla* Pall. (Gentianaceae), commonly known as "qin-jiu", is distributed mainly in China and Siberia [1]. The root of *G. macrophylla* is an antirheumatic herb widely used in China. However, the herb "qin-jiu" has multiple sources; it is mainly derived from *G. macrophylla*, but is also obtained from *G. crassicaulis*, *G. dahurica* and *G. straminea* [2]. Data show that *G. macrophylla* has a stronger antirheumatic effect compared to the other species of "qin-jiu" due to its higher alkaloid concentration [3]. Moreover, safety and reliability of herbal medications is an important issue [4]. It has been shown that *G. macrophylla* contains the least toxic alkaloids from all other *Gentiana* species [3].

When the external morphological characteristics of material medicines are removed due to powdering or processing such as shredding, the only possibility to authenticate the herbal medicines is by means of using a molecular marker. In fact, the use of DNA as a molecular marker has turned out to be the most powerful tool for species identification [4]. According to DNA analysis of herbal medicines, many studies have described different techniques based on restriction site polymorphic fragments (RFLP) [5], random amplified polymorphism DNA (RAPD) [6], and others [7], [8], [9] with the aim of identifying medicinal materials. However, these techniques are not able to detect reliably a specific species in a mixture, which is widespread in Chinese medicine. Real-time polymerase chain reaction technology is based on the detection and quantification by a high-quality optical detection instrument of a fluorescence reporter included within a specific fluorogenic probe [10].

The main aim of this study was to achieve a relative quantification procedure to identify and quantify *G. macrophylla.*

Materials and Methods

Sources of samples

In addition to *Gentiana macrophylla*, *G. crassicaulis*, *G. dahurica.* and *G. straminea*, the other four Gentianaceae species were used as reference materials for the validation of the method. They are *Halenia elliptica*, *Swertia franchetiana*, *S. erythrostica* and *S. tetraptera*, which are usually being sold as "qin-jiu" on the market. *G. macrophylla* was the detected target species and the other seven species were reference species. **Table 1** lists all

Table 1 List of plant materials.

Taxon	Provenance	Voucher	Accession Numbers	Herbarium
Gentiana crassicaulis	Zhongdian, Yunnan, China	XCY2002002	DQ398636	KUN
G. dahurica	Menyuan, Qinghai, China	XCY0031	DQ398632	KUN
G. macrophylla	Qingling, Shanxi, China	Zhou L H 2 872	DQ398652	HNWP
G. straminea	Haibei, Qinghai, China	XCY2001008	DQ317491	KUN
Halenia elliptica	Ping'an, Qinghai, China	XCY1998002	AF346012	HNWP
Swertia erythrostica	Ping'an, Qinghai, China	XCY2001003	AF251122	HNWP
S. franchetiana	Xining, Qinghai, China	XCY2001004	AF255916	KUN
S. tetraptera	Ping'an, Qinghai,China	XCY2001005	AF346013	KUN

Table 2 Primers and TaqMan probes.

Name		Sequence (5′-3′)
G. macrophylla	Forward primer: F-QJ (ITS3)	5′-GCATCGATGAAGAACGCAGC-3′
	Reverse primer: R-QJ (ITS4)	5′-TCCTCCGCTTATTGATATGC-3′
	TaqMan probe: QJ	5′-(FAM)CTGTCGCGCGTTTCCCCGTCGGAT-3′
5.8S	Forward primer: F-5.8S	5′-GGCAACGGATATCTCGGCTCTC-3′
	Reverse primer: R-5.8S	5′-GCGTGACGCCCAGGCAGACGT-3′
	TaqMan probe: 5.8S	5′-(FAM)CATCGATGAAGAACGTAGCGAA-3′

taxa included in this study, together with voucher information, collection sites and GenBank accession numbers for the ITS sequences. Plant materials were identified by the authors. Voucher specimens are housed in the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN), and the Northwest Plateau Institute of Biology, Chinese Academy of Sciences (HNWP). To prepare the processed samples, medicinal herbs were collected from the fields and processed by grinding, slicing and dry-frying. Five individuals were investigated from each species.

DNA extraction, TaqMan probes and primer design

DNA was extracted from fresh tissue or processed samplers (shredded pieces) according to a modified CTAB procedure [11]. All primers and fluorogenic probes were designed using the Primerselect software (DNAstar) following the information provided. Primers and TaqMan probes were chosen to be consistent with the alignment of nrDNA ITS sequences collected from GenBank. The sequences of primers and probes are listed in **Table 2**. The probes were labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) on the 5′-end. The fluorescent quencher dye, 6-carboxytetramethylrhodamine (TAMRA), was located on the 3′-end of the probes. All of the primers and probes were purchased from Applied Biosystems. Multiple alignments were carried out using the Megalign program (DNAstar).

Real-time PCR conditions

Amplification was performed using a total reaction volume of 25 μL in a MicroAmp Optical 96-well reaction plate (Applied Biosystems). Real-time reactions were carried out with TaqMan Universal Master Mix (Applied Biosystems) containing the primers and probes described in **Table 2**. Reactions were run on the ABI Prism 7000 sequence detection system (Applied Biosystems) with the following thermal conditions: 50 °C for 2 min, 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR efficiency was calculated, from each linear regression of standard curves, using the equation $[10^{1/\text{slope}} - 1] \times 100$.

Relative quantification method

This method uses an unknown amount of standard, but it compares the relative amount of the target sequence to any of the reference values. The target and endogenous control amplifications were carried out in separate tubes in triplicate. Finally, for each reaction, a C_t variation ($\Delta C_t = C_t^{\text{target}} - C_t^{\text{ref}}$) for the target was calculated from the C_t values. At the end, ΔC_t value was transformed to a percentage ($\%\text{target} = 2^{-\Delta Ct} \times 100$).

Statistical analysis

Linearity tests were developed three times with different individuals. Each C_t value was obtained by the means of three replicates with a standard deviation of < 0.2.

Supporting information

Comparison of primers and probes of the 5.8S detection TaqMan system with different Gentianaceae species, comparison of probes of the *G. macrophylla* detection TaqMan system with different Gentianaceae species, pairwise distances of total character differences between taxa based on 5.8S and ITS2 and selectivity of the TaqMan systems (C_t-values by 200 ng DNA) are available as Supporting Information.

Results and Discussion

As target genes for both real time PCR detection systems (for *G. macrophylla* and for references), the nuclear ribosomal DNA (nrDNA) genes were selected. The nrDNA units, separated by intergenic spacers, consist of the 18S, 5.8S and 26S coding regions in plants. The internal transcribed spacers 1 and 2 (ITS1 and ITS2) are located between the 18S and 5.8S, 5.8S and 26S coding regions, respectively [12]. Along with nrDNA genes, the ITS region is quite variable, which helps to distinguish phylogenetic relationships among species, and the 5.8S gene region is highly conserved among the angiosperm, which helps in alignment [11]. To establish a *G. macrophylla*-specific system gene, a region unique for *G. macrophylla* was of highest interest; for establishing a general herbs system, highly conserved gene regions were ideal. As a target site for the specific detection of *G. macrophylla*

a 230 bp fragment of ITS2 of nrDNA was selected. The ITS2 sequence is highly variable amongst the Gentianaceae species and highly unique for *G. macrophylla* showing that it is suitable for a specific detection system (**Table 2S**, Supporting Information). For general herbs detection system, the conserved nrDNA gene 5.8S sequence was investigated. The 5.8S sequences of sample species were aligned to design a general herbs detection system. As a consequence of this alignment, a highly conserved region of 164 base pairs was found and two conserved primers and a specific probe were designed (**Table 2S**, **Fig. 1S**, Supporting information). The calculated homologies of the chosen sequences were 100% except for *Halenia* and *Swertia* genera at 97.6%, 98.8%, respectively.

To obtain a specific and reliable TaqMan methodology, a polymorphic site was placed for *G macrophylla* in the middle of the probe. The primer that flanks the target was located close to the probe by means of the Primer Express program. In this way, we have developed a specific detection system to detect *G. macrophylla* (QJ system).

Both detection systems were tested for their selectivity and cross-reactions with those Gentianaceae species listed in **Table 1**. The general herbs detection system (5.8S system) is applicable to a broad spectrum of every species listed in this work, whereby the detected C_t values were between 35 and 40 cycles. The specific *G. macrophylla* detection system (QJ system) has no cross-reactivity with other related species, although a slight signal was revealed by some individuals from *Gentiana* genus species. This fact was negligible compared to the strong signals measured for *G. macrophylla* individuals.

We concluded that the systems are specific to their targets. Moreover, in agreement with the character of 5.8S gene described above and some preliminary tests with other seed plants (data not shown), the 5.8S system could be used as reference gene to carry out relative quantification methods with other seed plants species.

The relative quantification method does not use a known amount of standard but compares the relative amount of the target sequence to any of the reference values. Thus, we have designed a relative quantification method (QJ method) to detect and quantify *G. macrophylla* by comparing the relative amount of the target sequence (QJ system) with a reference (5.8S system). At the end of the reaction, we obtained two C_t values for this method: one for the target and another one for the reference. After the relative quantification equations, described under Materials and Methods, had been applied, a C_t variation that could be transformed to a percentage was obtained. To apply correctly these equations, it was necessary to validate the detection methods through testing of the linearity and efficiency of two TaqMan systems involved in the methodology.

To test the linearity, the resulting C_t values were plotted versus the logarithm of the DNA concentration. **Fig. 1** and **Fig. 2** show the linearity test with intact DNA obtained from fresh samples and degraded DNA obtained from processed samples, respectively. The sensitivity range of the system was determined by using 10-fold dilutions of DNA template, and the linearity was maintained from 200 to 0.020 ng of template (4 orders of magnitude). No significant variation in the detection limit between processed samples and fresh samples was detected.

A PCR efficiency of 100% is ideally achieved when the slopes are close to the theoretical value of –3.32. According to the slopes shown in **Fig. 1** and **Fig. 2**, an efficiency of nearly 100% was estimated for the QJ detection method. In fact, according to the equation $[10^{1/\text{slope}} - 1] \times 100$, the calculated efficiency ranged from 90 to 100%.

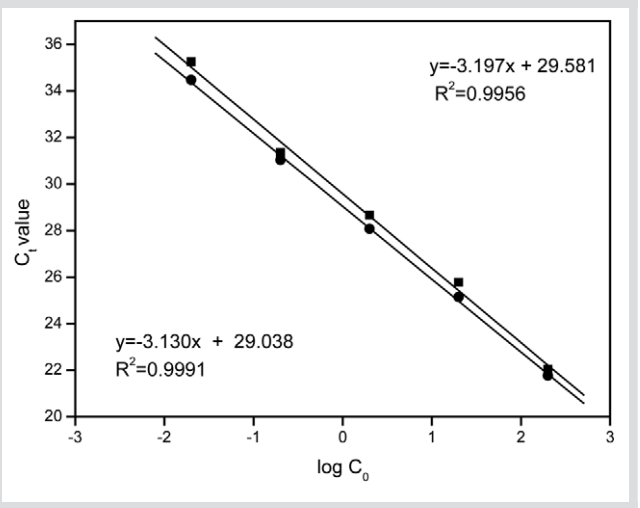

Fig. 1 Linearity test with intact DNA as template: QJ (■) and 5.8S (●) specific TaqMan systems belong to the *G. macrophylla* specific quantification method using DNA from *G. macrophylla*. C_t values are plotted versus the logarithm of the DNA concentration. Voucher specimens are housed in KUN and HNWP.

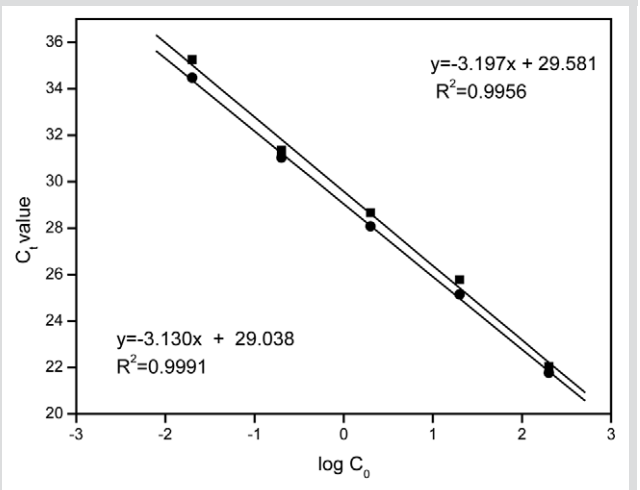

Fig. 2 Linearity test with degraded DNA as template: QJ (■) and 5.8S (●) specific TaqMan systems belong to the *G. macrophylla* specific quantification method using DNA from *G. macrophylla*. C_t values are plotted versus the logarithm of the DNA concentration. Voucher specimens are housed in KUN and HNWP.

Real-time PCR does not require post-PCR sample handling, thus preventing contamination and resulting in much faster and higher throughput assays. Although costly apparatus and equipment are required, the methodology described here is a one-step protocol which is carried out during the amplification reaction. The relative quantification does not require precise quantification of DNA due to the absence of standard curves, allowing more samples to be analyzed per plate, saving costs.

This method allows the detection and quantification of *G. macrophylla* in mixtures. In addition, it is also suitable to detect the presence or absence of this species in herbal medicines labeled as *G. macrophylla*.

Acknowledgements

This research was supported by the National Basic Research Program of China (973 Program, 2007CB411601 to DZ Li), the Natural Science Foundation of China (NSFC 30770153 to CY Xue) and the Natural Science Foundation of Yunnan (NSFN, 2006C 0050 M to CY Xue).

References

1 *Ho TN, Pringle JS.* Gentianaceae. In: Wu ZY, Raven PH, editors. Flora of China; Vol. 16 Beijing and Missouri Botanical Garden: Science Press; 1995: 1 – 139
2 The state Pharmacopoeia Commission of the PRC. Pharmacopoeia of the People's Republic of China. Beijing: Chemical Industry Press; 2005: 190 – 1
3 *Tan R, Wolfender JL, Zhang L, Ma W, Fuzzati N, Marston A et al.* Acyl secoiridoids and antifungal constituents from *Gentiana macrophylla.* Phytochemistry 1996; 42: 1305 – 13
4 *Zhao ZZ, Hu Y, Liang ZT, Yuen JPS, Jiang ZH, Leung KSY.* Authentication is fundamental for standardization of Chinese Medicines. Planta Med 2006; 72: 856 – 74
5 *Ngan F, Shaw PC, But PPH, Wang J.* Molecular authentication of *Panax* species. Phytochemistry 1999; 50: 787 – 91
6 *Lim W, Mudge KW, Weston LA.* Utilization of RAPD markers to assess genetic diversity of wild populations of North American ginseng (*Panax quinquefolium*). Planta Med 2007; 73: 71 – 6
7 *Lau DTW, Shaw PC, Wang J, But PPH.* Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of ribosomal DNA. Planta Med 2001; 67: 456 – 60
8 *Liu ZhQ, Wang YQ, Zhou KY, Han DM, Yang XG, Liu XH.* Authentication of Chinese crude drug, Gecko, by allele-specific diagnostic PCR. Planta Med 2001; 67: 385 – 7
9 *Xue HG, Zhou SD, He XJ, Yu Y.* Molecular Authentication of the Traditional Chinese Medicinal Plant *Euphorbia pekinensis*. Planta Med 2007; 73: 1 – 3
10 *Holland PM, Abramson RD, Watson R, Gelfand DH.* Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc Natl Acad Sci USA 1991; 88: 7276 – 80
11 *Doyle JJ, Doyle JL.* A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 1987; 19: 11 – 5
12 *Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ.* The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann Missouri Bot Gard 1995; 82: 247 – 77