Differentiation of the Traditional Chinese Medicinal Plants *Euphorbia humifusa* and *E. maculata* from Adulterants by TaqMan Real-Time Polymerase Chain Reaction

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Abstract

DNA sequence analysis of the rDNA internal transcribed spacer 1 (ITS1) and TaqMan real-time polymerase chain reaction were exploited for their applications in the differentiation of the traditional Chinese medicinal plants *Euphorbia humifusa* and *E. maculata* from three related adulterants *E. hypericifolia*, *E. atoto* and *E. prostrata*. The data demonstrated that variations in the ITS1 regions were very low at the intra-species level but extremely high at the inter-species level, so that they could be easily distinguished at the DNA level. The sequence difference allowed an effective and reliable differentiation of *E. humifusa* and *E. maculata* from the adulterants by TaqMan real-time PCR.

Key words

Traditional Chinese medicinal plants · *Euphorbia humifusa* · *Euphorbia maculata* · Euphorbiaceae · TaqMan real-time polymerase chain reaction

Abbreviations

ITS: internal transcribed spacer

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

"Dijincao" (Herba Euphorbiae Humifusae) is an important traditional Chinese medicine that has commonly been used for treating dysentery and colitis in many Asian countries for thousands of years [1]. According to the Chinese Pharmacopoeia (2005 edn.), only *E. humifusa* and *E. maculata* are listed as the origin of "Dijincao". However, there are at least 3 adulterants, such as *E. hypericifolia*, *E. atoto* and *E. prostrata*, bearing the name "Dijincao" on the market. This situation might cause inconsistent therapeutic effects, or even jeopardize the safety of consumers. Obviously, the accurate identification of these medicinal materials is a prerequisite for the quality control of traditional Chinese medicines. An effective method to distinguish *E. humifusa* and *E. maculata* from their adulterants is important to the healthy development of the herbal industry. Micromorphological characteristics of the lower epidermis and the non-glandular hair of seeds in these *Euphorbia* species have been examined and used in the identification of the source plants [2]. Unfortunately, those methods are always problematic because they are rather time-consuming and only few people possess the experience to exercise them. A simple, sensitive, and species-specific method for identification of "Dijincao" is therefore urgently needed.

Recent advances in DNA technology have led to the development of real-time polymerase chain reactions, at the forefront of which is the TaqMan technology [3] (the principles of TaqMan technology are detailed in the Supporting Information). The TaqMan process allows gel-free product detection during PCR using fluorescent probes. In this study, we have developed a species-specific TaqMan real-time PCR probes procedure to distinguish *E. humifusa* and *E. maculata* from the adulterants based on the sequences of the nuclear ribosomal DNA (nrDNA) internal transcribed spacer 1 (ITS1) region.

The ITS1 region from 31 dried leaf samples of 5 *Euphorbia* species was amplified and sequenced (**Fig. 2S** Supporting Information). The sequence data showed that variations in the ITS1 regions were very low at the intra-specific level but extremely high at the inter-specific level (The variations at the intra-specific and inter-specific level of five *Euphorbia* species are listed in detail in the Supporting Information), so that they could be easily distinguished at the DNA level. Based on the multiple sequence alignment of the ITS1 sequences, primers and probes for the TaqMan system were designed and synthesized for the authentication of *E. humifusa* and *E. maculata*, and for distinguishing them from adulterants (**Table 1**).

We tested several commercially prepared crude drugs purchased in the market. The *E. humifusa* species-specific fluorescently labeled TaqMan probe failed to detect *E. maculata* and adulterants. Because there were several base pair mismatches within *E. maculata* and the adulterants amplicons, the probe formed unstable hybrids that dissociated during the annealing stage, so they could not be cleaved during the cycle, as a result fluorescence could not be recorded (**Fig. 1A**). Furthermore, even when amplification products were obtained from herbs other than *E. maculata*, they were not detected by the *E. maculata*-specific TaqMan probe (**Fig. 1B**). These results demonstrated that our method could authenticate *E. humifusa* and *E. maculata*. Alternatively, for *E. humifusa* and *E. maculata* products which are in the form of powder or shredded material, the additional probes which hybridize with the three adulterant species (**Table 1**) might be needed to determine if they are mixed with adulterants (see analysis of a mixture of *E. humifusa*, *E. maculata* and adulterants, Supporting Information). Species-specific TaqMan real-time PCR did not require post-PCR sample handling, thus preventing contamination and resulting in much faster and higher throughput assays. Moreover, recently real-time PCR had also been used for the semiquantification of beef in food [4]. Future research should be focused on testing this method with the possibility of quantifying plant species within mixtures of herbal medicines.

Materials and Methods

Experienced botanists collected plant specimens from Yunnan, Sichuan and Guangdong provinces (**Table 1S** Supporting Information). All specimens were deposited in the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN), Kunming, P. R. China. Plant DNA was extracted essentially

Sequence	Primer	%GC
Euphorbia humifusa		
5′-TGGCCCTTCACAATGCTTTTA-3′	forward primer	43
5′-GGGGAGGCCGGTCTTTTCA-3′	reverse primer	63
5′-(FAM)-CGGCGTGCCTCGGTAGC-3′	TaqMan probe	76
E. maculata		
5′-CGAGGGGTCAAGAGCTGGTTATGC-3′	forward primer	58
5′-CGCGCCGGGGGTTTGTTAGA-3′	reverse primer	65
5′-(FAM)-CACAAGGCCTCGATCGGGGTTTTA-3′	TaqMan probe	54
E. hypericifolia		
5′-ACAGCCTCGATCGGGGTATTATGG-3′	forward primer	54
5′-CGAGGCGACCGAGCTTTTTATTTA-3′	reverse primer	46
5′-(FAM)-GCCAGGCCTGCACACCATAATAC-3′	TaqMan probe	57
E. atoto		
5′-ATGGCAGCCTCGGTCCTTCATAG-3′	forward primer	57
5′-CGTTTCCAGGGCAATCGAGATAGC-3′	reverse primer	54
5′-(FAM)-GCCCCGATCGAGGCCCTATGA-3′	TaqMan probe	67
E. prostrata		
5′-AACGAGGGGTCCGAAGCTATTGTG-3′	forward primer	54
5′-AAGGCACCTTGGGAGAAAG-3′	reverse primer	53
5′-(FAM)-CCCCGATCGAGGCCCTGTGA-3′	TaqMan probe	70

Table 1 Sequences of the TaqMan probes and forward, reverse primers of 5 *Euphorbia* species

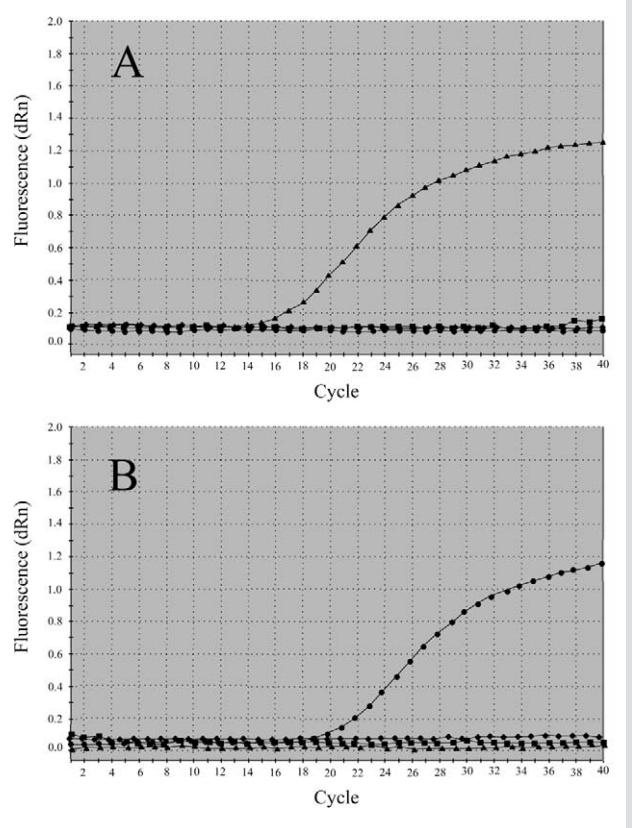

Fig. 1 Real-time PCR assay. **A** Even when amplification products were obtained from herbs other than *Euphorbia humifusa*, they were not detected by the *E. humifusa*-specific TaqMan probe. **B** Even when amplification products were obtained from herbs other than *E. maculata*, they were not detected by the *E. maculata*-specific TaqMan probe. ■, no template control; ▲, *E. humifusa*; ●, *E. maculata*; ◆, adulterant.

as described in [5]. Plant rDNA was amplified using a pair of primers ITS5 and ITS2 [6]. The PCR reaction was conducted essentially as described in [7]. Sequences were determined by using the PCR products with the two primers (ITS5, ITS2). Automated DNA sequencing was run on an ABI 3700 Sequencer (Applied Biosystems, Inc.) by United Gene Holdings, Ltd.

All primers and fluorogenic probes were designed and synthesized by Sangon Co. (**Table 1**). The probes were labeled at the 5′ end with the fluorescent reporter dye 6-carboxyfluorescein (FAM) and the non-fluorescent quencher at 3′end. 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 1**. 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 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

The sequences were aligned and compared using the Clustal 1.81 program and analyzed using the MEGA version 3.1 program.

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