Application of LightCycler Polymerase Chain Reaction and Melting Curve Analysis to the Authentication of the Traditional Chinese Medicinal Plant Cimicifuga foetida

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Abstract
DNA sequence analysis of rDNA internal transcribed spacer (ITS) and fluorescence melting curve analysis of LightCycler real-time polymerase chain reaction products were exploited for their applications in the authentication of the traditional Chinese medicinal plant Cimicifuga foetida from four substitutes: C. heracleefolia, C. dahuurica, C. acerina, and C. simplex. According to the melting temperature - which is a function of the GC/AT ratio, length, and nucleotide sequences of the amplified product - C. foetida was differentiated from C. heracleefolia, C. dahuurica, C. acerina, and C. simplex. Melting curve analysis offers a rapid and reliable method for the authentication of the traditional Chinese medicinal plant C. foetida.

Keywords
traditional Chinese medicinal plant, Cimicifuga foetida, Ranunculaceae, LightCycler, real-time polymerase chain reaction, fluorescence melting curve analysis

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

Fig. 1 Real-time LightCycler PCR assay on five Cimicifuga species using the primers ITS5S and ITS4 to amplify a DNA fragment from the ITS region. O = no template control; □ = C. foetida; ● = C. heracleefolia; △ = C. dahuurica; ○ = C. simplex; × = C. acerina.
237, 432, 441, 497, and 505 (Fig. 15, Supporting Information). As expected, those fragments of the ITS region among Cimicifuga species have different lengths, nucleotide sequences, and GC/AT ratios and thus may have different melting temperatures (Fig. 1). In order to assess the reproducibility of this method for the authentication of C. foetida, a total of 10 independent experiments were conducted over a period of 5 months. The mean melting temperatures were 79.54°C [standard deviation (SD) = 1.57°C; n = 10] for C. foetida, 82.48°C (SD = 1.82°C; n = 10) for C. heraclefolia, 85.05°C (SD = 1.91°C; n = 10) for C. dahurica, 91.43°C (SD = 1.65°C; n = 10) for C. simplex, and 91.89°C (SD = 1.80°C; n = 10) for C. acerina (Fig. 1). The difference in melting temperatures between C. foetida and substitutes was statistically highly significant according to the t-test (p < 0.001).

We tested four commercially prepared crude drugs purchased in the market. Three samples were recognized as C. foetida, and one sample was not C. foetida (Fig. 2). These results demonstrate that melting curve analysis by the method presented in this paper could be reliably used to identify C. foetida. Alternatively, for C. foetida products that were in the form of powder or shredded slices, the melting curve analysis assay was capable of detecting whether they were a mixture of C. foetida and substitutes (see Supporting Information). Although the SYBR Green I dye binds to double-stranded DNA, melting curve analysis allows one to differentiate non-specific products of amplification, as all non-specific products, such as primer dimers that can be produced when little or no template is present, melted at temperatures below 75°C [13]. Compared with other methods, the LightCycler assay allows a more rapid differentiation of Cimicifuga species, as the assay can be performed within 3–6 h, including DNA extraction from botanic samples.

Materials and Methods

Experienced botanists collected plant specimens from China (Table 1, Supporting Information). All specimens were deposited in the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, P.R. China. Plant DNA was extracted essentially as described in [14]. Plant rDNA was amplified using the primer pair ITS5 and ITS4 [15]. The PCR reaction was conducted essentially as described in [16]. Sequences were determined by using the PCR products with the two primers (ITS5 and ITS4). Automated DNA sequencing was run on an ABI 3700 sequencer (Applied Biosystems, Inc.) by United Gene Holdings, Ltd.

Real-time PCR was performed by hot-start PCR using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) in a LightCycler. The 20-μL reaction mixture contained 1× LightCycler FastStart DNA Master SYBR Green I, 2 mM MgCl2, 10 μM of each primer, and 2 μL of template. The amplification program with ITS4 and ITS5 included an initial denaturation step at 95°C for 8 s and 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 8 s. The temperature transition rate was 20°C. After amplification, a melting curve was acquired by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 67°C, keeping it at 67°C for 30 s, and then slowly heating at 0.1°C/s to 95°C. The program used with primers ITS4 and ITS5 differed only by the duration of the extension step, which was 30 s instead of 8 s. Fluorescence was measured through the slow heating phase. For improved visualization of the melting temperatures (Tm), melting peaks were derived from the initial melting curves [fluorescence (F) versus temperature (T) by plotting the negative derivative of fluorescence over temperature-dF/dT versus dT]. Melting curves were used to determine the specific PCR products.

Supporting Information

The detection mixture of Cimicifuga foetida and adulterants; the aligned sequences of rDNA ITS from Cimicifuga heraclefolia, C. dahurica, C. foetida, C. simplex, and C. acerina; and the locality and sample size of plant samples and sequence data are available as Supporting Information.

Acknowledgments

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Bibliography
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Erratum

Biosynthesis of Salvonin A: Overexpression and Biochemical Characterization of Carboxy Methyltransferase from EST of Salvia divinorum Clands
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One of the authors' names is misspelt. The correct name is Laurenzi, A.