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Entire industrial chain botanical origin authenticity control of ginseng formula granule products using simple PCR-based identification

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ABSTRACT

Ginseng formula granule is derived from the root of *P. ginseng*, and is one of the most important Chinese industrial herbal products. Discriminating between *P. ginseng* and its closely-related substitutes, *P. quinquefolius* (American ginseng) and *P. notoginseng* (Notoginseng) in formula granule is a critical issue in the ginseng industrial chain. The industrial chain begins with the ginseng crop, which undergoes a series of herbal preparation procedures—processing, extraction, concentration, desiccation, and granulation. During these processes, morphological appearances and microscopic characteristics are destroyed, and phytochemical profiles vary greatly by extract manipulation, formulation process, and storage conditions. Therefore, a uniform authenticity control method is necessary for the critical stages in the entire industrial formula granule production process. In this study, a simple allele-specific identification method was established for uniform botanical origin authenticity control of ginseng drugs, extracts, and formula granule. *P. ginseng*-specific primers and non-*P. ginseng*–specific primers from high copy number 18S rDNA were employed for ginseng authenticity identification. They were used to generate 166 bp fragments from the original plant, crude drug, extract, and formula granule. The method was capable not only of distinguishing *P. ginseng* from related species, but also of detecting adulterants in ginseng products. This study applies a uniform DNA marker and techniques for quality control in the entire ginseng granule industrial chain.

1. Introduction

Chinese herbal medicines (CHMs) are derived from botanical, animal, or mineral sources. They play a vital role in healthcare in Asia countries, and their use is increasing in Western countries. According to China's National Bureau of Statistics, the global herbal industrial output of CHMs was estimated to be USD $107 billion in 2017, and with an annual growth rate of 37.9% from 2011 (Ghisleni et al., 2016; Liu et al., 2015). Botanical medicines are the most important CHMs in China. They account for more than 90% of all CHM types and exhibit significant biological activity in phytochemicals and pharmaceuticals (Abdel-Rahman et al., 2011).

Accurate identification of botanical medicine at the species level is extremely difficult for some materials, especially during downstream material processing in the industrial chain (Jiang et al., 2016a). To protect the vital interests of patients, guaranteeing the safety of CHMs is a fundamental need that requires distinguishing botanical medicines from their inferior substitutes, adulterants, or counterfeits. For instance, inaccurate identification of herbal materials has caused several serious safety issues that have attracted global attention. As highlighted in recent reports, misidentification of *Aristolochia* and related plants that contain aristolochic acids caused kidney failure, as well as cancers of the liver and urinary tract (Gold and Slone, 2003; Luciano and Perazella, 2015; Ng et al., 2017). Products that substituted American ginseng (*Panax quinquefolius*) for Korean ginseng (*P. ginseng*) have also been reported (Wallace et al., 2012). The nature of the pharmacological effects in those CHMs are different, because Korean ginseng is "warm" and usually used to treat a "yang deficient" conditions, whereas American ginseng is "cool" and is usually used to treat a "yin-deficient" conditions (Chan et al., 2000). Substituting one for the other can place a

Abbreviations: rDNA, ribosomal DNA; bp, base pair; CHM, Chinese herbal medicine; SNP, single nucleotide polymorphism; COXI, cytochrome c oxidase subunit I; ITS, internal transcribed spacer; CTAB, cetyltrimethylammonium bromide; mtDNA, mitochondrial DNA; cpDNA, chloroplast DNA

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Table 1
Herbal extracts and formula granules used in this study.

NO.	Samples	Origin	Voucher
1	Ginseng granules	CR	1507001S, 1408001S, 1509001S, 1507001S, 1504001S, 1401001W, 1501001S, 1509002S, 1706002S, 1601001W, 1701001S, 1704001S, 1702001S
2	Ginseng granules	BTC	1700631, 17013592
3	Ginseng granules	JTP	1706038, 1706083, 20171106001, 1701120, 2017110685, 2017110691
4	Ginseng granules	Yifang	7060701, 7032361
5	Ginseng granules	Neo	1604055
6	Ginseng granules	Spring	161017
7	American ginseng granules	CR	1602001S, 1606001S, 1405001S, 1702001S, 1509001W, 1703001S, 1704005S,
8	American ginseng granules	JTP	1611803, 1704806, 1508803
9	American ginseng granules	Yifang	6095031, 7071421, 6086892, 7026202, 5060721, 412383T
10	American ginseng granules	BTC	17003152, 16008833
11	American ginseng granules	Spring	160209
12	Notoginseng granules	CR	1504001W, 1502001S, 1504001S, 1307001S, 1504001S, 1406002S, 1706002S, 1708001W, 1501001S
13	Notoginseng granules	BTC	1619122
14	Notoginseng granules	JTP	1704117, 1707187, 1708093
15	Notoginseng granules	Yifang	7062881
16	Notoginseng granules	Spring	170711
17	Ginseng extraction	CR	170102C, 7170801C, 7171001C, 7170802C
18	American ginseng extracts	CR	161201C, 161202C, 160601C
19	Notoginseng extracts	CR	5171101C, 5171102C, 5171103C

CR: China Resources Co., Ltd. BTC: Beijing Tcmages Pharmaceutical Co. Ltd. JTP: Jiangyin Tianjiang Pharmaceutical Co., Ltd. Yifang: Guangdong Yifang Pharmaceutical Co., Ltd. Neo: Sichuan Neo-Green Pharmaceutical Technology Development Co., Ltd. Spring: Spring 9 Department of modern traditional Chinese Medicine Co., Ltd.

patient at risk. In addition, products with adulterants are often morphologically and chemically similar to the authentic product.

Derived from the root of *P. ginseng*, ginseng formula granule is one of the most important Chinese herbal industrial products. To convert botanical materials into formula granule, an industrial chain begins with the crude ginseng drug and undergoes a series of herbal preparation procedures—processing, heat reflux extraction, vacuum concentration, spray drying, and granulation. During these processes, the morphological appearance, microscopic characteristics, and phytochemical profiles vary greatly because of extract manipulation, formulation process, and storage conditions. In addition, morphological appearances and some microscopic characteristics are destroyed during extraction and granulation; thus, traditional identification methods fail at these stages. Consequently, it is hard to establish a uniform authenticity control method in the formula granule industrial process.

In recent years, DNA-based molecular approaches have become a popular species identification tool for their high specificity, robustness, and reliability from the original plant to the commercial products (Madesis et al., 2014; Mishra et al.,2016; Jiang et al., 2018). Several DNA-based methods have been applied to identify the authenticity of ginseng in different industrial stages, including the original plant, powder, decoction, extract, and numerous commercial ginseng preparations (Niu et al., 2011; Wang et al., 2011; Jung et al., 2014; Lo et al., 2015). Ginseng formula granule is the final product in the industrial chain. At this stage, identification with DNA-based molecular tools is relatively difficult—hot temperature extraction and drying procedures fragment the DNA, but species identification requires an excessively long amplification region (Lo and Shaw, 2018). Fortunately, in several recent studies, DNA fragments shorter than 200 bp were amplifiable from highly processed materials, including decoctions and formula granules, using a series of DNA extraction and purification procedures (Kumeta et al., 2014; Jiang et al., 2017b). Therefore, a PCR-based method could serve as a uniform botanical origin authenticity control for the entire ginseng formula granule industrial chain.

In this study, a rapid and reliable allele-specific PCR identification method was developed as a uniform tool for botanical origin authenticity control of ginseng drugs, extract, and formula granule in the entire industrial chain. *P. ginseng*-specific primers and non-*P. ginseng* ginseng–specific primers were employed to identify authentic species and adulterants in the same sample. The method was proven capable of distinguishing *P. ginseng* from related species and detecting adulterants in ginseng products.

2. Materials and methods

2.1. Samples

Information identifying plant samples and crude drug materials that were tested in this study are shown in Table S1 (273 sample batches). The crude drugs (authentic *P. ginseng* and the closely related species) were obtained from different herbal markets in China and were identified morphologically by experts according to methods described in Chinese Pharmacopoeia 2015. These were used as reference materials to establish molecular identification methods. Samples were stored at −20 °C until use to prevent degradation of the DNA. To remove potential contamination, all samples were washed with 75% alcohol and sterilized water prior to DNA extraction. Herbal extracts and formula granules were collected from six different granule companies (Table 1, Fig. 1). All tested samples were deposited in the National Resource Centre for Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China.

2.2. Genomic DNA extraction

Plant and crude drug materials were frozen in liquid nitrogen and ground to a fine powder with a MM 400 Mixer Mill (Retsch Technology GmbH, Haan, Germany). Genomic DNA was extracted from approximately 30 mg of drug powder using a DNeasy Plant Mini Kit (QIAGEN, CA, USA) according to the manufacturer's protocol.

In total, 50 mg of formula granule samples was dissolved in 1500 µL of DNA Binding Buffer (Thermo Scientific, Waltham, USA) by vortex and incubated at 56 °C for 20 min. Undissolved debris was removed by centrifugation at 5000 × *g* for 2 min. 1000 µl of supernatant was added twice to G2 Purification Columns (GeneJET Gel Extraction Kit, Thermo Scientific, Waltham, USA) and centrifuged at 12,000 × *g* for 1 min. Then the manufacturer's instructions were followed to obtain 50 µl genomic DNA, and kept at −20 °C before use. The concentration of the isolated DNA and the ratios of the absorbances at 260 nm–280 nm (OD_{260}/OD_{280} ratio) and 260 nm–230 nm (OD_{260}/OD_{230}) were measured with a NanoDrop ND-1000 spectrophotometer (Gene, Hong Kong, China).

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