ORIGINAL ARTICLE

A nucleotide signature for the identification of Pinelliae Rhizoma (*Banxia*) and its products

Tianyi Zhang[1,2] · Fusheng Xu[1] · Markus Ruhsam[3] · Li Feng[1,2] · Miao Zhang[4] · Zhengwei Wang[5] · Xumei Wang[1,2]

Abstract

Background Ensuring the authenticity of raw materials is a key step prior to producing Chinese patent medicines. *Pinellia ternata* (Thunb.) Breit. is the botanical origin of Pinelliae Rhizoma (*Banxia*), a traditional Chinese medicine used to treat cough, insomnia, nausea, inflammation, epilepsy, and so on. Unfortunately, authentic Pinelliae Rhizoma is often adulterated by morphologically indistinguishable plant material due to the insufficient regulatory procedures of processed medicinal plant products. Thus, it is important to develop a molecular assay based on species-specific nucleotide signatures and primers to efficiently distinguish authentic Pinelliae Rhizoma from its adulterants.

Methods and results The ITS2 region of 67 Pinelliae Rhizoma and its common adulterants were sequenced. Eight single nucleotide polymorphisms within a 28–43 bp stretch of ITS2 were used to develop six primer pairs to amplify these species-specific regions. We assayed 56 Pinelliae Rhizoma products sold on the Chinese market, including medicinal slices, powder and Chinese patent medicines, which revealed that about 66% of products were adulterated. The most common adulterants were *Pinellia pedatisecta* (found in 57% of the assayed products), *Arisaema erubescens* (9%), *Typhonium giganteum* (2%) and *Typhonium flagelliforme* (2%).

Conclusions A severe adulteration condition was revealed in the traditional medicine market. The species-specific nucleotide assays developed in this study can be applied to reliably identify Pinelliae Rhizoma and its adulterants, aiding in the authentication and quality control of processed products on the herbal market.

Keywords *Pinellia ternata* · Chinese patent medicine · Nucleotide signature · Degraded DNA · Identification

Abbreviations
CPM   Chinese patent medicine
SNP   Single nucleotide polymorphism
ITS   Internal transcribed spacer
TCM   Traditional Chinese medicine

Tianyi Zhang and Fusheng Xu have contributed equally to this article.

✉ Xumei Wang
   wangxumei@mail.xjtu.edu.cn

1   School of Pharmacy, Xi'an Jiaotong University, Xi'an 710061, China

2   Key Laboratory of Qiyao Resources and Anti-Tumor Activities, Shaanxi Administration of Traditional Chinese Medicine, School of Pharmacy, Xi'an Jiaotong University, Xi'an 10061, China

3   Royal Botanic Garden Edinburgh, Edinburgh EH3 5LR, UK

4   Lixian Spring Pharmaceutical Co. Ltd., Longnan 742200, China

5   Shanghai Chenshan Botanical Garden, Shanghai 201602, China

Introduction

Traditional Chinese medicine (TCM) has been used for health care in China and other countries such as North and South Korea, and Japan for 1000s of years. According to the processing method, TCM can be divided into Chinese medicinal raw materials, slices and Chinese patent medicines (CPMs) [1]. CPMs are herbal medicinal products developed according to the theory of TCM, which consist of various plant or animal ingredients, and are manufactured in a ready-to-use form such as capsules, tablets, granules, pills or oral liquids [2]. The therapeutic benefits of some CPMs have been validated scientifically [1]. The market value of CPMs reaches tens of billions of US dollars annually in China alone. CPMs, such as Lianhuaqingwen capsules, are used in combination with chemical medicines for

the management of COVID-19, and proposed as adjunctive and therapeutic option to fight the public health emergency by national and provincial guidelines in China [3]. However, it may take many years before medicinal plants, especially woody ones, can be harvested, so it can be difficult to satisfy the market demand for some Chinese medicinal plant materials. As the discrepancy between supply and demand is increasing, so is the temptation to substitute original medicinal material with adulterated raw materials [4], affecting the efficacy and safety of the TCM product.

Traditionally, the authentication of TCM mainly relies on organoleptic parameters which are an expert-dependent technique [5]. However, it is difficult to authenticate the raw materials of CPMs in the form of tablets, capsules or oral liquids which consist of many different, often highly processed, ingredients from plants or animals. In the past few decades, analytical chemistry techniques such as Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas chromatography (GC) have been used for the quality assurance of CPMs [6]. These methods can complement morphological identification to some extent, but have limitations as closely related species might have the same or very similar compounds. For example, although arginine can be used as the chemical indicator to distinguish *Pinellia ternata* from *Typhonium flagelliforme*, it is not possible to discriminate between *P. ternata* and *P. pedatisecta* [7]. In such instances, molecular tools like DNA barcoding can be an effective tool for rapidly and accurately identifying plants [8, 9]. The non-coding *trnL-trnF*, *trnH-psbA*, *psbK-psbI* and *atpF-atpH* plastid intergenic spacers and the multicopy nuclear internal transcribed spacer (ITS) are widely used to distinguish authentic plant material from adulterants [8, 10]. Many studies have shown that ITS2 is a useful genomic region which can be used to identify herbal medicines [10–12], although it is difficult to amplify highly processed samples [13, 14]. For example, ITS2 could not be amplified from Angelicae Sinensis Radix extracts or decoctions and from some CPMs in the form of oral liquids or injection samples [1, 15] as it can be very difficult to amplify fragments more than a few hundred base pairs long from degraded DNA. Short species-specific nucleotide signatures [16] could be an alternative tool to detect fraudulent product substitutions, adulteration or the inclusion of unlabelled ingredients.

Pinelliae Rhizoma (*Banxia*), derived from the dried tuber of *Pinellia ternata* (Thunb.) Breit. (Araceae), is a well-known pharmacopoeia-recorded medicine in China [6]. Pinelliae Rhizoma was first recorded in *Shen Nong Ben Cao Jing* (Shen Nong's herbal classic) 2000 years ago and is now part of 432 traditional CPMs (Chinese Patent Medicine Prescription Database) and 3036 prescriptions (TCM Prescription Database). Modern research has shown that Pinelliae Rhizoma possesses many pharmacological properties such as being antitussive, antiemetic, expectorant and antitumor [17, 18]. However, as the raw material Pinelliae Rhizoma is toxic [19], excessive or long-term use of this herbal medicine can cause renal and hepatic injuries [18, 20]. Therefore, the processed products of Pinelliae Rhizoma, namely Pinelliae Rhizoma Praeparatum (*Fabanxia*), Pinelliae Rhizoma Praeparatum cum Zingibere et Alumine (*Jiangbanxia*), and Pinelliae Rhizoma Praeparatum cum Alumine (*Qingbanxia*), are generally used as described in the Chinese Pharmacopoeia [6]. Pinelliae Rhizoma is usually applied as powder, capsule, tablet or decoction in a wide range of clinical applications. For example, Banxia Houpu Decoction is used for the treatment of depression-related diseases [21] and Xiao-Qing-Long-Tang has been widely used for the treatment of allergic diseases [22].

Although *P. ternata* naturally occurs across mainland China except Inner Mongolia, Heilongjiang and Tibet, the production of *P. ternata* does not match the market demand for Pinelliae Rhizoma. Thus, many adulterants have appeared on the market including two *Pinellia* species (*P. pedatisecta* Schott and *P. cordata* N. E. Brown), three *Typhonium* species [*T. giganteum* Engl., *T. divaricatum* (L.) Decne. and *T. flagelliforme* (Lodd.) Blume], and three *Arisaema* species [*A. heterophyllum* Blume, *A. amurense* Maxim. and *A. erubescens* (Wall.) Schott] [23, 24]. *P. ternata* can be easily distinguished from its adulterants using leaf and flower morphology, however the medicinally active underground parts (tubers) are extremely similar. For example, Pinelliae Rhizoma is spheroidal in shape, while the dried tuber of *P. pedatisecta* is also spheroidal with several small bulbs alongside. If small bulbs of *P. pedatisecta* are not yet formed or removed during processing, the tubers will be very difficult to distinguish from Pinelliae Rhizoma based on gross morphology. Even microscopic traits such as the needle-like calcium oxalate crystals in the tubers of *P. ternata* are of limited use as these also occur in *P. pedatisecta* and *T. flagelliforme* [24]. TLC is also insufficient for identifying Pinelliae Rhizoma reliably because similar compounds exist within the known adulterant *P. pedatisecta* [7, 25]. Therefore, the development of a rapid and reliable method for the authentication of Pinelliae Rhizoma and its adulterants is urgently needed to safeguard the quality and safety of this important CPM.

Zheng et al. [26] employed the plastid spacers *psbK-psbI* and *atpF-atpH* to authenticate *P. ternata* from related species. The results indicated that *psbK-psbI* had higher identification success than *atpF-atpH*. Although Zhang et al. [23] suggested ITS2 as a DNA barcode for identifying Pinelliae Rhizoma from its adulterants, CPM often only contain very small amounts of degraded DNA and it can be very difficult to carry out PCR amplification of regions > 200 bp in length like *psbK-psbI* (usually > 400 bp) and ITS2 (usually > 200 bp) successfully.

However, several studies have shown that a "mini-barcode" with a length ranging from 100 to 200 bp can be used to amplify degraded DNA [27, 28]. For instance, Lo et al. [29] successfully PCR amplified target products from TCM material which had been boiled for 120 min. Shokralla et al. [30] screened processed fish products using a mini-barcode and reported a success rate of 88.6% compared to a 20.5% success rate using the full-length DNA barcode *CO1* (~650 bp). More recently, nucleotide signatures, which target one or more nucleotides unique to one species, have been used [15, 16, 31]. Generally, mini barcodes take the whole sequencing region (usually > 100 bp) into consideration to identify differences among species, whereas nucleotide signatures are based on species-level nucleotide polymorphisms inside shorter regions (commonly < 50 bp). Nonetheless, not all species necessarily have species-specific nucleotides due to incomplete lineage sorting.

The aim of this study is (1) to develop a nucleotide signature for the reliable identification of adulterants in CPM containing Pinelliae Rhizoma and (2) to authenticate processed Pinelliae Rhizoma products including tablets, pills, granules and decoctions (oral liquids) from the Chinese market.

Materials and methods

Reference samples

A total of 67 reference samples including 34 *Pinellia ternata* individuals and 33 individuals from six species commonly found as adulterants of Pinelliae Rhizoma were collected from botanical gardens, forest reserves and medicinal plant nurseries in China (Supplementary Table 1). Young leaves were collected in silica gel for DNA extraction. Vouchers were deposited at the herbarium of Xi'an Jiaotong University (Xi'an, China). We also downloaded 284 ITS2 sequences of the genera *Pinellia*, *Typhonium* and *Arisaema*, and 17 ITS sequences of *Pinellia* from GenBank. All samples were used for the development of a nucleotide signature of Pinelliae Rhizoma and its adulterants (Supplementary Table 2).

Pinelliae Rhizoma products sold on the Chinese market

A total of 36 medicinal slices of Pinelliae Rhizoma were purchased from online stores and brick-and-mortar drugstores from Shaanxi, Shanxi, Shandong, Henan, Gansu and Liaoning provinces of China (Table 1).

Twenty batches of CPM containing Pinelliae Rhizoma, including Huoxiang Zhengqi pills (capsules or decoctions), Xiaochaihu granules, Baohe pills, Qingfei Huatan pills, Naoliqing pills, etc. were purchased from different drugstores (Table 2), and the declared compositions of different Chinese patent medicines are shown in Supplementary Table 3. All the CPMs contained between 5 and 15 ingredients.

DNA extraction

Total DNA was extracted from silica gel-dried leaves (30 mg) using the Plant Universal Genomic DNA Kit (Tiangen Biotech, Co., Ltd., Beijing, China), according to the manufacturer's instructions.

About 150–200 mg of medicinal slices, granules, pills, capsules, and tablets samples were tested from each product. DNA of medicinal slices, granules, pills, capsules, tablets was extracted using a modified CTAB extraction method. All samples were surface-cleaned with 75% alcohol, and then grinded into fine powder in liquid nitrogen. The powder was moved to three Eppendorf tubes which were cycled through the following steps separately: 700 μL of pre-wash buffer (1 M Tris–HCl, pH 8.0; 0.5 M EDTA, pH 8.0; 5 M NaCl; 2% PVP-40; 0.4% β-mercaptoethanol) was used to wash the powder several times until the supernatant was clear and colourless, and a centrifugation was performed at 7500×g for 5 min at room temperature. 800 μL preheated nuclear lysate (2% CTAB (w/v); 1 M Tris HCl, pH 8.0; 0.5 M EDTA, pH 8.0; 5 M NaCl; 0.4% β-mercaptoethanol) was mixed with the obtained precipitate in the preceding step. The mixture was subsequently put into a water bath at 65 °C for 3 h and centrifuged at 7500×g for 5 min at room temperature. Next, 70% methanol was added to the precipitate and stored at −20 °C for 1 h, followed by another centrifugation step at 7500×g for 5 min at room temperature. Then a second round using 800 μL preheated nuclear lysate was carried out following the steps above, except that the mixture was kept in the water bath for 20 min only. At room temperature, the liquid was mixed with an equal volume of chloroform: isoamylalcohol (24:1), and centrifuged at 10,000×g for 10 min at 4 °C. This step was repeated 1–2 times to increase the DNA yield. The upper aqueous layer was mixed with two volumes of ice-cold isopropanol, kept at −20 °C for 1 h and then centrifuged at 7500×g for 10 min at 4 °C. The precipitate (DNA pellet) was washed with 70% ethanol twice and air dried. Finally, the DNA pellet was re-suspended in 100 μl sterilized double distilled water.

For liquid samples, such as Huoxiang Zhengqi Shui, about 800 μL of the liquid samples were transferred into 2.0 mL tubes adding 700 μL of pre-wash buffer. Extractions then followed the same steps as solid samples.

DNA of three samples per product was extracted and concentrated into one tube. Visual inspection of DNA bands on a 1.0% agarose combined with an ultraviolet spectrophotometer (ND-2000, NanoDrop Technologies, Wilmington,

Table 1 Identification results of medicinal slices of Pinelliae Rhizoma

Sample No.	Sample name	Sample type	Collection site	Identification results
YC01	*Qingbanxia*	Medicinal slices	Xi'an, Shaanxi	*Pinellia ternata, Pinellia pedatisecta*
YC02	*Jiangbanxia*	Medicinal slices	Xi'an, Shaanxi	*Pinellia pedatisecta*
YC03	*Fabanxia*	Medicinal slices	Xi'an, Shaanxi	*Pinellia pedatisecta*
YC04	*Qingbanxia*	Medicinal slices	Xi'an, Shaanxi	*Pinellia ternata, Pinellia pedatisecta*
YC05	*Jiangbanxia*	Medicinal slices	Xi'an, Shaanxi	*Pinellia pedatisecta*
YC06	*Fabanxia*	Medicinal slices	Xi'an, Shaanxi	*Pinellia pedatisecta*
YC07	*Qingbanxia*	Medicinal slices	Xi'an, Shaanxi	*Pinellia pedatisecta*
YC08	*Qingbanxia*	Medicinal slices	Xi'an, Shaanxi	*Pinellia ternata*
YC09	*Qingbanxia*	Medicinal slices	Xi'an, Shaanxi	*Pinellia ternata*
YC10	*Qingbanxia*	Medicinal slices	Xi'an, Shaanxi	*Pinellia ternata*
YC11	*Fabanxia*	Medicinal slices	Xi'an, Shaanxi	*Pinellia ternata*
YC12	*Jiangbanxia*	Medicinal slices	Huozhou, Shanxi	*Arisaema erubescens*
YC13	*Fabanxia*	Medicinal slices	Huozhou, Shanxi	*Pinellia pedatisecta*
YC14	*Fabanxia*	Medicinal slices	Jinan, Shandong	*Pinellia ternata, Pinellia pedatisecta*
YC15	*Fabanxia*	Medicinal slices	Xinxiang, Henan	*Pinellia ternata, Pinellia pedatisecta*
YC16	*Jiangbanxia*	Medicinal slices	Taiyuan, Shanxi	*Pinellia ternata*
YC17	*Qingbanxia*	Medicinal slices	Taiyuan, Shanxi	*Pinellia ternata*
YC18	*Fabanxia*	Medicinal slices	Taiyuan, Shanxi	*Pinellia ternata*
YC19	*Qingbanxia*	Medicinal slices	Taiyuan, Shanxi	*Pinellia ternata*
YC20	*Qingbanxia*	Medicinal slices	Taiyuan, Shanxi	*Pinellia ternata*
YC21	*Fabanxia*	Medicinal slices	Taiyuan, Shanxi	Pinellia ternata
YC22	*Jiangbanxia*	Medicinal slices	Taiyuan, Shanxi	*Pinellia ternata*
YC23	*Qingbanxia*	Medicinal slices	Huozhou, Shanxi	*Pinellia ternata, Pinellia pedatisecta*
YC24	*Fabanxia*	Medicinal slices	Taiyuan, Shanxi	*Pinellia pedatisecta*
YC25	*Qingbanxia*	Medicinal slices	Taiyuan, Shanxi	*Pinellia ternata*
YC26	*Jiangbanxia*	Medicinal slices	Taiyuan, Shanxi	*Pinellia ternata, Pinellia pedatisecta*
YC27	*Jiangbanxia*	Medicinal slices	Taiyuan, Shanxi	*Pinellia pedatisecta*
YC28	*Jiangbanxia*	Medicinal slices	Longnan, Gansu	*Pinellia pedatisecta, Arisaema erubescens*
YC29	*Jiangbanxia*	Medicinal slices	Longnan, Gansu	*Typhonium flagelliforme*
YC30	*Qingbanxia*	Medicinal slices	Longnan, Gansu	*Pinellia pedatisecta*
YC31	*Qingbanxia*	Medicinal slices	Shenyang, Liaoning	*Arisaema erubescens*
YC32	*Fabanxia*	Medicinal slices	Shenyang, Liaoning	*Pinellia ternata, Pinellia pedatisecta*
YC33	*Qingbanxia*	Medicinal slices	Shenyang, Liaoning	*Arisaema erubescens*
YC34	*Fabanxia*	Powder	Shenyang, Liaoning	*Pinellia ternata*
YC35	*Qingbanxia*	Medicinal slices	Shenyang, Liaoning	*Pinellia pedatisecta*
YC36	*Fabanxia*	Medicinal slices	Shenyang, Liaoning	*Arisaema erubescens*

DE, USA) were used to determine the quality and quantity of DNA.

PCR amplification of ITS region

Universal ITS or ITS2 primers (Supplementary Table 4) were used to amplify the corresponding region of all assayed samples. PCR was performed in a 25 µL reaction system containing 12.5 µL of 2 × PCR Master buffer (HT-biotech Co., China), 1.0 µL of each primer (2.5 µM), 2 µL (about 50 ng) of DNA templates and filled with double-distilled water. The reactions were performed in a thermal cycler (VeritiTM 96-Well Thermal Cycler, Applied Biosystems Co., USA) using the conditions as listed in Supplementary Table 5. PCR products were run on a 1.2% agarose gel in 0.5 × TBE buffer and visualized by ethidium bromide under ultraviolet light using a Bio-Rad Gel Documentation System (Universal Hood II system, Yuanye Bio Co. Ltd., Beijing, China). Negative controls (no template DNA added) were included in all PCR and sequencing experiments. Successful PCR amplifications were sent to Tsingke Biotechnology Co., Ltd. (Xi'an, China) for Sanger sequencing, and the raw data

Table 2 Identification results of Chinese patent medicines containing Pinelliae Rhizoma

Sample No.	Sample name	Sample type	Batch No.	Identification result
1	Xiaochaihu Keli	Granule	ZEA1854	*Pinellia ternata, Pinellia pedatisecta*
2	Huoxiang Zhengqi Shui	Oral liquid	180621	*Pinellia ternata, Pinellia pedatisecta*
3	Guilong Kechuannning Pian	Tablet	180403	*Pinellia ternata, Pinellia pedatisecta*
4	Erchen Wan	Pill	1808	*Pinellia ternata, Pinellia pedatisecta*
5	Baohe Wan	Pill	18030	*Pinellia ternata, Pinellia pedatisecta*
6	Qingfei Huatan Wan	Pill	281317	*Pinellia ternata*
7	Juhong Wan	Pill	160901	*Pinellia ternata, Pinellia pedatisecta, Typhonium giganteum*
8	Huoxiang Zhengqi Koufuye	Oral liquid	17040443	*Pinellia ternata, Pinellia pedatisecta*
9	Naoliqing Wan	Pill	181007	*Pinellia ternata, Pinellia pedatisecta*
10	Huoxiang Zhengqi Wan	Pill	201610042	*Pinellia ternata, Pinellia pedatisecta*
11	Xiangsha Yangwei Wan	Pill	16A25	*Pinellia ternata, Pinellia pedatisecta*
12	Zhike Wan	Pill	280073	*Pinellia ternata*
13	Huoxiang Zhengqi Diwan	Droplet pill	170311	*Pinellia pedatisecta*
14	Qingfei Huatan Wan	Pill	291480	*Pinellia ternata*
15	Huoxiang Zhengqi Shui	Oral liquid	180602	*Pinellia pedatisecta*
16	Qingfei Huatan Wan	Pill	271779	*Pinellia ternata*
17	Baohe Wan	Pill	18E57	*Pinellia pedatisecta*
18	Naoliqing Wan	Pill	181010	*Pinellia ternata*
19	Huoxiang Zhengqi Shui	Oral liquid	180445	*Pinellia pedatisecta*
20	Huoxiang Zhengqi Jiaonang	Capsule	ZFB1708	*Pinellia ternata*

were edited and assembled manually using Geneious v10.1 (Biomatters Ltd., Auckland, New Zealand).

Development of primers for species-specific regions

All available ITS2 sequences of *Pinellia ternata* and its adulterants, i.e. species of the genera *Pinellia*, *Typhonium* and *Arisaema*, consisting of our sequenced reference samples as well as samples downloaded from GenBank (Supplementary Table 2), were annotated and delimited using the Hidden Markov Model (HMM)-based method [32] to eliminate the 5.8 S and 28 S rDNA regions.

The remaining ITS2 regions were then aligned using MEGA 7.0 via the MUSCLE alignment method [33, 34] to identify species-specific nucleotide signatures and to design primers which amplify the identified species-specific regions. *Pinellia ternata* samples showed great individual variation and the complete ITS region was therefore aligned.

Species identification for each sequence was executed utilizing Basic Local Alignment Search Tool (BLAST) against GenBank and a local nucleotide signature library for a specific species with a minimum BLAST cutoff of 98% identity for a top match.

Six primer pairs for species-specific regions, i.e. HZ-F/HZ-R, DJL-F/DJL-R, LTJ-F/LTJ-R, BYJ-F/BYJ-R, TNX-F/TNX-R and BX-F/BX-R were designed via Primer Premier

6.0 software (Premier Co., Canada) to amplify *Pinellia ternata* and its adulterants (Supplementary Table 4). PCR conditions are listed in Supplementary Table 6.

Authentication of Pinelliae Rhizoma products sold on the Chinese market

All reference samples were screened with all newly developed primers to test their species-specific nature. PCR conditions were the same as that in Supplementary Table 6 and amplicons were sent to Tsingke Biotechnology Co., Ltd (Xi'an, China) for Sanger sequencing.

Results

Development of nucleotide signatures and primer pairs of species-specific regions for Pinelliae Rhizoma and its adulterants

PCR amplification of ITS2 for all 67 reference samples was successful. Aligned ITS2 sequences of *P. pedatisecta* were 258 bp long (Supplementary Fig. 1a). Sequence analyses of *P. pedatisecta* and its closely related species uncovered two single nucleotide polymorphism (SNP) sites which were used to define a *P. pedatisecta*-specific 28 bp nucleotide signature (5′-ACAGGACCGA CCGTGAAGAA CCCAGT
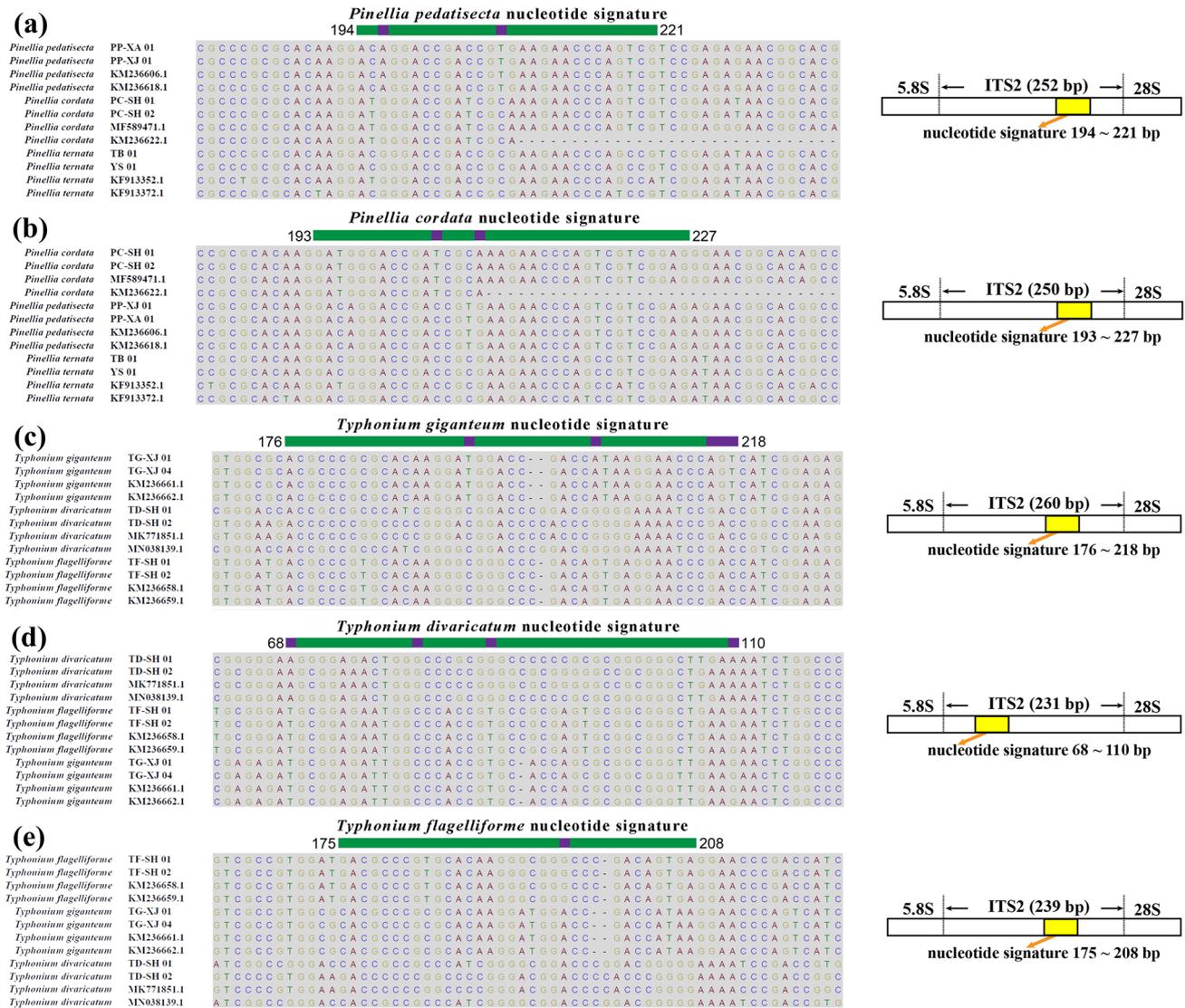
Fig. 1 ITS2 sequence alignment and SNPs of *Pinellia pedatisecta* (**a**), *P. cordata* (**b**), *Typhonium giganteum* (**c**), *T. divaricatum* (**d**) and *T. flagelliforme* (**e**).The highlighted regions represent the nucleotide signature and the marked bases represent the SNP sites of each signature, while the lines represent alignment gaps

CG-3′) (Fig. 1a). BLAST results demonstrated that this nucleotide signature was unique to *P. pedatisecta* (Supplementary Table 5). The nucleotide signatures for the other seven adulterants, including *P. cordata*, *T. giganteum*, *T. divaricatum*, *T. flagelliforme*, *A. heterophyllum*, *A. amurense* and *A. erubescens*, were developed similarly. This resulted in a 35 bp signature (5′-GATGGGACCG ATC GCAAAGA ACCCAGTCGT CGGAG-3′) for *P. cordata* (Fig. 1b), a 41 bp signature (5′-ACGCCCGCGC ACAAGG ATGG ACCGACCATA AGGAACCCAG T-3′) for *T. giganteum* (Fig. 1c), a 43 bp signature (5′-AGSGGARACT GGG CCCSCGG G SCSCSSGSG CSGSGGGCTK RAA-3′) for *T. divaricatum* (Fig. 1d), a 33 bp signature (5′-GACGCC CGTG CACAAGGGCG GGCCCGACAG TGA-3′) for *T.*

flagelliforme (Fig. 1e), a 32 bp (5′-CTGGGCGATT AAC GGTGAGT GGTGGACGAC GC-3′) for *A. heterophyllum* (Fig. 2a), a 43 bp (5′-CGAGTGGTGG ACAATGCTCA TCGTCGCCGT AGTGCACGCC CGT-3′) for *A. amurense* (Fig. 2b), and a 35 bp (5′-GTCATCGTCG CCGTGCCCGC GCGCAAGGAC GGGCC-3′) for *A. erubescens* (Fig. 2b). These nucleotide signatures are species-specific and were not present in any other species (Supplementary Table 7).

To amplify the regions with the species-specific nucleotide signatures, the following primer pairs were designed for *P. pedatisecta* and *P. cordata* (HZ-F/HZ-R, 167 bp), *T. giganteum* (DJL-F/DJL-R, 195 bp), *T. divaricatum* (LTJ-F/ LTJ-R, 167 bp) and *T. flagelliforme* (BYJ-F/BYJ-R, 190 bp). The primer pair TNX-F/TNX-R was designed to amplify
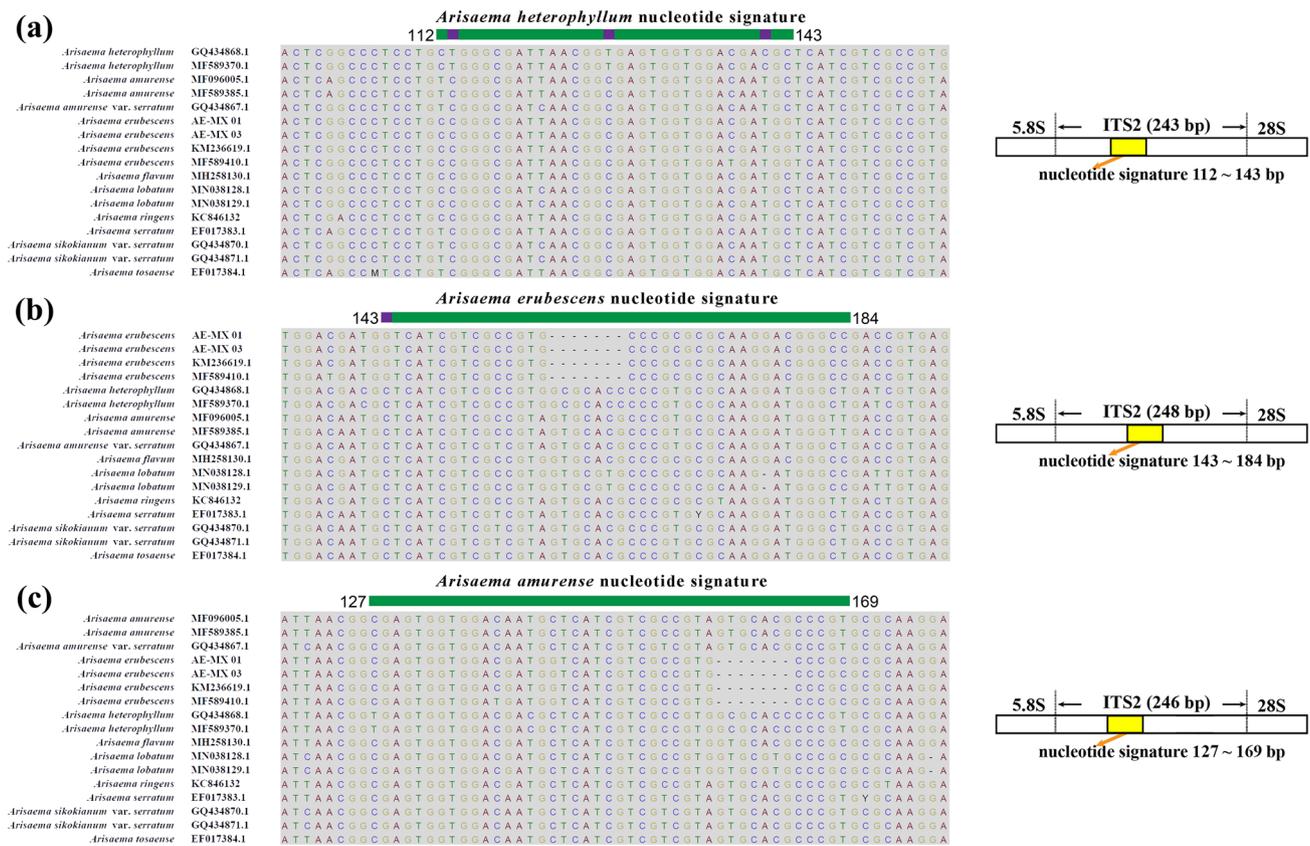
Fig. 2 ITS2 sequence alignment and SNPs of the three nucleotide signatures for *Arisaema* spp. The highlighted regions represent the nucleotide signature and the marked bases represent the SNP sites of each signature, while the lines represent alignment gaps

three *Arisaema* species including *A. heterophyllum*, *A. amurense* and *A. erubescens*, the lengths of their amplicons ranged from 154 to 161 bp (Supplementary Table 4). Additionally, as the ITS1 sequences are more conservative than the ITS2 sequences in *P. ternata* (Supplementary Fig. 2), a total of 93 ITS1 sequences (34 *P. ternata* reference samples and 59 *P. ternata* sequences downloaded from GenBank) were analyzed. A short species-specific primer BX-F/BX-R was designed for *P. ternata* to amplify a 138 bp region (Supplementary Table 4).

All primers developed except for BX-F/BX-R are located outside the species-specific nucleotide signature, i.e. PCR products need sequencing to ascertain the species at hand. In contrast, the primer, BX-F/BX-R for *P. ternata* is anchored on the species-specific SNPs, and the presence of a PCR product confirms the presence of *P. ternata*.

Screening of reference material with the newly developed primers

Six primer pairs targeting the species-specific regions were verified before market survey. We used our reference samples to check the species-specific nature of the PCR

amplifications (the conditions were the same as that in Supplementary Table 6).

The *P. ternata* specific primer pair amplified only *P. ternata* samples and no PCR product was observed in any of the other species (Fig. 3a). The other five primer pairs targeting the species-specific nucleotide signatures of *P. pedatisecta*, *P. cordata Typhonium* and *Arisaema* species amplified successfully all respective reference samples (Fig. 3b).

Market survey of adulteration via nucleotide signature and specific primers

We authenticated 36 products from the Chinese market, which according to the label, should only contain slices of Pinelliae Rhizoma. For this purpose, we used the six species-specific nucleotide signatures developed in this study (the sequences are listed in Supplementary Datasheet 1; Fig. 4a, b). In 23 products (64%) we found the nucleotide signatures of the following adulterants: *P. pedatisecta* (n=17), *A. erubescens* (n=4), both *P. pedatisecta* and *A. erubescens* (n=1), and *T. flagelliforme* (n=1) (Table 1). The other five possible adulterants, i.e. *P. cordata*, *T. giganteum*, *T.*

(a)

(b)
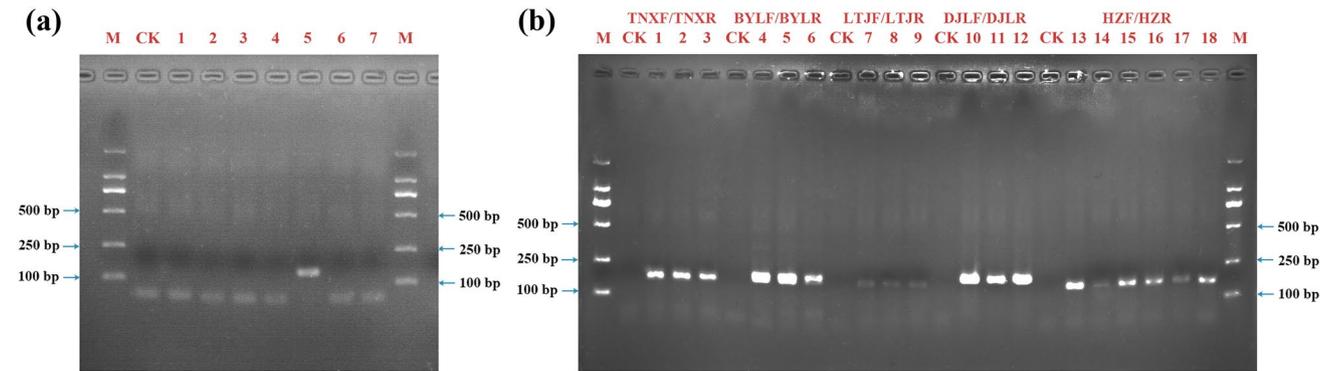
Fig. 3 Gel image of the PCR amplification. **a** Reference samples from six adulterants and one *Pinellia ternata* reference sample by primer pair BX-F/BX-R. M, marker lane; CK, negative control; 1, *Arisaema erubescens*; 2, *Typhonium divaricatum*; 3, *T. giganteum*; 4, *T. flagelliforme*; 5, *Pinellia ternata*; 6, *P. cordata*; 7, *P. pedatisecta*. **b** Five different primer pairs used for the reference individuals, respectively.

The primers are listed above the corresponding lanes. Numbers 1–3, 4–6, 7–9, 10–12, 13–15 and 16–18 represent amplicons from the samples of *Arisaema erubescens*, *Typhonium flagelliforme*, *T. divaricatum*, *T. giganteum*, *Pinellia pedatisecta* and *P. cordata*, respectively. M and CK represent marker and negative control lanes, respectively

(a)

(b)

(c)

(d)
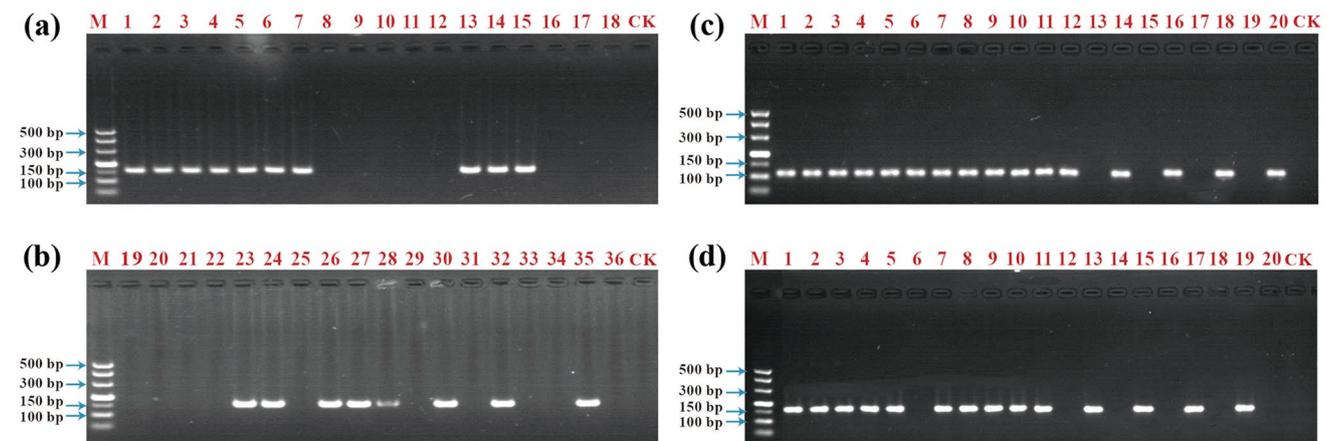
Fig. 4 Representative gel images using species-specific primers. The primer pair HZ-F/HZ-R (**a**, **b**) was used to amplify slice samples. Lanes 1–36 of (**a**) and (**b**) are corresponding to the numbers in Table 1. Chinese patent medicines containing Pinelliae Rhizoma

with the primer pairs BX-F/BX-R (**c**), and HZ-F/HZ-R (**d**) are also shown. Lanes 1–20 of (**c**) and (**d**) are corresponding to the numbers in Table 2. *M* marker; *CK* negative control

divaricatum, *A. heterophyllum* and *A. amurense* were not detected in any of the Pinelliae Rhizoma products.

We also tested 20 CPM products which should, amongst other ingredients, also contain Pinelliae Rhizoma. Pinelliae Rhizoma could be amplified specifically via the primer pair BX-F/BX-R from Qingfei Huatan pills. No amplicons were obtained using the other five newly developed primers, suggesting that no adulterants were present in the three batches of Qingfei Huatan pills (CPM 6, 14 and 16). Another example is Juhong (CPM 7) consisting of 15 ingredients, of which Pinelliae Rhizoma was successfully amplified by the specific primer pair BX-F/BX-R. However, a visible band was detected using primer pair HZ-F/HZ-R (Fig. 4d), which suggests the presence of adulterants in the assayed Juhong pills.

Only 6 of the 20 assayed CPMs (30%) contained Pinelliae Rhizoma and no adulterant. In contrast, 10 of the 20 CPMs (50%) contained Pinelliae Rhizoma and at least one adulterant. In 4 out of 20 CPMs (20%) only an adulterant of Pinelliae Rhizoma could be amplified (Table 2). PCR products obtained via the primer pairs BX-F/BX-R and HZ-F/HZ-R for the targeted species are shown in Fig. 4c and d. Four CPMs (Nos. 13, 15, 17, and 19) were substituted with *P. pedatisecta*, and 10 CPMs (Nos. 1-5, 7-11) contained *P. ternata* as well as *P. pedatisecta*. In addition, both the adulterants *P. pedatisecta* and *T. giganteum* were detected in one batch of CPM (No. 7). Interestingly, different batches from the same manufacturer contained different adulterants. For example, among the three batches from one manufacturer (Nos. 2, 15, and 19), one batch

comprised a mixture of Pinelliae Rhizoma and *P. pedatisecta*, whereas the other two only contained *P. pedatisecta*. In total, *P. pedatisecta* (14 out of 20, 70%) was the most common adulterants in products labeled as containing Pinelliae Rhizoma. *Pinellia cordata*, *T. divaricatum*, *T. flagelliforme*, and three *Arisaema* species were not present in any of the assayed CPMs (Table 2).

In summary, out of 56 Pinelliae Rhizoma products sold on the Chinese market, about 66% of the products were adulterated. The most common adulterants were *P. pedatisecta* (found in 57% of the assayed products), *A. erubescens* (9%), *T. giganteum* (2%) and *T. flagelliforme* (2%).

Discussion

In this study, we developed primers to amplify species-specific sequences of common adulterants of the TCM medicinal product Pinelliae Rhizoma. The results showed that there is considerable adulteration of products containing Pinelliae Rhizoma as only 30% (6 out of 20) of authenticated CPMs contained the correct species *Pinellia ternata*. *Pinellia pedatisecta* was the most common adulterant (32 out of 56, 57%) of Pinelliae Rhizoma products both on the market for medicinal materials and CPMs, followed by *A. erubescens* (5 out of 56, 9%) and *Typhonium* spp. (2 out of 56, 4%). As the price of authentic Pinelliae Rhizoma is much higher than that of its adulterants [35], there is an incentive for the adulteration of products containing Pinelliae Rhizoma. With the increasing popularity of TCM in the western world, the demand for Chinese medicinal materials will put even further pressure on some species, increasing the incentive for species substitution and adulteration of TCM products. Recent studies have shown that adulteration of TCM products is common. For example, Ruhsam and Hollingsworth [10] reported that 36% of Siberian Ginseng (*Eleutherococcus senticosus*) products also contained other *Eleutherococcus* species and that only 50% (five out of ten) of *Rhodiola* supplements had the target species *R. rosea* in them. Equally, Jiang et al. [36] identified that 8 out of 27 (30%) Schisandrae Chinensis Fructus (*Wuweizi*) crude drug samples were adulterated and one out of 8 CPMs contained adulterants. Equally, a market survey on Cistanches Herba (*Roucongrong*) indicated 36.4% adulteration, suggesting that adulteration is common in TCM [16].

PCR is considered as a sensitive and specific method for trace analysis [37]. However, it should be noted that some factors, including DNA quality, primer affinity, PCR amplification and sequencing of amplicons, can lead to the problem in PCR analysis [38]. In other words, PCR failure cannot be fully excluded in our study, which might be the reason for different authentication results from different batches (Table 2), especially considering the slightly lower successful rate of PCR reaction targeting ITS region than chloroplast regions [39]. Another limitation is the requirement of Sanger sequencing when amplicons can be obtained with more than one primer pairs, i.e. positive results in both HZ-F/HZ-R and BX-F/BX-R. Besides, mixed signals could be found in certain sites as a result of multiple adulterations [40], which might influence the determination on species. Chen et al. [1] developed a chip-based colorimetric detection approach to distinguish the PCR products of ginseng CPM from its possible adulterants based on G-quadruplex integrated hybridization chain reaction. Illumina midi-barcodes were applied to the identification of bees by Lanner et al., providing another option for molecular authentication [41]. In future, the authentication of CPMs containing Pinelliae Rhizoma may benefit from similar systems.

The nucleotide signatures developed in this study provide a set of tools to authenticate Pinelliae Rhizoma products on the market. This will help suppliers of raw materials and producers of TCM products to ensure that what is stated on the label also matches what is in the product. In turn, this will increase the safety of consumers and the efficacy of the medicinal product.

Conclusion

We newly developed six primers to authenticate Pinelliae Rhizoma from the adulterants based on species-specific region based on ITS region. Market survey revealed severe adulteration conditions in the slices and the CPMs of *Banxia*. Over 60% products have adulterants, most of which is *Pinellia pedatisecta* (57% of the assayed products), followed by *Arisaema erubescens* (9%). We hope that the method in this study could help authentication, quality control, and supervision of Pinelliae Rhizoma products, leaving a better TCM market in the future,

Declarations

Conflict of interest The authors have no relevant financial or non-financial interest to disclose.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to publish We hereby declare that we participated in this research and the paper's development. We have read its final version and give consent for the publication.

References

1. Chen R, Dong J, Cui X et al (2012) DNA based identification of medicinal materials in Chinese patent medicines. Sci Rep 2:958. https://doi.org/10.1038/srep00958
2. Wu L, Chen Y, Ma Y et al (2020) Clinical practice guideline on treating influenza in adult patients with Chinese patent medicines. Pharmacol Res 160:105101. https://doi.org/10.1016/j.phrs.2020.105101
3. Ni L, Zhou L, Zhou M et al (2020) Combination of western medicine and Chinese traditional patent medicine in treating a family case of COVID-19. Front Med 14:210–214. https://doi.org/10.1007/s11684-020-0757-x
4. Shaheen S, Ramzan S, Khan F, Ahmad M (2019) Preventive measures to stop adulteration in marketed herbal samples. Springer, Cham, pp 57–63. https://doi.org/10.1007/978-3-030-28034-5_6
5. Joshi K, Preeti C-G, Warude D, Patwardhan B (2004) Molecular markers in herbal drug technology. Curr Sci 87:159–165
6. National Pharmacopoeia Committee (2020) Pharmacopoeia of the People's Republic of China (2020 edition). China Medical Science and Technology Press, Beijing
7. Zhu YH, Xiang FL, Ren Y et al (2016) Identification and medication safety of *Pinellia ternata* and its adulterants. Gansu Med 35:943–945. https://doi.org/10.15975/j.cnki.gsyy.2016.12.029
8. Hollingsworth PM, Forrest LL, Spouge JL et al (2009) A DNA barcode for land plants. Proc Natl Acad Sci 106:12794–12797. https://doi.org/10.1073/pnas.0905845106
9. Cui XY, Li WJ, Wei JH et al (2020) Assessing the identity of commercial herbs from a Cambodian market Using DNA barcoding. Front Pharmacol 11:244. https://doi.org/10.3389/fphar.2020.00244
10. Ruhsam M, Hollingsworth PM (2018) Authentication of Eleutherococcus and Rhodiola herbal supplement products in the United Kingdom. J Pharm Biomed Anal 149:403–409. https://doi.org/10.1016/j.jpba.2017.11.025
11. Liow HL, Ahmad Tarmizi AA, Jahari PNS et al (2021) DNA barcoding for authentication of *Orthosiphon stamineus* herbal medicinal product using ITS2 nuclear marker originating from Malaysia. IOP Conf Ser Earth Environ Sci 736:12034. https://doi.org/10.1088/1755-1315/736/1/012034
12. Ma Y, Li J, Tian M et al (2020) Authentication of Chinese prickly ash by ITS2 sequencing and the influence of environmental factors on pericarp quality traits. Ind Crops Prod 155:112770. https://doi.org/10.1016/j.indcrop.2020.112770
13. Newmaster SG, Grguric M, Shanmughanandhan D et al (2013) DNA barcoding detects contamination and substitution in North American herbal products. BMC Med 11:222. https://doi.org/10.1186/1741-7015-11-222
14. Jones L, Twyford AD, Ford CR et al (2021) Barcode UK: a complete DNA barcoding resource for the flowering plants and conifers of the United Kingdom. Mol Ecol Resour 21:2050–2062. https://doi.org/10.1111/1755-0998.13388
15. Liu Y, Wang XY, Wang LL et al (2016) A nucleotide signature for the identification of American ginseng and its products. Front Plant Sci 7:319. https://doi.org/10.3389/fpls.2016.00319
16. Wang XY, Xu R, Chen J et al (2018) Detection of Cistanches Herba (*Rou Cong Rong*) medicinal products using species-specific nucleotide signatures. Front Plant Sci 9:164. https://doi.org/10.3389/fpls.2018.01643
17. Mao RJ, He ZG (2020) Pinellia ternata (Thunb.) Breit: a review of its germplasm resources, genetic diversity and active components. J Ethnopharmacol 263:113252. https://doi.org/10.1016/j.jep.2020.113252
18. Wu JJ, Cheng ZX, He SG et al (2015) Pinelliae Rhizoma, a toxic Chinese herb, can significantly inhibit CYP3A activity in rats. Molecules 20:792–806. https://doi.org/10.3390/molecules20010792
19. Cui MN, Zhong LY, Zhang DY et al (2020) Study progress on Pinelliae Rhizoma processed by replication method. China J Chi Mat Med 45:1304–1310. https://doi.org/10.19540/j.cnki.cjcmm.20200207.305
20. Zhai XY, Zhang L, Li BT et al (2019) Discrimination of toxic ingredient between raw and processed *Pinellia ternata* by UPLC/Q-TOF-MS/MS with principal component analysis and T-test. Chin Herb Med 11:200–208. https://doi.org/10.1016/j.chmed.2019.03.007
21. Chen ZY, Xie DF, Liu ZY et al (2020) Identification of the significant pathways of Banxia Houpu decoction in the treatment of depression based on network pharmacology. PLoS ONE 15:e0239843. https://doi.org/10.1371/journal.pone.0239843
22. Kim MH, Hong SU, Kim HT et al (2019) A multicenter study on the efficacy and safety of So-Cheong-Ryong-Tang for perennial allergic rhinitis. Complement Ther Med 45:50–56. https://doi.org/10.1016/j.ctim.2019.05.018
23. Zhang YQ, Song M, Sun W et al (2014) Identification of Pinelliae Rhizoma and its adulterants based on ITS2 sequence. World Sci Technol Tradit Chin Med Mater Med 16:1725–1729. https://doi.org/10.11842/wst.2014.08.008
24. Luo M, Xu G, Ren XY et al (2018) Research progress on identification methods of *Pinellia ternata* and its adulterants. LISHIZHEN Med Mater Med Res 29:2739–2741. https://doi.org/10.3969/j.issn.1008-0805.2018.11.066
25. Jing Y, Lai YY, Chen H et al (2019) Study on the identification of Pinelliae rhizoma and Pinelliae pedatisectae rhizoma based on the characteristic component triglochinic acid. RSC Adv 9:11774–11780. https://doi.org/10.1039/c9ra01626k
26. Zheng DS, Zhang JY, Guo QS (2013) cpDNA non-coding sequence analysis of *Pinellia ternata* and its related species. Chin Tradit Herb Drugs 44:881–886
27. Paradiso L, Little DP (2021) Authentication of garlic (*Allium sativum* L.) supplements using a trnL^UAA mini-barcode. Genome 64:1021–1028. https://doi.org/10.1139/gen-2021-0001
28. Xie XN, Ye HT, Cai X et al (2021) DNA mini-barcodes, a potential weapon for conservation and combating illegal trade of Pangolin. Trop Conserv Sci 14:1–10. https://doi.org/10.1177/19400829211017361

29. Lo YT, Li M, Shaw PC (2015) Identification of constituent herbs in ginseng decoctions by DNA markers. Chin Med 10:1. https://doi.org/10.1186/s13020-015-0029-x

30. Shokralla S, Hellberg RS, Handy SM et al (2015) A DNA mini-barcoding system for authentication of processed fish products. Sci Rep 5:15894. https://doi.org/10.1038/srep15894

31. Wang XY, Liu Y, Wang LL et al (2016) A nucleotide signature for the identification of Angelicae Sinensis Radix (Danggui) and its products. Sci Rep 6:34940. https://doi.org/10.1038/srep34940

32. Keller A, Schleicher T, Schultz J et al (2009) 5.8S-28S rRNA interaction and HMM-based ITS2 annotation. Gene 430:50–57. https://doi.org/10.1016/j.gene.2008.10.012

33. Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054

34. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797. https://doi.org/10.1093/nar/gkh340

35. Li JY, Xu RC, Wang PF, Lin YJ (2015) Identification study of Rhizoma Pinellia, adulterate Rhizoma Pinellia and Rhizoma Typhonii Flagelliformis. Asia Pac Tradit Med 11:23–26. https://doi.org/10.11954/ytctyy.201524010

36. Jiang W, Ren L, Guo M et al (2019) Detecting *Schisandrae Chinensis Fructus* and its Chinese patent medicines with a nucleotide signature. Genes 10:397. https://doi.org/10.3390/genes10050397

37. Postollec F, Falentin H, Pavan S et al (2011) Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiol 28:848–861. https://doi.org/10.1016/j.fm.2011.02.008

38. Parveen I, Gafner S, Techen N et al (2016) DNA barcoding for the identification of botanicals in herbal medicine and dietary supplements: strengths and limitations. Planta Med 82:1225–1235. https://doi.org/10.1055/s-0042-111208

39. Pawar RS, Handy SM, Cheng R et al (2017) Assessment of the authenticity of herbal dietary supplements: comparison of chemical and DNA barcoding methods. Planta Med 83:921–936. https://doi.org/10.1055/s-0043-107881

40. Ivanova NV, Kuzmina ML, Braukmann TW et al (2016) Authentication of herbal supplements using next-generation sequencing. PLoS ONE 11:e0156426. https://doi.org/10.1371/journal.pone.0156426

41. Lanner J, Curto M, Pachinger B et al (2019) Illumina midi-barcodes: quality proof and applications. Mitochondrial DNA A 30:490–499. https://doi.org/10.1080/24701394.2018.1551386

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.