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Full Length Research Paper

Rapid identification of the medicinal plant *Taraxacum formosanum* and distinguishing of this plant from its adulterants by ribosomal DNA internal transcribed spacer (ITS) based DNA barcode

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Original identification of medicinal plants is essential for quality control. In this study, the internal transcribed spacer 2 (ITS2) nuclear ribosomal DNA served as a DNA barcode and was amplified by allele-specific PCR. This approach was exploited to differentiate *Taraxacum formosanum* from five related adulterants. Using a set of designed PCR primers, a highly specific 223 bp PCR product of *T. formosanum* was successfully amplified by PCR. However, no similar DNA fragment was amplified from any of the other adulterants. This indicates that, our allele specific primers have high specificity and can accurately discriminate *T. formosanum* from its adulterant plants.

Key words: Medicinal plant, polymerase chain reaction (PCR), authentication, *Taraxacum formosanum*, traditional Chinese medicinal, internal transcribed spacers 2 (ITS2).

INTRODUCTION

Taraxacum formosanum (TF) kitamura is a traditional Chinese medicine used in Taiwan and China. A whole plant extract of *Taraxacum*, commonly also known as herbal tea, is a popular folk drink and has a number of functional ingredients. It is traditionally used by herbalists and doctors for the treatment of boils, sores, inflammation of the eye, urethral infection, lung and breast abscesses, acute appendicitis and jaundice (Li et al., 1992). *Taraxacum* has been found to have antibacterial, antifungal, antileptospiral and antiviral effects, all of which have been reported by previous studies (Li et al., 1992; Ou et al., 1994; Zhao et al., 2006).

Taraxacum officinale, *Ixeridium laevigatum*, *Youngia japonica*, *Ixeris chinensis* and *Emilia sonchifolia* var. *javanica* are very commonly misused in place of *T. formosanum,* because they have very similar morphological characteristics or because some of the key diagnostic features have been removed during processing. Especially, there is a high risk of exchange of TF specimens with its adulterants in the herbal medicine market (Cao et al., 1997; Tong et al., 1999; Yuan et al., 2001; Zhao et al., 2006). A histological approach using microscopy together with chemical profiling by high pressure liquid

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Abbreviations: PCR, Polymerase chain reaction; **ITS**, internal transcribed spacer; **HPLC**, pressure liquid chromatography; **RAPD**, random amplified polymorphic DNA; **PCR-RFLP**, polymerase chain reaction-restriction fragment length polymorphism; **AFLP**, amplified fragment length polymorphism.

chromatography (HPLC) is often used to authenticate TF and identify its adulterants and these approaches have been documented in a previous study (Committee of Chinese Medicine and Pharmacy, 2002). However, environmental and other factors such as growth, harvest time and storage condition can affect the morphological features and chemical composition of *T. formosanum*. This can result in difficulties when discriminating the various species (Zhang et al., 2007). Various DNA based methods for plant identification have been developed, for instance, random amplified polymorphic DNA (RAPD) analysis, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), amplified fragment length polymorphism (AFLP) and authentication by sequencing of ITS regions (Zhang et al., 2001; Ha et al., 2002; Ding et al., 2002; Kaundun et al., 2003). Identification at a DNA level has a number of advantages. These include higher effectivity, sensitivity, reliability and easy standardization when compared with other more conventional methods.

DNA barcoding using a highly variable DNA region is a powerful technique when employed to distinguish organisms or species (Chaseb et al., 2005). The ITS of nuclear ribosomal DNA (nrDNA) and some non-coding or coding regions of plastid genomes such as *rbc*L, *mat*K, *rop*C1, *ycf*5 and *psb*A-*trn*H have been widely used as barcode candidates for plant species identification (Hon et al., 2003; Kress et al., 2005; Pang et al., 2010). Up to this day, a few molecular DNA techniques, such as RAPD analysis and PCR-RFLP have been used to identify or authenticate TF and these have allowed this plant to be distinguished from its common misused adulterants (Cao et al., 1997; Yuan et al., 2007). Nonetheless, the RADP and RFLP approaches still have some disadvantages, such as their low reproducibility and a complex and time-consuming procedure.

The aim of this study was to employ the highly specific ITS regions of TF as a DNA barcode for developing an allele-specific primer set that would allow the rapid identification of TF from its adulterants. This successfully established ITS-based PCR approach is more convenient than other molecular methods and using this approach will make it feasible to create an identification kit for TF and its adulterants in the future. The DNA barcoding methodology described in this study is the first available for the identification of TF.

MATERIALS AND METHODS

Plant source

Plants samples of *T. formosanum*, *T. officinale*, *I. laevigatum*, *Y. japonica*, *I. chinensis* and *E. sonchifolia* var. *javanica*, were collected from the China Medical University (CMU) medicinal plant garden (Taichung, Taiwan) and were identified by Associate Professor Chao-Lin Kuo of the CMU. The plant voucher specimens were deposited at the School of Chinese Medicine Resources (SCMR) of CMU.

DNA extraction from plants

Dried leaves of *T. formosanum*, *T. officinale*, *I. laevigatum*, *Y. japonica*, *I. chinensis* and *E. sonchifolia* var. *javanica*, were collected and ground with liquid nitrogen into powder. Total DNA from each plant was extracted from the homogenized plant tissue by the Geneaid genomic extraction mini kit (Geneaid, Taipei, Taiwan) according to the manufacturer's instructions (Huang et al., 2010). The concentration of total plant DNA obtained was determined by spectrophotometer (NanoVue™, GE Healthcare, USA), and then the samples were stored at -20°C until required.

Internal transcribed spacer (ITS) sequence alignment and design of the primers

For sequence alignment, the ITS regions of *T. formosanum*, *T. officinale*, *I. laevigatum*, *Y. japonica*, *I. chinensis* and *E. sonchifolia* var. *javanica*, were identified by BLAST and subjected to multiple-wised sequence alignment. The GenBank accession numbers of *T. formosanum*, *T. officinale*, *I. laevigatum*, *Y. japonica*, *I. chinensis* and *E. sonchifolia* var. *javanica* used in this study were AY862577, AY862576, AY862582, AY862580, AY862578 and EU057987, respectively. Sequence alignment was carried out by Clustal W2 (http://www.ebi.ac.uk/clustalw/index.html) (Li, 2003). Oligonucleotide primers for PCR, F3-TF (5'-GCGTCGCCCCCCATCATAG-3') and B3-TF (5'-GGTCGAAGCATCATCCTAAG-3'), were designed based on the intraspecific variation and interspecific divergence of the aligned sequence of the ITS2 within *T. formosanum* and its adulterants. The sequence and the target positions of the primers are shown in Figure 1.

Determination of intraspecific variation of internal transcribed spacer (ITS)

The intraspecific variation was determined by calculating the ratio of numbers of aligned different nucleotides base pairs to total aligned nucleotide base pairs within ITS1 and ITS2, respectively.

Polymerase chain reaction (PCR) amplification and sequencing

In total, 50 ng of genomic DNA of *T. formosanum* were used as template in PCR. The PCR was carried out in a 25 µl reaction mixture containing 1× Pro-taq buffer (10 mM Tri-HCl, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100, pH 9.0), 0.4 mM dNTPs, 5 pmole each of F3-TF and B3-TF primers, 1U Pro-taq™ DNA polymerase (Protech, Taiwan). The conditions used for PCR was 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min and a final extension cycle at 72°C for 10 min. The PCR product was separated by 2% agarose gel electrophoresis and detected by observation of the presence of visible DNA bands after ethidium bromide staining. Using 300 ng of amplified PCR product as insert DNA and 50 ng of pGEM™-T vector, the PCR amplified was DNA TA cloned according to the manufacturer's instructions (Promega, USA) using T4 DNA ligase at 4°C overnight. The ligation mixture was then transformed into competent *Escherichia coli* Top10 cells for subsequent plasmid purification and DNA sequencing.

Sensitivity and specificity of the polymerase chain reaction (PCR) amplification

To determine PCR sensitivity, different amounts of extracted genomic DNA were used as template for the PCR reaction in the

Figure 1. Species-specific primers designed for identifying *T. formosanum* based on the nuclear ribosomal DNA (nrDNA) ITS2 region sequences among six plant species. (A) Forward primer F3-TF; (B) backward primer B3-TF; (*) represents aligned nucleotide that are identical to upper sequence; (-) represents a gap in the aligned sequence; accession numbers of GenBank EF114672, AY862576, AY862582, AY862580, AY862578 and EU057987 represents *T. formosanum*, *T. officinale*, *I. laevigatum*, *Y. japonica*, *I. chinensis* and *E. sonchifolia* var. *javanica*, respectively.

presence of the F3-TF and B3-TF primers. The specificity of the PCR using the F3-TF and B3-TF primers was examined using extracted genomic DNA from *T. officinale*, *I. laevigatum*, *Y. japonica*, *I. chinensis* and *E. sonchifolia* var. *javanica* as the negative controls.

RESULTS

Analysis of internal transcribed spacer (ITS2) sequence alignment

To design the allele-specific primers for identifying TF, the ITS regions of the nrDNA of TF and its adulterants, *T. officinale* (TO), *I. laevigatum* (IL), *Y. japonica* (YJ), *I. chinensis* (IC) and *E. sonchifolia* var. *javanica* (ES) available from the GenBank were collected and aligned. The ITS sequences of one TF sample (GenBank accession no: AY862577), four TO samples (GenBank accession no: AY862583, AY862576, AY548211 and L48337), two IL samples (GenBank accession no: EU363588, AY862582), two YJ samples (GenBank accession no: AY862580, AJ633294), two IC samples (GenBank accession no: EU363587, AY862578) and one ES samples (GenBank accession no: GQ434537, FJ980356, EU057987 and EF108405) were used to determine the species-specific conservative DNA region. Based on the interspecies and intraspecies sequence variation of ITS2, the allele-specific primers for TF, F3-TF and B3-TF, were designed using NCBI primer blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Figure 1).

Development of allele specific primers for authentication of *T. formosanum*

To examine the primer specificity for TF, extracted genomic DNA of TF and its adulterants were individually used as template to perform the species-specific PCR for

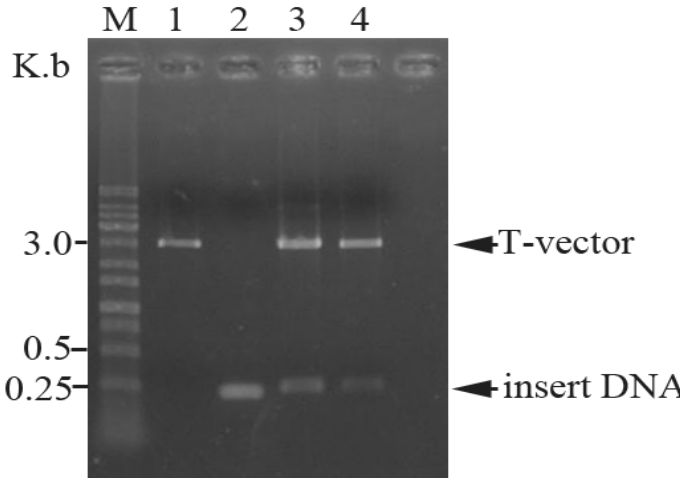

Figure 2. Agarose gel electrophoresis of PCR products from species-specific primer amplification using F2-TF and B3-TF, which were designed for identifying *T. formosanum* based on its nuclear ribosomal DNA (nrDNA) ITS2 region sequence. Lanes 1 to 6 represents *T. formosanum*, *T. officinale*, *I. laevigatum*, *Y. japonica*, *I. chinensis* and *E. sonchifolia* var. *javanica*, respectively;lane 7, blank; M,DNA marker.

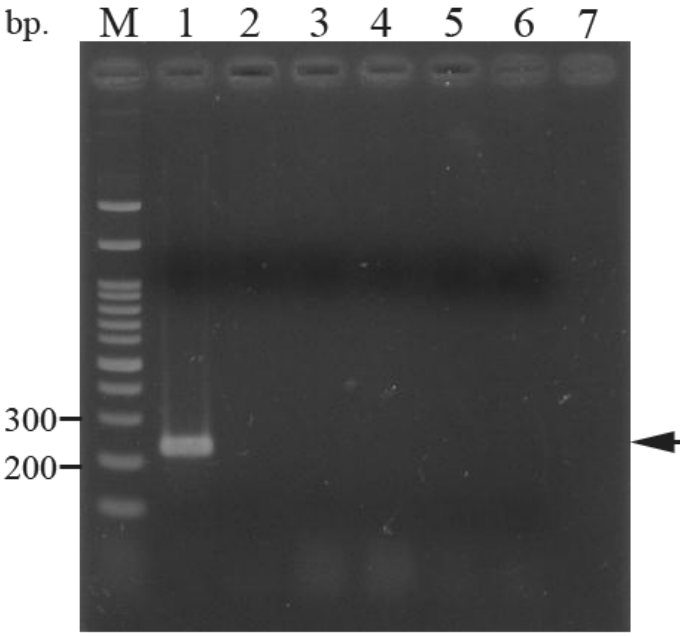

Figure 3. Cloning of the *T. formosanum* PCR amplified DNA fragment of 250 bp into pGEM-T vector. Lane M, 100 bp DNA ladder marker; lane 1, insert DNA (250 bp of DNA fragment amplified by species-specific PCR); lane 2, pGEM-T vector only; lane 3, 250 bp of insert DNA was cloned into pGEM-T vector and the recombinant plasmid digested at two sites with *EcoRI*

TF identification. As shown in Figure 2, an abundant DNA fragment of 223 bp, approximately the size amplified, was detected by agarose gel electrophoresis when genomic DNA of TF was added to the PCR reaction. In contrast,

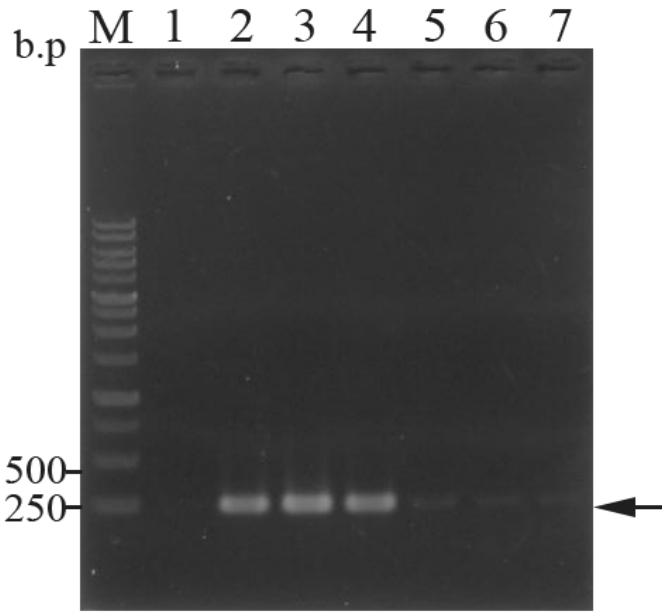

Figure 4. Determination of the PCR sensitivity using allele-specific primers. The allele-specific primers, F2-TF and B3-TF, were used with genomic DNA from *T. formosanum* for the PCR reaction. The PCR reactions were performed after adding various amounts of *T. formosanum* genomic DNA. Lane M, 100 bp DNA ladder marker; lane 1, negative control; lane 2 to 7 were amplified with 100 ng, 20 ng, 1 ng, 100, 10 and 1 pg of extracted genomic DNA, respectively.

PCR reactions involving genomic DNA template of the adulterants, *T. officinale*, *I. laevigatum*, *Y. japonica*, *I. chinensis* and *E. sonchifolia* var. *javanica*, did not result in any PCR product. These results indicate that the PCR primers designed for TF are suitable for amplifying the target nucleic acid of TF and are able to discriminate between TF and its adulterants. To further confirm the identity of the amplified PCR product in the TF sample, the PCR product was cloned and sequenced. As shown in Figure 3, the PCR product cloned into pGEM-T vector gave a plasmid, approximately 3.25 kb in size. After sequencing, the obtained sequence of the PCR product was 99.5% identical to the ITS2 sequence of TF (GenBank accession no: AY862577), which confirms that the species-specific PCR specifically detects TF.

Sensitivity of PCR for authentication of *T. formosanum*

To evaluate the sensitivity of the PCR method for authenticating TF genomic DNA, extracted TF genomic DNA was serially diluted 10-fold and used as template DNA. Genomic DNA amounts ranging from 1 ng to 1 fg were used in the PCR reaction. After performing the PCR reaction, the 223 bp of PCR product was detected over the DNA range 1 ng to 1 pg as shown in Figure 4. No PCR product was detected by DNA electrophoresis when less than 1 pg of template DNA was added to the PCR

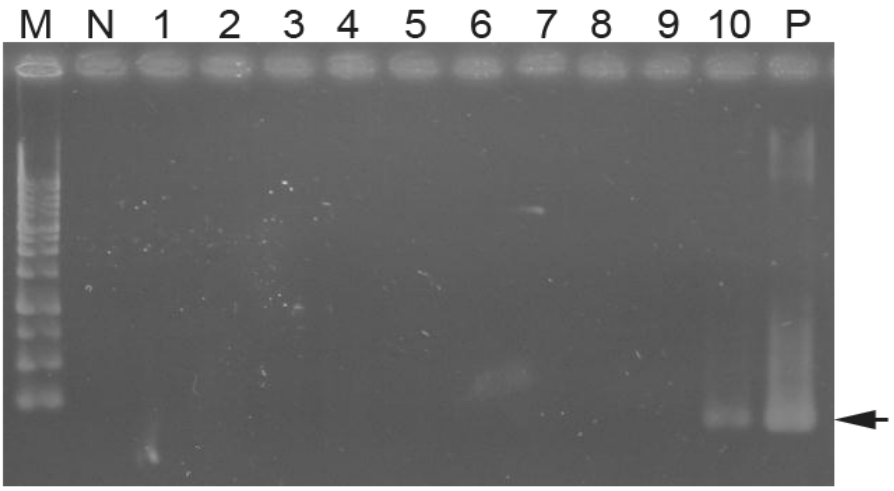

Figure 5. Species-specific PCR for identifying *T. formosanum* among 10 collected commercial plant species. Lane M, 100 bp DNA ladder marker; lanes 1 to 10 PCR amplification products using DNA extracted from 10 collected commercial samples that were reputed to be *T. formosanum*; lane N, negative control; lane P, positive control.

reaction. Therefore, the sensitivity of species-specific PCR for authenticating TF was determined to be 1 pg. These results suggested that, this newly established PCR assay using allele-specific primers is both highly specific and sensitive when authenticating TF genomic DNA from its common misused adulterants.

Application of PCR to the authentication of *T. formosanum* from an herbal market

Based on the stated results, we applied this species-specific PCR in practice by examining 10 commercially obtained TF plants purchased from different herbal markets in Taiwan. After DNA extraction and performing the PCR reaction, the desired PCR product was amplified from one of 10 crude samples of TF plant (Figure 5). These results indicate that most of the commercial specimens of TF were significantly adulterated; that the PCR assay for identifying TF material works well and shows high selectivity; that this approach is able to amplify any species-specific DNA present; and that when TF was mixed with its adulterants, this specific PCR product of TF could be detected.

DISCUSSION

In this study, we have successfully developed an allele-specific PCR method for sensitive and specific identification of TF. Herein, the allele-specific PCR primer was designed based on the specific DNA region of ITS2 of TF. The results of sequence alignment revealed that, the ITS2 of TF showed higher interspecies variation than intraspecies variation. However, ITS1 did not show significantly different sequence divergence across the *Taraxacum* species between ITS1 (4.70%) and ITS2 (4.88%). Therefore, the ITS2 sequence was chosen and the conservative DNA regions of ITS2 were aligned and are presented in Figure 1. The interspecies variation of ITS2 among TF and its adulterants ranged from 27.02 to 59.45%, which is very useful when designing allele-specific primers.

After establishing the allele-specific PCR method, it was also showed to be applied in practice for identifying TF medicinal plant. Thus, the approach employed in this work was proved to be both sensitive and specific in the further studies (data not shown). The length of the ITS regions seems to vary with the medicinal plants sample and, if necessary, can be sequenced during the process of identification. The length of the ITS1-5.8S rDNA-ITS2 fragments ranged from 550 to 850 bp (Hon et al., 2003; Kress et al., 2005; Pang et al., 2010). Direct sequencing of the ITS regions is frequently employed when these regions are amplified by PCR with various universal primer sets. However, a lack of truly universal primers for amplification of ITS regions from plants has limited their usefulness when this methodology is applied to species identification (Kress et al., 2005). In contrast, using suitable target DNA sequence information from the GenBank, it is possible to examine the interspecies and intraspecies variation of specimens and this information can be used to design species-specific primers. In this study, the newly established species-specific PCR for medicinal identification is highly effective, simple and quite easy to apply and this will allow more effective high-throughput authentication than directly sequencing. Additionally, widely used traditional Chinese medicine materials are usually processed into dried slices for long-term storage or transportation. DNA degradation is

common during this process. The presented species-specific PCR should be able to overcome the problems associated with this processing, because in the future the length of amplified PCR product can be adjusted during primer designing to increase the probability of the PCR succeeding. Other advantages of this approach are its time-effectiveness and lower cost compared with the other methods such as DNA sequencing. Based on the stated findings, it would seem that, this established PCR assay should have worldwide applications in medicinal plant identification (Xue et al., 2006; Li et al., 2007).

In conclusion, this investigation has successfully developed a species-specific PCR system for identifying TF that is rapid, cost-effective and highly specific. It targets the DNA barcode of ITS2 and distinguishes TF from its adulterants. It is an easy, useful and standard method that can be developed for use with many herbal ingredients. Therefore, in the future, species-specific PCR should become a powerful tool for quality control for the authentication of herbal plants in the market place.

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