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DNA barcodes for discriminating the medicinal plant *Isatis indigotica* Fort. (Cruciferae) and its adulterants

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ABSTRACT

Isatis indigotica Fort. (Cruciferae) is a biennial medicinal plant. In order to protect the decreasing natural genetic resources of *I. indigotica*, three candidate DNA barcodes (ITS2, *trnL-F* and *rbc*L) were employed to establish an accurate and effective identification system for *I. indigotica*. The results demonstrated that all three candidate DNA barcodes have performed very well in *I. indigotica*. The interspecific genetic distances were obviously greater than the intraspecific distance among *I. indigotica* as indicated by ITS2, *trnL-F* and *rbc*L. Sequence alignment analysis of *I. indigotica* genotypes revealed that four SNPs (54, 108, 146 and 181 bp) located in ITS2, three (2, 30, 709 bp) in *trnL-F* and one (531 bp) in *rbc*L, respectively. UPGMA phylogenetic tree constructed from *trnL-F* and *rbc*L could allote *I. indigotica* to the correct corresponding genus, whereas *rbc*L could not distinguish *I. indigotica* from its adulterants. Meanwhile, UPGMA tree of ITS2 could accurately identify *I. indigotica* from its adulterants according to the corresponding species. Consequently, it can be concluded that ITS2 is a more suitable and accurate DNA barcode for identifying *I. Indigotica* and its adulterants than *trnL-F* and *rbc*L.

1. Introduction

Isatis indigotica Fort., a biennial herbaceous plant of the family Cruciferae, has a long history of use as a medical plant in China (Deng et al., 2008; Fortune, 1846). The root and leaf of *I. indigotica* are Isatidis Radix (Ban-lan-gen) and Isatidis Folium (Da-qing-ye), which are the famous traditional Chinese medicine (Chinese Pharmacopoeia Committee, 2010).

Approximately 79 species of *Isatis* are primarily distributed in the northern hemisphere, especially in central Europe, the Mediterranean region and the central and western Asia (Al-Shehbaz et al., 2006). There are 4 species of *Isatis* growing in China (Hsuan et al., 2009). Isatidis Radix and Isatidis Folium have long been considered as primary raw materials for many TCM and newly developed drugs, such as “Ban-lan-gen granules” (Chinese Pharmacopoeia Committee, 2010). Thus, it is not surprising that a large number of domestic and international pharmaceutical companies are deeply interested in these plants.

Due to its high market demand, overexploitation in the past decades caused a rapid decline in the natural resource of *I. indigotica*, hence the roots and leaves of other species in the genus *Isatis*, including *Isatis minima* and *Isatis oblongata* are commonly treated as the adulterants of Isatidis Radix or Isatidis Folium to be used as medicine (Hao et al., 2013), which may result in a series of inconsistent therapeutic effects and quality problems in the herbal medicine industry. Accurate identification plays an important role in the first key step for developing quality security assurance measures for TCM. It is difficult to identify the overly similar phenotypic characteristics based on morphology, and the result is not highly accurate. Furthermore, assessing relationships based solely on morphology is very likely to lead to erroneous conclusions (Moazzeni

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et al., 2007; Stoker, 1997), while cytological identification is overly dependent on the availability of experts and requires a great deal of time and labor (Al-Shehbaz et al., 2006). Therefore, the development of accurate and reliable method for the identification *I. indigotica* appears to be extremely urgent.

Recently, DNA barcoding technology has attracted much attention as a burgeoning molecular detection technology (CBOL Plant Working Group, 2009) that uses a short standardized DNA region to discriminate plant species (Chen et al., 2010; Shiran et al., 2014; Yang et al., 2012). This technique has been applied for the authentication of medicinal herbs of TCM and some candidate regions were proposed (Albach et al., 2007; Guo et al., 2011; Kress and Erickson, 2008). This study for the first time provided a reliable and effective method for the identification of *I. indigotica* and its adulterants through sequencing and analyzing the three candidate DNA barcodes from the chloroplast genome: the *trnL* intron and *trnL-F* spacer (*trnL-F*), the ribulose-1,4-bisphosphate carboxylase large subunit gene (*rbc*L) and the second internal transcribed spacer (ITS2) of nuclear ribosomal DNA.

2. Materials and methods

2.1. Plant materials

The *I. indigotica* specimens were collected from Shanxi, Anhui, Henan, Hebei, Inner Mongolia, Gansu, Yunnan and Xinjiang provinces, while *I. minima* and *I. oblongata* were collected in Altay of Xinjiang provinces (Table 1). Silica gel-dried leaves were obtained from 5 individuals from each population. The species were identified by Professor Qiaosheng Guo of the Institute of Chinese Medicinal Materials, Nanjing Agricultural University. The voucher samples were planted in Nanjing Agricultural University.

2.2. DNA extraction

Genomic DNA was extracted from leaf samples using the modified CTAB procedure (Doyle and Doyle, 1987).

2.3. PCR amplification and DNA sequencing

The universal primer pairs (Chen et al., 2010; Taberlet et al., 1991) and polymerase chain reaction (PCR) amplification programs for the ITS2, *trnL-F* and *rbc*L sequences used in this study are provided in Table 2.

The components of the PCR amplification mixtures for the three markers were as follows: 1× buffer (Mg^{2+}), 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 U of *Taq* DNA polymerase (TaKaRa Biotechnology Co. Ltd., Dalian, China), 0.5 μM each primer and 20 ng of genomic DNA, in a total volume of 25 μL. PCR amplification of the three markers was performed with a PTC200 thermal cycler (Bio-Rad, U.S.A.). The PCR products were examined electrophoretically using 1.0% agarose gels. All PCR products were purified using the PCR DNA recovery kit UNIQ-10 (Sangon, China).

Purification of the PCR products was sequenced in both directions using the above PCR primers on an ABI 3730 DNA Sequencer (Applied Biosystems, U.S.A.) by the Beijing Genomic Institute (BGI) (China).

2.4. Sequence alignment and data analysis

The raw DNA sequences were assembled and compiled with SeqMan software (DNA STAR package; DNAStar Inc., Madison, WI, USA). To verify whether these three DNA candidate barcodes were effective competent for family or genus assignment in authenticating *I. indigotica*, we downloaded the ITS2, *trnL-F* and *rbc*L sequences of the different genera of the family Cruciferae from GenBank for analysis. Alignment of the sequences was performed with Clustal X V2.0 software (Thompson et al., 1997). Genetic distances were calculated using the Kimura-2-Parameter (K-2-P) model.

Unweighted pair group method with arithmetic mean (UPGMA) trees based on the ITS2, *trnL-F* and *rbc*L regions were constructed with MEGA5.0 (Tamura et al., 2011). Bootstrap support (BS) analyses of individual clades of 1000 replications were performed to show (Felsenstein, 1985), and gaps were treated as “missing”.

Table 1
Plant materials used in the present study.

Species	Origins	Sampling size	Voucher ID	Location
I. indigotica	Yuncheng (YC), Shanxi	5	*IIN-1*	N35°02′,E110°98′
	Bozhou (BZ), Anhui	5	*IIN-2*	N33°85′,E115°78′
	Xinxiang (XX), Henan	5	*IIN-3*	N35°30′,E113°90′
	Shijiazhuang (SJZ), Hebei	5	*IIN-4*	N38°05′,E114°52′
	Baotou (BT), Inner Mongolia	5	*IIN-5*	N40°65′,E109°83′
	Tianshui (TS), Gansu	5	*IIN-6*	N34°58′,E105°72′
	Dali (DL), Yunnan	5	*IIN-7*	N25°60′,E100°23′
	Wulumuqi (WL), Xinjiang	5	*IIN-8*	N43°82′,E87°62′
I. minima	Aletai (ALT), Xinjiang	5	*IMI*	N47°85′,E88°13′
I. oblongata	Aletai (ALT), Xinjiang	5	*IOB*	N47°70′,E86°85′