Suitable DNA Barcoding for Identification and Supervision of *Piper kadsura* in Chinese Medicine Markets

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**Abstract:** *Piper kadsura* is a vine-like medicinal plant which is widely used in clinical treatment. However, *P. kadsura* is often substituted by other materials in the markets, thereby causing health risks. In this study, 38 *P. kadsura* samples and eight sequences from GenBank, including a closely-related species and common adulterants were collected. This study aimed to identify an effective DNA barcode from four popular DNA loci for *P. kadsura* authentication. The success rates of PCR amplification, sequencing, and sequence acquisition of *matK* were 10.5%, 75%, and 7.9%, respectively; for *rbcL* they were 89.5%, 8.8%, and 7.9%, respectively; ITS2 rates were 86.8%, 3.0%, and 2.6%, respectively, while for *psbA-trnH* they were all 100%, which is much higher than for the other three loci. The sequences were aligned using Muscle, genetic distances were computed using MEGA 5.2.2, and barcoding gap was performed using TAXON DNA. Phylogenetic analysis showed that *psbA-trnH* could clearly distinguish *P. kadsura* from its closely related species and the common adulterant. *psbA-trnH* was then used to evaluate the fake proportions of *P. kadsura*. Results showed that 18.4% of *P. kadsura* samples were fake, indicating that adulterant species exist in the Chinese markets. Two-dimensional DNA barcoding imaging of *P. kadsura* was conducted, which was beneficial to the management of *P. kadsura*. We conclude that the *psbA-trnH* region is a powerful tool for *P. kadsura* identification and supervision in the current medicine markets.

**Keywords:** *Piper kadsura*; DNA barcoding; *psbA-trnH*; medicine market supervision

1. **Introduction**

*Piper kadsura* (Choisy) Ohwi is a vine-like medicinal plant found mostly in the Fujian, Zhejiang, and Guangdong provinces of China. The stem part of *P. kadsura* is a traditional medicine in China known as “haifengteng”, and is harvested in summer or autumn. According to Chinese medicinal theory, *P. kadsura* is generally used to dredge meridian, expel wind-dampness, and relieve limb pain [1]. It is also used for cooking and improving digestive function in Japan, because its fruit is similar to pepper. To date, various constituents have been isolated from *P. kadsura*, including amides, lignans, terpenes, and cyclohexanes, which possess anti-human hepatitis B virus, anti-platelet activating factor, anti-insect feeding, and anti-inflammatory activities [2,3]. *P. kadsura* has been widely used in medical treatment and has attracted considerable attention because of its many functions.
P. kadsura as sold in medicine markets is always dried or sliced, which makes the identification of its traditional morphological characteristics difficult. Given this, it is frequently replaced by other herbs in clinical treatment, causing potential medical problems, thereby eroding consumers' confidence. Several closely related species and other adulterants of P. kadsura are usually adopted as alternative herbs in many situations. *Piper wallichii*—a closely related species of *P. kadsura* that has no anti-inflammatory action—is often used as a substitute for *P. kadsura* in Zhejiang, Fujian, and Hunan provinces. Both species have the same name and are obtained in the same location. *P. kadsura* is commonly substituted by *Kadsura heteroclite* in Guangxi and Guangdong provinces, which exhibits different healing effects. The application of alternatives, to a certain extent, influences the curative effect. In extreme cases, this behavior may lead to major medical accidents. Therefore, the correct identification of *P. kadsura* is especially critical, and a practical and effective method is urgently needed.

DNA barcoding makes use of short but specific DNA tags or “barcodes”—parts of genes present in all living things—to distinguish one species from another [4]. “Barcode” is a term first coined by Hebert [5]. Many DNA barcodes have been searched. Among them are four loci—namely, *matK*, *rbcL*, *ITS2*, and *psbA-trnH*—which are the most well-known in the identification of medicinal plants. The *matK* gene of 1084 plant species was studied, and it could be used as a standard DNA barcode for flowering plants [6]. Chase et al. concluded that *rbcL* sequence variation is appropriate for phylogenetic analysis at the taxonomic level of seed plants [7]. Chen et al. concluded that *ITS2* could be a good DNA barcode for the authentication of medicinal plants and their closely related species. They also demonstrated that *psbA-trnH* is excellent for species identification [8]. Combination with the above loci for use in species authentication has been widely investigated [9–13]. In this study, we utilized DNA barcoding to discriminate *P. kadsura* species from its closely related species and common adulterants. This technique demonstrated that *psbA-trnH* is the best sequence for *P. kadsura* identification.

DNA barcoding has been increasingly used for supervision of the medicine market [14–16]. Chen et al. established a barcode system, called traditional Chinese Medicine Database, based on a combination of the *ITS2* and *psbA-trnH* barcodes [17]. The system contains 78,847 sequences belonging to 23,262 medicinal species, and covers more than 95% of the herbs in pharmacopeia, including those of China, Japan, Korea, India, the USA, and Europe. The sequences provide consumer protection and safety. In the present study, we used *psbA-trnH* to supervise commercial *P. kadsura* products in the current markets. Results indicated that adulterants are present, and that *psbA-trnH* is a powerful identification tool for *P. kadsura*. Portable equipment based on DNA barcoding technology is desired in the future.

2. Results

2.1. Success Rates of PCR Amplification, Sequencing, and Sequence Acquisition

Unsuccessful sequences require repeat experiments or PCR amplification reconstruction to ensure data reliability. The success rate of PCR amplification refers to the rate of samples with evident DNA straps in all the text samples. The success rate of sequencing is the rate of samples with high-quality sequences in all sequences. Sequence acquisition rate is the product of the success rate of PCR amplification and the success rate of sequencing. As shown in Figure 1, all three factors of four loci were investigated. Among the 38 samples, only four samples could be amplified with the *matK* locus. The amplification rates of *rbcL* and *ITS2* were 89.5% and 86.8%, respectively; however, the success rates of sequencing were 8.8% and 3.0%, respectively. We attempted to perform repeat experiments for the two loci, but the results remained unsatisfactory. Thus, the sequence acquisition rate of *ITS2* was 2.6%; *matK* and *rbcL*, 7.9%; and *psbA-trnH*, 100%. Given that the sequence acquisition rate of *psbA-trnH* was much greater than *ITS2*, *matK*, and *rbcL*, *psbA-trnH* was chosen as the DNA barcode for *P. kadsura*.
The minimum interspecific distance of 0.004 between P. kadsura and its adulterants varied from 0.004 to 0.426. The interspecific distance between P. kadsura and Actinidia chinensis was larger than the maximum intraspecific distance, proving that psbA-trnH is a useful identification tool (Table 1).

### Table 1. Genetic distances within and between species.

| K2P Genetic Distances       | Genetic Distance |
|-----------------------------|------------------|
| Intra-specific distances    | 0.000            |
| Inter-specific distances    | 0.004            |
| Inter-specific distances    | 0.318            |
| with Piper wallichii        | 0.426            |
| with AC (Actinidia chinensis) |                 |
| with Kadsura heteroclita    |                 |

2.3. Barcoding Gap Assessment

Barcoding gap is an important index to determine whether or not a DNA barcode is suitable. Barcodes should exhibit a “barcoding gap” between *intra* and *interspecific* divergences [6]. The divergence distributions were classed in 0.002 distance units to evaluate whether or not the gap exists. With psbA-trnH in the P. kadsura and its adulterants matrix, there were no overlaps, and the distributions of *intra* and *interspecific* divergence were well separated, forming an evident gap, which is a positive proof for psbA-trnH identification ability (Figure 2).

**Figure 1.** The success rates of PCR amplification, sequencing, and sequence acquisition.

**Figure 2.** Relative distribution of interspecific divergence between congeneric species and intraspecific distances for psbA-trnH locus. K2P: kimura-2-parameter.
2.4. Neighbor-Joining (NJ) Tree Identification

An NJ tree was constructed using 46 psbA-trnH sequences (Figure 3). A total of 31 P. kadsura sequences clustered into one clade with the P. kadsura sequence downloaded from GenBank, whereas its adulterants clustered into other clades. Surprisingly, adulterants were neither P. wallichii nor K. heteroclite. Seven samples from Hebei Anguo (AG), Henan Yuzhou (YZ), and Anhui Bozhou (BZ) were identified as Actinidia chinensis stem. In addition to common adulterants, other fake samples that could not be easily identified by morphology were also present. Thus, DNA barcoding is necessary for authentication. We conclude that the NJ tree could effectively identify P. kadsura from its adulterants, including its closely related species P. wallichii, which further indicates that psbA-trnH is a suitable DNA barcode for P. kadsura identification.

Figure 3. Phylogenetic analysis of psbA-trnH regions for P. kadsura and its adulterants.

2.5. Proportions of Adulterant Species

Product adulteration and ingredient substitution are common as species of a lower market price are used to replace those of a higher price. The frequency of product mislabeling in herbal markets is estimated at 14% to 33% [18]. In this study, 38 psbA-trnH sequences were searched to
evaluate adulterant proportions of *P. kadsura* in the current markets. The analysis of *psbA-trnH* region identification showed that 18.4% of samples were different from their commercial names. No fake *P. kadsura* samples were found in Hunan Shaoyang (SY), Heilongjiang Haerbin (HB), or Shanxi Xian (XA). The fake sample rates for AG and BZ were 18.2% and 20%, respectively, whereas YZ obtained the highest rate at approximately 50% (Figure 4). The existence of adulterants poses health risks and seriously delays the development of traditional medicine.

![Figure 4](image_url) The adulterant rate observed for 38 *P. kadsura* samples.

### 2.6. DNA Barcoding and Two-Dimensional DNA Barcoding Image

DNA barcoding technology aims to identify species, similar to the barcoding of goods in the supermarket. Based on the written code and open-source PHP QR Code coding method [19], the *psbA-trnH* sequences of *P. kadsura* were converted into two-dimensional DNA barcoding images (Figure 5). Two-dimensional DNA barcoding is the combination of two-dimensional technique and DNA barcoding, which is beneficial for the information conversion of DNA barcoding. Different colors represent different nucleotides, and numbers represent the length of the sequence. The sequence of *P. kadsura* can be obtained by scanning the two-dimensional code of the mobile terminal and then sending this sequence to a DNA barcoding database for identification, which makes identification more convenient and quick and facilitates the management of *P. kadsura*. This method can be applied to the identification of other medicinal herbs, to provide a new technical means for the protection of clinical safety.

![Figure 5](image_url) DNA barcoding and two-dimensional DNA barcoding image of *psbA-trnH* sequences for *P. kadsura* (A-T-C-G).

### 3. Discussion

#### 3.1. Efficacy of *psbA-trnH* for Identification

The non-coding *psbA-trnH* spacer is the most variable plastid region in angiosperm, and it remains the leading candidate for plant DNA barcoding. The *psbA-trnH* spacer satisfies the criteria deemed appropriate for a plant barcode, including short length (often <500 bp) that allows for easy DNA extraction and amplification, high interspecific variability and divergence, and universal flanking
primers [20–22]. The psbA-trnH intergenic spacer region could be used as a barcode to distinguish various Dendrobium species and differentiate Dendrobium species from other adulterating species [23]. Kress et al. indicated that the rate of PCR success of psbA-trnH with standard primers is 95.8% (46 of 48 genera), which is the highest in the nine loci searched [9]. The psbA-trnH region ranked first in divergence value in six of the eight genera, and in 11 of the 14 species pairs, compared with the other eight plastid regions [24]. Moreover, psbA-trnH alone showed the highest rate of identification (90%), much higher than rbcLa and matK [25]. Ma et al. concluded that psbA-trnH is a powerful DNA marker for medicinal Pteridophytes identification [26]. In the present study, the psbA-trnH region showed the highest success rates of PCR amplification and sequencing, as well as sequence acquisition rate in P. kadsura, which was far higher than the other three loci. The inter-specific divergence of psbA-trnH was much greater than intra-specific, creating an evident barcoding gap. In addition, the psbA-trnH sequence could clearly identify P. kadsura from its adulterants, including closely related species, according to NJ tree description. The two-dimensional DNA barcoding image of psbA-trnH sequences for P. kadsura also verify the importance of DNA barcoding. Our research demonstrated that psbA-trnH is a useful tool for the identification of P. kadsura.

3.2. Opportunities and Challenges of DNA Barcoding in Medicine Markets

DNA barcoding is a novel system designed to provide rapid, accurate, and automated species identification by using short, standardized gene regions as internal species tags [27]. In recent years, DNA barcoding has played an increasingly significant role in the identification of Chinese medicines. Several studies were conducted to apply DNA barcoding for supervision in medicine markets. Han et al. used barcoding to investigate the proportions and varieties of adulterant species in traditional Chinese medicine (TCM) markets. A total of 1436 samples representing 295 medicinal species from seven primary TCM markets were supervised. The results showed that of the successfully generated samples, approximately 4.2% were adulterants. They suggest that a DNA barcode platform should be established for TCM market investigation [28]. Zhao et al. indicated that the ITS2 barcode could effectively identify Acanthopanax cortex, and DNA barcoding is a convenient tool for medicine market investigation [29]. In the current study, 18.4% of P. kadsura samples were not genuine. Uncommon adulterants, such as A. chinensis, also existed. Three of the six markets were identified with fake medical materials. Certainly, psbA-trnH is a favorable control monitor for P. kadsura quality. Thus, the current TCM markets should be regulated through a DNA barcoding method, considering the consumer’s trust and clinical safety.

However, many aspects were considered in the determination of medicine quality. For instance, in the pharmacopoeia of China, medicinal effective ingredients are an important index. Whether the herbs that were identified as genuine using DNA barcoding meet the qualification of medicinal effective ingredients is also a significant topic. In addition, morphological methods still play a fundamental role in medicine identification. Thus, morphological and chemical information is necessary to integrate DNA barcoding and achieve maximum efficiency for medicinal material identification [28].

4. Experimental Section

4.1. Plant Materials

Many medical materials are dried in medicine markets. To determine the efficacy of DNA barcoding identification, we collected 38 dried P. kadsura samples from six approved national herbal medicine markets from six provinces in China, including 11 samples from Hebei Anguo (AG), 5 samples from Hunan Shaoyang (SY), 4 samples from Heilongjiang Haerbin (HB), 8 samples from Henan Yuzhou (YZ), 5 samples from Shanxi Xian (XA), and 5 samples from Anhui Bozhou (BZ) (Table 2), which could reflect P. kadsura quality condition in the current market. All 38 specimens were deposited in Beijing Forestry University. One P. kadsura sequence, two P. wallichii sequences, and five K. heteroclite sequences were also downloaded from GenBank (Table 3).
Table 2. The list of 38 samples used in this study. AG: Hebei Anguo; BZ: Anhui Bozhou; HB: Heilongjiang Haerbin; SY: Hunan Shaoyang; XA: Shanxi Xian; YZ: Henan Yuzhou.

| Sl No. | Latin Name | Place of Collection | Sample No. | Identification Result |
|--------|------------|---------------------|------------|-----------------------|
| 1      | *P. kadsura* | AG                  | HF001AG01  | Genuine               |
| 2      | *P. kadsura* | AG                  | HF002AG02  | Genuine               |
| 3      | *P. kadsura* | AG                  | HF003AG03  | Genuine               |
| 4      | *P. kadsura* | AG                  | HF004AG04  | Genuine               |
| 5      | *P. kadsura* | AG                  | HF005AG05  | Genuine               |
| 6      | *P. kadsura* | AG                  | HF006AG06  | Genuine               |
| 7      | *P. kadsura* | AG                  | HF007AG07  | Fake                  |
| 8      | *P. kadsura* | AG                  | HF008AG08  | Genuine               |
| 9      | *P. kadsura* | AG                  | HF009AG09  | Fake                  |
| 10     | *P. kadsura* | AG                  | HF010AG10  | Genuine               |
| 11     | *P. kadsura* | AG                  | HF011AG11  | Genuine               |
| 12     | *P. kadsura* | SY                  | HF012SY01  | Genuine               |
| 13     | *P. kadsura* | SY                  | HF013SY02  | Genuine               |
| 14     | *P. kadsura* | SY                  | HF014SY03  | Genuine               |
| 15     | *P. kadsura* | SY                  | HF015SY04  | Genuine               |
| 16     | *P. kadsura* | SY                  | HF016SY05  | Genuine               |
| 17     | *P. kadsura* | HB                  | HF017HB01  | Genuine               |
| 18     | *P. kadsura* | HB                  | HF018HB02  | Genuine               |
| 19     | *P. kadsura* | HB                  | HF019HB03  | Genuine               |
| 20     | *P. kadsura* | HB                  | HF020HB04  | Genuine               |
| 21     | *P. kadsura* | YZ                  | HF021YZ01  | Fake                  |
| 22     | *P. kadsura* | YZ                  | HF022YZ02  | Fake                  |
| 23     | *P. kadsura* | YZ                  | HF023YZ03  | Fake                  |
| 24     | *P. kadsura* | YZ                  | HF024YZ04  | Genuine               |
| 25     | *P. kadsura* | YZ                  | HF025YZ05  | Genuine               |
| 26     | *P. kadsura* | YZ                  | HF026YZ06  | Genuine               |
| 27     | *P. kadsura* | YZ                  | HF027YZ07  | Genuine               |
| 28     | *P. kadsura* | YZ                  | HF028YZ08  | Fake                  |
| 29     | *P. kadsura* | XA                  | HF029XA01  | Genuine               |
| 30     | *P. kadsura* | XA                  | HF030XA02  | Genuine               |
| 31     | *P. kadsura* | XA                  | HF031XA03  | Genuine               |
| 32     | *P. kadsura* | XA                  | HF032XA04  | Genuine               |
| 33     | *P. kadsura* | XA                  | HF033XA05  | Genuine               |
| 34     | *P. kadsura* | BZ                  | HF034BZ01  | Genuine               |
| 35     | *P. kadsura* | BZ                  | HF035BZ02  | Genuine               |
| 36     | *P. kadsura* | BZ                  | HF036BZ03  | Genuine               |
| 37     | *P. kadsura* | BZ                  | HF037BZ04  | Genuine               |
| 38     | *P. kadsura* | BZ                  | HF038BZ05  | Fake                  |

Table 3. The list of accessions downloaded from GenBank.

| No. | Latin Name | Genus Name | Genebank No. |
|-----|------------|------------|--------------|
| 1   | *P. kadsura* | *Piper* Linn. | AB331285.1 |
| 2   | *P. kadsura* | *Piper* Linn. | KM055222.1 |
| 3   | *P. wallichii* | *Piper* Linn. | KM055221.1 |
| 4   | *K. heteroclita* | *Kadsura* Kaempf. ex Juss. | KP690026.1 |
| 5   | *K. heteroclita* | *Kadsura* Kaempf. ex Juss. | KP690025.1 |
| 6   | *K. heteroclita* | *Kadsura* Kaempf. ex Juss. | KP690024.1 |
| 7   | *K. heteroclita* | *Kadsura* Kaempf. ex Juss. | KP690023.1 |
| 8   | *K. heteroclita* | *Kadsura* Kaempf. ex Juss. | KP690022.1 |

4.2. DNA Extraction, Amplification, and Sequencing

The samples were first scraped and then wiped with 75% ethanol to prevent fungal contamination. Total DNA extraction was achieved using a plant genomic DNA kit (Tiangen, Beijing, China),
based on the Hexadecyltrimethy Ammonium Bromide (CTAB) approach. The primers used for all four regions and the PCR conditions were as follows: ITS2: S2F, ATGCGATACTTGGTGTGAAT; S3R, GACGCTTCTCCAGACTACAAT; PCR conditions: 94 °C 5 min, 40 cycles at 94 °C 30 s, 56 °C 30 s, 72 °C 45 s, and 72 °C 10 min. \( \text{psbA-trnH:} \) fwd. PA, GTTATGCATGAACGTAATGCTC; rev. TH, CGCGCATGTTGGATTCACAATCC; PCR conditions: 94 °C 5 min, 30 cycles at 94 °C 1 min, 55 °C 1 min, 72 °C 1.5 min, and 72 °C 10 min. \( \text{matK:} \) 390F, CGATCTATTCATTCAATATTTC; 1326R, TCTAGCACACGAAAAGTCGAAGT; PCR conditions: 94 °C 5 min, 30 cycles at 94 °C 1 min, 48 °C 30 s, 72 °C 1 min, and 72 °C 10 min. \( \text{rbcL:} \) 1f, ATGTACCACAAACAGAAAC; 724r, TCGCATGTACCTGCAGTAGC; PCR conditions: 95 °C 2 min, 34 cycles at 94 °C 1 min, 55 °C 30 s, 72 °C 1 min, and 72 °C 10 min [8].

4.3. Sequence Alignment and Genetic and Phylogenetic Analyses

CodonCode Aligner 4.2.7 (CodonCode Co., Centerville, MA, USA) was used to trim and assemble raw trace files. BLAST analysis was performed using the nucleotide database at National Center for Biotechnology Information (NCBI). Intra- and inter-species genetic distances were obtained with the kimura-2-parameter (K2P) model of MEGA 5.2.2 [30]. Intra- and inter-species pairwise divergences were calculated as a barcoding gap by using TAXON DNA [31]. A neighbor-joining (NJ) tree based on phylogenetic analysis was constructed, with 1000 replicate bootstrap tests to identify \( P. \) kadsura via MEGA 5.2.2 [30].

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

TCM: Traditional Chinese medicine
K2P: Kimura 2-Parameter
NJ: Neighbor-joining

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