Journal of Clinical and Nursing Research, 2024, Volume 8, Issue 3
http://ojs.bbwpublisher.com/index.php/JCNR
Online ISSN: 2208-3693
Print ISSN: 2208-3685

Analysis of the Authenticity of Stone Medicinal Plants by DNA Barcoding

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Abstract: The genus Pyrrosia belongs to the family Polypodiaceae and are medium-sized epiphytic ferns, where the dried leaves of *Pyrrosia lingua*, *Pyrrosia sheareri*, *Pyrrosia lanceolata*, and *Pyrrosia calvata* are commonly used in medicinal practice. In this study, the authenticity of the collected medicinal plant samples of Shiwei was identified with the help of DNA barcoding technology using the internal transcribed spacer 2 (ITS2) as the identifying sequence. The experimental samples were analyzed using the basic local alignment search tool (BLAST) and the authenticity of the samples was further verified with the results of similarity comparison. The results proved that the sequences of the experimentally collected samples of *Pyrrosia lingua*, *Pyrrosia sheareri*, *Pyrrosia lanceolata*, and *Pyrrosia calvata* had a similarity of more than 97% when compared with the corresponding sequences that were uploaded on the Internet.

Keywords: Polypodiaceae plants; DNA barcoding technology; ITS2

Online publication: March 29, 2024

1. Introduction

Pyrrosia is a stone reed belonging to the water keel family, including Lushan stone reeds, felt hair stone reeds, stalked stone reeds, Beijing stone reeds, and southwest stone reeds. The traditional methods of identifying Chinese herbal medicines are time-consuming and laborious and cannot be identified in large quantities. Hence, there is an urgent need to discover new techniques for an accurate and rapid method to verify the authenticity of medicinal plants.

To ensure the safety and effectiveness of traditional Chinese medicine (TCM) in clinical use, the DNA barcode molecular identification method ensures the accurate identification of TCM phytobenthic species [1,2]. In the pre-application stage of this technology, it was first researched and tested among many plant samples. Chen et al. [3] validated the identification technology method through several experiments. They established the universal barcode for medicinal plants as the internal transcribed spacer 2 (ITS2) sequence, based on the accurate identification results of the basal species of Maitake, Chuanxiong, and Serpentina [4–6]. These are commonly used Chinese herbs, which have been repeatedly verified through the mixing of forgeries. The development of DNA barcoding technology serves as another milestone in the scientific and technological advancement of rapid and accurate identification of medicinal plants. After all, the DNA of different medicinal plants each has its specific characteristics, hence the use of DNA barcode technology can replace the traditional

analysis and identification of Chinese herbal medicines and prevent the subjective judgment of the controversy brought about by the invention of Linnaeus after the famous researcher put forward the dual-name method of plant taxonomy has made a breakthrough in research, regarded as the "big science project" [7]. Doorduin *et al*. identified and classified 17 species of *Jacobaea vulgaris* from the Asteraceae family using barcodes of the corresponding genes in the chloroplast genome [8]. Parks *et al*. used chloroplast genome sequencing to conduct corresponding experiments on 37 samples of the genus Pinus, which not only demonstrated the phylogenetic and population genetics properties of the chloroplast genome but also proposed that the chloroplast genome could be recognized as a barcode exclusive to taxonomic attributes in plant identification [9]. Nock *et al*. also used the total DNA in plant genes as templates for identification and sequencing and used the chloroplast sequences of plants of proximate congeneric species as reference splicing templates. From this, the full sequence of the chloroplast genome gene expression was obtained, which indicated that taking the gene sequences of the reference proximate species can reduce the corresponding sequencing costs, and thus can be applied in the identification technology of proximate species [10].

This technique was applied to identify the medicinal use of a variety of medicinal plants, such as the genus Chonglou [11] and Artemisia [12], for research experiments. DNA barcode technology is based on the role of genetic information in the artificial cultivation of medicinal plant resources and species identification. This is because the artificial cultivation of TCM plant resources, the environmental conditions including biological, physical, and chemical nutrient ratios, etc. are different from the wild [13]. To some extent, these plants will exhibit different individual traits. Not only can DNA barcode technology accurately identify the species origin but also from the genetic point of view for the protection of plant resources and species [14]. For the safe use of medicines and the standardization of market regulations, DNA barcode technology is now one of the commonly used technical methods for the identification of Chinese herbal medicines, including animal-based and seed-based Chinese medicines [15,16]. There have been cases where non-legitimate pharmaceuticals were passed off as legitimate pharmaceuticals. This kind of counterfeiting behavior greatly endangers the health of consumers. Based on the principle of DNA barcode technology, Li *et al*. carried out the identification of ultrafine slices of TCM from different sources and different parts of the ingredients. They proved that this technology does not affect the identification of the ultrafine slices of TCM that have completely lost the characteristics of their fresh plant morphology [17]. It also guarantees the accuracy and outcome of the clinical use of the medicines. In the identification of the reliability of the source of each component in proprietary Chinese medicines (pCms), there is no standardized protocol in the traditional methods. Hence, researchers have utilized the application of DNA barcode technology in multi-component TCM mixing systems [18]. This technology has been studied in TCM including stomach-calming powder and Long Dan Xie Gan Wan by Zhang *et al*. [19] and Xin *et al*. [20] respectively. By using DNA barcode technology, the corresponding composition of the drugs can be detected, which illustrates that DNA barcode can be utilized for the inspection of raw materials to ensure the accuracy and effectiveness of the medicine, standardize market regulations, and promote the modernization of the TCM industry.

2. Experimental materials and instruments

2.1. Sample source

In this study, a total of 40 samples of Pyrrosia plant medicinal materials were collected, including stalked *Pyrrosia lingua*, *Pyrrosia sheareri*, *Pyrrosia lanceolata*, and *Pyrrosia calvata* in 4 batches each, from Wuxi County of Chongqing, Cangxi County of Sichuan, Luo Man Village of Fuji County of Guangxi, and Anshun Market of Guizhou, respectively. All experimental materials were freshly collected from the soil with varying origins for each batch. The samples were collected above 1,000 meters of geographic isolation and the tests

were conducted using their fresh newly-grown leaves.

2.2. Reagents and drugs

Table 1 shows the reagents and drugs used in DNA barcoding.

2.3. Instruments used

Table 2 shows the list of instruments used in this study.

Table 1. Reagents and drugs used in DNA barcoding

Reagent/Drug	Manufacturer
Kit	Beijing Tiangen Biochemical Technology Co., LTD
Trihydroxymethyl aminomethane	MDBIO Inc Company
Ethylenediaminetetraacetic acid (EDTA)	MDBIO Inc Company
Polyvinylpyrrolidone 40 (PVP40)	BIOSHARP Company
β-mercaptoethanol (BME)	AMRESCO Company
Spanish agarose, Biowest Agarose	Original package imported from Spain
Nucleic acid dye, Gelview	Beijing Shengtech Biotechnology Co., Ltd
DM2000 DNA marker	Beijing Kangwei Century Biotechnology Co., LTD
Boric acid; saline sedative; Homberg's sedative salt; boracic acid	Sinopharm Group Chemical Reagent Co., LTD
Chloroform; trichloromethane; formyl trichloride	Sinopharm Group Chemical Reagent Co., LTD
Iso-amyl alcohol	Sinopharm Group Chemical Reagent Co., LTD
Avantin	Sinopharm Group Chemical Reagent Co., LTD
Absolute ethyl alcohol	Sinopharm Group Chemical Reagent Co., LTD
Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA2H_2O$)	Sinopharm Group Chemical Reagent Co., LTD
Sodium hydroxide (NaOH)	Sinopharm Group Chemical Reagent Co., LTD
Sodium chloride (NaCl)	Sinopharm Group Chemical Reagent Co., LTD
Primer dry powder	Shanghai Shenggong Biological Engineering Co., Ltd
Polymerase chain reaction (PCR) mix	Beijing Edlai Biotechnology Co., LTD

Table 2. List of instruments used for DNA barcoding

Instrument name and model number	Manufacturer
One in 100,000 electronic balance	BT125D
Scientz-48 high-throughput tissue grinder	Ningbo Xinzhi Biotechnology Co., LTD
Liquid transfer gun	Dalong Xingchuang Experimental Instrument Co., LTD
HH-2 digital display constant temperature water bath pot	Changzhou Aohua Instrument Co., LTD
D2012 High-speed centrifuge	SeroCzech United States
A DH-II DNA mixing instrument	Ningbo Xinzhi Biotechnology Co., LTD
DYY-6C electrophoresis cell	Beijing Liuyi Instrument Factory
GL-3120 UV detection lamp	Jiangsu Haimen Qinbel Instrument Manufacturing Co., LTD
Nucleic acid dye, Gelview	Beijing Shengtech Biotechnology Co., Ltd
Applied Biosystems 2720 thermal cycler	Thermo Fisher Scientific
BCD-183KF1 refrigerator	TCL company

2.4. Empirical method

2.4.1. Extraction of total DNA

2.4.1.1. Composition of the SDS lysate solution

The sodium dodecyl sulfate (SDS) lysate solution comprised of 1.5% SDS, 1 mol/L Tris-HCl (pH 8.0), 50 mmol/L EDTA, and 500 mmol/L NaCl. The Tris-EDTA (TE) buffer (pH 8.0) comprised of 10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L EDTA-Na (pH 8.0). The preparation of the reagents and extraction of total DNA was carried out as follows.

(1) One mol/L Tris-HCl (pH 8.0): 60.5 g of Tris-HCl was weighed and placed in a measuring flask containing 200 mL of double distilled water and dissolved completely. The pH was adjusted to 8.0 with concentrated HCL. The solution was then condensed to 500ml.

(2) 50 mmol/L EDTA (pH 8.0): 93.06 g of EDTA-Na was weighed and placed in a measuring flask containing 200 mL of double distilled water and dissolved completely. The pH was adjusted to 8.0 by adding NaOH powder, and finally, the volume was fixed to 500 mL.

(3) The DNA of *Pterocarpus indicus* was extracted from 500 mg of the collected leaves of stone weed plants and placed in 5 mL Eppendorf (EP) tubes with corresponding numbers. All sampling tools were sterilized with 75% ethanol. The leaves were washed with 70% ethanol, rinsed well with double distilled water repeatedly, and placed on a high throughput tissue grinder set at 50 Hz for 120 s.

(4) After grinding, 2 mL of SDS lysate was added to the sample tube and mixed using a mixer for 3 min, then centrifuged at 10,000 r/min for 10 min. The supernatant was discarded.

(5) The remaining precipitate was lysed with 2 mL of SDS lysis solution and kept in a water bath at 65°C for 1 h. The reaction system was turned up and down every 10 min to ensure homogeneity.

(6) The centrifuge tube was removed and let cool naturally to room temperature. Then, 2 mL of chloroform: isoamyl alcohol: ethanol (80:4:16) was added and shaken gently. The tube was then centrifuged at 10,000 r/min for 10 min. A pipette gun was used to transfer the supernatant into another renumbered centrifuge tube and the steps above were repeated twice.

2.4.2. PCR amplification and sequencing

In this study, the ITS2 sequence was used as the identification sequence and combined with the standard sequence to identify the basal origin of the collected samples of Pyrrosia plants, and the sequences were amplified by using a thermal cycler (PCR instrument), concerning the Guidelines for the molecular identification of DNA barcodes of traditional Chinese medicinal herbs. In terms of the primers for PCR amplification, the forward primer used for the amplification of the ITS2 sequence was ITS2F.

2.4.2.1. Configuration of the PCR amplification system

The primer masterbatch was prepared by centrifuging the dry primer powder at 5000 rpm for 3 min. A pipette gun was then used to inject double distilled water quantitatively according to the instructions, where it was then vigorously shaken to ensure homogeneity. The powder was once again centrifuged at 5000 rpm for 3 min to obtain the primer masterbatch (100 M). In this experiment, the commonly used primers of the ITS2 sequence were selected for amplification and the PCR reaction system was configured into, as shown in **Table 3**.

Table 3. Configuration of the PCR reaction system

System	Ingredient	Amount (μL)	Total system (μL)
The 2F-3R system	2×Taq PCR Master Mix	12.5	2.5
	Forward primer	1.0	
	Reverse primer	1.0	
	Double distilled water	8.5	
	DNA template	2.0	

2.5. Sequencing results of the medicinal plants of Pyrrosia

2.5.1. DNA extraction, PCR amplification, and sequencing efficiencies

The amplification success rate of ITS2 in 40 samples was 97%, and the sequencing success rate was 90%. Repeated sequencing showed that the sequencing still showed messy overlapping peaks and exhibited low sequencing quality. A total of 33 sequences of ITS2 were obtained respectively.

2.5.2. Sequence characterization

After PCR amplification and sequencing, a total of 33 sequences of ITS2 were obtained. The length of the ITS2 sequences of Pyrrosia and its relatives was 228 bp after comparison, and there were 58 conserved sites and 169 variant sites, with an average G+C content of 68.7%, as shown in **Table 4**.

Table 4. Sequence characterization of 4 batches of samples of Pyrrosia

Sample code	ITS2 sequence length (bp)	Mean interspecific genetic distance	G+C content (%)
YBSW	216	0.000–0.127	68.52
LSSW	218	0.005–0.155	70.84
GSW	218	0.005–0.147	66.97
SW	216	0.000–0.155	68.52

2.6. Analysis of the identification results of medicinal plants

2.6.1. Analysis of DNA identification results

A total of 7 ITS2 sequences were obtained after sequence splicing, and the results of the BLAST method showed that the sequences of the 4 batches of samples were 96% similar to the ITS2 sequences of the existing stone weed plants in the database of the DNA barcode identification system of TCM herbs. This indicated that all of the samples of the stalked stone weed could be used as medicinal herbs in TCM.

3. Conclusion

Since the young leaves of the stone weed plant contain higher DNA content and can be easily extracted, we tried to use the freshest leaves of the plant in DNA extraction. The temperature and humidity were controlled to preserve the leaves to reduce the loss of DNA content and improve the efficiency of the extraction.

Disclosure statement

The author declares no conflict of interest.

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