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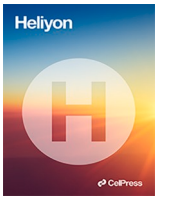

DNA from herbs can be obtained from air and authenticated by polymerase chain reaction

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ABSTRACT

DNA barcoding of herbs allows accurate species authentication. However, the DNA of herbs are often not easily PCR amplified due to co-extraction of inhibitors. Methods have been developed to improve DNA extraction to reduce contaminants. These methods usually require toxic chemical treatments or expensive commercial kits and are labor intensive. In this report, we collected the air passed from the herbs and directly amplified the DNA obtained. Results showed that DNA could be obtained, and it was PCR amplifiable. Sequencing of the amplified DNA allowed species authentication. This DNA collection method is applicable to herbs from different plant tissues. It has the advantages of reducing the use of toxic substances and more economical.

1. Introduction

Worldwide, herbs are used in food and medicine. For examples, St. John's Wort is often found in tea bags, tablets or capsules [1]. Perilla Leaves, Hairy Fig Roots, Lotus Seeds, Gordon Euryale Seeds and Babury Wolfberry Fruit are common components of soup and food recipes [2]. The use of authentic herbs is important to ensure the beneficial effects and to avoid poisoning. Substitution frequently occurs due to the confusion of names and appearances [3], and the use of cheaper herbs [4] to reduce the cost. For example, herbal tea containing toxic Japanese Star Anise *Illicium anisatum* L. instead of non-toxic Chinese Star Anise *Illicium verum* Hook.f. has caused seizures in infants [5]. The fruit of *Lycium barbarum* L., which is used to make juices, jams, bakery products and energy bars [6], is used as a medicinal material in China [7]. However, it is substituted by cheaper *Lycium* species from time to time [8] and the medicinal value is diminished.

DNA barcoding of herbs allows accurate species authentication [9]. However, the efficiency of DNA barcoding is reduced if the extracted DNA is of poor quality [10] as such DNA can result in PCR inhibition. The problems include the co-extraction of impurities such as polyphenols and polysaccharides [10] and insufficient DNA templates [11]. DNA extraction conventionally requires the lysis of cells, the solubilization of DNA, and the chemical and enzymatic removal of contaminants such as lipids, RNA and proteins [11] which demands time and skill. Researchers have developed various methods to improve the concentration and purity of the extracted DNA [10]. Recent advances mainly focus on chemically modified protocols and newly developed commercial kits with special consideration of selected herbs. Some popular kits used by researchers include DNeasy Plant Pro Kit (Qiagen) and DNeasy Mericon Food Kit (Qiagen)

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[12]. However, they are in general costly.

It is known that DNA of animals in zoo could be obtained from the air [13] and Varicella-Zoster Virus DNA in air collected by cellulose filters could be directly PCR amplified [14]. In this study, we set forth to explore whether the DNA of herbs can be obtained from the air for direct PCR amplification.

2. Methods and materials

2.1. Samples

Dried herbs of different plant parts that are commonly used as food or health enhancement were selected. Samples were obtained from shops in Hong Kong, coded and stored at LDS YYC R&D Centre for Chinese Medicine. The plant parts, species names, and codes are shown in Table 1. Each herb was obtained from two different shops for the purpose of replication. Photos of samples are presented in Appendix A.

2.2. Obtaining DNA of herbs

The samples were made into fine powder with an electric blender. One hundred grams of powder of each sample was transferred into a 250 ml round bottom flask with a stirrer bar. The flask was then sealed with a rubber stopper. A hole was drilled open with an electric drill for the insertion of a plastic duct. The duct was inserted into the flask near the sample powder. A DNA binding silica column from the Broad-spectrum Plant Rapid Genomic DNA Kit (Biomed, China) was applied to the opening of the duct and connected to vacuum for the collection of the air. A syringe needle was punctured through the stopper, and the stirrer plate was turned on to allow air flow and stirring of sample during the operation of the vacuum setup.

An appropriate amount of glass wool was applied to prevent the powder adhering to the DNA filter. (Fig. 1A). The filter was photographed to confirm the absence of herbal powder (Fig. 1B). In addition, a blank was set by collecting air without sample to ensure that the setup was not contaminated. Collection of air for each sample lasted for 3 h.

Between each sample collection, the plastic duct was removed and rinsed with water and absolute ethanol thoroughly. The site of the DNA column connecting to the duct was rubbed with ethanol using cotton sticks and then dried. The flask was thoroughly washed with detergent and water and dried. A new DNA column and a clean flask were used for each sample. After the collection of air from each sample, 41–50 μL of pre-warmed sterilized ultrapure water was poured into the column to recover the DNA from the filter.

Table 1
Sample information.

Herb	Uses in food	Major content	Health benefit	Code
Goji Berry (*Lycium*/枸杞子)	Direct ingestion, soup, energy bar, fruit juice herbal tea, and health supplement	Vitamin A, vitamin C, fiber, and iron	Improve vision, immune functions and digestion; and, provide iron for the production of red blood cells [15]	T5743, T5752
Lotus Seed (*Nelumbo*/蓮子)	Moon cake, congee, soup, curry, popcorn, and herbal tea	Carbohydrate, calcium, potassium, proteins, and vitamin B-1, -3, -5, -6, -9 [16]	Reduce inflammation, prevent obesity, and protect the liver [17]	T5706, T5754
Euryale Seed (*Euryale*/芡實)	Congee and soup	Starch, protein, potassium, calcium, zinc, manganese, phosphorus, and magnesium [18]	Provide phosphorus for nucleic acid synthesis and maintenance of living cells, promote bone health, and provide energy	T5705, T5753
Milkvetch Root (*Astragalus*/黃芪)	Tea, soup, and liquid drop	Choline, polysaccharides, saponins, glucuronic acid, folic acid, and astraisoflavanin [19]	Repair cells, improve strength, reduce blood lipids, detoxification, and manage blood sugar [20]	T5721, T5755
Hairy Fig (*Ficus*/五指毛桃)	Soup	Phytonutrients, organic acids, amino acids, essential minerals, and vitamins A and C [21]	Improve functions of lung and spleen, and improve mobility and energy [21]	T5659, T5666
White Mulberry Root Bark (*Morus*/桑白皮)	Fish soup	Flavonoids, benzofurans, stilbenoids, triterpenes, phenolic acids, and coumarin [22]	Synergistic with other herbs in soups to relieve cough [22]	T5758, T5759
Radish Seed (*Raphanus*/萊服子)	Soup	Glucosinolates and derivatives, anthocyanins, and ascorbic acid [23]	Ease digestion and egestion, and relieve coughing [23,24]	T5742, T5760
Purple Perilla Leaf (*Perilla*/紫蘇葉)	Soup and meal	Amino acids, polyphenols, essential minerals, bioactive compounds, and fatty acids [25]	Anti-inflammatory actions, reduce lipid levels, and improve respiration [26,27]	T5663, T5724
Menthol leaf (*Mentha*, 薄荷葉)	Soup, candy, and meal	Iron, menthol, tannins, and flavonoids [28]	Relieve diarrhea, respiration difficulty, and infections [28]	T5677, T5725
Lily Bulb (*Lilium*, 百合)	Soup and meal	Starch, sucrose, amino acids, and ferulic acid [29]	Relieve pneumonia symptoms [29]	T5761, T5762
Longan Pulp (*Dimocarpus*, 龍眼)	Soup, tea, juice, and dessert	Vitamin C, potassium, carbohydrate, flavonoids, alkaloids, and carotenoids [30]	Improve cognitive functioning and blood component production [30]	T5763, T5764

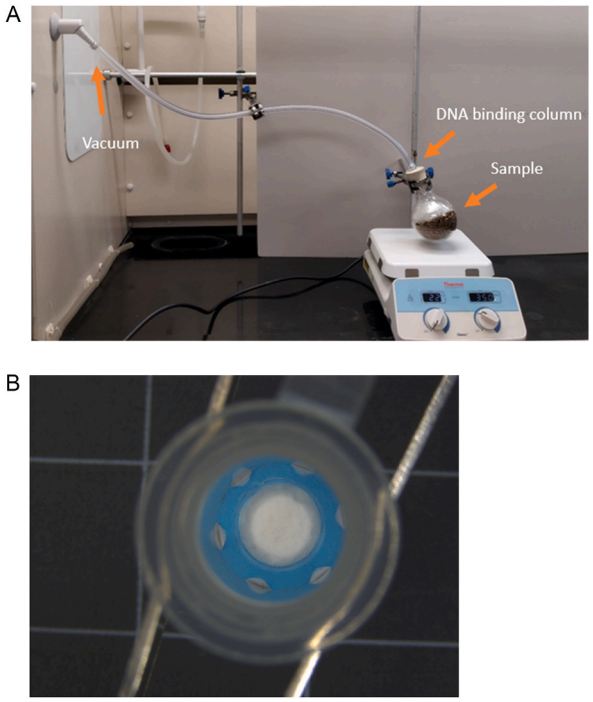

Fig. 1. A Setup for collection of air from samples; B. View of the DNA binding filter after collecting air from T5761 (Grid = 1 cm * 1 cm).

2.3. Measurement of DNA concentration and purity

The DNA concentration (in ng/μl), A260/A280 and A260/A230 ratios, were measured using Thermo Nanodrop OneC (Thermo Fisher Scientific, USA) following the instructions provided by the manufacturer (Table 2).

2.4. PCR amplification, gel electrophoresis and DNA sequencing

A PCR master mix reaction comprised 3 μl of 10 × PCR buffer, 2.4 μl of 2.5 mM dNTPs, 3 μl of 2.5 mM $MgCl_2$, 1.5 μl of 10 μM of each

Table 2
Concentration and purity of obtained DNA.

Herb	Code	Concentration ng/μl	A260/A280
Goji Berry (*Lycium*/枸杞子)	T5743	35	1.69
	T5752	54	1.44
Lotus Seed (*Nelumbo*/蓮子)	T5706	13	2.19
	T5754	13.9	1.88
Euryale Seed (*Euryale*/芡實)	T5705	11	2.38
	T5753	12.9	2.52
Milkvetch Root (*Astragalus*/黃芪)	T5721	25.7	2.30
	T5755	15.6	2.52
Hairy Fig (*Ficus*/五指毛桃)	T5644	6.3	2.96
	T5666	12	1.96
White Mulberry Root Bark (*Morus*/桑白皮)	T5758	22.8	1.60
	T5759	14.4	1.25
Radish Seed (*Raphanus*/萊服子)	T5742	12.1	2.01
	T5760	18.7	2.34
Purple Perilla Leaf (*Perilla*/紫蘇葉)	T5724	27.3	1.00
	T5766	81.7	1.01
Menthol leaf (*Mentha*, 薄荷葉)	T5677	13	1.51
	T5725	16	2.21
Lily Bulb (*Lilium*,百合)	T5761	19.0	1.55
	T5762	25.4	1.31
Longan Pulp (*Dimocarpus*, 龍眼)	T5763	75.6	1.41
	T5764	30.9	1.17
	Average =	25.3	1.83

primer, 3 μL of DNA template, 0.2 μl of 5 U/μL Taq DNA polymerase, and double distilled Milli-Q (Merck Millipore) Ultrapure Water filling up to 30 μl. The PCR amplification was performed using VeritiTM Thermal Cycler (Applied Biosystems, USA) or T100TM Thermal Cycler (Bio-Rad, USA). PCR amplification protocols at the *psbA-trnH*, *rpoC1*, *rps14*, *rbcL* and *ITS2* regions are presented in Appendix B. Positive and negative controls were applied for each PCR amplification. Positive control was the DNA of *Datura stramonium* L. extracted by the first author.

Gel electrophoresis was conducted for visualizing amplicons with 1.5% 1x TAE gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) under blue light. Gel purification was performed using a DNA gel purification kit (Biomed, China). Sanger sequencing was performed by BGI Hong Kong. Subsequently, Consensus sequences between the forward and reverse sequences were generated and identities elucidated by blast search on GenBank. ClustalW multiple alignment with the combined DNA sequences was conducted using BioEdit version 7.0.5.3 (10/28/05) to assess the nucleotide differences between species.

3. Results and discussion

3.1. DNA obtained from air

The concentration and A260/A280 of obtained DNA are shown in Table 2.

3.2. PCR amplification and DNA sequencing

Samples were PCR amplified at either *ITS2*, *psbA-trnH*, *rbcL*, *rpoC1* or *rps14* region. A representative gel electrophoresis photo is presented in Fig. 2A(a - d) and B(a - c). The consensus sequence combined using forward and reverse sequences of the Sanger sequencing platform were blasted. The matched sequence in Genbank and the Latin name of the herb were identical to the input sequence and the tested herb (Table 3). The GenBank accession numbers of the reference sequences used for the comparison are shown in Table 3. All tested samples were therefore correctly identified (Table 3). For each herb species, two individual samples were obtained from two different herb shops and PCR was performed at two DNA regions. This ensured the accuracy in the species identification.

3.3. Advantages of herb authentication by the amplification of DNA from air

Past research on the authentication of processed or dried herbs showed low DNA purity and concentration could impede PCR

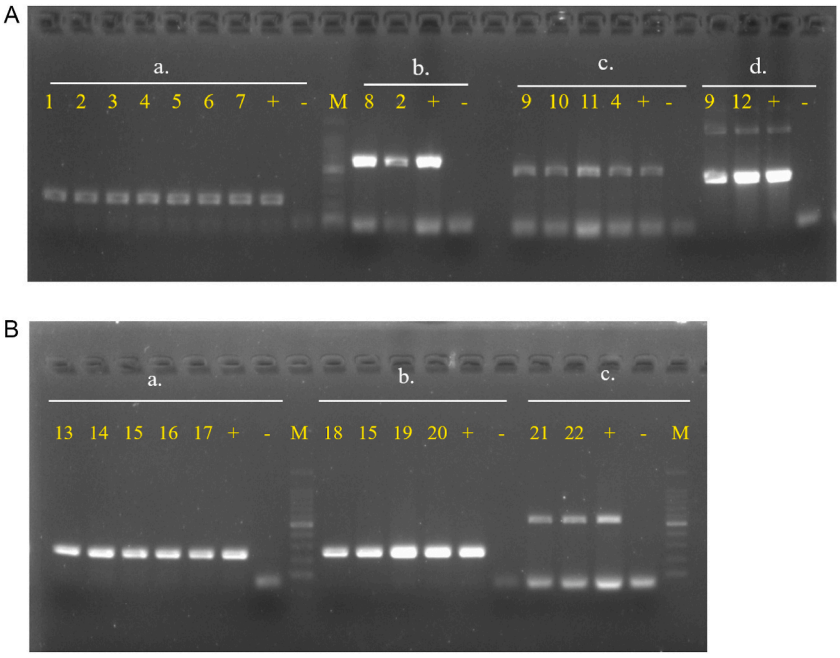

Fig. 2. A PCR amplification with primers a. rbcL1/rbcLB b. rbcLaF/rbcLaR c. ITS2F/ITS3R d. ITSu3/ITSu4 at *rbcL* and *ITS2* regions. 1: T5705 2: T5758 3: T5719 4: T5724 5: T5742 6: T5743 7: T5706 8: T5666 9: T5753 10: T5759 11: T5760 12: T5721 +: positive control -: negative control M: 100 bp ladder. A full, non-adjusted image can be found in the supplementary materials. B. PCR amplification with primers a. ccF/ccR, b. ffF/ffR and c. psbAF/trnHR at *rps14*, *rpoC1* and *psbA-trnH* regions respectively M: 100 bp ladder. 13: T5644 14: T5763 15: T5761 16: T5677 17: T5754 18. T5766 19. T5725 20. T5752 21. T5764 22. T5762 +: positive control -: negative control M: 100 bp ladder. A full, non-adjusted image can be found in the supplementary materials.

Table 3
Sample identification by BLASTn comparison with DNA sequences in Genbank.

Herb	Code	Species	DNA region	Accession number, Coverage, Similarity
Goji Berry	T5743	*Lycium* sp.	*rbcL*	NC_042204.1, 100%, 100%
	T5752	*Lycium* sp.	*rpoC1*	MN866909.1, 100%, 100%
Lotus Seed	T5706	*Nelumbo nucifera*	*rbcL*	MN068956.1, 100%, 100%
	T5754	*Nelumbo nucifera*	*rps14*	NC_025339.1, 100%, 100%
Euryale Seed	T5753	*Euryale ferox*	*ITS2*	JF976313.2, 100%, 100%
	T5705	Mesangiospermae[a]	*rbcL*	OP142688.1, 100%,100%
Milkvetch Root	T5721	*Astragalus membranaceus*	*ITS2*	MN224270.1, 100%, 100%
	T5719	*Astragalus* sp.	*rbcL*	NC_047381.1, 100%, 100%
Hairy Fig	T5644	*Ficus* sp.	*rps14*	MT093220.1, 100%, 100%
	T5666	*Ficus* sp.	*rbcL*	MN102667.1, 100%, 100%
White Mulberry Root Bark	T5759	*Morus alba*	*ITS2*	MK087936.1, 100%, 100%
	T5758	*Morus alba*	*rbcL*	MK526206.1, 100%, 100%
Radish Seed	T5760	*Raphanus sativus*	*ITS2*	KX709357.1, 100%, 100%
	T5742	*Raphanus* sp.	*rbcL*	MN216800.1, 100%, 100%
Purple Perilla Leaf	T5724	*Perilla frutescens*	*ITS2*	MH711603.1, 100%, 100%
	T5766	*Perilla frutescens*	*rpoC1*	NC_030757.1, 100%, 100%
	T5724	*Perilla frutescens*	*rbcL*	MH711603.1, 100%, 100%
Menthol leaf	T5725	*Mentha* sp.	*rpoC1*	NC_047475.1, 100%, 100%
	T5677	*Mentha* sp.	*rps14*	NC_047475.1, 100%, 100%
Lily Bulb	T5761	*Lilium formosanum*	*rps14*	MT261162.1, 100%, 100%
	T5761	*Lilium formosanum*	*rpoC1*	MT261162.1, 100%, 100%
	T5762	*Lilium formosanum*	*psbA-trnH*	AB331270.1, 100%, 100%
Longan Pulp	T5764	*Dimocarpus longan*	*psbA-trnH*	OK052822.1, 100%, 100%
	T5763	*Dimocarpus longan*	*rps14*	MK726005.1, 100%, 100%

[a] Adulterant species found in sample T5705.

efficiency. SDS- [31] or CTAB-chemical treatments [32], and better-quality commercial extraction kits [12] were frequently applied for reducing contaminants and increasing DNA yield during the extraction of DNA, sometimes combined with the use of tangible tools such as magnetic bead [33] or dipstick [34]. These methods resulted in high PCR efficiency. However, the preparation of chemical solutions and tangible tools, and the extraction processes, including cell lysis, separating DNA from protein, DNA precipitation and purification, could be labor intensive. Moreover, CTAB and SDS are toxic [35,36] and the commercial kits are in general costly. Our method is straightforward and can be further streamlined by automation and running several setups at the same time.

In this study, we collected PCR-amplifiable DNA with the use of a simple air collection setup and basic silica DNA column. We recovered the DNA using sterilized ultrapure water and centrifugation without further purification. The average DNA concentration and A260/A280 were 25.3 ng/μl and 1.83 respectively. Several herbs had low A260/A280 ratio might be due to the trapping of volatile oil or other contaminants in the DNA binding column. *Perilla* species contains significant essential oils [37]. The readings implied satisfactory concentration and purity of DNA, comparable to that of established DNA isolation methods [12,34]. Samples could be amplified to 500 bp (Fig. 2A(a - d) and 2B(a - c)), indicative of good DNA integrity. Our method helps reducing the need of the design of primers for the production of fragmented short amplicons.

Despite several samples had low A260/A280 reading due to higher protein content, the DNA was PCR amplified and sequenced, and the results enabled taxonomic resolution (Appendix C). Genus and species information were provided at the plastid regions *rbcL*, *psbA-trnH*, *rps14* or *rpo14*; and the ribosomal *ITS2* region. The choice of the DNA regions is based on the suggestion by Mishra and colleagues, that easily PCR-amplifiable and align-able DNA regions such as *rbcL*, can provide a basic framework; and DNA regions with high sequence variation such as the *ITS2* region can provide satisfactory species differentiation results for plants [38]. An adulterant species of Family Mesangiospermae was discovered in a sample of Euryale Seed (Family Nymphaeaceae) at the *rbcL* region, showing the power of species differentiation using this approach.

Our method can be applied to products from bark, fruit, whole plant, root, bulb, leaf, aerial part, and seed. This showed the versatility of this approach in obtaining PCR amplifiable DNA.

3.4. Future directions

3.4.1. Suggested improvements

Although we have shown that this method is applicable to a number of herbs for different parts of the plants, more data will be needed for extending the method to other herbs, including those which have been heavily processed. Different types of filters with varied pore sizes may be explored to increase the trapped DNA. More DNA may be obtained by increasing the surface area of DNA binding and the increase of air flow passing through the column. Further streamlining of this setup could be enabled by the development of an automated device with multiplex function. With all these improvements, DNA from air will provide an efficient method for obtaining herbal DNA, which will eliminate a big hurdle in molecular authentication.

3.4.2. Potential application on the authentication of products

It has been shown recently that metabarcoding of airborne pollens collected from stations was able to identify 168 species from 56

plant families [39]. It is worth trying if metabarcoding of DNA from air could be used to identify a mixture of herbal products. This may facilitate the discovery of contaminants. The data obtained could then be documented on a block chain platform, such as HerBChain [40], for quality assurance in the product manufacturing.

Author contribution statement

Hiu-Lam Ngai: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Hung Kay Lee: Conceived and designed the experiments; Contributed reagents and materials; analyzed and interpreted the data.

Pang-Chui Shaw: Conceived and designed the experiments; Analyzed and interpreted the data.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18946.

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