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Application of DNA Molecular Identification Method to Distinguish Ejiao and its Adulterants

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ABSTRACT

Objective: To identify Ejiao and its adulterants at the DNA level by using DNA molecula marker. Ejiao (*Asini Corii Colla*) is a commonly used medicinal material. However, its adulteration is a serious concern. Due to the morphological characteristics of *Asini Corii Colla* and its adulterants, traditional identification techniques often complex and professional, which is not conducive to the circulation management and safety of the medicinal materials. To improve the distinction between *Asini Corii Colla* and its adulterants accurately, this study identified and its adulterant samples based on the *CytB* sequence. Sequence characteristics, Basic Local Alignment Search Tool (BLAST) application, genetic distance, construction of phylogenetic tree showed the *CytB* sequence to accurately identify *Asini Corii Colla* from its adulterants. Furthermore, in this study, we designed a specific primer, based on the CytB sequence, and established a PCR detection system for rapid, sensitive, and specific identification of *Asini Corii Colla*. Compared to DNA barcoding technology, this method has shorter detection time, stronger specificity, and higher sensitivity, which lays the foundation for the rapid identification of *Asini Corii Colla*.

1. Introduction

Colla Corii Asini (Ejiao in Chinese), which is made from fresh or dry skin of *Equusasinus L*. after dehairing, also known as donkey skin glue. It is a precious Chinese traditional medicine widely used in China for thousands of years. As recorded in many classic monographs of Chinese traditional medicine and in ancient books, Ejiao displays great efficacy in enriching blood and staunching bleeding, being mainly used for the treatment of gynecological diseases, such as menoxenia and post-partum uterine bleeding [1]. It is no surprise that multiple pharmaceutical factories manufacture Ejiao under different brand in Chinese traditional medicine market. To market supervision department, there are many problems related to quality control of Ejiao, a major issue is its manufacturer identification. There are many manufacturers of Ejiao using different raw materials, and the quality of their products varies wildly, affection medical efficacy and also price. There is no consensus on which product is best, due to complex composition of medicinal materials of Ejiao and the lack of relevant standards and regulatory system in Chinese Pharmacopoeia.

Ejiao is used to treat blood deficiency and chlorosis, dizziness, heart palpitations, upset and insomnia, muscle weakness, weakness, internal movement, lung dryness, cough, and hemoptysis [2]. It has high medicinal and edible value. However, as prices have been rising, more and more adulteration and fraud have disrupted market order and adversely affected consumers' health.

The preparation process of Ejiao includes more than

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ten processes such as airing the skin of donkey, scraping, soaking, gutting, peeling, concentrating, gelling, cutting, airing and rubbing, etc. After that, Ejiao is further refined into liquid formulation, etc. Enzymatic hydrolysis, purification, drying and other complicated manufacturing processes are implemented in production [3]. The traditional methods of authenticity identification of Ejiao, such as macroscopical identification, microscopic identification and physical and chemical identification, depend on personal experience and professional knowledge [4]. Meanwhile, the preparation process of other animal skin glues is similar to that of Ejiao, and their appearance traits are similar as well, thus it is extremely difficult to distinguish Ejiao from other animal skin glues.

At present, Traditional Chinese medicinal materials are authenticated in three methods. The first method is macroscopical identification, which can only be applied to appearance identification but is very hard to authenticate some processed medicinal materials their appearance had been changed. The second method is microscopic identification, which is used to observe and authenticate microscopic features of tissue structure of Traditional Chinese medicinal materials, but cannot identify some Traditional Chinese medicinal materials having similar basic structure. The third method is physical and chemical identification, which is applied to identify effective components via physical and chemical methods, such as chromatography and spectrometry, but has low resolution and poor specificity to near-source chemical components [5].

With the development of biotechnology, the species identification methods are also constantly developed. It has gone through several stages such as morphological identification, cell-level identification, biochemical analysis identification, immunological identification, and molecular marker identification. For morphological identification, cell-level identification, biochemical analysis identification and immunological identification, there are some defects that may lead to failure or incorrect identification [6-7].

Molecular Marker Identification: This is an approach to detect the differences in DNA fragments between individuals or between populations to achieve the goal of identification. Molecular markers have many advantages: it is not affected by the environment or other factors, high polymorphism, and can provide complete genetic information, etc. The most commonly applied technologies of molecular markers are: Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphism DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Microsatellite DNA (STR), Single Nucleotide Polymorphism (SNP), and etc. [7-9].

DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species. It was first proposed in 2003 by researchers at the University of Guelph in Ontario, Canada [10]. The short genetic sequence from a standard part of the genome works efficiently to identify species the way a supermarket scanner distinguishes products using the black stripes of the Universal Product Code. Thus it was given the name "DNA barcode". DNA barcodes vary among individuals of the same species, but only to a very minor degree. If the DNA barcode region is effective, the minor variation within species will be much smaller than the differences among species.

Mitochondrial DNA (mtDNA) is the genetic material that exists outside the nucleus in eukaryotic cells. It has a simple molecular structure. It does not undergo recombination with nuclear DNA and has no identical sequence with nuclear DNA. It has multiple copies, has a rapid evolutionary rate, and follows maternal inheritance. *Cytochrome b* gene (*Cyt b*) is one of the genes that is coded by mtDNA, and its gene product plays an important role in electron transfer in the respiration chain. *Cyt b* gene has a moderate evolutionary rate and a clear evolutionary pattern that makes it suitable for the studies on the phylogenetic evolution at the intra- and interspecific levels [11-13].

In the present study, to implement the feasible DNA barcode technique in the identification of Ejiao and its adulterants. Several Ejiao products had been collected on the market and tested their authenticity with approach of LC-MS/MS, according to Chinese Pharmacopoeia. Then, by comparing different genomic extraction methods, a suitable way for donkey-hide gelatin was optimized to obtain the genome with higher purity. Because mitochondrial genes are the only self-replicating genome in eukaryotes, its number via duplicate is thousands of times higher than that of nuclear genes, furthermore, it is more stable and resistant to degradation than nuclear genomes, the mitochondrial gene *CytB* gene was selected as the molecular marker to detect the collected samples in the study. Meanwhile, Bioinformatics analysis would be performed after obtaining the sequence by sequencing. Compared the legal detection with Chinese Pharmacopoeia, the *CytB* sequence could accurately identify Ejiao from its adulterants. This method has shorter detection time, stronger specificity, and higher sensitivity, which lays the foundation for the rapid identification of Ejiao. The study provides a theoretical basis for the establishment of standard, and provides technical support for further improving the molecular detection field of Ejiao.

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2. Experimental Materials and Reagents

Reagents

Formic acid, ammonium bicarbonate, NaCI, EDTA, chloroform, isoamyl alcohol and other reagents were purchased from Sinopharm Reagent Co., Ltd. β-mercaptoethanol, proteinase K, equilibrated phenol, C helex-100, Agarose, DNA polymerase (Phanta™ Super-Fidelity DNA Polymerase Vazyme), DNA marker (Toneker 2000), DNA dye (Gel RED™ Nucleic Acid Gel Stain, Biotium), etc were purchased from Wuhan Shengya Biotechnology Ltd.

Experimental instruments

Electronic balance, Electro-thermostatic water bath, Ultrasonic, high performance liquid chromatography - mass spectrometry (HPLC-MS, LC 2OA -API 4000). Eppendoff, refrigerated centrifuge, ultraviolet spectrophotometer. PCR amplifier (Hangzhou Jingge Scientific Instrument Co., Ltd., K 640), electrophoresis apparatus (Beijing Liuyi Instrument Factory, DYY-2C), Gel electrophoresis imaging system (ChemiDocMP, Bio-Rad, USA).

Chromatography Column

Shiseido (S201204-11, column length: 150 mm, column diameter 2.1 mm).

3. Results

3.1 The Legal Identification

Ejiao occupies an important position in China's health product market, with an output value of nearly 500 billion yuan. Genuine Ejiao is made from the hide of Equus animus L. Due to the shortage of raw materials, the price of Ejiao is continually rising, adulteration occurs from time to time. The main ingredient of adulteration is cow hide glue. The legal identification method of Ejiao includes two parts. One is observation of the characteristics of Ejiao, and the other is comparing the consistency of the chromatographic peaks between sample and reference medicinal materials by using high performance liquid chromatography - mass spectrometry (HPLC-MS).

Sample collection

A total of 11 different samples used in this study are all purchased from Chinese market and enterprises, and stored at -20℃ after collection. 8 out of 11 Ejiao products were obtained from 7 manufacturers, one donkey hide was provided by Hubei Kang Pharmaceutical Co., Ltd. one cowskin came from slaughterhouse in Wuhan. The source of samples showed in the Table 1 below.

Table 1. The sample collection

Sample	Manufacturer	Batch productin
Y1	Hubei A Pharmaceutical Co., Ltd	150108
Y2	Shijiazhuang A Pharmaceutical Co., Ltd	140409
Y3	Shandong A Pharmaceutical Co., Ltd	1505018 1
Y4	Shandong B Pharmaceutical Co., Ltd	140811
Y5	Shandong B Pharmaceutical Co., Ltd	20150505
Y6	Shandong C Pharmaceutical Co., Ltd	20141101
Y7	Shandong D Pharmaceutical Co., Ltd	20141130
Y8	Shandong E Pharmaceutical Co., Ltd	20150611

Character identification

In order to identify the authenticity of Ejiao products, firstly, according to the 2015 edition of Chinese Pharmacopoeia, the character of Ejiao was identified by observation. The sharp of genuine Ejiao should be cuboid or cube. The color is brown to dark brown with luster. The material is hard and brittle with bright cross section, and the fragments are brown and translucent to light. The smell is weak and taste is slightly sweet.

Ejiao component identification

According to the "Chinese Pharmacopoeia" 2015 edition, whether the collected samples are authentic Ejiao depends on if the consistency has been fulfilled or not by comparing chromatographic peaks between sample and reference medicinal materials which was detected by HPLC-MS.

The result of the legal identification for these 8 collected Ejiao products are shown in the Table 2 below. They are all genuine products.

Table 2. The result of the legal identification

Number	Character identification	Ejiao component identification
Y1	qualified	qualified
Y2	qualified	qualified
Y3	qualified	qualified
Y4	qualified	qualified
Y5	qualified	qualified
Y6	qualified	qualified
Y7	qualified	qualified
Y8	qualified	qualified

The development of chromatography- spectrum technology has established the fingerprint information of various traditional Chinese medicines, which can provide abundant information for the identification and quality evaluation of traditional Chinese medicines. Legal identification of Ejiao implements this method because of its high specificity and effectiveness. However, this method

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requires the use of large and expensive instruments, which is complicated to operate and has high requirements for the professional level of experimental personnel.

3.2 The Molecular Identification Test of Ejiao

The molecular identification test of Ejiao requires the extraction of DNA. The preparation process of Ejiao product mainly includes multiple steps such as donkey skin pretreatment, peeling, filtration, concentration, and gelation, which takes a long time. During this lengthy process, the degradation of DNA is very serious, increasing the difficulty of molecular detection. In order to obtain a higher concentration of DNA, a variety of extraction methods have been tested. After obtaining the DNA with higher purity, *CytB* gene was selected as the molecular marker, and the Ejiao samples were amplified, sequenced and analyzed.

3.2.1 Separating and Purifying DNA

Sodium Dodecyl Sulfonate (SDS) is a plasma surfactant, which can dissolve the cell membrane and nuclear membrane protein, rupture the cell membrane and nuclear membrane to release nucleic acid, and depolymerize the nuclear protein in the cell and denature the protein precipitation. Chelex-100 is a chemical chelating resin composed of styrene and divinylbenzene copolymer. It contains pairs of iminodiacetate ions, which can chelate polyvalent ions, especially, have higher affinity and chelation for high-valent metal ions. Glass milk technology uses the principle of combining DNA and silica under high-concentration salt conditions and eluting under low-salt conditions, so as to achieve the purpose of separating and purifying DNA [14].

There is a high amount of protein in Ejiao. Proteinase K is added during the extraction process to hydrolyze and digest proteins, especially histones that bind to DNA. Proteinase K requires a suitable temperature (65 ℃) and proper time to hydrolyze proteins. On one hand, With the increase of reaction time, the protein hydrolyzes more thoroughly, on the other hand, a too long hydrolysis time will lead to partial nucleic acid depletion and proteinase K hydrolyzes in a poor ability. For improving the efficiency of DNA extraction, the time of proteinase K action should be optimized. Finally, three ways were tried for DNA extraction of donkey-hide gelatin.

Method a

Use proteinase K and Chelex-100 in DNA extraction, and purified DNA with glass milk.

(1) Transfer 200 mg Ejiao sample to a microfuge tube. Add 400 μL of digestion buffer (pH 8.0, consisting of 10 mmol/L Tris-HCI, 25 mmol/L EDTA, 100 mmol/L NaCI and 0.5% SDS) and 380 μL of 15% Chelex-100, invert the capped tubes to mix the contents. Incubate the solution at 50-60 ℃ water bath, occasionally inverting the tubes to make the solution completely dissolved. (2) Cooled the solution to room temperature, and added 20 mg/mL proteinase K 20 μL, mixed and incubated the solution in 56℃ for 1 hour. (3) Centrifuge the solution at 12000 r/min for 10 min. Transfer the supernatant fluid to a new EP tube by aspiration. And add an equal volume of chloroform. Mix thoroughly, centrifuge at 12000 r/min for 10 minutes, and carefully transfer the supernatant to a new EP tube. (4) Add 5-20 μL glass milk and two times volumes of DNA Binding Buffer (6 mol/L NaCl), Incubate the solution at 50-60 ℃ water bath. Mix sample several times during incubation by inverting tube. (5) Incubate at room temperature for 5 minutes, and invert several times during this time. (6) Centrifuge the solution at 4000 r/min for 1 minute, and discard the supernatant. (7) Wash the DNA precipitate twice with 70% ethanol. Remove as much of the 70% ethanol as possible, using an aspirator. Store the pellet of DNA in an open tube at room temperature until the last visible traces of ethanol have evaporated. Add an appropriate amount of deionized water to completely dissolved DNA. Store the DNA solution at -20℃ .

Method b

Use SDS alkaline lysis method and more than once use chloroform and isoamyl alcohol in DNA extraction.

(1) Transfer 200 mg Ejiao sample to a microfuge tube. Add 1ml of SDS extraction /lysis buffer (pH 7.2, 500 mmol/L NaCl, 100 mmol/L Tris-HCl, 50 mol/L EDTA, 100 mmol/L β- mercaptoethanol, 20% SDS) and 50 μL of 20 mg/mL proteinase K, invert the capped tubes to mix the contents. Mixed and incubated the solution in 56℃ for 1 hour. (2) Centrifuge the solution at 12000 r/min for 1 minute, transfer the supernatant to a new EP tube. (3) Add an equal volume of equilibrated phenol, and gently mix the solution by slowly turning the tube end-over-end. Centrifuge the solution at 12000 r/min for 10 minutes, transfer the aqueous (upper) phase to a new EP tube. (4) Add an equal volume of phenol - chloroform - isoamyl alcohol (25: 24: 1), gently mix the solution by slowly turning the tube end-over-end. Centrifuge the solution at 12000 r/min for 10 minutes, transfer the aqueous (upper) phase to a new EP tube. (5) Add 0.2 volume of 10M NaAc and 2 volumes of ethanol, then swirl the tube until the solution is thoroughly mixed, Store the DNA solution at -20℃ until the DNA forms a precipitate. (6) Centrifuge the solution at 12000 r/min for 10 minutes, and discard the supernatant. Wash the DNA precipitate twice with 70% ethanol.

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Remove as much of the 70% ethanol as possible, using an aspirator. Store the pellet of DNA in an open tube at room temperature until the last visible traces of ethanol have evaporated. Add an appropriate amount of deionized water to completely dissolved DNA. Store the DNA solution at -20℃ .

Method c

Use Deep-processed product genome extraction kit in DNA extraction.

The kit was purchased from Shanghai Shenggong Biological Co., Ltd. Ejiao sample was grinded with liquid nitrogen. DNA was extracted using Shenggong Deep-processed product genome extraction kit in accordance with the manufacturer's protocol, and then was dissolved in 100 μL distilled water.

3.2.2 Comparison and Analysis of DNA Extraction Methods by Measuring the Concentration of the DNA

Measure the concentration of the DNA by Ultraviolet spectrophotometry to test the absorbance at 260 nm and 280 nm. If the value is about 1.8 to 1.9 at A260/A280, that means the purity of DNA is well. If the value is greater than 1.9 at A260/A280, that indicated the DNA may mix with some RNA. If the value is less than 1.8 at A260/A280, that suggested the DNA solution may contain some contamination of phenol or protein. Take sample Y 4 as an example, the absorbance is shown in the Table 3 below.

Table 3. Comparison and analysis of DNA extraction methods by measuring the concentration of the DNA

	Method a	Method b	Method c
*A*260/*A*280	1.846	1.751	1.802

By Comparison and analysis of DNA extraction methods, the DNA extracted in the method B had residues of phenols or proteins that were not removed. Method a and c may get higher purity DNA solution. Considering the simplicity of the operation, the method c was adopted, while increasing enzymatic reactions time of Proteinase K.

3.2.3 Selection of Molecular Markers and Sample Detection

Design of primers

Because mitochondrial genes are the only self-replicating genome in eukaryotes, and copy number is thousands of times higher than that of nuclear genes, it is more stable and resistant to degradation than nuclear genomes. Many studies have reported the use of mitochondrial *CytB* and 16SrRNA gene sequence analysis to identify animal species. Due to the serious degradation of DNA during the processing of Ejiao, mitochondrial genes are selected as molecular markers. 3 pairs of primers were designed for screening, as shown in the following Table 4 (primers are synthesized by Wuhan Aoke Dingsheng Biotechnology Co., Ltd.):

PCR amplification

The mitochondrial DNA *CytB* sequence was amplified by using the primers in Table 4. The amplification reaction system for *CytB* (30 μl) contained 1 μl of 1U/μl DNA Polymerase, 6 μl of 5×PCR SF buffer (with 10mM $MgSO_4$), 1 μl of 10mmol/L dNTPs, 1 μl of each of forward and reverse primers(10pmol/L), 1μl (about 10 ng) of DNA template and 19 μl of triple-distilled water.

The amplification procedures for *COI* were started with predenaturing at 94℃ for 1 min, followed by 5 cycles of denaturing at 94 ℃ for 1 min, annealing at 45 ℃ for 1.5 min and extension at 72 ℃ for 1.5 min, and 35 cycles of denaturing at 94 ℃ for 1 min, annealing at 50 ℃ for 1.5 min and extension at 72℃ for 1.5 min; the amplification was completed by holding the reaction mixture at 72 ℃ for 10 min to allow complete extension of PCR products.

The amplification procedures for *16sRNA* were started with predenaturing at 94℃ for 2 min, followed by 40 cycles of denaturing at 94℃ for 1 min, annealing at 55℃ for 1 min and extension at 72℃ for 1.5 min; the amplification was completed by holding the reaction mixture at 72 ℃

Table 4. The design of primers

		The design of primer
CytB	Forward primer（5'-3'）	CCA TCC AAC ATC TCA GCA TGA TG AAA
	Reverse primer（5'-3'）	GCC CCT CAG AAT GAT ATT TGT CCT CA
COI	Forward primer（5'-3'）	GGT CAA CAA ATC ATA AAG ATA TTG
	Reverse primer（5'-3'）	TAA ACT TCA GGG TGA CCA AAA AAT
16sRNA	Forward primer（5'-3'）	GCC TAT ATC AGA ACG AAT ACT CAC
	Reverse primer（5'-3'）	CAT GCC TGT GTT GGG TTA A

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for 10 min to allow complete extension of PCR products.

The amplification procedures for *CytB* were started with predenaturing at 94℃ for 2 min, followed by 40 cycles of denaturing at 94℃ for 1 min, annealing at 53℃ for 1 min and extension at 72℃ for 1.5 min; the amplification was completed by holding the reaction mixture at 72 ℃ for 10 min to allow complete extension of PCR products.

Detect PCR product by electrophoresis

Detect PCR product by electrophoresis with 1.2% agarose, we found that we found that only *CytB* gene can be amplified, and other primers cannot be used to obtain fragments in the amplification procedure.

CytB fragment of the sample genome in amplification. As shown in the Figure 1 below (1.2 % agarose).

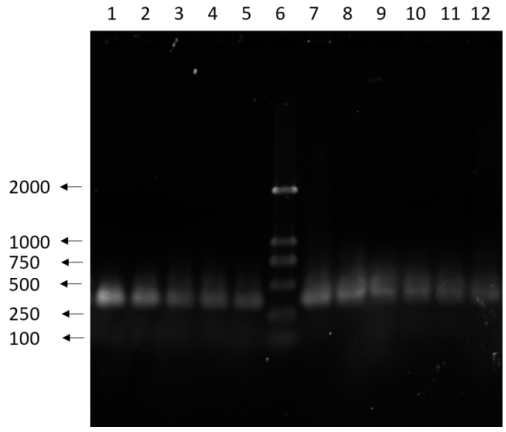

Figure 1. Detect PCR product by electrophoresis (1 Y1, 2 Y2, 3 Y3, 4 Y4, 5 Y5, 6 marker, 7 Y6, 8 Y7, 9 Y8, 10 donkey hide, 11 one cowskin, 12 Ejiao reference medicinal material)

3.2.4 Sequence Comparison

Recovered the band of *CytB* from agarose gel, then sequenced the DNA, the sequence comparison result is shown in the Figure 2 below:

3.2.5 Phylogenetic Analysis

The phylogenetic tree result is shown in the Figure 3 below:

The phylogenetic tree shows that the *CytB* fragment of the sample and the cowhide are different dramatically and can be clearly distinguished. It is confirmed that the *CytB* gene can be used as a molecular marker to identify the donkey skin components of Ejiao and its products.

4. Discussion

A larger sample collection is needed to further test the effectiveness of the method. From an economic point

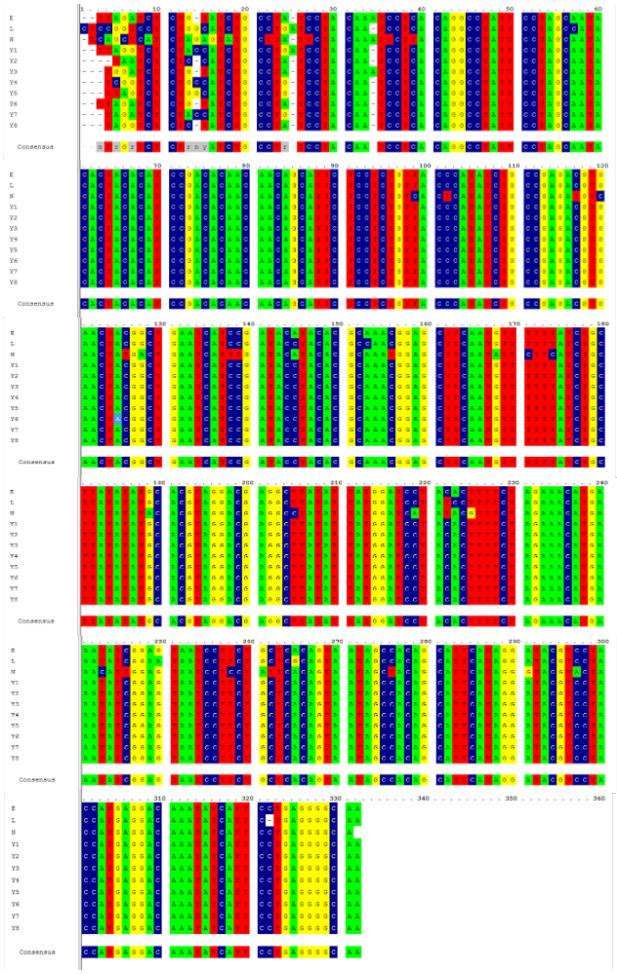

Figure 2. the sequence comparison of *CytB* (E, Ejiao reference medicinal material; L, donkey hide; N, cowskin, Y1-YB, samples)

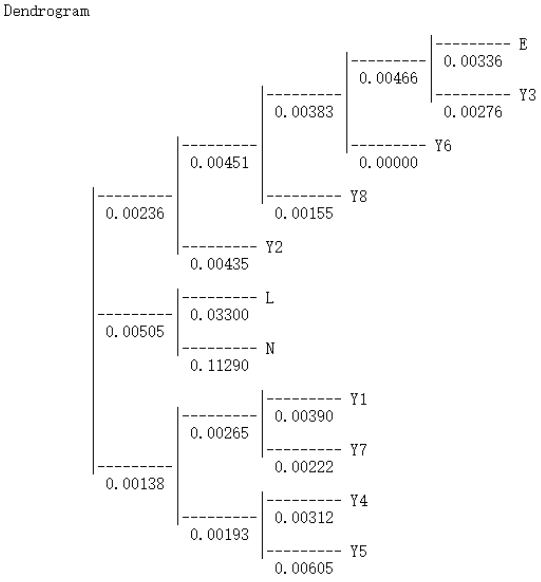

Figure 3. The phylogenetic tree (E, Ejiao reference medicinal material; L, donkey hide; N, cowskin, Y1-YB, samples)

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of view, the counterfeit manufacturers of Ejiao will use cheaper raw material, cowhide instead of donkey skin, to product Ejiao. So this project mainly focuses on detection method for distinguishing the cowhide from donkey skin. In addition, horse skin, mule skin, calfskin, calfskin, goat skin, etc. many other skins could be the raw material. The detection method needs to be further improved to enhance its applicability.

Although this project has initially completed the detection method for distinguishing Ejiao and its adulterants by the molecular marker gene (*CytB*), molecular identification technology cannot replace the existing detection technology. Species identification can ensure the "authenticity" of drugs, while the content determination of active ingredients or active parts can ensure the "effectiveness" of drugs, both of which are indispensable steps in drug quality control. Although molecular technology identification has broad application prospects, it will also may encounter various challenges in the research. This technology cannot completely replace other existing identification technology methods, such as microscopy, physics and chemistry, chromatography and spectroscopy. Long storage time and improper storage condition will cause such phenomena as infestation, discoloration or mildew of Chinese herbal medicines, decoction pieces and Chinese patent medicines, which will seriously affect their quality and efficacy. This kind of situation also could affect the results of identification in molecular technology. For example, the DNA degradation too serious to be successful obtained its bar code sequence, or the extracted DNA may be contaminated by fungi due to mildew. Therefore, DNA barcoding technology should be combined with other methods to comprehensively evaluate the drug quality if the above situations are encountered in the actual drug testing work.

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