Research Note

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Sequence Analysis Based on ITS1 Region of Nuclear Ribosomal DNA of *Amomum villosum* and Ten Species of *Alpinia*

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ABSTRACT

Ripe fruits of *Amomum villosum* Lour. are important traditional Chinese medicines and food spices. Due to similar morphological and anatomical characteristics, fruits from some *Alpinia* species are used clinically as substitutes or adulterants of Fructus Amomi. In this study, we determined the nrDNA ITS1 (internal transcribed space 1) sequences from *Amomum villosum* and ten *Alpinia* species, which are adulterants of Fructus Amomi. The ITS1 sequences of these species were 175~179 bp in length, and the G+C contents were 53.37~56.74%. Sequence alignment revealed that there were 55 polymorphic nucleotide sites among the tested species. The base substitution were 0.57~9.60% within the ten *Aplinia* species, but 17.61~21.59% between *Amomum villosum* and the *Alpinia* species. These molecular data indicated that ITS1 sequences can be used to differentiate Fructus Amomi and its substitutes or adulterants from various *Alpinia* species.

Key words: *Amomum villousm*, Fructus Amomi, *Alpinia*, nrDNA ITS1

INTRODUCTION

Amomum villosum Lour. (Zingiberaceae) is an aromatic plant distributed mainly in Guangdong, Yunnan, Fujian and Guangxi Province of China[(1)]. The ripe fruits of *A. villosum*, named "Sha-ren" in Chinese, have been used as traditional medicines and food spices in China for centuries[(2)]. Its main function is to treat dyspepsia, gastric disease, vomiting and diarrhea[(3)]. Experimental bioassays have demonstrated that the extracts of Fructus Amomi help improving the gastrointestinal milieu[(4)] and show obvious analgesic and anti-inflammatory effects[(5,6)].

According to the Chinese Pharmacopoeia (2005), Fructus Amomi is derived from *A. villosum*[(3)]. However, our literature search and survey of the commercial crude drugs of Fructus Amomi revealed that the fruits of some *Alpinia* species from the same family, such as *A. japonica*, *A. pinnanensis* and *A. guinanensis*, are also used clinically as substitutes or adulterants in China[(7-9)]. However, improper usage may cause inconsistent therapeutic effects, or even jeopardize the safety of consumers. Therefore, accurate identification of Fructus Amomi is important for its safety and efficacy.

The morphological and anatomical characteristics of the fruits from these *Alpinia* and Fructus Amomi species are very similar[(10,11)]. Since the chemical constituents of these species are complicated and still unknown, a chemical analysis method for the quality control of Fructus Amomi has not yet been developed. In recent years, the DNA-based polymorphism assay has been more closely adapted for the identification of herbal medicines, and developed to be a more effective, accurate, reliable and sensitive technology[(12,13)]. Samples of *A. villosum* from different habitats and adulterants have been analyzed by RAPD (random amplified polymorphic DNA)[(14,15)]. Furthermore, the ITS (internal transcribed space) sequence of *A. villosum* was also amplified and determined. The results showed that the ITS1 sequence can be used to confirm its production area and determine its adulterants from *Amomum villosum* var. *xanthioides* and *Amomum longiligulare*[(16,17)]. In the present study, we determined and compared the ITS1 sequences amplified from *A. villosum* and ten *Alpinia* species, which are adulterants of Fructus Amomi, the results indicated that

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several polymorphic sites may be used as DNA markers for the identification of this herbal medicine.

MATERIALS AND METHODS

I. *Materials*

The plants of *A. villosum* and ten *Alpinia* species were collected from different regions in China (Table 1). The species were identified carefully according to the morphological characteristics. Voucher samples were deposited in Department of Pharmacognosy, China Pharmaceutical University.

II. *DNA Extraction and Purification*

The air-dried leaves of the samples were grounded into powder with mortar and pestle in liquid nitrogen. Total DNA was extracted from 100 mg of powdered sample using a modified CTAB (cetyltrimethylammonium bromide) procedure[18] and 1.2 mL of CTAB extraction buffer (1% CTAB; 50 mM Tris-HCI, pH 8.0; 10 mM EDTA; 0.7 M NaCl; 0.14% 2-mercaptoethanol) was added. The suspension was incubated in a water bath at 56°C for 30 min with occasional shaking. After centrifugation, the supernatant was extracted with equal volume of chloroform/isoamyl alcohol (24:1). After centrifugation at 12000 rpm for 15 min, duple volume of absolute alcohol was added to the upper aqueous layer. After centrifugation at 12000 rpm for 15 min, the liquid was discarded. Then, the deposit was washed with 70% ethanol twice, air-dried and dissolved with 55 μL duple-distilled water for the next process of purification. The total DNA preparation was purified using a PCR Preps DNA Purification Kit (Watson, China) according to the protocol supplied by the manufacturer.

III. *PCR Amplification*

The primers used for PCR amplification were designed based on the primers for *Diseae* species (Orchidaceae)[19] and the known rDNA ITS sequences of Zingiberaceae plants. Nucleotide sequences of the primers were as follows: 5'-ATTGAATGGTCCGGTGAAGTGTTCG-3' (P1) and 5'-AATTCCCCGGTTCGCTCGCCGTTAC-3' (P2). The forward primer P1 was located at the flanking region of the 3' end of 18S and the reverse primer P2 was located at the flanking region of the 5' end of 26S. PCR amplification was performed using 70-80 ng of total DNA as a template in 30 μL of reaction mixture, consisting of 3.0 μL 10 × PCR buffer, 0.15 mM of dNTPs (Sangon, China), 0.20 mM of each primer, and 1.0 U of Taq Polymerase (Promega, USA). Amplification was carried out under the following conditions: pre-cycling at 94°C for 4 min, 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 1 min and elongation at 72°C for 3 min, and then final elongation at 72°C for 3 min. PCR products were detected by 1.5% agarose gel (containing 1 μg/mL ethidium bromide) electrophoresis.

IV. *Sequencing*

The purified PCR products by a PCR Preps DNA Purification Kit (Watson, China) served as the template. The sequencing reaction was performed using the Big Dye™ Terminator RR Mix (Perkin Elmer, USA) with the same primers as in PCR amplification. The thermal cycling conditions was 95°C for 30 s, 35 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. After puri-

Table 1. Samples used in DNA sequence analysis

Code	Species	Voucher	Locality	GenBank accession
1	*Amomum villosum* Lour.	ZHAO97011	Yangchun, Guangdong	EF488008
2	*Alpinia japonica* (Thunb.) Miq.	ZHAO98004	Conghua, Guangdong	AF254474
3	*Alpinia pinnanensis* T. L. Wu & Senjen	ZHAO98005	Wuming, Guangxi	AF254470
4	*Alpinia guinanensis* D. Fang & X. X. Chen	ZHAO98021	Nanning, Guangxi	AF254477
5	*Alpinia tonkinensis* Gagnep.	ZHAO98011	Nanning, Guangxi	AF254473
6	*Alpinia blepharocalyx* K. Schum. var. *glabrior* (Hands-Mazz.) T. L. Wu	ZHAO98006	Wuming, Guangxi	
7	*Alpinia suishaensis* Hayata	ZHAO98002	Conghua, Guangdong	AF254465
8	*Alpinia stachyoides* Hance	ZHAO98015	Yangchun, Guangdong	
9	*Alpinia polyantha* D. Fang	ZHAO98023	Guangzhou, Guangdong	AF254475
10	*Alpinia pumila* Hook. f.	ZHAO98017	Guangzhou, Guangdong	AF254472
11	*Alpinia jianganfeng* T. L. Wu	ZHAO97002	Guangzhou, Guangdong	EF488011

fication, the sequencing products were analyzed on an automated DNA sequencer (ABI PRISM™ 310 Genetic Analyzer, USA). The obtained sequences were edited and aligned with the Clustal W program. The nucleotides sequence data of ITS1-5.8S-ITS2 region were deposited in the GenBank nucleotide sequence database with the accession numbers shown in Table 1. The borders of ITS1, 5.8S, ITS2 regions were determined by comparison with the known sequences of Zingiberaceae plants.

RESULTS AND DISCUSSION

A comparison of alignments of the ITS1 sequences from *Amomum villosum* and ten *Alpinia* species is shown in Figure 1. The lengths and base compositions of the sequences were measured, and the numbers of transitions/transversions and substitution percentage were summarized.

For all the species, the lengths of the ITS1 sequences were 175~179 bp. The G+C contents of the sequences from ten *Alpinia* species were 53.37~55.93%, and that of *Amomum villosum* was slightly higher at 56.74%. The *Aplinia* species are more closely related to each other than to *Amomum villosum*. In fact, the minimum difference (base substitution percentage) between these ten *Aplinia* species was 0.57% (between *A. japonica* and *A. pumila*), and even the maximum was only 9.60% (between *A. tonkinensis* and *A. jianganfeng*). On the other hand, the difference between *Amomum villosum* and the *Alpinia*

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      1                                                                   70
1     TTGTGAGAGC  ACTGAACAAT  GGATGATTGT  GAACGTGTCA  ACATGCCCCT  CTACTTGGCC  CCATTTT--G
2     ...A......  .T.....G.C  .....G....  ...T......  ..G.......  T.TG...-..  ....G.....
3     ...A......  .T.....G.C  .....GA...  ...T......  ..G.......  T.TG...-..  .T..G.....
4     ...A......  .T.....G.C  .....GC...  ...T......  ..GC......  T.CGC..-..  ....G.-...
5     ...A......  .T..G..G.C  .....GC...  ...T......  ..GC......  A.CGC..-..  ....G.....
6     ...A......  .T.....G..  .....GC...  ...T......  ..GC......  T.CGC..-..  ....G..GT.
7     ...A......  .T.....G.C  .A....C...  ...T......  ..G.......  T.CGC..-..  ....G.....
8     ...A......  .T.....G.C  .A...G....  ...T......  ..G.......  T.TG...-..  ....G.....
9     ...A......  .T.....G.C  .....GC...  ...T......  ..G.......  T.TG...-..  ....G.....
10    ...A......  .T.....G.C  .....-....  ...T......  ..G.......  T.TG...-..  ....G.....
11    ...A......  .T.....G.C  ..........  ...T......  ..G....A..  T.TG...-..  ....G.....

      71                                                                  140
1     GTGGCCGACT  GACCATAGCT  CGGTGCGATC  GGCACCAAGG  AACAATGAAC  TCAGAAGCAG  AGGG-CCCTC
2     .CA.....T.  ....G.....  ..........  T.........  ..........  .........-  ..........
3     .CA.....T.  ....G.....  ..........  T.........  ..........  .........-  ..........
4     .CA.TT..T.  ..T.G.....  ..........  T.........  ..........  .........T  .T........
5     .CA.TT..T.  ..T.G.....  ..........  T.........  ..........  .........T  ....G.....
6     .CA.TT..T.  ..T.G.....  ..........  T.........  ..........  .........T  ....T....T
7     .CA...T..T.  ....G.....  ..........  T.........  ..........  ..........  ..........
8     .CA...T..T.  ..T.G.....  ..........  T.........  ..........  ..........  ..........
9     .CA...T..T.  ....G.....  ..........  T.........  ..........  ..........  ..........
10    .CA.....T.  ....G.....  ..........  T.........  ..........  ..........  ...A......
11    .CA.....T.  ....G.....  ..........  T.........  ..........  ..........  ..........

      141                                                 183
1     GGTGTGCCCG  GGG-AGCCCA  CT-GCGTCAG  AGACGGTCGG  AAT
2     A.C....G..  A.....G...  A....A..G.  ...T.CCTCA  ...
3     A.C....G..  A.....G...  A....A..G.  ...T.CCTCA  ...
4     A.C....G..  ......G...  G....A..G.  ...T..CTCA  ...
5     A.C...TG..  ......G...  A....A..G.  ...T..CTCA  ...
6     A.C....G.A  ..-...G...  A....A..G.  ...T.CCTCA  ...
7     A.C....G..  A.....G...  A....A..G.  ...T.CCTCA  ...
8     A.C....G..  ......G...  A.T..A..G.  ...T.CCTCA  ...
9     ..C....GT.  A..G..G...  A....A..G.  ...T.CCTCA  ...
10    A.C....G..  A.....G...  A....A..G.  ...T.CCTCA  ...
11    A.C....G..  A.....G...  A....A..G.  C..T.CCTCA  C..
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Figure 1. Alignments of ITS1 sequences of *Amomum villosum* and ten *Alpinia* species. Dots represent sequence identity with sample 1, and dashes indicate deletions.

1. *Amomum villosum*; 2. *Alpinia japonica*; 3. *Alpinia pinnanensis*; 4. *Alpinia guinanensis*; 5. *Alpinia tonkinensis*; 6. *Alpinia blepharocalyx* var. *glabrior*; 7. *Alpinia suishaensis*; 8. *Alpinia stachyoides*; 9. *Alpinia polyantha*; 10. *Alpinia pumila*; 11. *Alpinia jianganfeng*

species was 17.61~21.59%, which was obviously much greater. As demonstrated in these molecular data, the ITS1 sequences may serve as a useful tool for comparison between intra-genus or inter-genus samples.

Sequence alignment of the ITS1 from these eleven species revealed that there are 55 polymorphic nucleotide sites and almost every species had their own specific variable bases. Therefore, these polymorphic sites can be used as DNA markers not only to distinguish *Amomum villosum* from *Aplinia* species but also to identify the original species of the raw material.

For correct identification of biological materials, DNA profiling has several advantages over morphological and chemical analyses because genotypes rather than phenotypes are directly assayed; consequently, the result is not affected by environmental factors. The present investigation indicates that DNA authentication is reliable in differentiating *Amomum villosum* from the ten *Alpinia* species. The selected ITS1 sequences may be useful to identify Fructus Amomi and its substitutes or adulterants from various *Alpinia* species. Based on our findings, the prospects of establishing methods to identify the crude drugs of Fructus Amomi are promising.

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