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Identification of Saposhinkoviae Radix in Concentrated Chinese Medicine Preparations by Nested PCR and DNA Sequencing Methods

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ABSTRACT

Saposhinkoviae Radix, the dried root of *Saposhinkovia divaricata* (Turcz.) Schischk., is widely used in Chinese medicine preparations. This paper reports on the development of a nested polymerase chain reaction (nested PCR) and DNA sequencing method able to detect the presence of authentic Saposhikoviae Radix in Chinese medicine preparations. Forty concentrated preparation samples in 17 formulas containing a total of 54 kinds of raw materials were purchased. DNA samples were completely digested with a lysis buffer containing proteinase and sarcosyl, extracted with organic solutions and CTAB, and then purified using a commercial kit (QIAGEN GmbH, Germany) that contained silica membrane to absorb the DNA. The internal transcribed spacer (ITS) regions of 40 preparations were amplified by nested PCR. All PCR products were analyzed by auto-sequencer. We compared resultant sequences with the ITS sequences obtained from the standard and Genbank databases. Saposhinkoviae Radix in all 40 preparations tested were identified as *Saposhinkovia divaricata*. These results indicate the above is a valid method by which to identify the presence of authentic Saposhinkoviae Radix in Chinese medicine preparations.

Key words: *Saposhinkovia divaricata*, nested PCR, internal transcribed spacer (ITS)

INTRODUCTION

Because Chinese medicine, prepared in the traditional manner, must be boiled for a long time before being consumed, it is not suited to the pace of modern life. Pre-prepared, concentrated Chinese medicine, known as "scientific Chinese medicine", have therefore gained in popularity. For "scientific Chinese medicine", Chinese medicine raw materials are ground, boiled, filtrated, concentrated, dried and then blended with an excipient. Different formulas require different Chinese medicine raw material components and the complexity of resultant herbal ingredients complicate identification of constituent components.

Physical analytical methods (such as histology and micrography) are difficult to use effectively on ground herbal ingredients. Chemical analytical methods (such as thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) for chemical quality standardization)[1] may deliver inconclusive readings on such preparations. Therefore, developing new analytical methods to authenticate ingredients used in Chinese medicine preparations is necessary.

DNA analysis is an increasingly important tool in Chinese medicine raw materials identification. This method of analysis is more reliable than chemical and physical analyses because, unlike phenotypes, a plant's genotype is unaffected by either different growth environments or its use in different medical preparations. Molecular techniques of DNA analysis, such as DNA Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), PCR-Sequencing, and PCR-RFLP, have been used successfully to identify Chinese medicine raw materials[2]. Many genes have been used as DNA markers for herb identification and phylogenetic analysis[3-6]. Among these, the internal transcribed spacer (ITS) varies between species of the same plant genus and has been generally used as the DNA molecule marker of choice.

At present, using molecular techniques to identify the presence of a specific herb in concentrated preparations is seldom reported. This may be due to the fact that the concentration process used at pharmaceutical factories leaves few intact cells and DNA in finished preparations, making extracting and detecting DNA using molecular biology methods unproductive. However, as PCR can be applied to amplify the DNA in concentrated preparation, PCR-sequencing methods may offer a viable approach. Once amplified by PCR, the DNA marker can be sequenced out for herb identification. Only one DNA marker for a single herb can be amplified at a time because this sequencing method cannot differentiate one herb from another in preparations.

According to the Chinese Medicine Pharmacopoeia, true Saposhinkoviae Radix is the dried root of

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Saposhinkovia divaricata (Turcz.) Schischk. When consumed, it acts as an antihypertensive, analgestic and anticonvulsive[7-9]. Saposhinkoviae Radix is widely used in Chinese medicine preparations. In this report, we propose a modified method by which DNA can be extracted from concentrated Chinese medicine preparations. Using nested PCR and Sequencing methods, we amplify the ITS gene for use as a marker to identify the presence in such preparations of true Saposhinkoviae Radix.

MATERIALS AND METHODS

I. *Standard and Samples*

A sample quantity of authentic Saposhinkoviae Radix was purchased from the Pharmaceutical Industry Technology and Development Center (Taiwan) for use as the standard. Forty samples of Chinese medicine preparations with Saposhinkoviae Radix noted on the ingredient label were purchased from traditional Chinese (herbal) pharmacies in Taiwan. Ten unverified herb samples of herbs imported from Mainland China claimed to be Saposhinkoviae Radix were also collected.

II. *Primers*

We amplified the ITS region for herb samples using PCR with primer 18F (5'-GTGAACTGCG-GAAGGATCTTG-3') and 28R (5'-CCGCCTGACCT-GRGGTC-3'; R = A/G). This primer pair was designed based on the conserved region of the 18S and 26S rRNA genes and wobble theory. For preparation samples, we amplified the ITS region by PCR with primers F3 (5'-ACCCGCTAACACGTCAACAAT-3') and R2 (5'-AAGCGCACAGA GTGTGTGCT-3'), and then the PCR products were re-amplified by nested PCR with primer F1 (5'-AACAATTTGGGCAAGCGTCG-3'') and R4 (5'-GGATCTCTCTAAGATGACGAG-3'). These two primer pairs were designed in accordance with the ITS sequence identified in the standard specimen.

III. *DNA Extraction*

We modified our DNA extraction method from that published by Saghai-Maroof *et al*.[10]. First, we ground the herb samples using a grinder. One hundred milligrams powder of all samples was respectively placed in a 2.0-mL micro-centrifuge tube, digested in 1 mL lysis of buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 1% *N*-lauroyl sarcosine sodium salt (sarcosyl), and 1 mg/mL proteinase K) and incubated at 56˚C for 1 hr. The sample solution was extracted with 1 mL of phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) mixture solution and centrifuged at 12,000×g for 5 min. The aqueous layer was mixed with 0.2 mL of 5 M NaCl and 0.15 mL of 10% hexadecyltrimethylammonium cromide (CTAB) in 0.7 M NaCl, and further incubated at 56˚C for 15 min. The solution was extracted with a 1 mL of chloroform/isoamyl alcohol (24:1, v/v) mixture solution and centrifuged at 12,000×g for 5 min. The aqueous solution was then transferred into another 2.0-mL micro-centrifuge tube. DNA in the solution was precipitated by adding 0.7 mL of isopropanol and 0.1 mL of 3 M sodium acetate, after which the solution was centrifuged at 12,000×g for 5 min. Precipitated DNA was air-dried, and dissolved in 0.1 mL of sterile, distilled water. A PCR purification kit (QIAGEN GmbH, Germany) was used to purify the dissolved DNA, with the silica membrane provide able to absorb DNA through the high concentration of chaotropic salt in solutions. The purified DNA product was reserved for further PCR analysis.

III. *Polymerase Chain Reaction and ITS Fragment Amplification*

All purified DNA were used as the PCR amplification template. Amplification was performed in 50 μL of solution by incubating 1 μL of DNA with 5 μL of 10× Taq buffer, 1.0 μL of 50 μM PCR primer, 1 μL of 10 mM dNTP, 2 units of Taq polymerase, and sterilized distilled water. A negative-control (no template) was included in each reaction. Mixtures containing the DNA extracted from herb samples were amplified by 30 cycles of PCR, each cycle consisting of a 30 sec denaturation step at 94˚C, a 30 sec annealing step at 58˚C, and a 30 sec extension step at 72˚C. The mixtures containing DNA from preparation samples were amplified by 25 cycles of PCR, each cycle consisting of a 30 sec denaturation step at 94˚C, a 30 sec annealing step at 56˚C, and a 30 sec extension step at 72˚C. One microliter of the resultant PCR product was taken as a template for nested PCR. Nested PCR conditions were set as 25 cycles of an initial DNA denaturation step at 94˚C for 30 sec, an annealing step at 59˚C for 30 sec, and an extension step at 72˚C for 30 sec. Five microliter of final PCR products were analyzed by electrophoresis in a 2% agarose gel. After electrophoresis in 0.5× TBE buffer (Tris-Boric acid-EDTA) at 100 V for 30 min, the gel was stained with ethidium bromide (0.5 mg/mL) before being photographed under ultraviolet (UV) illumination.

IV. *Sequencing*

We commended the final PCR products to sequencing analysis by Mission Biotech (Taiwan) using the Applied Biosystems 3730 (ABI, USA). Sequence data were compared with the Genbank database and the ITS sequence of the standard.

RESULTS AND DISCUSSION

The goal of this project was to identify the genetic sequences in standard and herb samples and then use sequence data to design primers that could help identify

authentic Saposhinkoviae Radix in our preparation samples. Our extraction method was modified from previously published approaches in order to conserve more DNA during the extraction processes. We used a commercial kit in place of an alcohol washing procedure to eliminate DNA loss and improve DNA purity. We found that this method, originally applied in the extraction of herbal DNA from Chinese medicine preparations, suited to the extraction of DNA from the herbs themselves. The PCR products of herb samples were all about 600 bp size (ITS1-5.8S-ITS2). Sequence data and the result of Genbank database searches, shown in Table 1 and Figure 1, indicate only one sequence from the herb samples matches the sequence of *Saposhinkovia divaricata*. All herb samples can be identified by histological methods and micrography in order to determine their scientific name. We will submit sequence data to Genbank in the future.

The presence of 10 to 20 herbal ingredients in Chinese medicine preparation samples may confound efforts to detect the presence of any single one ingredient. All con-

Table 1. The sequences of raw material samples compared with Genbank database

	Sample	Results of Genbank searching	Sequence similarity
	Standard	*Saposhnikovia divaricata*	99%[a]
S3	Ko Lu Tzu	*Smyriopsis aucheri*	89%
S4	Fang Feng	*Angelica cartilagino*	97%
S5	Chuan Fang Feng	*Smyriopsis aucheri*	89%
S6	Kuan Fang Feng	*Saposhnikovia divaricata*	99%[a]
S8	Chuan Fang Feng	*Sphallerocarpus gracilis*	100%[b]
S9	Chuan Fang Feng	*Smyriopsis aucheri*	89%
S10	Cultured Kuan Fang Feng	*Peucedanum terebinthaceum*	95%
S11	Cultured Fang Feng	*Sphallerocarpus gracilis*	100%[b]
S12	Han Fang Feng	*Smyriopsis aucheri*	89%
S13	Shui Fang Feng	*Carum carvi*	99%[a]

The Accession number of each result in Genbank is *Saposhnikovia divaricata*-AY548221, *Smyriopsis aucheri*-AF077909, *Angelica cartilagino*-AY548222, *Sphallerocarpus gracilis* -AF073677 and AF073678, *Peucedanum terebinthaceum*-AY548232, and *Carum carvi*-AF077878.

[a]Sequences of S, S6 with *Saposhnikovia divaricata*-AY548221 and S13 with *Carum carvi*-AF077878 were one different nucleotide at 5.8S rRNA gene, the similarity was 99%.

[b]Comparsion between sequences of ITS1 and ITS2 because of no sequence data of 5.8S rRNA gene of *Sphallerocarpus gracili* in Genbank.

Table 2. A list of concentrated preparation samples, sample amounts, and complete list of herbal ingredients

Sample	Sample amount	Complete listing of herbal ingredients
Gou Terng San	3	Akebiae Caulis, Allii Fistulosi Bulbus,
Chuan Chiong Char Tyau San	3	Anemarrhenae Rhizoma, Angelicae Dahuricae Radix, Angelicae Laxiflorae Radix, Angelicae Sinensis Radix,
Jing Fang Bai Du Tang	3	Asari Heterotropoidis Herba, Atractylodis Lanceae Rhizoma,
Shiau Feng San	3	Atractylodis Macrocephalae Rhizoma, Arctii Fructus, Aurantii Fructus, Bupleuri Chinensis Radix, Cassiae Semen,
His Kan Ming Mu San	3	Chuanxiong Rhizoma, Cimicifugae Dahuricae Rhizoma,
Shyr Wey Bay Dwu Tang	3	Cinnamomi Lignum, Citri Sinensis Exocarpium , Coptidis Rhizoma, Cyathulae Radix,
Ching Shang Fang Feng Tang	3	Dendranthemae Morifolii Flos, Ephedrae Herba,
Dwu Hwo Jih Sheng Tang	3	Eucommiae Cortex, Forsythiae Fructus, Fritillariae Ussuriensis Bulbus, Gardeniae Fructus,
Jen Ren Hwo Minq Yiin	3	Gentianae Crassicaulis Radix, Ginseng Radix,
Jing Jieh Lian Chyau Tang	2	Glycyrrhizae Radix, Ligustici Rhizoma, Lonicerae Japonicae Flos, Loranthi Ramulus,
Shin Yi San	2	Magnoliae Biondii Flos, Menthae Herba,
Fang Feng Tung Sheng San	2	Notopterygii Rhizoma et Radix, Ophiopogonis Radix, Paeoniae Alba Radix, Peucedani Radix,
Ching Yann Lih Ger Tang	2	Pinelliae Ternatae Rhizoma, Platycodi Radix,
Tang Kuei Yin Tzu	2	Polygoni Multiflori Radix, Pruni Cortex,
Ching Liang Yin	1	Rehmanniae Radix, Rhei Rhizoma, Saposhinkoviae Radix, Schizonepetae Herba, Scutellariae Radix, Sesami Semen,
Sheau Shiuh Minq Tang	1	Sophorae Flavescentis Radix, Theae Folium, Tribuli Fructus,
Ching Kong Gau	1	Trichosanthis Radix, Unicariae Cum Uncis Ramulus, Viticis Trifoliae Fructus, Zingiberis Rhizoma

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                                                                                                                60
AY548221          TCGAATCCTGCAACAGCAGAATGACCCGCTAACACGTCAACAATTTXGGGCAAGCGTCGG
Standard          ------------------------------------------------------------
Preparations      XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX-------------------------
S03,S05,S09,S12   -----G-----GXT-----T-------------TA--X-----CA-C--------X--AC
S04               -------------------------T-T---------------------------C---
S06               ------------------------------------------------------------
S08,S11           -----------TCT---G--------T-T-----T--XT-A--CA-C-----------A-
S10               -------------T--------------------T---G-A-----A--------A----
S13               -----C-----GAT-------C------------T--XT-A--CA-C--------X--AT

                                                                                                               120
AY548221          GGGGCCTCGGTCTCCTGTCTGCGAACCCTTGGTAGGTGGCCACTCCCGGGTGGCCACTGG
Standard          ------------------------------------------------------------
Preparations      ------------------------------------------------------------
S03,S05,S09,S12   ----AT-T---TCCTC-----T----------------------TT-CA---A----C--
S04               ---A---------C------------------C--------------------------
S06               ------------------------------------------------------------
S08,S11           -----A-A----CT----T-----T---AA-----T--T--C---AT----TT-A--CA-
S10               -------------C-------T---T--C----------A--------A-------TC--
S13               ----AT--CT--CCA---TG--------CA-----------G-C-T----------TC--

                                                                                                               180
AY548221          CCTTCAAAATCATTCGGGCGCGGAATGCGCCAAGGACCTTAAAAACTGAATTGTACGTCC
Standard          ------------------------------------------------------------
Preparations      ------------------------------------------------------------
S03,S05,S09,S12   ---ATG-------C----------------------AAA-----X--------X------
S04               -----A-------------------------------------X---------G-----
S06               ------------------------------------------------------------
S08,S11           --AA-T------AC-------T--T-----------AA----T-X-G-------T---TA
S10               ---A-A--------T----------------------A------X---------------
S13               ---A-A----A---------T---------------AA------X--------X-T-A--

                                                  ITS1◄► 5.8S                   240
AY548221          GTATCCCGTTAGXCGGGCAGCGGCGTCATTCTAAAACACAAXCGACTCTCGACAACGGAT
Standard          ------------------------------------------------------------
Preparations      ------------------------------------------------------------
S03,S05,S09,S12   -CTCT-X-------------T----------C---------A------------------
S04               ------T-------------------------------------------------------
S06               ------------------------------------------------------------
S08,S11           -CT--T----C------A---AA-----G---G--------AT--------G--------
S10               ----------G-----------------------------G--------------------
S13               ACT-T---A---T-----T-T--------------------A------------------

                                                                                                               300
AY548221          ATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAG
Standard          ------------------------------------------------------------
Preparations      ------------------------------------------------------------
S03,S05,S09,S12   ---C--------------------------------------------------------
S04               ------------------------------------------------------------
S06               ------------------------------------------------------------
S08,S11           ---C-------T-------------------------------------------------
S10               ------------------------------------------------------------
S13               ------------------------------------------------------------

                                                                                                               360
AY548221          AATCCCGTGAACCGTCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCACTAGGCTGAGGG
Standard          -------------A----------------------------------------------
Preparations      -------------A----------------------------------------------
S03,S05,S09,S12   -------------A-------------------------T-------------CA----
S04               -------------A-------------------------------T--------------
S06               -------------A----------------------------------------------
S08,S11           -------------A-T--------------------------------------------
S10               -------------A-----------------------------------T------------
S13               -------------A-----------------------------------T----T------
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                                    5.8S<►ITS2                                  420
AY548221            CACGTCTGCCTGGGTGTCACGCATCGTCTTTGCCCACAGACCACTCACACCTGXAGAAGX
Standard            ------------------------------------------------------------
Preparations        ------------------------------------------------------------
S03,S05,S09,S12     --------------------------G--X--T-C--X---------T-TAAT--G---
S04                 ------------------------------------A---------------------
S06                 ------------------------------------------------------------
S08,S11             ---------T---------------T-G--X----TTGAC-A-A-G-AT-T-CA-AG--A
S10                 ------------------------------X------GA-----C---------------
S13                 ---------------------T-----G--X----C--X---------T---CTG-G---

                                                                            480
AY548221            TTGTGTAGGTTTGGGGGCGGAAACTAGCCTCCCGTACCTTGTCGTGCGGTTGGCGGAAAA
Standard            ------------------------------------------------------------
Preparations        ------------------------------------------------------------
S03,S05,S09,S12     C-XXXCC-----------------G---------G-----AT------------C----
S04                 -----CC-----------------G-----------A---------------T-----
S06                 ------------------------------------------------------------
S08,S11             --T---------T------T-----G---------G--C---T------------T----
S10                 -----CC--G------------T--G--------------------------T------
S13                 C-A-TCC-----A----------T-G---------------CG------A----AC----

                                                                            540
AY548221            ACGAGTCTCCGGCGACGGATGTCGCGACATCGGTGGTTGTXAAAAGACCCTCTTCTCTTG
Standard            ------------------------------------------------------------
Preparations        ------------------------------------------------------------
S03,S05,S09,S12     G-----------------C--T-----------------A-----XXX-C---G-----
S04                 -----------------C---------------------------------G-----
S06                 ------------------------------------------------------------
S08,S11             -T------AT--T--T-----------------------ATG------TCT--G-----
S10                 T-----------------C----T-------------------------------G-----
S13                 GT---------AT------C----T-----T---------T------ACT----G-----

                                                                            600
AY548221            TCGCGCGAATCCTCGTCATCTTAGAGAGATCCAGGACCCTTAGGCAGCACACACTCTGTG
Standard            ------------------------------------------------------------
Preparations        --------------------------------XXXXXXXXXXXXXXXXXXXXXXXXXXX
S03,S05,S09,S12     ----A------TGG--TGC-----T---C--G---------G-----------T-G----
S04                 ------A-------------------------------------------------
S06                 ------------------------------------------------------------
S08,S11             ---T-T----G-C-----C-----TC--C--G-T-------G---GC----A---T----
S10                 -T-------------------------C-------------------------------
S13                 --A--T------C----------TG------T-T--T-----A--GA------T-G----

AY548221            CGCTTCG
Standard            -------
Preparations        XXXXXXX
S03,S05,S09,S12     ----C--
S04                 -------
S06                 -------
S08,S11             -----T-
S10                 -------
S13                 ----C-A
```

Figure 1. Sequence alignment of ITS regions and 5.8S rRNA gene from samples and *Saposhnikovia divaricata*-AY548221 in Genbank. A hyphen (–) indicates that the nucleotide is identical to the uppermost sequence. An "X" indicates a gap in the data.

centrated preparation samples, sample amounts, and complete list of herbal ingredients are shown in Table 2. We tried to apply nested PCR to detect Saposhinkoviae Radix. The ITS length for all herbs were similar and we could not differentiate them by using electrophoresis after PCR only. DNA sequencing is necessary to certify results. ITS of Saposhinkoviae Radix was amplified specifically by PCR and nested PCR to get an amount of DNA adequate for sequencing.

The 54 herbs in our preparation samples (Table 2) included Apiaceae plants such as *Saposhinkovia divaricata* and others. We downloaded and analyzed sequence data

for Apiaceae herbs from Genbank, finding that the ITS regions in Apiaceae plants are similar. Designed primers were used to differentiate the ITS sequences of 54 herbs. Based on the acquired sequences and Genbank data, we designed three forward and four reverse primers and tried different combinations for PCR and nested PCR. Primarily, primer 18F and 28R were used for first round PCR, with another designed primer pair used for second round PCR. Using primer 18F and 28R designed from the conserved region of the 18S and 26S rRNA genes, we amplified the ITS of all herbs in samples in first round PCR. Sequencing analysis was then confounded at mixed sequences. Each round of PCR needed to use specific primers. The electrophoresis pattern of nested PCR products from different samples is shown in Figure 2.

We tested each combination of primer pairs to amplify the DNA of every sample by PCR-nested PCR in order to obtain a single final product for each. Resultant products were used to obtain clear sequence data from sequence analysis. All nested PCR products were analyzed by autosequencer and compared with the sequences of the standard and the Genbank database. Only one combination (F3-R2 and F1-R4) was able to amplify a single DNA fragment, which was subsequently used to obtain clear sequence data.

The result of sequence analysis indicates that the sequences of all nested PCR products mirrored that of the standard (Figure 1). The Saposhinkoviae Radix in preparation samples was identified as *Saposhinkovia divaricata*. Therefore, all samples of Chinese medicine preparations selected for analysis in our test contained true Saposhinkoviae Radix, as indicated on their labels.

In conclusion, this paper reports on the development of a DNA extraction method combined with nested PCR-sequencing method that was used to identify Saposhinkoviae Radix in 40 discrete Chinese medicine preparations. Nested PCR, using the combination of primer pairs (F3-R2 and F1-R4), was able to amplify the ITS of true Saposhinkoviae Radix in 17 formulas of Chinese medicine preparations containing 54 herbs. This study model may be further developed and applied in the identification of other herbal ingredients in concentrated Chinese medicine preparations.

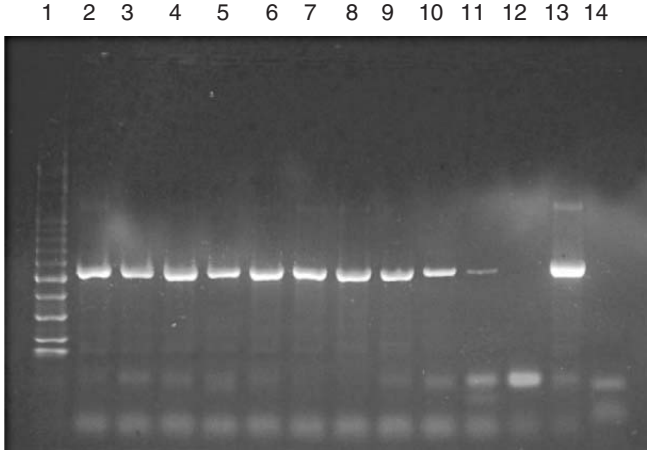

Figure 2. Nested PCR product of preparation samples and the standard. Lane 1: 100 bps ladder; Lane 2~10: concentrated preparation samples 1~9; Lane 11: traditional preparation sample (Chien Pu Wan); Lane 12: concentrated preparation sample (Fu Yuan Huo Hsieh Tang) without Saposhinkoviae Radix; Lane 13: Standard; Lane 14: Blank (no template).

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