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Application of Inter Simple Sequence Repeat (ISSR) Marker for Typing of *Saccharomycopsis fibuligera* Isolated from Loog-pang, Kao-mag and Satho

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ABSTRACT

The D1/D2 26S rDNA sequence was shown to be applicable for yeast species identification. The size of this region was 600 bp and the identification results showed more than 98% similarity of all isolates compared to the sequences in the Genbank database. This research also included a small survey for ISSR variation in the yeast *Saccharomycopsis fibuligera*. Eight of twelve UBC (University of British Columbia) primers gave 80 reproducible patterns. The size of the ISSR products ranged from 300 to about 2000 bp. Among the eight primers used in this study, the primers in the UBC group (880/888/889) gave the most informative profiles for grouping and distinguished 11 *S. fibuligera* isolates into six groups. The two isolates in groupVI were further divided from others by UBC 891. Only one of the primers in the UBC group together with UBC 891 was enough to discriminate the 11 *S. fibuligera* isolates into seven groups. These results indicated that the ISSR marker could be successfully applied to study intra-specific variation and that there were genetic variations in the yeast species of *S. fibuligera* from different kinds of food products in Thailand.

Key words: *Saccharomycopsis fibuligera*, ISSR (inter simple sequence repeat), UBC primers, loog-pang, kao-mag, satho

INTRODUCTION

Rice wine is a popular and traditional beverage in Asia. Loog-pang (mold bran starter) or traditional starter flat cakes are used as a fermentation starter for the production of Thai traditionally fermented alcoholic products, such as satho (rice wine) and kao-mag (alcoholic, sweetened rice). Starter cultures for traditional alcoholic beverages in other Asian countries are peh-chu in China, ragi tapai in Indonesia, nuruk in Korea and bubod in the Philippines (Jeyaram *et al.,* 2008). These starters are made by mixing rice flour or glutinous rice with microorganisms (yeasts, molds and bacteria), herbs and spices including garlic, pepper, chili and cinnamon. There are previous reports on strain selection (Dung *et al.,* 2006), characteristics (Dung *et al.,* 2007),

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identification and enzymatic activity (Tsuyoshi *et al.,* 2005) for microorganisms present in products and starters. *Saccharomycopsis fibuligera* has been commonly found in both major types of loog-pang. Loog-pang lao and loog-pang kao-mag are used as starters for the fermentation of satho and kao-mag, respectively. *S. fibuligera* was found to have a strong amylase activity in both products. The enzymatic and molecular characteristics of *Saccharomycopsis* alpha amylase were studied by Matsui *et al.* (1990) and Gonzalez *et al.* (2008). However, not all *S. fibuligera* strains have the ability to produce quality beverages or foods. Specific strains of this yeast are needed for specific products.

The use of molecular markers has provided important advances in the identification and differentiation of yeasts at both species and strain levels. PCR-based methods are of specific interest for the identification and study of genetic variation because they are simple and fast. In recent years, the identification of yeasts at the species level was based on the analysis of the DNA sequence of the divergent D1/D2 domain at the 5′ end of the large subunit (26S) rDNA (Cadavid *et al.,* 2008; Jespersen *et al.,* 2005). The analysis of this domain and the two ribosomal, intergenic, spacer regions (ITS1 and ITS2) are now well established for yeast identification (Kurtzman and Robnett, 1997). Molecular identification of yeast isolates from traditional alcoholic products is performed by mt-RFLP, ITS-RFLP and PFGE (Jeyaram *et al.,* 2008; Cadavid *et al.,* 2008). Inter Simple Sequence Repeat (ISSR) markers are also used for a PCR-based method. They are arbitrary, multi-loci markers produced by PCR amplification with microsatellite primers. This technique is advantageous because no prior genomic information is required (Bornet and Branchard, 2001). ISSR markers are highly polymorphic and useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Reddy *et al.,* 2002). Moreover, they are easy and quick to both develop and use. Because of their high level of polymorphism and reproducibility, this fingerprinting is widely used, especially in plants (Bornet *et al.,* 2002), but only a few reports are available for yeasts.

The present study focused on the differentiation of strains of *S. fibuligera* that is the dominant species used to produce loog-pang. The species was identified by its morphology. Sequencing of the D1/D2 domain was used to confirm the identification. Variations within species or subspecies were examined by determination of their patterns of PCR products amplified by the ISSR method.

MATERIALS AND METHODS

Yeast isolation

Six reference strains were purchased from the Microbiological Resources Centre (Thai Institute of Scientific and Technological Research) and five strains were isolated from various sources (Table 1). Yeast strains were isolated by a dilution method. One gram of each sample was mixed with 9 ml normal saline solution and diluted to 10^6. Yeast in 10^4-10^6 dilutions was isolated on Dichloran Rose-Bengal Chloramphenicol (DRBC) Agar at 28°C. Yeast collection was focused on yeast-like fungi using colony morphology. All yeast strains were grown on YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) for DNA analysis. The purified yeasts were maintained on YM slant agar (0.3% w/v yeast extract, 0.3% w/v malt extract, 0.5% w/v peptone, 1% w/v glucose, 1.5% w/v agar) at 4°C.

DNA extraction

DNA isolation was performed by the modified method of Legras and Karst (2003). The yeast isolates were grown in 10 ml of YPD for 24 h at 28°C and then 3 ml of the culture was centrifuged (5000 rpm, 4°C, 5 min). The pellet was suspended and washed in sterile water three

Table 1 *Saccharomycopsis fibuligera* strains used in this study.

Strains	Sources
TISTR 5033	Rice wine (Satho)
TISTR 5096	Rice wine (Satho)
TISTR 5097	Mold bran starter (Loog-pang)
TISTR 5118	Mold bran starter (Loog-pang)
TISTR 5330	Mold bran starter (Loog-pang)
TISTR 5034	Mold bran starter (Loog-pang)
SF1	Satho submerge state
SF2	Kao-mag (white sticky rice)
SF3	Kao-mag (black sticky rice)
SF4	Kao-mag
SF5	Mold bran starter (Loog-pang)

times. Then 300 μl of lysis buffer (10mM Tris-HCl pH8, 1mM EDTA, 0.1M NaCl, 2% Triton X-100, 1% SDS), 0.3 g of glass beads (diameter 0.5 mm) and 400 μl of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) were added to the pellet. After vortexing for three min followed by centrifugation at 12,000 rpm for 5 min, the upper phase was pipetted into a new tube and chloroform/isoamyl alcohol (24:1) was added in equal volume. After centrifugation at 12,000 rpm for 5 min, cold 100% ethanol was added to the supernatant for precipitation. The precipitated DNA was washed in cold 70% ethanol before drying and resuspending in 50 μl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

PCR amplification

PCR amplification was performed as described by Jespersen *et al*. (2005). The D1/D2 domain of the 5′ end of the large subunit (26S) rDNA was amplified by the following primers: NL1 (5′ -GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′ -GGT CCG TGT TTC AAG ACG G-3′). The 20 μl reaction volume contained 2 μl of 10X *Taq* buffer, 10 pmoles of primers, 0.08 mM of deoxyribonucleoside triphosphate (dNTP) mix, 2mM of $MgCl_2$, 0.5 U of *Taq* DNA polymerase (Fermentas) and 200 ng of template DNA. The amplification was performed in an automatic thermal cycler (GeneAmp PCR 9700; Applied Biosystems). The PCR program consisted of the following steps: an initial denaturation at 94 °C for 3 min, 36 cycles of 94°C for 2 min, annealing at 52°C for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 7 min, holding at 4°C. Amplification was confirmed by electrophoresis on 1.5% agarose gel 200V for 30 min, staining with ethidium bromide and photographing under UV light using Gel doc (1000 system Biorad).

Direct PCR sequencing

The amplicons were purified by precipitation with sodium acetate and 95% ethanol. The purified products were sequenced directly using Terminator Ready Reaction Premix (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems) following the manufacturer’s instructions. The cycle sequencing was amplified by use of primer NL1 or NL4. The reaction mix contained 1 μl of primer (5 μm), 4 μl of Terminal premix, 1 μl of PCR product (180-200 μl) and 4 μl of deionized water under the following conditions: preheat at 95°C for 10 min, then 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. After cleaning the cycle sequencing products, they were mixed with loading buffer. Samples were denaturated at

94°C before running in a capillary ABI PRISM 310 DNA sequencer (Applied Biosystems). The sequences were compared to the sequences reported in GenBank (http://www.ncbi.nlm.nih.gov/) using a basic BLAST search for yeast species identification.

ISSR analysis

Twelve UBC (University of British Columbia) primers (Table 2) were used for ISSR-PCR analysis. The reaction was performed in 15 µl of a PCR mixture containing 1.5 µl of 10xbuffer, 0.27 µM of primer, 1 mM of deoxyribonucleoside triphosphate (dNTP) mix, 1.25 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (fermentas), and 60 ng of template DNA. The reaction solution was then adjusted with deionized water. The amplification was performed in an automatic thermal cycler with the following thermal cycling program: an initial denaturation at 95°C for 2 min, 40 cycles of 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 40 s, with a final extension at 72°C for 8 min, holding at 4°C. Patterns of ISSR products were performed by electrophoresis on 1.5% agarose in 1xTBE buffer before staining by ethidium bromide and then photographing under UV light.

The ISSR amplified bands were scored for presence and absence as 0 and 1, respectively. A phylogenetic tree was constructed by combining data on ISSR products amplified by the eight primers. The ISSR data were analyzed with the Phylip package software. Genetic distances were estimated using the Restdist program. A dendogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA) with 1000 resampling bootstrap analysis.

RESULTS AND DISCUSSION

ISSR markers are useful tools for studying the population biology of plants and animals. This is the first report on the use of ISSR PCR-fingerprinting to discriminate *Saccharomycopsis fibuligera* isolates from loog-pang, kao-mag and satho. *S. fibuligera*, commonly associated with traditional fermented food and beverages, has not been detected in every state of fermentation. In general, it is involved in the amylolytic fermentation before alcohol-producing yeasts adopt the fermentation process.

Table 2 Sequences and UBC primers used in this study.

UBC primer no.	DNA sequences	Tm (1mNa$^+$) (°C)
UBC 878	$(GGAT)_4$	59.81
UBC 880	$(G(GA)_2)_3$	61.10
UBC 888	$BDB(CA)_7$	50.17
UBC 889	$DBD(AC)_7$	50.17
UBC 890	$VHV(GT)_7$	50.17
UBC 891	$HVH(TG)_7$	50.17
UBC 811	$(GA)_8C$	63.50
UBC 818	$(CA)_8G$	63.50
UBC 841	$(GA)_8YC$	62.29
UBC 848	$(CA)_8RG$	62.29
UBC 857	$(AC)_8YG$	62.29
UBC 868	$(GAA)_6$	57.67

UBC = University of British Columbia primer
B, D, H, R, V, Y = IUB code

At present, there are classical morphology-based and molecular methods for the identification of *S. fibuligera*. Morphologically, *S. fibuligera* is characterized by multilateral budding, septate hyphae and ascospore shape variation (Kurtzman and Blanz, 1998). However, phenotypic traits may lead to misclassifications and may be less sensitive than molecular identification methods.

Yeasts isolated from loog-pang, kao-mag and satho in a submerged state (SF1-SF5) (Table 1) were selected. Only isolates that could be morphologically reassigned to *S. fibuligera* were used for identification by sequence analysis. The identification results from sequences in the D1/D2 domain of the LSU rRNA gene gave 600 nucleotides as Kurtzman and Robnett reported in 1997 and 2003. The sequences of all isolates were compared to the D1/D2 sequences maintained in the GenBank database. Sequence analysis of the D1/D2 domain resulted in a good resolution at the species level. There was more than 98% similarity in all isolates and they could clearly be identified as *S. fibuligera*.

Twelve of the UBC primers were initially screened for ISSR analysis. Eight primers (UBC 880, UBC 888, UBC 889, UBC 890, UBC 891, UBC 811, UBC 818 and UBC 857) generated 80 reproducible polymorphic bands with an average of 10 bands per primer across 11 *S. fibuligera* isolates. The ISSR product sizes ranged from 300 to around 2,000 bp (Figure 1). The other four primers (UBC 878, UBC 841, UBC 848 and UBC 868) failed to produce bands (data not shown). Primer UBC* (818/857) classified 11 *S. fibuligera* strains into four groups: group A (TISTR 5033), group B (TISTR 5096) group C (TISTR 5097 and TISTR 5034) and group D (TISTR 5118, TISTR 5330, SF1, SF2, SF3, SF4 and SF5) (Table 3).

Primers UBC 811, UBC 890 and UBC 891 classified 11 *S. fibuligera* into five groups but each primer sorted different yeast strains into different groups. Primer UBC 811 generated group

Table 3 Grouping of *S. fibuligera* isolates by ISSR profiles.

UBC primer no.	Isolates											No. of groups
	TISTR 5033	TISTR 5096	TISTR 5097	TISTR 5118	TISTR 5330	TISTR 5034	SF1	SF2	SF3	SF4	SF5	
UBC*	A	B	C	D	D	C	D	D	D	D	D	4
891	A	B	C	D	D	C	E	E	E	D	E	5
811	A	B	C	D	D	C	D	D	D	E	D	5
890	A	B	C	E	E	C	D	E	E	E	E	5
UBC**	A	B	C	D	D	C	E	D	D	F	D	6
group	AAAAA (I)	BBBBB (II)	CCCCC (III)	DDDED (VI)	DDDED (VI)	CCCCC (III)	DEDDE (IV)	DEDED (VII)	DEDED (VII)	DDEEF (V)	DEDED (VII)	

DNA pattern = A, B, C, D, E and F
UBC* = UBC818 and UBC857
UBC** = UBC880, UBC888 and UBC889

A (TISTR 5033), group B (TISTR 5096), group C (TISTR 5097 and TISTR 5034), group D (TISTR 5118, TISTR 5330, SF1, SF2, SF3 and SF5) and group E (SF4). This primer could be used as a unique fingerprinting primer to distinguish SF4 from the others (Figure 1). Primer UBC 890 generated group A (TISTR 5033), group B (TISTR 5096), group C (TISTR 5097 and TISTR 5034), group D (SF1) and group E (TISTR 5118, TISTR 5330, SF2, SF3, SF4 and SF5). Primer UBC 891

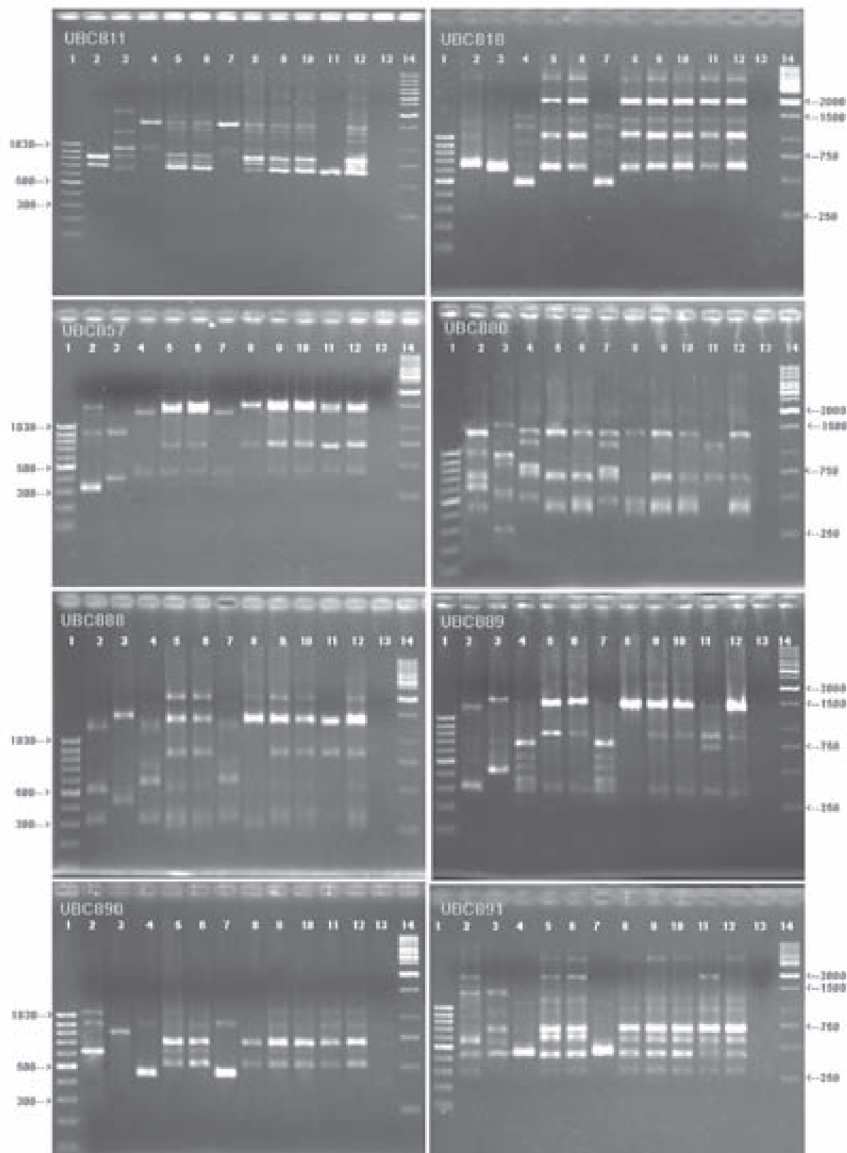

Figure 1 Comparison of the electrophoresis profiles amplified by 8 primers (UBC811, UBC 818, UBC857, UBC880, UBC888, UBC889, UBC890 and UBC891) obtained for 11 *S. fibuligera* isolates. Lanes 1 and 14 = 100 bp DNA Ladder and 1kb DNA Ladder (GeneRulerTM MBI Fermentas), respectively. Lanes 2-7 = *S. fibuligera* strains of TISTR 5033, 5096, 5097, 5118, 5330 and 5034, respectively. Lanes 8-12 = SF1-SF5 and Lanes 13-14 = negative control.

generated group A (TISTR 5033), group B (TISTR 5096), group C (TISTR 5097 and TISTR 5034), group D (TISTR 5118, TISTR 5330 and SF4) and group E (SF1, SF2, SF3 and SF5). Primer UBC** (880/888/889) gave the highest number of groups (6): group A (TISTR 5033), group B (TISTR 5096) group C (TISTR 5097 and TISTR 5034), group D (TISTR 5118, TISTR 5330, SF2, SF3 and SF5), group E (SF1) and group F (SF4).

The 11 yeast isolates were divided into seven groups by the analysis of ISSR DNA patterns from the eight primers: group I (TISTR 5033), group II (TISTR 5096), group III (TISTR 5097 and TISTR 5034), group IV (SF1), group V (SF4), group VI (TISTR 5118 and TISTR 5330) and group VII (SF2, SF3 and SF5). All eight primers generated unique ISSR patterns for each isolate in group I, group II and group III. This result indicated that any one of the primers was sufficient to differentiate these three groups from the others. In group IV (SF1) and group V (SF4), not only primers in the UBC** group exhibited unique banding profiles, but these two groups could also be separated from other groups by distinct bands using primers 890 and 811, respectively. Group VI (TISTR 5118 and TISTR 5330) could be distinguished by using only primer UBC 891, which produced an intense band and is suggested for use as a molecular marker.

The repeated analyses showed that only the faintest DNA bands were not reproducible. The dendogram showed mixing distributions in different groups with strong bootstrap values in most branches (Figure 2). It is interesting to note that there were genetic variations in *S. fibuligera* detected in this study even in the same product. Some yeast isolates were not clustered according to origin.

The 11 representatives of *S. fibuligera* were widely distributed in various products and frequently found in loog-pang, which is an important source for kao-mag and satho production. The clustering of some isolates was not consistent with the origin of the samples. Isolated yeasts from the same products were often placed in different groups or clades. Both TISTR 5096 and TISTR 5033 isolated from rice wine could be distinguished from each other and other

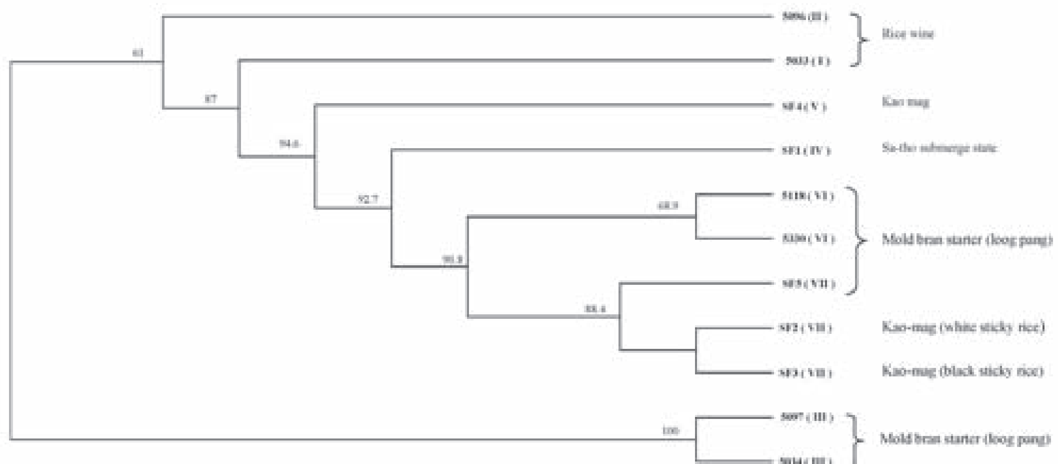

Figure 2 Phylogenetic tree showed the relation among species of *Saccharomycopsis fibuligera*. Genetic distance was obtained from ISSR patterns. The numbers given at nodes are the percentage of frequencies with which a given branch appeared in 1000 bootstrap replications (values <50% not shown).

groups by any of the eight primers. Although SF4 was isolated from the same product type as SF2 and SF3, this isolate showed clear differentiation from both of these isolates.

Group VI (TISTR 5118 and TISTR 5330) and group VII (SF2, SF3 and SF5) had the closest genetic relationship. The analysis of most primers in both groups produced similar patterns. Although primer UBC 891 showed a similar pattern between group VI (TISTR 5118 and TISTR 5330) and group V (SF4), group VI could be separated from group V (SF4) and group VII (SF2, SF3 and SF5) by using primers UBC 811 and UBC 891, respectively (Table 3). In addition, SF5 isolated from loog-pang was placed into the same group as SF2 and SF3 isolated from kao-mag belonging to the same producer but using different types of rice. Isolates TISTR 5097, TISTR 5118, TISTR 5330, TISTR 5034 and SF5 were isolated from the same product type, but these strains were distributed into three groups: group III (TISTR 5097 and TISTR 5034), group VI (TISTR 5118 and TISTR 5330) and group VII (SF5).

The ISSR-PCR technique, allowing discrimination within *S. fibuligera* species, can be performed within 2 hr for PCR and turned out to be useful in monitoring *S. fibuligera* in the fermentation process. The results of this study support the recommendation to use ISSR markers for routine discrimination of yeast isolates.

CONCLUSIONS

The sequence analysis of the D1/D2 domain of the 26S large subunit has been described as an effective method for yeast identification and used as reference for correct strain identification. Since 1998, more than 500 species of ascomycetous yeasts have been analyzed in this variable region making work on the yeast identification relatively easy.

This study presented the first study using molecular methods to determine the genetic diversity among *S. fibuligera* strains isolated from loog-pang, kao-mag and satho. The analysis revealed that ISSR markers give unique, specific and highly reproducible patterns and are universal, quick and easy to apply. No sequence knowledge is required for ISSR analysis. Although similar at the phenotypic level, *S. fibuligera* isolates showed genetic variation. Sequence analysis and ISSR marker techniques are rapid and precise. ISSR markers are a good choice for *S. fibuligera* DNA fingerprinting in addition to conventional identification techniques.

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