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ORIGINAL ARTICLE

Sakae Horisawa · Yoh Sakuma · Shuichi Doi

Qualitative and quantitative PCR methods using species-specific primer for detection and identification of wood rot fungi

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Abstract Species-specific oligonucleotide primers for detecting wood rot fungi, *Gloeophyllum trabeum*, *Trametes versicolor*, *Coniophora puteana*, and *Serpula lacrymans*, and the primer detecting a group of related fungi to *G. sepiarium* were developed. These primer sequences were picked up from the internal transcribed spacer region between small-subunit rDNA and large-subunit rDNA. The species selectivities of the developed primers were checked. Real-time polymerase chain reaction (PCR) was carried out using these highly specific primers to quantitatively detect at least of 0.01 ng genome DNA of the target species. This quantitative PCR was also used to differentiate the target species DNA from mixed species DNA. A PCR-based technique using the species-specific primers would be applicable to multiple-sample assay in diagnosis of wood decay and to investigation of environmental fungal populations.

Key words Species-specific primer · Wood rot fungi · Quantitative PCR · Wood decay

S. Horisawa (✉)
Department of Environmental Systems Engineering, Faculty of Engineering, Kochi University of Technology, 185 Miyanokuchi, Kami, Kochi 782-8502, Japan
Tel. +81-887+57-2519; Fax +81-887-57-2520
e-mail: horisawa.sakae@kochi-tech.ac.jp

Y. Sakuma
Graduate School of Science and Engineering, Ehime University, Matsuyama 790-8577, Japan

S. Doi
Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Ibaraki, Japan

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Introduction

In the field of wood preservation and in research on wood decay, it is important to identify the causal fungi of decay. In conventional methods, wood rot fungi have been identified based on the morphology of the fruit bodies, spores, and mycelial growth conditions. However, the appraisers are required to have specific knowledge and experience to identify fungi using the conventional methods. As such, it is difficult to avoid occasional misidentification of fungi. In particular, the identification of wood rot fungi invading in wood becomes very difficult when no fruit body is obtained. In addition, the appraisers must detect wood rot fungi grown on the surface and distinguish it from other fungi such as mold.

Recently, several biomolecular techniques have been developed to detect and identify fungi.[1,2] Protein assay such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) have been used to detect diversity among species and particular proteins. An immunological method has been developed as a sensitive diagnostic method and has been used in practical testing.[3–6] Fourier transform infrared (FT-IR) spectroscopy has been reported as a sensitive tool for detection of biomolecules or metabolites.[7,8] DNA is a useful chemical form that can be used to detect and identify wood rot fungi in decayed wood or the environment because it is a relatively stable and amplifiable biomolecule. Random amplification of polymorphic DNA (RAPD) analysis[9] and amplified fragment length polymorphism (AFLP) analysis[10,11] have been reported to produce genome DNA fingerprints for individual diversity. These DNA detection techniques are sensitive because the DNA is amplifiable by PCR.

For detecting basidiomycetes, small-subunit (SSU) and large-subunit (LSU) rDNA, internal transcribed spacer (ITS) region, intergenic spacer (IGS) region, and β-tubulin are employed due to their species-specific sequences as target genes.[12–15] PCR using species-specific primers can be used to detect and identify wood rot fungi with great sensi-

tivity. Suhara et al. and Schmidt and Moreth developed species-specific primers for detecting *Phelebia brebispora*, *Serpula lacrymans*, and *Coniophora puteana*.[16–18] Quantitative PCR using species-specific primers is able to detect target species of fungi both qualitatively and quantitatively.[19–22] In the present study, species-specific primers for detecting *Gloeophyllum sepiarium*, *Gloeophyllum trabeum*, and *Trametes versicolor*, in addition to the reported species mentioned above, were developed. The quantitative capabilities of the species-specific primers were also examined.

Materials and methods

Fungal strains and DNA extraction

Coniophora puteana NBRC6275, *C. puteana* FPRI-m, *Gloeophyllum sepiarium* NBRC4944, *G. sepiarium* NBRC6267, *Gloeophyllum trabeum* NBRC6430, *G. trabeum* NBRC6509, *Serpula lacrymans* NBRC8697, *S. lacrymans* NBRC30955, *Trametes versicolor* NBRC30340, and *T. versicolor* NBRC30388 were adopted as representative strains for making and checking species-specific primers. *Fomitopsis palustris*, *Gloeophyllum striatum*, *Gloeophyllum ungulatum*, *Lentinus lepideus*, *Aspergillus restrictus*, *Chaetomium globosum*, *Cladosporium cladosporioides*, and *Cladosporium herbarum* were employed as negative control strains (Table 1).

The DNA of each fungal strain was extracted by a modified method previously reported.[10] Mycelia of test strains were collected from their growing mycelial mat on potato dextrose agar (PDA) plates. To each fungus, approximately 0.1 g of mycelia was placed in a 2.0-ml microtube and 500 µl of TE buffer [10 mM Tris-HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 9.0] and 10 µl of mercaptoethanol were added. Sample mycelia were ground for 2 min with a 7-mm zirconia bead at a frequency of 25 Hz, using a mixer mill grinder (Tissuelyser, Qiagen, Valencia, CA, USA). Three hundred microliters of TE-saturated phenol and 100 µl of 10% SDS were added to each tube and incubated at 50°C for 30 min with reversing at 5-min intervals to keep the two phases thoroughly mixed. The tubes were centrifuged at 11 000 *g* and 4°C for 10 min, and the supernatant was collected. DNA was extracted from the supernatants with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1). The DNA was precipitated with 2-propanol and sodium acetate and collected by centrifugation. The DNA pellets were rinsed with 70% ethanol followed by drying. The DNA was dissolved in 5 ml TE containing RNase and purified on a column (Qiagen, Valencia, CA, USA).

Primer design and species-specific PCR

An ITS region was selected as a target sequence for species-specific PCR using a universal primer ITS 1 as a forward primer.[23] Reverse primers were designed from a species-specific sequence in the ITS II region of each species, respectively (Fig. 1). The sequence of test strains and sequence information of objective and related species in the Genbank/DDBJ/EMBL database were aligned by ClustalW[24] and species-specific sequences were searched by sight. The primers were designed with interrelation to the forward primer ITS 1 and the melting temperature (Tm) using primer-designing software Primer3.[25] The primer sequences and prospective lengths of the amplicons are shown in Table 2.

Table 1. Tested strains

Species	Strain name	Synonym
Basidiomycetes		
Gloeophyllum trabeum	NBRC6430	
G. trabeum	NBRC6509	
Gloeophyllum sepiarium	NBRC4944	
G. sepiarium	NBRC6267	
Trametes versicolor	NBRC30340	
T. versicolor	NBRC30388	
Coniophora puteana	NBRC6275	
C. puteana	FPRI-m	
Serpula lacrymans	NBRC8697	
S. lacrymans	NBRC30955	
Fomitopsis palustris	NBRC30339	
Gloeophyllum ungulatum	NBRC6431	
G. ungulatum	NBRC6507	
Gloeophyllum striatum	NBRC30341	*Gloeophyllum abietinum*
G. striatum	NBRC6429	*G. abietinum*
Lentinus lepideus	NBRC30750	*Gloeophyllum subferrugineum*
L. lepideus	NBRC4957	*G. subferrugineum*
Deuteromycetes		
Aspergillus niger	NBRC9642	
Aspergillus restrictus	NBRC31385	
A. restrictus	NBRC7101	
Chaetomium globosum	IAM8059	
C. globosum	NBRC6347	
Cladosporium cladosporioides	NBRC6348	
Cladosporium herbarum	IAM 5517	

A PCR mixture was prepared from Takara Ex Taq (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The mixture contained 10 ng of template DNA, each primer at a concentration of 0.5 pM, each dNTP at a concentration of 200 nM, 1.2 U/100 µl of DNA polymerase, and Expand reaction buffer with 1.5 mM $MgCl_2$. PCR was performed with a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following parameters: an initial denaturization of 5 min at 95°C, 30 cycles of 30 s at 95°C for denaturization, 30 s at 48°C, 56°C, and 60°C for annealing, 80 s at 72°C for extension, and a final extension of 7 min at 72°C. All PCR products were stored at 4°C until used. Aliquots of PCR products were examined by running them on 1.0% agarose gel.

Quantitative PCR using species-specific primer

Real-time PCR was set up using a 20-µl volume, with 0.01-ng, 0.1-ng, 1-ng, and 10-ng samples of genome DNA from *G. sepiarium*, *G. trabeum*, and *T. versicolor* added to 20 µl of SYBR Premix Ex Taq Perfect Real Time (Takara Bio, Shiga, Japan), 0.2 µM concentrations of each primer, and ROX dye at the concentrations indicated in the manufacturer's directions. The following program was performed on the Applied Biosystems 7000 real-time PCR system: an initial denaturization step at 95°C for 5 min followed by 40 cycles of denaturization at 95°C for 5 s, annealing and extension at 60°C for 41 s. All the real-time PCR was carried out in triplicate by using three reaction wells for each template DNA. The threshold cycle (Ct) was determined automatically by the instrument.

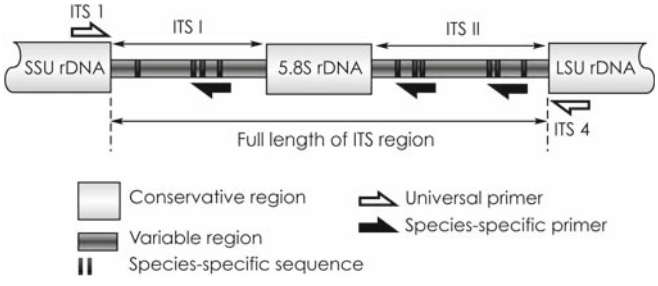

Fig. 1. Schematic diagram of the internal transcribed spacer (*ITS*) region between small subunit (*SSU*) rDNA and large subunit (*LSU*) rDNA, which includes ITS I, ITS II, and 5.8S rDNA

Quantitative detection of target species by species-specific PCR

Quantitative PCR for detecting target species was conducted using species-specific primer Gtr-R1 or Tve-R1 from mixed-genome DNA. The mixed-genome DNA solution, except for target species, was prepared at a concentration of 10 ng/µl, involving 1 ng/µl of each genome DNA (*G. sepiarium* NBRC6267 and NBRC4944, *C. puteana* NBRC6275 and FPRI-m, *S. lacrymans* NBRC8697 and NBRC30955, *F. palustris* NBRC30339, *G. ungulatum* NBRC6431, *G. striatum* NBRC30341, and *L. lepideus* NBRC30750). The real-time PCR for detecting *G. trabeum* was conducted using species-specific primer Gtr-R1 and a total of 10 ng DNA template, which was prepared from 0.01 ng, 0.1 ng, 1 ng, and 10 ng of *G. trabeum* NBRC6430 added to mixed-genome DNA, respectively. The PCR detecting *T. versicolor* was conducted using species-specific primer Tve-R1 and 10 ng of the mixed DNA solution added to 0.01 ng, 0.1 ng, 1 ng, and 10 ng DNA of *T. versicolor* NBRC30340, respectively. Other conditions of this real-time PCR were the same as those described above.

Results

Screening species-specific primer

The full length of ITS regions from the tested fungal strains in Table 1 were amplified by PCR using the universal primers ITS 1 and ITS 4[23] to assess whether these primers were available, and a predicted length of PCR product was obtained for each strain (data not shown). Based on these results, the primer ITS 1 was employed as a forward primer for species-specific PCR in the present study. The specificity

Table 2. List of universal and species-specific primers

Primer name	Sequence (5′–3′)	Tm (°C)	Primer site[a]	Target species	Product length[b] (bp)
ITS 1	tccgtaggtgaacctgcgg	64.5	–	(Universal)	–
ITS 4	tcctccgcttattgatatgc	57.8	–	(Universal)	–
Gtr-R1	cgattagcagctgatccactaag	58.7	ITS II	*G. trabeum*	548
Gtr-R2	tagcagctgatccactaagg	55.8	ITS II	*G. trabeum*	544
Gse-R1	ccaagcccaccacagttaat	59.9	ITS II	*G. sepiarium*	412
Gse-R2	attacattctgagactaca	41.7	ITS I	*G. sepiarium*	189
Sla-R1[c]	tgttgtcttgcgacaacg	58.4	ITS II	*S. lacrymans*	586
Sla-R2	gaggagccgatgaacaagag	54.3	ITS II	*S. lacrymans*	476
Cpu-R1	tttaccaatgcttttcagaggag	59.8	ITS II	*C. puteana*	500
Cpu-R2	gagcgtctcttgcgagac	57.2	ITS I	*C. puteana*	169
Tve-R1	atcacactgagagccgatcc	58.4	ITS II	*T. versicolor*	487
Tve-R2	ctgagagccgatccgtacgg	65.3	ITS II	*T. versicolor*	493

[a] See Fig. 1
[b] Predicted length by polymerase chain reaction using the primers of ITS 1 and species-specific primers
[c] Sla-R1 was described by Schmidt and Moreth[17]

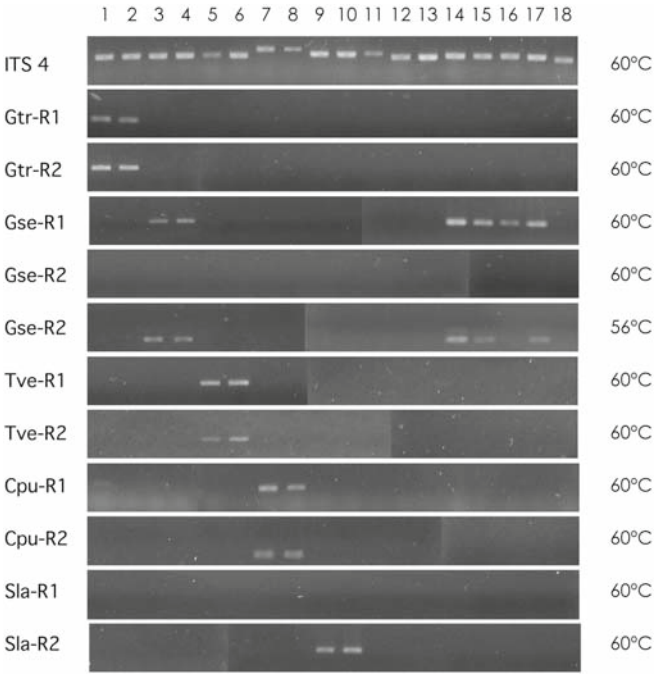

Fig. 2. Specificity of the primers designed for detecting the wood rot fungi, *Gloeophyllum trabeum*, *Gloeophyllum sepiarium*, *Trametes versicolor*, *Coniophora puteana*, and *Serpula lacrymans*. The *numbers* on the *top* indicate the template DNA used in each lane: 1, *G. trabeum* NBRC6430; 2, *G. trabeum* NBRC6509; 3, *G. sepiarium* NBRC6267; 4, *G. sepiarium* NBRC4944; 5, *T. versicolor* NBRC30340; 6, *T. versicolor* NBRC30388; 7, *C. puteana* NBRC6275; 8, *C. puteana* fpri-m; 9, *S. lacrymans* NBRC8697; 10, *S. lacrymans* NBRC30955; 11, *Fomitopsis palustris* NBRC30339; 12, *Gloeophyllum striatum* NBRC6431; 13, *G. striatum* NBRC6507; 14, *Gloeophyllum ungulatum* NBRC30341; 15, *G. ungulatum* NBRC6429; 16, *Lentinus lepideus* NBRC30750; 17, *L. lepideus* NBRC4957; 18, *Aspergillus niger* NBRC9642. The primers used in each panel are shown on the *left*. The annealing temperatures are shown on the *right*

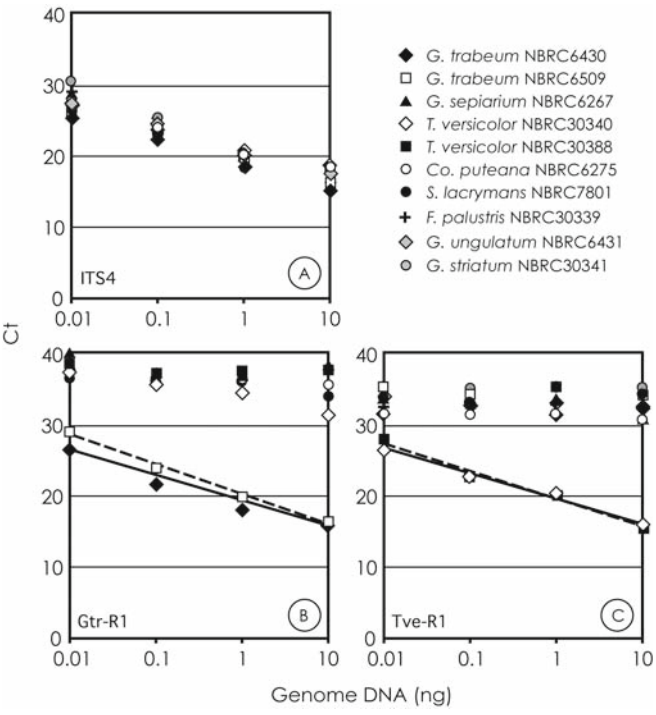

Fig. 3A–C. Standard curves obtained by quantitative polymerase chain reaction (PCR) from tenfold serial diluted DNA of *G. trabeum*, *G. sepiarium*, *G. striatum*, *G. ungulatum*, *T. versicolor*, *Fomitopsis palustris*, *C. puteana*, *S. lacrymans*, and *L. lepideus.* **A** Real-time PCR using the universal primer ITS 1 and ITS 4; **B** using the ITS 1 and the species-specific primer Gtr-R1; and **C** using the ITS 1 and the Tve-R1

of the developed species-specific primer was checked for the target genome DNA. Some of these results are presented in Fig. 2. The primers that had high specificities to the target species were: Gtr-R1 and Gtr-R2 for *Gloeophyllum trabeum*; Tve-R1 and Tve-R2 for *Trametes versicolor*; Cpu-R1 and Cpu-R2 for *Coniophora puteana*; and Sla-R2 for *Serpula lacrymans*. These primers amplified each portion of the ITS region, but only target species.

No primer having high specificity for *Gloeophyllum sepiarium* was produced. PCR using the Gse-R1 detected not only *G. sepiarium* but also *Gloeophyllum ungulatum* and *Lentinus lepideus.* Gse-R2 did not amplify any fragment from the tested strains at the annealing temperature of 60°C, but amplified a part of the ITS region from *G. sepiarium*, *G. ungulatum*, and *L. lepideus* at an annealing temperature of 56°C because of the low Tm of 47°C. No product was obtained by the primer Sla-R1.[17] The reason for this result seems to be the differences in PCR conditions, including differences in the type of polymerase, buffer compositions, and thermal-cycle program effects on the reaction products. None of the primers developed for detecting wood rot fungi responded to any of the deuteromycetes tested.

Quantitative PCR

The real-time PCR using the universal primer and the species-specific primer was performed with various amounts of target genome DNA. The Ct values were plotted against the amount of genome (Fig. 3). The Ct value is defined as the actual PCR cycle when the fluorescence signal increases above the background threshold. To construct a standard curve, genome DNA extracted from tested basidiomycete strains was serially diluted tenfold and was applied to quantitative PCR using the universal primer pair, ITS 1 and ITS 4 (Fig. 3A). In this PCR, the detection limit was estimated to be more than 0.01 ng of genome DNA and the coefficient correlations were at least 0.96 in the standard curve of tested basidiomycete strains. A similar curve was obtained in the real-time PCR using the primers ITS 1 and Gtr-R1 from the genome DNA of *G. trabeum* NBRC6430 at amounts of 0.01–10 ng (Fig. 3B), and also the primer ITS 1 and Tve-R1 from *T. versicolor* NBRC30340 (Fig. 3C). However, the Gtr-R1 and the Tve-R1 primers did not amplify any products other than those of the target species.

The quantitative PCR assay to detect the target species from mixed-genome DNA was examined (Fig. 4). The Gtr-R1 and Tve-R1 detected each target species quantitatively, and the curves of Ct against the template amounts were similar to the standard curve obtained in the PCR using the universal primer pair. The two species-specific primers did

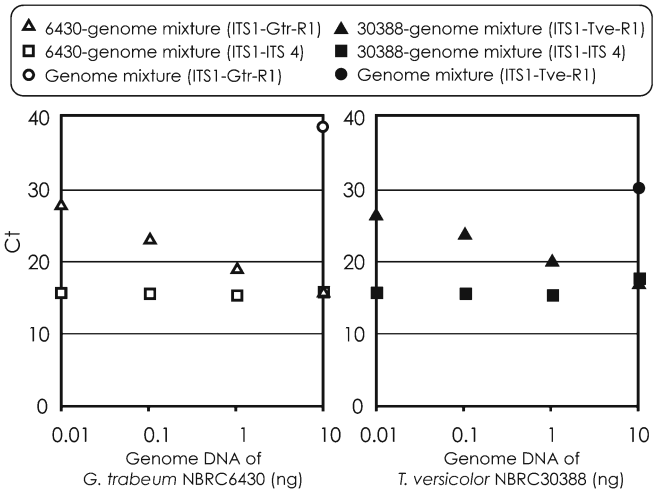

Fig. 4. Quantitative detection by real-time PCR using the species-specific primer. A total of 10 ng of DNA was added to each reaction as template. Template DNA consisted of target species DNA (*G. trabeum* NBRC6430 or *T. versicolor*) and mixed-genome DNA of wood rot fungi, which consisted of *G. sepiarium* NBRC6267 and NBRC4944, *C. puteana* NBRC6275 and FPRI-m, *S. lacrymans* NBRC8697 and NBRC30955, *F. palustris* NBRC30339, *G. ungulatum* NBRC6431, *G. striatum* NBRC30341, and *L. lepideus* NBRC30750. *Filled* and *open squares* show PCR using the universal primer pair of the ITS 1 and ITS 4. *Filled* and *open* triangles and *circles* show PCR using the ITS 1 and the species-specific primer (Gtr-R1 or Tve-R1)

not amplify the regular products from the mixed DNA without the target. The template containing the target genome DNA and mixed DNA (10 ng in a total amount) presented the same Ct values in the PCR using the universal primer pair (ITS 1 and ITS 4) regardless of the amount of target genome DNA.

Discussion

The ITS region of eukaryotes, which is between SSU rDNA and LSU rDNA, is an intron and easily accumulates gene mutations. The ITS region is highly variable between species in both sequence and length; it therefore can be used for examination of the intraspecies and interspecies relation. The ITS sequence provides useful information for identifying organic species. In the present study, species-specific primers based on the ITS sequence were developed to classify and identify the species of wood rot fungi, and the effectiveness of these primers was examined. Primers were developed to detect each fungal species, *Coniophora puteana*, *Gloeophyllum trabeum*, *Serpula lacrymans*, and *Trametes versicolor*, respectively.

The species-specific primer was designed as a reverse primer and the universal one was employed as a forward primer. The specific primers were picked from the ITS II region in order to obtain PCR products at lengths of around 300 bp for clear detection in agarose gel electrophoresis. The specificity of the primer should be a most important consideration. Other sequences for discriminating taxonomic groups have the potential to make the species-specific primer.

The Gse-R1 primer was imperfect in detecting only the target species, although it was able to discriminate a group of some fungal species. A strategy that detects some group or genera of fungi is also effective in the diagnosis of wood decay. Identification and classification of *S. lacrymans* by means of ITS sequence variation has been discussed in previous articles and the differences between the European and the Japanese strains were indicated.[10,13,26] To design the species-specific primers, as much sequence information of the ITS from target and related species as possible should be acquired because intraspecific variation might be found in this region of some species.[27,28] In addition, the PCR-based assay would show the dissimilar results due to that condition.

Combined use of the species-specific primers and real-time PCR technology allowed us to identify species rapidly and to estimate with sensitivity the quantity of wood rot fungi (minimum 10 ng). This technique is able to quantitatively detect target fungal species from a mixed-genome DNA pool. It would be applicable to multiple-sample assay in diagnosis of wood decay and to investigation of environmental fungal populations.

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