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RESEARCH NOTE

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Development of Molecular Markers to Detect *Diaporthe* spp. from Decayed Soybean Seeds

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ABSTRACT

Soybean is one of the world's most widely cultivated food crops, and soybean seeds are supplied from national seed resources in Korea. However, the transmission of seed-borne diseases through infected soybean seeds is problematic. Among these diseases, soybean seed decay is caused by *Diaporthe* spp. Infecting the pods, and the infected seeds show rotting symptoms. Most diseased seeds are removed during the selection process; however, it is difficult to distinguish infected seeds that do not display symptoms. Hence, a sequence-based method was devised to screen *Diaporthe*-infected seeds. Based on the nuclear ribosomal internal transcribe spacer (ITS) region of the pathogen, a primer was designed to distinguish the infection from other soybean seed pathogens. As a result of the comparison between healthy and *Diaporthe*-diseased seeds by using the primers, *Diaporthe* was detected only in the diseased seeds. Therefore, it is possible to distribute healthy soybean seeds by detecting *Diaporthe*-diseased seeds at the genetic level using the *Diaporthe*-specific primers.

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Soybean (*Glycine max*(L.) *Merr*) is a major crop worldwide and a source of protein and oil [1]. As the area under soybean cultivation gradually increases, the damage caused by diseases also increases. Soybean seeds are severely infected by *Diaporthe* species, showing symptoms including stem cancer and seed decay [2,3]. The diseased seeds are smaller and lighter than healthy seeds, and the germination rate also decreases, thus, it severely decrease soybean yield, quality, and stability [4]. It is important to remove the diseased seeds before sowing; however, it is difficult to discern the *Diaporthe*-infected seeds in the early-infection stage, and the diseased seeds are also cultivated. Therefore, in this study, we developed specific primers to detect *Diaporthe* spp. and *Diaporthe eres*-infected seeds before sowing, and separate it from healthy seeds.

Diseased soybean seeds were obtained from Agricultural Research and Extension Services, Agricultural business establishment, and Agricultural Technology Center in various locations in South Korea (Table 1). Fungal pathogens were isolated using the method described by Nguyen (2014) with modifications [5]. Briefly, diseased-seeds were surface-sterilized using 70% ethanol for 30 s and 1% NaClO for 30 s, washed with sterile distilled water three times, and air-dried on sterile filter paper (Whatman, No.2). The seeds were incubated on water agar at 25 °C for 7 days. The hyphal tips were transferred to acidified potato dextrose agar (APDA, pH 4.5) and incubated for 30 days for conidia production. The single fungal spores were isolated using a microscope, incubated on APDA at 25 °C for 7 days and then transferred to potato dextrose agar (PDA) at 25 °C for 7 days. The gDNA of isolated fungi was extracted using the Qiagen DNeasy mini plant kit standard protocol. The internal transcribed spacer (ITS) region was amplified *via* the polymer chain reaction (PCR) using universal primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [6]. The PCR cycling conditions were: initial denaturation at 94 °C for 30 s, 30 cycles of 55 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplified sequences were analyzed by Macrogene Inc (Seoul, South Korea). The sequence results were firstly aligned with reference species obtained from a BLAST search in the NCBI database (http://www.ncbi.nim.nih.gov/RefSeq/) using DNA-Star, and phylogenetic trees were generated using MEGA X. In total, 22 fungi were isolated from decayed soybean seeds and identified as *Cercospora* spp., *Fusarium* spp., *Alternaria alternata*, *Corynespora cassiicola*, and *Diaporthe* spp., including *D. eres* and *D. longicola* (Table 1).

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Table 1. List of fungal isolates obtained from soybean seeds with decay symptom.

	Scientific Name	Strain name	Cultivar	Source	Location
1	*Cercospora* sp.	C01	Daewonkong	Seed	Myryang
2	*Cercospora* sp.	C02	Daewonkong	Seed	Myryang
3	*Cercospora* sp.	C03	Daechan	Seed	Myryang
4	*Cercospora* sp.	C04	Seinpung	Seed	Jeonju
5	*Fusarium* sp.	MY06	Daechan	Seed	Myryang
6	*Fusarium* sp.	SC09	Mix	Seed	Sunchang
7	*Fusarium* sp.	G11-1	Mix	Seed	Gyeong-gido
8	*Fusarium solani*	SC11	Mix	Seed	Sunchang
9	*Fusarium oxysporum*	SC18	Mix	Seed	Sunchang
10	*Alternaria alternate*	MY14	Daechan	Seed	Myryang
11	*Corynespora cassiicola*	MY07	Daechan	Seed	Myryang
12	*Corynespora cassiicola*	SC43	Mix	Seed	Sunchang
13	*Diaporthe* sp.	HC1-1	Unknown	Stem	Jeonju
14	*Diaporthe* sp.	G3	Mix	Seed	Gyeong-gido
15	*Diaporthe* sp.	G11-2	Mix	Seed	Gyeong-gido
16	*Diaporthe* sp.	SC33	Mix	Seed	Sunchang
17	*Diaporthe* sp.	SC42	Mix	Seed	Sunchang
18	*Diaporthe* sp.	KJ04	Unknown	Stem	Gimje
19	*Diaporthe eres*	D01	Daewonkong	Seed	Myryang
20	*Diaporthe eres*	D02	Daechan	Seed	Myryang
21	*Diaporthe longicola*	KHT01	Mix	Seed	Gimje
22	*Diaporthe longicola*	KHT02	Mix	Seed	Gimje

To determine the molecular detection region for *Diaporthe* spp., ITS, elongation factor-α (EF-α), calmodulin (CAL), and b-tubulin (TUB) gene were tested [7]. The nucleotide sequences of amplified EF-α, CAL, and TUB were highly variable in length based on each fungal isolate, it was difficult to find conserve nucleotides of genus *Diaporthe*. Therefore, in this study, ITS region was used for design specific primers to distinguish *Diaporthe* spp., from *Cercospora* spp., *Fusarium* spp., *A. alternate*, and *C. cassiicola*.

The consensus sequences flanking the 18S rRNA and 28S rRNA sequences of *D. eres*, *D. angelicae* (KJ590735.1), *D. cuppatea* (KC343057.1), *D. ganjae* (KC343112.1), *D. longicola* (KJ590729.1), *D. lusitanicae* (KC343136.1), *D. melonis* (KC343142.1), *D. phaseolorum* (KJ590738.1), *D. sojae* (KJ590715.1), *D. subordinaria* (KC343213.1), *Diaporthe* sp. (KC343203.1), *Alternaria alternata* (KU254607.1), *Fusarium solani* (NR_163531.1), *Botrytis cinerea* (MW820601.1), *Cercosproa kikuchii* (NR_119616.1), and *Sclerotinia sclerotiurum* (MG818952.1) were compared to design ITS-based detection primers for *Diaporthe* spp. (Figure 1B). Three candidate primer sets, i.e. D_sp1, D_sp2, and D_sp3, were designed (Table 2). The specificity was assayed using ten soybean seed-borne pathogens, i.e. four *Diaporthe* spp., *Fusarium* spp., *F. oxysporum*, *F. solani*, *R. solani*, *A. alternata,* and *S. sclerotiurum*. The culturing of the ten pathogens, as well as the gDNA extraction and PCR amplification, were conducted as described above. The PCR products were electrophoresed on a 1.5% agarose gel stained with GelRed nucleic acid in TAE and then visualized under a UV-light using a Bio-Rad Gel document system (Bio-Rad, Hercules, CA, USA). The PCR amplicon (386 bp) was only observed in *Diaporthe* species but not in the tested *Alternaria*, *Fusarium*, *Butrytis*, *Cercospora,* and *Sclerotinia* species. In addition, cross-reactivity of the primer sets was not observed (Figure 1). Among the candidate primer sets, the D_sp3 (forward/reverse) had the highest sensitivity, and it was used for further testing.

The designed D_sp3 primer for molecular detection of *Diaporthe* species was applied to separate *Diaporthe*-diseased seeds from healthy seeds. For the preparation of the artificial diseased seeds, the surface was disinfected with 70% ethanol for 30 s and 1% NaClO for 30 s, washed with sterile distilled water three times, and air-dried on sterile filter paper. Seeds were inoculated with the mycelia and spores of *Diaporthe* spp. (SC42, HC1-1, G3), *D. eres* (D02), *Alternaria alternata, Fusarium spp., F. oxysporum, F. solani, and Rhizoctonia solani* and incubated. The gDNA of diseased and healthy seeds were prepared as described above. The PCR was conducted by using ten-fold diluted gDNA (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg). The D_sp3 product was detected in *Diaporthe*-inoculated seeds, and the primer sensitivity was up to 10 pmol (Figure 1D,E). For the development of a molecular marker for *D. eres*, a phylogenetic tree was constructed based on ITS regions sequence alignment analysis of *Diaprothe* spp. (Figure 2A). The specificity of the D_eres primers to detect *D. eres* was confirmed (Figure 2C). Using the D_eres 3F3R primer set with the 10-fold dilution series of *D. eres* D02 gDNA consistently displayed detection limits of 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg, respectively, after 30 cycles of amplification (Figure 2D), while the D_eres 3F3R primer set had a sensitivity of up to 100 pg of gDNA. In order to test the specificity of the D_eres 3F3R primer set, *Diaporthe* spp., *Alternaria alternata, Fusarium* spp., *F. oxysporum, F. solani* and *Rhizoctonia solani*, were tested. The primer set showed specificity only in the *Diaporthe* spp. The primer set D_eres 3F3R was only amplified in the gDNA of seeds with symptoms of seed decay by *D. eres* (Figure 2E).

In this study, the primer set, D_sp3, was developed as a molecular detection marker for the soybean seed decay pathogen *Diaporthe* spp. The marker was specific to *Diaporthe* species in in silico assays and soybean seeds and did not detect *Fusarium* sp., *F. oxysporum*, *F. solani*, *R. solani*, *A. alternate,* or *S. sclerotiurum*. Soybean seed decay is caused by various *Diaporthe* species other than *D. eres*, and soybean disease caused by *D. sojae*, *D. longicolla, D. caulivora,* and *D. aspalathi* are continuously reported [8]. Many diagnostic kits for the detection of plant pathogenic viruses and bacteria

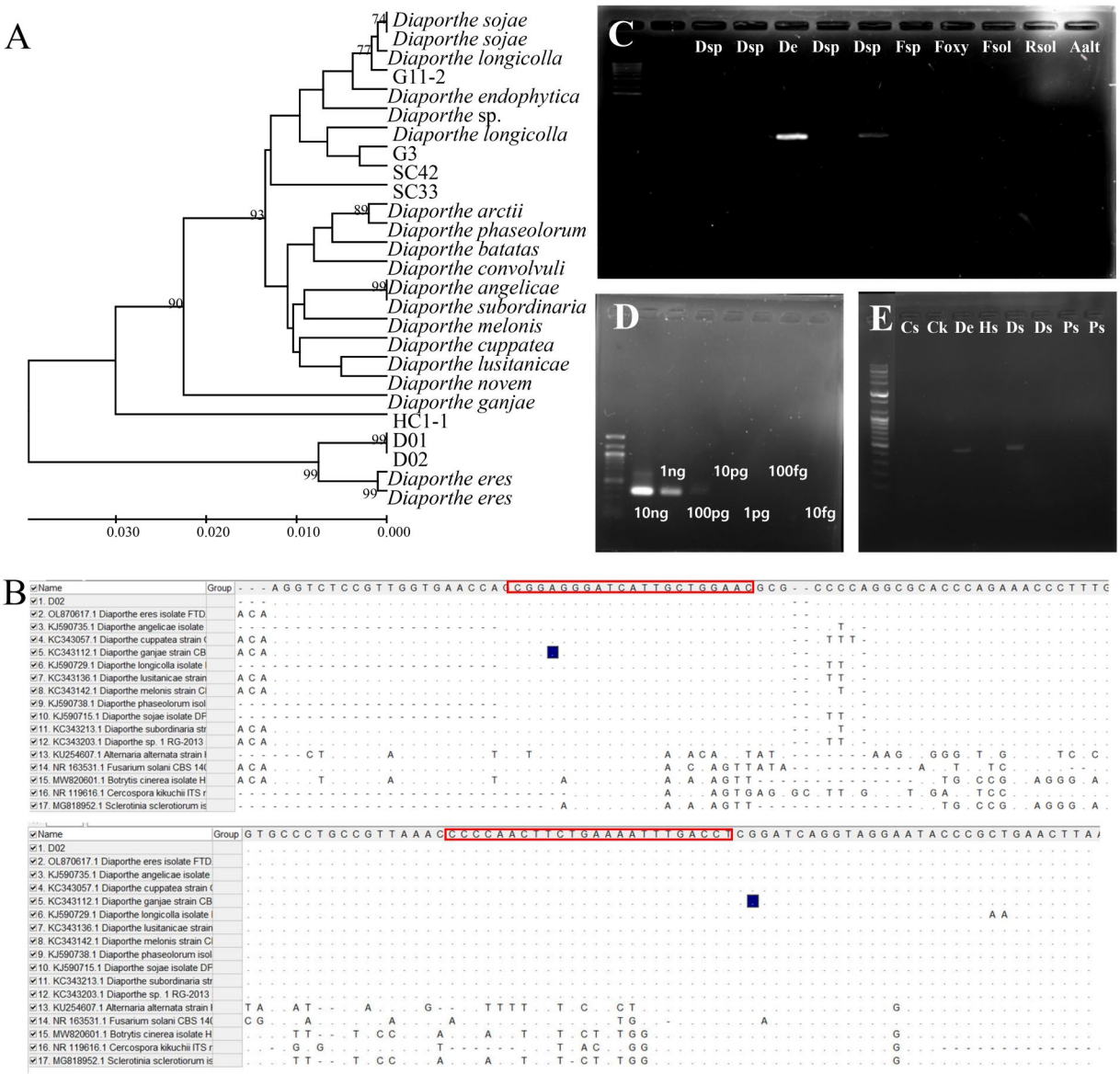

Figure 1. Maximum likelihood phylogenetic analysis of the *Diaporthe* species using ITS primers to develop a specific marker to detect *Diaporthe* spp. The bootstrap numbers represent the percent of 1000 replicates (only values greater or equal to 70% are shown) (A); specific detection of *Diaprothe* spp. from seven isolates, including *Cercospora kikuchii, B. cienrea, F. solani, Sclerotinia sclerotiorum,* and *Alternaria alternata* (B); lane M, 1000 bp DNA ladder; lanes dsp, *Diaporthe* sp.; lane De, *D. eres*; lane fsp, *Fusarium* sp.; lane foxy, *F. oxysporum*; lane fsol, *F. solani*; lane, *R. solani*; and lane aalt, *A. alternata* (C); Conventional PCR amplification of different amounts of *Diaporthe eres* DNA with the primers D_sp3F3R. M, 100 bp ladder marker; 10 ng; 1 ng; 100 pg; 10 pg; 1 pg; 100 fg; 10 fg. (D); detection of *Diaporthe* spp. using D_sp3F3R from *Cercospora sojina*, *C. kikuchii*, *D. eres,* and soybean seeds with decay and discoloration; lane Cs, *C. sojina* C01; lane Ck, *C. kikuchii*; lane De, *D. eres* D01; lane Hs, healthy seed; lanes Ds, seeds with decay symptoms; lanes Ps, brown and purple seeds. (E).

Table 2. Information on primers used in this study.

Target	Primer	Forward/reverse	Sequence(5-3)	Length	TM
Diaporthe sp.	D_sp1	F	AAACCCTTTGTGAACTTAT	19	50.4
	D_sp2	F	CATAAATGAATCAAAA	16	38
	D_sp3	F	GAGGGATCATTGCTGGAACGC	21	63.7
	D_sp1	R	CGGCAGGGCACCGCCAGGGCC	21	80.6
	D_sp3	R	CGAGGTCAAATTTTCAGAAGTTGG	24	59.1
Diaporthe eres	D_eres1	F	CCTCGGCGCTAGCTGGTCCT	20	69.7
	D_eres2	F	GTTGCCTCGGCGCTAGCTGGT	21	68.8
	D_eres3	F	TACTGTTGCCTCGGCGCTAGC	21	65.5
	D_eres1	R	GCGCAGTAGTTAAACCCTCGCT	22	63
	D_eres2	R	GGTTTAACTACTGCGCTCGG	20	60
	D_eres3	R	TTAACTACTGCTCGGGGTCCT	21	62.7

have been developed; however, sequence-based detection kits for plant fungal pathogens must be developed. It is especially important to detect seed-borne fungal pathogens during seed storage because the diseased seeds could spread in the fields, decreasing productivity. Therefore, the detection

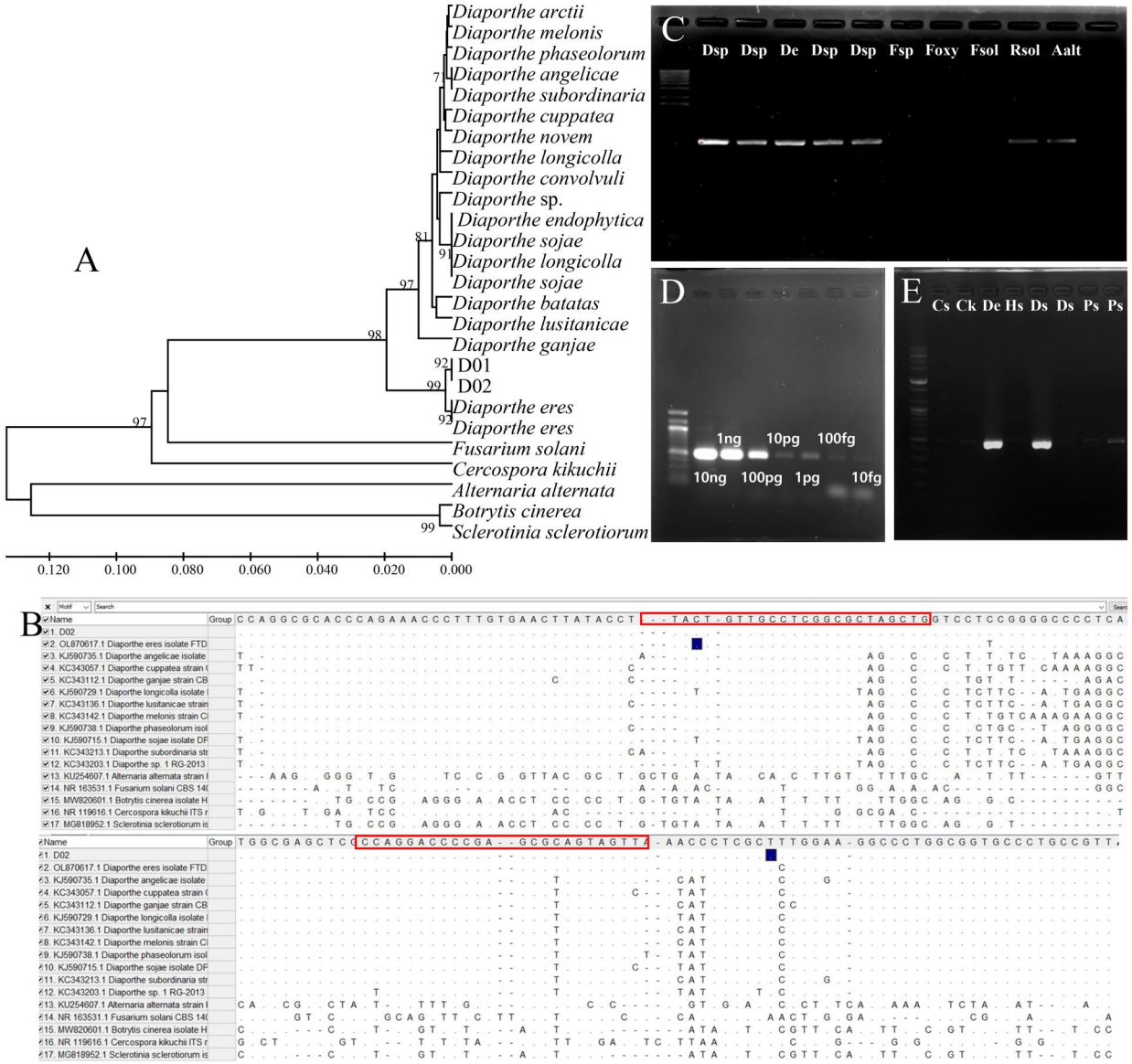

Figure 2. Maximum likelihood phylogenetic analysis of the *Diaporthe* species using ITS primers to develop a specific marker to detect *D. eres*. The bootstrap numbers represent the percent of 1000 replicates (only values greater or equal to 70% are shown) (a); specific detection of *D. eres* from seven isolates, including *Cercospora kikuchii, B. cienrea, F. solani, Sclerotinia sclerotiorum,* and *Alternaria alternata* (B); lane M, 1000 bp DNA ladder; lanes 1 and 2, *Diaporthe* sp.; lane 3, *D. eres*; lanes 4 and 5, *Diaporthe* sp.; lane 6, *Fusarium* sp.; lane 7, *F. oxysporum*; lane 8, *F. solani*; lane 9, *R. solani*; and lane 10, *A. alternata* (C); Conventional PCR amplification of different amounts of *D. eres* DNA with the primers D_eres 3F3R. M, 100 bp ladder marker; 1, 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, 1 pg; 6, 100 fg; 7, 10 fg. (D); detection of *Diaporthe* spp., D_sp3F3R from *Cercospora sojina*, *C. kikuchii*, *D. eres,* and soybean seeds with decay and discoloration; lane a, *C. sojina* C01; lane b, *C. kikuchii*; lane c, *D. eres* D01; lane d, healthy seed; lanes e and f, seeds with decay symptoms; lanes g and h, brown and purple seeds. (E).

marker identified in this study could be used to verify the integrity of seeds, and infected seeds could be separated from healthy seeds in an early stage. Moreover, since *Diaporthe* spp. can spread to the pod through the vessels in the stem, the designed primer could be applied to detect it in various plant tissues during the growing season. In the future, studies developing markers for direct use in the field for other seed pathogens, such as *Cercospora sojina, C. kikuchii*, and *Septoria glycine* will be conducted. Additionally, multiplex PCR markers combined with detection markers for all of these will be studied.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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