Mycobiology 30(2): 82-87 (2002)

The Determination of the Partial 28S Ribosomal DNA Sequences and Rapid Detection of *Phellinus linteus* and Related species

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(Received April 8, 2002)

Species of *Phellinus* were known to harmful fungi causing white pocket rot and severe plant disease such as canker or heart-rot in living trees in the West, but some species have been used to traditional medicines in the Orient for a long time. In this study the partial D1-D2 nucleotide sequences of 28S ribosomal DNA from 13 *Phellinus* strains were determined and compared with the sequences of 21 strains obtained from GenBank database. According to the neighbor-joining (NJ) method comparing the sequence data the phylogenetic tree was constructed. The phylogenetic tree displayed the presence of four groups. Group I includes *P. ferreus*, *P. gilvus* and *P. johnsonianus*, Group II contains *P. laevigatus*, *P. conchatus* and *P. tremulae*, Group III possesses *P. linteus*, *P. weirianus*, *P. baumii*, *P. rhabarbarinus* and *P. igniarius*, and Group IV comprises *P. pini*, *P. chrysoloma*. *P. linteus* and *P. baumii*, which were used mainly in traditional medicine, belong to the same group, but exactly speaking both were split into two different subgroups. To detect *P. linteus* only, we developed the PCR primer, D12HR. The primer showed the specific amplification of *P. linteus*, which is permitted to medicinal mushroom in the East. The results make a potential to be incorporated in a PCR identification system that could be used for the rapid identification of this species from its related species, *P. linteus* especially.

KEYWORDS: 28S rDNA, D1-D2 region, *Phellinus baumii*, *Phellinus linteus*, Phylogeny

There is an ancient legend; you can make even a dying person survive if you find a yellow loaf in a mulberry tree. It is found in the upper part of the stem in the dead trees and three or four year perennial plants. It has no taste and smell. It is called to Sangsin, Sang-i, Sanghwang in the Chinese encyclopedia for medicine and named to *Phellinus linteus* Teng as scientific name. In Korea, it is called to the 'Sanghwang-mushroom' and has been used a traditional oriental medicine to cure the stomachache and arthritis of knee.

Various culture studies were conducted to classify the genus *Phellinus* (Chi *et al.*, 1996, 1998; Choi, 1999; Song *et al.*, 1997). Molecular genetic techniques are becoming more important in the field of taxonomic and phylogenetic relationships among fungi. Nei and Li (1979) carried out restriction fragment length polymorphism (RFLP) analysis, but their methods are incorrect and variable in the band pattern on the agarose gel to be used for lower taxonomical level. The ribosomal DNA sequences have been frequently used in molecular systematics because they include both highly conserved (18S) and highly variable sequences (Non-transcribed spacers and Internal transcribed spacer) (Roderic and Edward, 1998), and so can reconstruct the phylogenetic relationships between both very distance and very closely related species. For the systematics at lower taxonomic levels, particularly the intergenic regions (IGR), the internal transcribed spacers (ITS), and the divergent domains of a large ribosomal subunit (28S rDNA), which were named D1-D7 in the model of Michot and Bachellerie (1987), could be used.

This study focused on delimitation of *P. linteus* and related species through comparing of the partial D1-D2 regions in 28S rDNA, which correspond to 25S rDNA molecule in yeast. Sequencing of the 25S rDNA molecule in yeast was performed at the same time as 18S rDNA in search for regions that were informative enough to deal with closer relationships. Several regions were tested including the 25S-635 regions, which was known as D2, V3, B region, or 25S-nucleotides 493 to 622, the 25S-1841 regions, which was known as 25S-nucleotide 1611 to 1835, and the D1-D2 region. The length of the sequenced position of region 25S-635 was extended to about 600 nucleotides establishing the use of the D1-D2 region for analysis at the species level (Kurzman and Robnett, 1997). Although the D1-D2 sequence might not distinguish sister species or varieties, Lachance *et al.* (1998) were able to differentiate varieties of *Metschnikowia continentalis*, which had been recognized on the basis of limited reproductive isolation. Using this D1-D2 region, Mannarelli and Kurzman (1998) designed specific primers that can be used in a PCR system for the identification of 14 species of human pathogenic yeasts. 28S rDNA may be reached to approximately 4 kbp. Therefore, the whole sequencing of 28S DNA costs expensive and much laborious. So the analysis of D1-D2 region that is inferred one of much variable regions should be done for fully understanding of *Phellinus* taxonomy with the analysis of the ITS sequence. In this study, we confirmed

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whether D1-D2 regions could be used for phylogenetic relationship of the *Phellinus* species and developed the *P. linteus* specific PCR primer.

Recently more and more fruiting body of *Phellinus* group has been imported from foreign countries because of anticipating to anti-tumor effect and desiring for health. These mushrooms called to Sanghwang are being sold at market places in too much high price. But exactly speaking, the term of sang-hwang mushrooms has been confused among *P. linteus*, *P. baumii*, and *P. igniarius*. Today most of mycologists in Asia consider *P. linteus* is true sang-hwang.

The objectives of present study are following: (1) reconstructing the phylogenetic relationship by using the D1-D2 divergent regions in a large ribosomal subunit (28S rDNA). (2) Designing PCR primer for detecting the *P. linteus*.

Materials and Methods

Fungal strains and cultivations. Most strains were supplied from KCTC and IMSNU and some strains were collected from Korean mountains and farms. The strains used in this study were arranged in Table 1. The strains were cultured with shaking in 100 m*l* of YM (Yeast-Malt extract), PD (Potato Dextrose), or ME (Malt Extract) broth at 25°C for two or three weeks. The mycelium was harvested by filtration or centrifugation, and stored in a freezer at −20°C. For the accurate phylogenetic comparison, the sequence data of 13 strains of *Phellinus* species were registered in the GenBank database (Table 1).

DNA extraction. Fungal DNA was extracted from each sample according to the benzyl chloride method (Zhu *et al.*, 1993). Approximately 0.1 g of the fungal pellets was suspended in 500 μl of Tris buffer (100 mM Tris-HCl, pH 8.0, 40 mM EDTA), 150 μl of 10% (w/v) sodium dodecyl sulphate (SDS) and 300 μl of benzyl chloride and then incubated at 50°C for 30 min. Treatment with phenol: chloroform: isoamylalcohol (25 : 24 : 1) and RNase (1 mg/m*l*) was carried out for the purification of the DNA and removal of RNA. Then adding 2.5 volumes of absolutely ice-cold ethanol precipitated the DNA. The pellet was washed with 2 volumes of 70% ethanol and resuspended in distilled water. The purified DNA was kept on −20°C before PCR amplification and sequencing.

Table 1. List of strains, sources and GenBank accession numbers

Scientific name	Strain no.*	Source	GenBank accession No.
P. linteus	ATCC 26710	ATCC	AF458461
P. gilvus	ATCC 26729	IMSNU**	AF458457
P. pini	ATCC 12240	IMSNU	AF458459
P. rhabarberinus	ATCC 26713	IMSNU	AF458466
P. linteus	IFO 6989	IMSNU	AF458462
P. johnsonianus	ATCC 60051	IMSNU	AF458458
P. linteus	CBS 454.76	KCTC	AF458464
P. weirianus	CBS 618.89	IMSNU	AF458465
P. igniarius	KCTC 6228	KCTC	AF458467
P. laevigatus	KCTC 6229	KCTC	AF458460
P. linteus	MPNU 7001	Hongcheon (Korea)	AF458463
P. baumii	MPNU 7004	Andong (Korea)	AF458468
P. baumii	MPNU 7005	Changwon (Korea)	AF458469

*ATCC : American Type Culture Collection, IFO : Institute for Fermentation, Osaka, CBS : Centraalbureau voor Schimmelcultures, KCTC : Korean Collection for Type Culture, MPNU : Mycological lab. in Pusan National University.
**IMSNU : Institute of Microbiology in Seoul National University.

PCR amplification and DNA sequencing. The nuclear rDNA corresponding to the D1-D2 region in 28S rDNA was amplified by PCR. The primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (Kurtzman and Robnett, 1997) were used. PCR procedures were carried out using a Perkin-Elmer GeneAmp PCR System 2400 under the following program: initial denaturation for 5 min at 95°C, 30 cycles of amplification (denaturation for 1 min at 95°C, annealing for 1 min at 45°C and extension for 1 min at 72°C) and final extension of 5 min at 72°C. The PCR products were obtained and excised by gel electrophoresis in a 1.6% agarose gel with the ethidium bromide in 0.5× TBE buffer. All PCR products yielded only a single visible band respectively (about 650 bp) and extracted by QIAGEN gel elution kits (Qiagen, Wartworth CA). The direct sequencing of the PCR products was performed using a Perkin-Elmer Applied Biosystems ABI 377 sequencer with a PRISM Dye Dideoxi Terminator Cycle Sequencing kit (Perkin Elmer) following the manufacturer's protocol (Smith *et al.*, 1986). NL1 and NL4 primer were used for sense and antisense sequencing, respectively. The DNA sequences were edited and assembled using the program CLONE MANAGER version 4.0 (Scientific & Educational Software, Stateline, PA).

Data analysis. The determined ribosomal DNA sequences were deposited in the GenBank and accession numbers were shown to Table 1. The distance matrix was calculated using NucML, and the phylogenetic relationships were inferred by the neighbor-joining method (Saitou and Nei, 1987). The strength of the internal branches from the resulting trees was statistically tested by a bootstrap analysis (Felsenstein, 1985) based on 1,000 bootstrap replications. The initial tree based on the neighbor-joining method was reconstructed using NJdist from the PHYLIP 3.5 software package.

***Phellinus linteus* specific probe design.** For detecting the *P. linteus*, D12HR was designed as antisense primer.

The region designing D12HR located between 520 nt and 540 nt of DI-D2 sites in 28S rDNA regions. The specificity of the *P. linteus* primer was tested with the NL1 sense primer (White *et al.*, 1990) in a nested fashion and expected PCR product size was about 550 bp. Low specificity of natural oligomers in the D1-D2 regions make us considering about transitional or transversional base change.

Results

G+C content and nucleotide length. The G+C content and nucleotide length of D1-D2 regions are shown in Table 2. Torres *et al.* (1990) found similar phenomenon in the G+C content of ITS regions in a wide range of organisms including fungi and called it "G+C balance". Taka-

Table 2. The nucleotide length and G+C content of the 28S rDNA D1-D2 regions

Fungal strain	Length*	G+C content (%)
P. gilvus ATCC 26729	600	49
P. johnsonianus ATCC 60051	642	48
P. igniarius KCTC 6228	637	52
P. laevigatus KCTC 6229	605	51
P. rhabarberinus ATCC 26713	618	54
P. baumii MPNU 7004	618	53
P. baumii MPNU 7005	633	53
P. weirianus CBS 618.89	641	53
P. linteus ATCC 26710	628	52
P. linteus IFO 6989	624	51
P. linteus CBS 454.76	611	52
P. linteus MPNU 7001	615	51
P. pini ATCC 12240	608	49

*Represents the length of nucleotide sequence (bp).

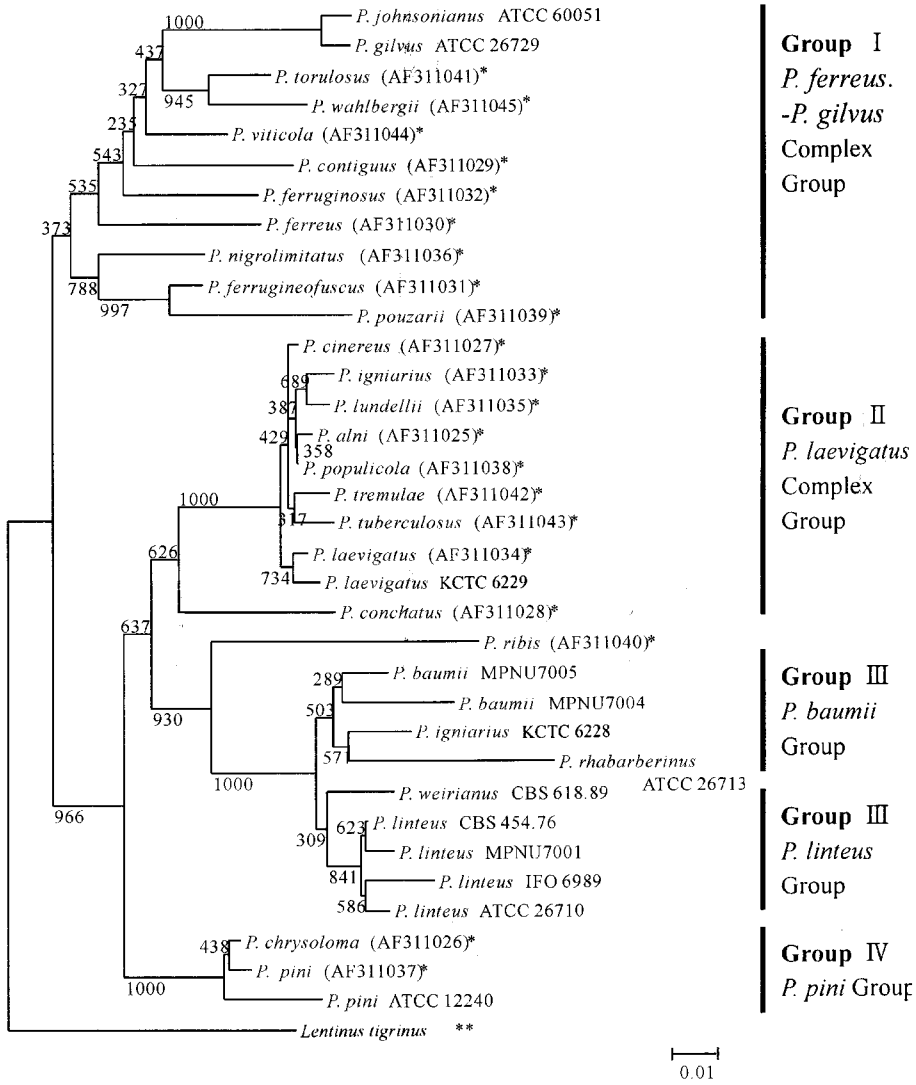

Fig. 1. Phylogenetic tree inferred from the analysis of D1-D2 region sequences in 28S rDNA. This is constructed by the Neighbor-joining method of PHYLIP 3.5. Bootstrap values of these data based on 1000 replications are shown at the internal nodes. The scale bar indicated the distance that corresponds to 0.01 sequence divergences. The strains cited from the GenBank database are represented to asterisk (*) symbol and outgroup to double asterisks (**).

matsu *et al.* (1998) suggested that the relatively low GC content of fungus might reflect the low optimum temperature of the fungus. ITS regions showed relatively broad GC content: 42.2~50.1% in ITS1 and 37.9~50.8% in ITS2 (Data not shown). In the case of D1-D2 region, the range of G+C content distributes from 48% to 54%. Group III has highest G+C content (51~54%). In turn Group II, *P. laevigatus*, has 51%, Group IV, *P. pini*, has 49%, and Group I, *P. gilvus* and *P. johnsonianus*, has 48 to 49% G+C content. G+C content was not proportional to the lengths of nucleotide of D1-D2 regions.

Phylogenetic analysis. Phylogenetic relationships inferred from D1-D2 region sequences in 28S rDNA of *P. linteus* and related species with the sequences of 21 strains cited from GenBank are shown in Fig. 1. This tree indicated that species of *Phellinus* are divided into four groups. Although Group I was combined with somewhat low bootstrap value, the other three groups was supported by bootstrap values between 93% and 100% but *P. conchatus* was ambiguous. Group I consisted of *P. gilvus*, *P. torulosus*, *P. ferreus*, etc. This group is poorly supported by 37% bootstrap value and may be divided into two groups at least. Group II included *P. laevigatus*, *P. tremulae*, *P. tuberculosus*, and etc. This group is tightly bound except *P. conchatus*. Group III contains *P. baumii*, *P. ribis*, *P. rhabarberinus*, *P. weirianus*, and *P. linteus*. Group IV was composed of *P. pini* and *P. chrysoloma*. Although *P. linteus* and *P. baumii* are the same group, that is supported by 100% bootstrap value, the two species can be divided into other subgroup, that is supported by 96% bootstrap value. This result is consistent with that of ITS analysis. Distance matrix is shown in Table 3.

Table 4. Designed primer sequence

Target species		Sequence	
P. linteus	Origin	5'CCGTCGCATACACTGGCT 3'	
	Reverse	5'AGCCAGTGTAT*GCGACGG 3'	
	D12HR	5'AGCCAGTGTACGCAACGG 3'	18 mer

*Transitional changed base.

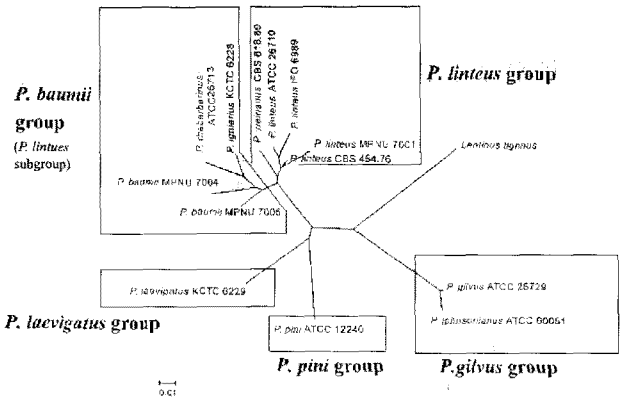

Fig. 2. Unrooted neighbor-joining tree of *Phellinus* species used in the certification of *P. linteus* specific PCR product. The scale bar indicates the distance of 0.01 unit.

Specificity of the primer D12HR. According to the D1-D2 region alignment data, the specific sites for *P. linteus* were chosen and *P. linteus*-specific PCR antisense primer, D12HR, was designed (Table 4). For accurate analysis of more related species, 13 *Phellinus* species were adapted in PCR system (Fig. 2). The natural base sequence did not differ significantly to design specific

Table 3. Distance matrix of 28S rDNA D1-D2 regions

	1*	2	3	4	5	6	7	8	9	10	11	12	13	14
1		0.0018	0.0399	0.0271	0.0271	0.0344	0.0235	0.0235	0.0307	0.0523	0.0325	0.0488	0.0307	0.0505
2	0.0108		0.0381	0.0253	0.0253	0.0325	0.0217	0.0217	0.0289	0.0505	0.0307	0.0470	0.0289	0.0487
3	0.0889	0.0926		0.0255	0.0309	0.0418	0.0345	0.0345	0.0381	0.0544	0.0364	0.0345	0.0417	0.0472
4	0.0814	0.0886	0.0582		0.0217	0.0290	0.0181	0.0181	0.0217	0.0416	0.0236	0.0380	0.0253	0.0307
5	0.1083	0.1083	0.0635	0.0741		0.0108	0.0036	0.0036	0.0108	0.0361	0.0090	0.0217	0.0108	0.0451
6	0.1103	0.1121	0.0655	0.0725	0.0090		0.0108	0.0109	0.0181	0.0398	0.0091	0.0290	0.0108	0.0524
7	0.1083	0.1083	0.0672	0.0705	0.0108	0.0127		0.0000	0.0072	0.0325	0.0090	0.0253	0.0072	0.0415
8	0.1049	0.1049	0.0599	0.0707	0.0036	0.0072	0.0072		0.0072	0.0307	0.0091	0.0236	0.0072	0.0416
9	0.1047	0.1047	0.0581	0.0741	0.0181	0.0217	0.0217	0.0145		0.0361	0.0163	0.0325	0.0144	0.0451
10	0.1264	0.1264	0.0672	0.0850	0.0415	0.0452	0.0451	0.0380	0.0325		0.0307	0.0434	0.0325	0.0632
11	0.1121	0.1103	0.0673	0.0725	0.0307	0.0308	0.0307	0.0290	0.0271	0.0271		0.0272	0.0054	0.0470
12	0.1031	0.0995	0.0582	0.0670	0.0163	0.0181	0.0127	0.0127	0.0145	0.0271	0.0181		0.0253	0.0633
13	0.1047	0.1065	0.0563	0.0687	0.0181	0.0217	0.0217	0.0163	0.0144	0.0325	0.0199	0.0090		0.0487
14	0.0812	0.0812	0.0817	0.1031	0.0866	0.0904	0.0903	0.0832	0.083	0.1047	0.0958	0.0868	0.0884	

The values on top upper right represent rates of transversion (A or G ↔ C or T) and the values on the lower left are rates of transition (A ↔ G; C ↔ T).

*1. *P. gilvus* ATCC 26729; 2. *P. johnsonianus* ATCC 60051; 3. *P. pini* ATCC 12240; 4. *P. laevigatus* KCTC 6229; 5. *P. linteus* ATCC 26710; 6. *P. linteus* IFO 6989; 7. *P. linteus* MPNU 7001; 8. *P. linteus* CBS 454.76; 9. *P. weirianus* CBS 618.89; 10. *P. rhabarberinus* ATCC 26713; 11. *P. igniarius* KCTC 6228; 12. *P. baumi* MPNU 7004; 13. *P. baumi* MPNU 7005; 14. *Lentinus tigrinus*.

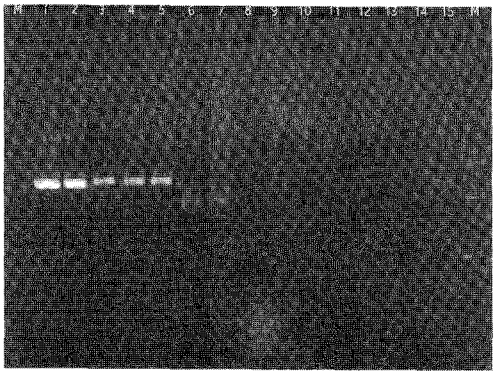

Fig. 3. PCR products for detecting *Phellinus linteus*. NL1-D12HR primer set was used. Annealing temperature is 55°C. M; size marker, Lane 1; *P. linteus* ATCC 26710, Lane 2; *P. linteus* IFO 6989, Lane 3; *P. linteus* CBS 454.76, Lane 4; *P. linteus* MPNU 7002, Lane 5; *P. linteus* MPNU 7001, Lane 6; *P. baumi* MPNU 7004, Lane 7; *P. baumi* MPNU 7005, Lane 8; *P. weirianus* CBS 618.89, Lane 9; *P. johnsonianus* ATCC 60051, Lane 10; *P. rhabarberinus* ATCC 26713, Lane 11; *P. pini* ATCC 12240, Lane 12; *P. gilvus* ATCC 26729, Lane 13; *P. igniarius* KCTC 6228, Lane 14; *P. nigricans* CBS 213.48, and Lane 15; *P. laevigatus* KCTC 6229.

species. The oligo probe of transitional changed two base lead to specific PCR band product. Since designed primer's homology to non-target regions is greater than 70%, we should focus also on modulation of general PCR condition and obtained the *P. linteus* specific distinct product (Fig. 3). However *P. baumii*-specific primer sets were not determined due to no existence of any clear available sites.

Discussion

In addition to ITS sequencing analysis, the sequencing of D1-D2 region was done to reconstruct and to design the species-specific DNA primer about the *Phellinus*. Certainly phylogenetic divergence was shown in 28S D1-D2 sequences analysis as well as ITS regions analysis although *P. linteus* and *P. baumii* were shown to similar evolutionary relationship. Phylogenetic diagram of 28S rDNA D1-D2 regions did not show any significant difference compared to that of ITS regions. However, the taxonomic position of *P. igniarius* was not clear. *Phellinus igniarius* AF311033 belongs to *P. laevigatus* complex group, but *P. igniarius* KCTC6228 belongs to *P. baumii* group as shown in Fig. 1. More strains of *P. igniarius* should be examined to classify accurately. *P. igniarius* and *P. johnsonianus* belong to different phylogenetic positions. *P. igniarius* included Group III with *P. laevigatus* and Group IV with *P. linteus* based on ITS and D1-D2 sequencing analysis, respectively. In the case of *P. johnsonianus*, it was in Group IV and Group I based on ITS and D1-D2 sequencing analysis, respectively. The length of 18S rDNA of *Phellinus* genus was estimated to be approximately 1800 bps. For more detailed phylogenetic relationship, it is necessary to examine this region.

Phellinus linteus and *P. baumii* belong to the same Group III, but they were not the same species. They could be divided to different subgroups. Most of all, establishment of type strain of *P. baumii* is need to be preceded. However our designed antisense primer, D12HR, can be adapted in rapid identifying of *P. linteus* with NL1 sense primer among the *Phellinus* species.

Anowledgement

This research was funded by the SGRP/HTDP (High-Technology Development Project for Agriculture and forestry) in Korea.

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