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Short Communication

PCR primers useful for nucleotide sequencing of rDNA of the powdery mildew fungi

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Four PCR primers that are useful to determine the nucleotide sequences of the rDNA of the powdery mildew fungi were newly designed. These primers provide both enough stability to work on a wide range of powdery mildews and enough specificity to eliminate contaminating DNA by PCR. DNA sequences of the rDNA ITS region were successfully obtained from specimens that were contaminated by other fungi. In addition, sequence results of the 18S and 28S rDNA were dramatically improved by using these primers in most of the specimens examined.

Key Words——Erysiphales; PCR primer; phylogeny; powdery mildew fungi; rDNA.

In order to reconstruct the evolutionary history of the powdery mildews, we have been trying to determine the nucleotide sequences of the rDNA for a large number of specimens of this fungal group. Since the powdery mildews are obligate parasites of plants that are unculturable on artificial media, DNAs are usually extracted from herbarium specimens or directly from fresh materials collected in the field. This results in frequent contamination of DNA by other fungi, plants and even insects or nematodes that were present in and around the powdery mildew infection, and eventually leads to failure of sequencing. Two PCR primers, PMITS1 and PMITS2, were designed based on the DNA sequences of 5′ end of the 18S rDNA and 3′ and of 28S rDNA in order to selectively amplify the rDNA of the powdery mildews from contaminated DNA extracts (Cunnington et al., 2001). Although these primers worked well on herbarium specimens, high annealing temperature (65°C) was required to eliminate contamination from the DNA by PCR. In this study, therefore, we newly designed four further PCR primers from the DNA sequences of the rDNA internal transcribed spacer (ITS) region. The ITS sequence is known to evolve at a rate that is close to the neutral rate of sequence evolution (Schlötterer et al., 1994), and thus it is variable between closely related taxa. However, the ITS region of the precursor molecules of rRNA was revealed to form a secondary structure including several stem-loop structures, and some conserved sequences are found in the stem regions (Van der Sande et al., 1992; Van Nues et al., 1995; Mai and Coleman, 1997; Takamatsu et al., 1998). This makes it possible to design, based on the ITS sequences, PCR primers that work on a wide range of the powdery mildews.

The DNA sequences and annealing sites of the primers are shown in Fig. 1. These primers were designed based on the conserved sequences of the ITS region from 19 powdery mildew taxa that were reported previously (Takamatsu et al., 1998). These primers should have both enough stability to work on a wide range of powdery mildew taxa and enough specificity to eliminate all other contaminating DNAs. We first examined stability of the primers. DNA fragments including ITS1, ITS2, and 5.8S sequences of the rDNA were amplified using universal primer set ITS1/P3 (Kusaba and Tsuge, 1995) from 32 powdery mildew taxa (Table 1) covering all major clades of the Erysiphales. These fragments were fractionated by electrophoresis in 1.5% agarose gel and recovered from the gel by use of the JETSORB kit (GENOMED) following the manufacturer's protocol. Primer sets PM3/P3, ITS1/PM4, PM5/P3, and ITS1/PM6, were tested. Universal primer set ITS1/ITS4 (White et al., 1990) was used as a positive control for each set of reactions. A negative control lacking template DNA was also included. PCR reaction was performed in a total reaction volume of 50 μl, including the following reagents: PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin); 200 μM of each deoxyribonucleotide triphosphate; 0.4 μl of each primer with 1 or 10 ng of the template DNA. Thermal cycling was performed in a thermal cycler PC-700 (ASTEC) under the following conditions: an initial denaturing step at 95°C for 2 min; then 30 cycles of 30 s at 95°C, 30 s at 52 or 55°C for annealing, and 30 s at 72°C for extension; and a final extension cycle of 7 min at 72°C. The annealing temperatures were determined by preliminary tests: 52°C for PM3/P3, PM5/P3, and ITS1/PM6; 55°C for ITS1/PM4 and ITS1/ITS4. The PCR products were subjected to electrophoresis in 2.0% agarose gel in TBE buffer and visualized by ethidium-staining. All of the primer sets tested successfully amplified the rDNA of the

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Table 1. PCR amplification of rDNA of powdery mildews and other fungi by newly designed primers.

Fungal species (Voucher/Isolate)	Primer set				
	PM3/P3	ITS1/PM4	PM5/P3	ITS1/PM6	ITS1/ITS4[a]
Powdery mildew[b]					
Arthrocladiella mougeotii (MUMH135)	+/+	++/++	++/+	++/++	++/++
Blumeria graminis f.sp. *agropyri*[c]	+/+	++/++	++/+	++/++	++/++
B. graminis f.sp. *bromi* (MUMH117)	+/+	++/++	+/+	++/++	++/++
B. graminis f.sp. *hordei*[c]	+/+	++/++	++/+	++/++	++/++
Brasiliomyces trina (MUMH113)	+/+	++/++	++/++	++/++	++/++
Cystotheca lanestris (MUMH114)	+/+	++/++	++/+	++/++	++/++
C. wrightii (MUMH137)	+/nt[d]	++/nt	++/nt	++/nt	++/nt
Erysiphe adunca (MUMH39)	+/+	++/++	++/++	++/++	++/++
E. australiana (MUMH134)	−/+	++/++	+/−	++/++	++/++
E. aquilegiae (MUMHs12)	+/+	++/++	++/++	++/++	++/++
E. friestii (MUMH6)	+/+	+/++	++/++	++/++	++/++
E. glycines (MUMH52)	+/+	++/++	++/++	++/++	++/++
E. gracilis (MUMH122)	+/+	++/++	++/++	++/++	++/++
E. huayinensis (MUMH30)	+/+	++/++	+/+	++/++	++/++
E. mori (MUMHs77)	+/+	++/++	++/++	++/++	++/++
E. pseudolonicerae (MUMH14)	+/+	+/++	++/++	++/++	++/++
E. simulans[c]	+/+	++/++	++/++	++/++	++/++
Golovinomyces cichoracearum (MUMH37)	+/+	+/++	++/+	++/++	++/++
G. orontii[c]	+/+	++/++	+/+	++/++	++/++
Leveillula taurica (MUMH125)	+/+	++/++	+/+	−/++	++/++
Neoerysiphe galeopsidis (MUMHs132)	+/+	++/++	+/+	++/++	++/++
Phyllactinia guttata (MUMH19)	+/+	++/++	++/++	++/++	++/++
Ph. guttata (MUMH35)	+/+	++/++	+/+	++/++	++/++
Pleochaeta shiraiana (MUMH36)	+/+	++/++	+/+	+/++	++/++
Podosphaera filipendulae (MUMHs43)	+/+	++/++	++/++	++/++	++/++
Po. fusca (MUMH68)	+/+	++/+	++/++	++/++	++/++
Po. longiseta (MUMH70)	+/+	++/+	++/++	++/++	++/++
Po. pannosa (MUMHs41)	+/+	++/++	++/++	++/++	++/++
Po. tridactyla (MUMHs62)	+/+	++/+	++/++	++/++	++/++
Sawadaea polyfida (MUMH47)	+/+	+/+	+/+	++/++	++/++
S. tulasnei (MUMH93)	+/+	++/+	++/++	++/++	++/++
Typhulochaeta japonica (MUMHs76)	+/+	++/++	++/++	++/++	++/++
Other fungus[e]					
Botrytis cinerea	−	−[f]	−[f]	−[f]	++
Colletotrichum sp.	−	−	−[f]	−	++
Cochliobolus miyabeanus (HE-7)	−	−	−[f]	−	++
Dendrophoma obscurans (5.29–5)	−	−	−	−	++
D. obscurans (5.30–5)	−	−	−	−	++
Fusarium oxysporum f.sp. *fragariae* (F2)	−	−	−	−	++
F. oxysporum f.sp. *fragariae* (F3)	−	−	−	−	++
Pythium aphanidermatum (Py-3)	−	−[f]	−[f]	−	++
Rhizoctonia solani AG-2-2 (PE-62)	−	−	−	−	++

a) Universal primer set used as a positive control.
b) Samples of 1 ng or 10 ng of DNA fragment amplified by ITS1/P3 were used as a template for each set of reactions. -: no visible DNA band; +: clear DNA band; ++: distinctly clear DNA band. MUMH: Mie University Mycological Herbarium, Japan., MUMHs: specimen sent by Dr. Y. Sato, Toyama Prefectural University to the Herbarium.
c) Voucher specimen not available.
d) nt: not tested.
e) Total DNA (1 μl) extracted by the method of Müller et al. (1992) was used as a template for each set of reactions.
f) Faint band differing from rDNA in size was detected.

PM3: 5'-GKGCTYTMCGCGTAGT-3'

PM4: 5'-CCGGCCCGCCAAAGCAAC-3'

PM5: 5'-TTGCTTTGGCGGGCCGGG-3'

PM6: 5'-GYCRCYCTGTCGCGAG-3'

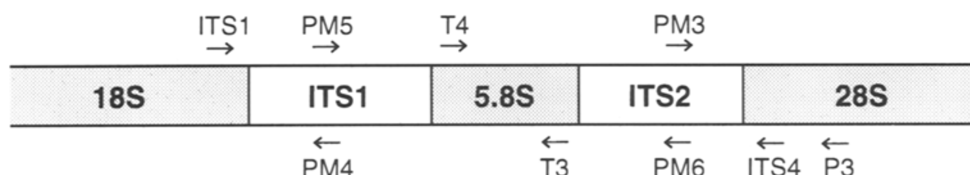

Fig. 1. Nucelotide sequences of the four PCR primers newly designed in this study and their annealing sites in the rDNA region. ITS1, ITS4, P3, T3, and T4 are universal primers used in this study.

32 powdery mildew taxa, although the amplification efficiency of PM3/P3 was lower than that of the other three primer sets. The primer sets PM3/P3 and PM5/P3 sometimes did not work on the DNA from *Erysiphe australiana* (McAlpine) U. Braun & S. Takamatsu (≡*Uncinula australiana* McAlpine). This result shows that all of the four primers are stable enough to work on a wide range of powdery mildew taxa.

Specificity of the primers was then investigated. Total DNA was extracted by the method of Müller et al. (1992) from mycelium (10 mg fresh weight) of the nine fungal isolates indicated in Table 1. Samples of 1 μl of the extracts were subjected to PCR amplification using the five primer sets and PCR conditions described above. Distinct DNA bands of expected size were detected by the positive control ITS1/ITS4 in all fungal isolates tested. No distinct DNA band was detected by the other four primer sets. Although faint DNA bands were found in some combinations of fungal isolates and primer sets, these bands were easily distinguished from the rDNA bands by their size. This suggests that the primers are specific enough to eliminate contaminating DNA by PCR.

The newly designed primers were used for sequencing of contaminated specimens. ITS1/P3 fragment, which was unsuccessfully subjected to sequencing, was diluted to 10 ng/μl with TE buffer (10 mM Tris-HCl, pH8.0; 1 mM EDTA) and reamplified using PM5/P3 and ITS1/PM6 as primer sets. The amplified PM5/P3 and ITS1/PM6 fragments were used as sequence templates, using ITS4 and T4 (Hirata and Takamatsu, 1996) as sequence primers for the former fragment, and ITS1 and T3 (Hirata and Takamatsu, 1996) for the latter. Nucleotide sequences were obtained by direct sequencing in an Applied Biosystems 373A sequencer. The sequence reactions were conducted using the PRISM Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) following the manufacturer's protocol. Twenty-seven powdery mildew specimens representing five teleomorphic and one anamorphic genera were used for the experiment (Table 2). When universal primer sets were used, sequencing failed due to overlapping signals in 20 of the 27 specimens. In the remaining 7 specimens, sequences from other fungi, e.g., *Ampelomyces*, *Alternaria* and *Cladosporium*, were obtained. By using the primers PM5 and PM6, the expected powdery mildew sequences of the ITS region were successfully obtained in 21 of the 27 specimens examined. Either the PM5/P3 or the ITS1/PM6 fragment was successfully sequenced in 3 specimens, and sequencing failed again in 3 specimens. This indicates that PM5 and PM6 are available for practical use in sequencing of the rDNA ITS region from contaminated specimens.

PM3 and PM4 were subjected to sequencing of the 28S and 18S regions, respectively. We tried to sequence the 28S rDNA of *Phyllactinia kakicola* Sawada (Vaucher: MUMH19) and *Erysiphe aquilegiae* DC. var. *rununculi* (Grev.) Zheng & Chen (MUMHs12) using universal primer sets, but failed several times. By using PM3 for PCR amplification of the specimens, we successfully obtained the 28S sequences. Sequence results of 18S and 28S regions of other specimens were also dramatically improved by using PM3 and PM4 for PCR amplification of the respective region. The sequences obtained and phylogenetic analyses based on them were reported elsewhere (Mori et al., 2000a, 2000b).

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Table 2. Practical use of PM5 and PM6 for sequencing of the rDNA ITS region of powdery mildew specimens that were failed in sequence using universal primer set[a].

Fungal species	Host	Voucher[b]	Contaminated by[c]	Fragment	
				PM5/P3	ITS1/PM6
Blumeria graminis	*Lolium multiflorum*	—[d]	Unknown	Failed	Failed
Erysiphe actinidiae	*Actinidia arguta*	MUMHs78	Unknown	OK[e]	OK
E. actinidiae	*Actinidia polygama*	MUMH457	Unknown	OK	OK
E. berberidicola	*Mahonia fortunei*	MUMH574	Unknown	OK	OK
E. betae	*Ambrina ambrosioides* var. *pubescens*	MUMH395	Unknown	OK	Failed
E. coriariae	*Coriaria japonica*	MUMH172	*Ampelomyces*	OK	Failed
E. divaricata	*Frangula alnus*	MUMH778	*Ampelomyces*	OK	OK
E. kusanoi var. *zelkowae*	*Zelkova serrata*	MUMH403	Unknown	OK	OK
E. ljubarskii var. *ljubarskii*	*Acer amoenum* var. *matsumurae*	MUMH475	Unknown	Failed	OK
E. necator var. *ampelopsidis*	*Vitis coignetiae*	MUMHs141	Unknown	OK	OK
E. prunastri	*Prunus domestica*	MUMH776	*Ampelomyces*	OK	OK
E. rodgersiae	*Rodgersia podophylla*	MUMHs69	Unknown	OK	OK
Erysiphe sp.	*Berchemiella berchemiafolia*	MUMH259	Unknown	OK	OK
Golovinomyces cichoracearum var. *cichoracearum*	*Physalis alkekengi* var. *franchetii*	MUMH441	Unknown	Failed	OK
G. cichoracearum var. *cichoracearum*	*Cirsium nipponicum* var. *yoshinoi*	MUMH592	Unknown	Failed	Failed
G. cichoracearum var. *cichoracearum*	*Saussurea nipponica* subsp. *hokurokuensis*	MUMH421	Unknown	OK	OK
Leveillula sp.	*Chamelaucium uncinatum*	VPRI20534	*Alternaria*	Failed	Failed
Oidium acalyphae	*Acalypha wiekesiana*	VPRI20226	*Cladosporium*	OK	OK
O. boroniae	*Boronia megastigma*	VPRI18337	Unknown	OK	OK
Oidium sp.	*Aster subulatus*	MUMH345	Unknown	OK	OK
Oidium sp.	*Aucuba japonica*	MUMH57	Unknown	OK	OK
Oidium sp.	*Indigofera psuedo-tinctoria*	MUMH898	Unknown	OK	OK
Oidium sp.	*Photinia glabra*	—[d]	Unknown	OK	OK
Oidium sp.	*Lycopersicon esculentum*	BPI746433	*Alternaria*	OK	OK
Oidium sp.	*Billardiera scandens*	VPRI20134	Unknown	OK	OK
Podosphaera pseudofusca	*Fatoua villosa*	MUMH346	Unknown	OK	OK
P. aphanis var. *aphanis*	*Geum nivale*	MUMH636	*Ampelomyces*	OK	OK

a) ITS1/P3 fragment, which was unsuccessfully subjected to sequencing, was diluted to 10 ng/μl by TE buffer, and reamplified using PM5/P3 and ITS1/PM6 as primer sets. The amplified DNA fragments were subjected to direct sequence.

b) MUMH: Mie University Mycological Herbarium, Japan; VPRI: Herbarium of the Institute for Horticultural Development, Australia; BPI: U.S. National Fungus Collection, Beltsville, MD, USA, MUMHs: specimen sent by Y. Sato, Toyama Prefectural University to MUMH.

c) Contaminating fungi were identified by database search using the obtained DNA sequence as a query. Unknown: Sequencing failed because of many overlapping signals.

d) Voucher specimen not available.

e) OK: Expected sequence of powdery mildew was obtained.

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