Nucleotide sequence diversity of rDNA internal transcribed spacers extracted from conidia and cleistothecia of several powdery mildew fungi*

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An improved protocol, including DNA extraction with Chelex, two amplifications with a nested primer set, and DNA purification by electrophoresis, made it possible to analyze

nuclear rDNA sequences of powdery mildew fungi using at most several hundred conidia or 20 cleistothecia. Nucleotide sequence diversity of the nuclear rDNA region containing the two internal transcribed spacers (ITS1 and ITS2) and 5.8S rRNA gene derived from conidia and cleistothecia was investigated for four kinds of powdery mildew fungi including two isolates of the same species. The results showed that the nucleotide sequences of the nuclear rDNA region were highly conserved between the teleomorph and the anamorph. Thus, the nucleotide sequence data obtained from either developmental stage can be used for phylogenetic studies of powdery mildew fungi. The nucleotide sequences of the 5.8S rRNA genes of the four species were highly conserved, but those of their ITS regions were variable. This suggests that the nuclear rDNA region is not suitable for phylogenetic studies of distantly related powdery mildew fungi, because too much sequence diversity exists within the ITS, and too little phylogenetic information is contained within the 5.8S rRNA gene. However, the ITS region will be useful for phylogenetic comparison of closely related species or intraspecies.

Key Words phylogeny; internal transcribed spacers; nucleotide sequence; powdery mildew.

Powdery mildew fungi cause serious diseases on a variety of cultivated plants such as cereal plants, vegetables, fruit trees, and ornamental plants. They belong to the family Erysiphaceae, which consists of 18 genera and 435 species worldwide (Braun, 1987), and 14 genera and 199 species in Japan (Nomura, 1992). The phylogeny of powdery mildew fungi has been discussed by Neger (1901), Blumer (1933), Katumoto (1973), Amano (1986) and Braun (1987). However, since these studies were based on the morphology of the fungi and their host range, a more objective analysis is still necessary to understand more precisely the

phylogenetic relationship amongst powdery mildew fungi. Recent advances in molecular techniques such as DNA-DNA hybridization, RFLP, RAPD-PCR, DNA sequencing and electrophoretic karyotyping, have made it possible to investigate the phylogeny of a variety of organisms at the molecular level (Bruns et al., 1991). However, most of these techniques are not applicable to powdery mildew fungi, because these obligately parasitic fungi cannot grow on artificial media and thus the amount of DNA obtainable from the target fungi is often insufficient for analysis using these techniques. Among the techniques developed by White et al. (1990), ribosomal DNA (rDNA) amplification and the direct sequencing of the PCR product seem the best for phylogenetic studies of powdery mildew fungi, because DNA sequences can be analyzed using only a small amount of fungal material.

Although powdery mildew fungi have been classified mainly on the basis of their teleomorphic characters, there are many species whose teleomorphic stages have not been found, or are rarely found. The lack of a teleomorphic state makes it difficult to discuss the phylogenetic relationships between anamorphic species and teleomorphic species. Recently, studies on phylogenetic relationships between anamorphic and teleomorphic species in several ascomycete genera have been performed using molecular analysis (Guadet et al., 1989; LoBuglio et al., 1993; Rehner and Samuels, 1994). Although single purified isolates were generally used for the analysis, it is necessary for obligate parasites such as powdery mildew fungi to use specimens collected in the field, because of the impossibility of culture on artificial media. However, the DNA extracted from the field specimens should be regarded as mixture of more than one isolate, since many species of powdery mildew fungi are known to be heterothallic (Abiko, 1985; Hiura, 1978; Homma, 1937; Schnathorst, 1959). Further, the DNA sequences derived from teleomorphic materials will not always be identical to those sequences derived from anamorphic materials of the same species, due to the recombination of DNA in teleomorphs as one possible reason for DNA sequence divergence. Thus, it is important to first investigate the nucleotide sequence diversity between the DNA derived from teleomorphic and anamorphic materials in order to study the phylogenetic relationships of powdery mildew fungi.

In this study, we developed techniques for nucelotide sequence analysis of the nuclear rDNA of powdery mildew fungi using a small amount of fungal material. Then, the nucleotide sequence diversity of the nuclear rDNA region covering two transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene was investigated by comparing the DNA derived from anamorphic (conidia) and teleomorphic (cleistothecia) materials for four species of powdery mildew fungi, including two isolates of the same species.

Materials and Methods

Sample sources The following powdery mildews were used in this study (Table 1): two isolates of Sphaerotheca cucurbitae (Jacz.) Z.Y.Zhao from Gynostemma pentaphyllum (Thunb.) Makino and Melothria japonica (Thunb.) Maxim.; Pleochaeta shiraiana (P.Henn.) Kimbr. et Korf on Celtis sinensis Pers. var. japonica (Planch.) Nakai; Phyllactinia moricola (P.Henn.) Homma on Morus bombycis Koidz.; and Uncinula adunca (Wallr.:Fr.) Lev. var. adunca on Salix ulpvina Anders. The conidial and cleistothecial stage specimens of Ph. moricola, Pl. shiraiana, and S. cucurbitae on M. japonica were collected in 1994 from the same individuals of respective host plants in Mie Prefecture, Japan. The conidia and cleistothecia of U. adunca var. adunca were collected from two neighboring host trees in the same day, and those of S. cucurbitae on G. pentaphyllum were collected in different parts of the prefecture, because we could not collect them from same plant individuals. Fungal DNAs were extracted from the fresh materials as soon as possible. The remaining specimens were dried and kept in the Mycological Herbarium of Mie University. The fungal species were identified according to the monograph of Nomura (1992).

DNA extraction Fungal DNA was extracted by the method of Walsh et al. (1991). Several hundred conidia or about 20 cleistothecia were added to 50 µl of 5% Chelex (Bio-Rad) in a 1.5-ml microcentrifuge tube and incubated at 56°C for several hours. After mixing vigorously, the extract was incubated in a boiling water bath for 8 min. The extract was mixed vigorously again, then centrifuged at 15,000 x *g* for 5 min. The supernatant was transferred to another tube and used as template DNA.

PCR amplification The nuclear rDNA region spanning the ITS1, ITS2 and 5.8S rRNA gene was amplified twice by PCR (polymerase chain reaction). Primers ITS5 (White et al., 1990) and P3 (Kusaba and Tsuge, 1995) were used for the first amplification, which 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 µM of each primer with 10 µl of the template DNA solution; and one unit of Tth DNA polymerase (Toyobo Co., Ltd.). The PCR reaction mixture was overlaid with 30 µl of mineral oil. The following thermal cycling conditions were performed in a thermal cycler PC-700 (ASTEC): an initial denaturing step at 95°C for 2 min; thermocycling for 30 cycles, where each cycle consisted of 30 sec at 95°C followed by 30 sec at 52°C for annealing, and 30 sec at 72°C for extension; and a final extension cycle of 7 min at 72°C. One microliter of the first amplification mixture was used for the second amplification using the nested primer set ITS1 (White et al. 1990) and P3. Components of the reaction mixture and the thermal cycle conditions for the second amplification were the same as for the first one. The PCR product from the second amplification was subjected to preparative electrophoresis in 1.5% agarose gel in TAE buffer. All amplifications yielded only a single visible DNA product. The DNA product band was excised from the ethidium bromide stained gel and purified using a JETSORB kit (GENOMED) following the manufacturer's protocol.

DNA sequencing Direct sequencing of PCR products was done in an Applied Biosystems 373A sequencer using a PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems) following the manufacturer's protocol. Six primers, ITS1, ITS2 (White et al., 1990), P3, T2 (5'GGGCATGCCTGTTCGAGCGT3'), T3 (5'ACGCTCGAACAGGCATGCCC3') and T4 (5'TCAACAACGGATCTCTTGGC3'), were used for sequencing in both directions (Fig. 1). The primers T2, T3 and T4 were designed based on the sequence of the 5.8S rRNA gene of Blumeria graminis (DC.) Speer f.sp. hordei (D84379).

Estimate of nucleotide sequence identity The nucleotide sequence data of the ITS1 and ITS2 regions and the 5.8S rRNA gene were subjected to pairwise alignment by the method of Lipman and Pearson (1985) using the program "GENETYX-MAC" (SOFTWARE

DEVELOPMENT CO., LTD.). The parameter sets were as follows: unit size to compare = 4, pick up location = 1. The percent identities were then estimated by the program.

Results

Double sequential amplification using nested primers The Chelex extraction method, which was developed for extraction of DNA from forensic materials (Walsh et al., 1991), was applied to powdery mildew materials. Although a single DNA band was found in every sample after the first amplification using the primer set ITS5/P3, the concentration of DNA was not enough for adequate sequencing results. Thus, the first PCR product was reamplified using the nested primer set ITS1/P3. This resulted in much more efficient amplification of DNA than when the primer set ITS5/P3 was used and yielded a higher amount of PCR product. The double sequential amplification yielded enough PCR product for adequate sequencing results.

PCR product purification Purification of the PCR product was another key step to obtain clear-cut sequence results. This was first attempted by the ultrafiltration tube method, using SUPREC-02 (TAKARA) without electrophoresis, but with unsatisfactory results. Instead, the product was electrophoresed through a 1.5% agarose gel. The DNA band of 500-600 nucleotides in length was excised from the agarose gel and purified by the JETSORB method, which was found to be superior to the SUPREC method for this purpose.

Sequence analysis of rDNA extracted from conidia and cleistothecia The ITS1, ITS2 and 5.8S rRNA gene sequences of rDNA extracted from conidia were identical with those from cleistothecia in Ph. moricola, Pl. shiraiana and the two isolates of S. cucurbitae. On the other hand, in U. adunca var. adunca, an insertion of guanine was found at nucleotide position 136 from the 5' end of ITS2 spacer region in the rDNA from cleistothecia compared with that from conidia (Fig. 2).

Diversity of rDNA sequences among tested taxa Table 2 shows the length of ITS1, ITS2 and the 5.8S rRNA gene of the powdery mildew fungi. The total lengths were 563, 557, 534 and 475 nucleotides in Ph. moricola, U. adunca var. adunca, Pl. shiraiana and S. cucurbitae,

respectively, indicating that the rDNA region is of variable length among the taxa, with a maximum difference in length of 88 nucleotides. This difference in nucleotide length derived from diversity in the ITS region, the 5.8S rRNA gene being the same length (154 nucleotides) in all tested taxa. The nucleotide sequence identities of the 5.8S rRNA gene were 96.8-100%, while those of ITS1 and ITS2 were 57.3-63.9 and 66.2-75.6%, respectively (Table3). This result indicates that the nucleotide sequence of the 5.8S rRNA gene is highly conserved among powdery mildew fungi, but that of the ITS region is variable. The nucleotide sequences of the two isolates of S. cucurbitae on G. pentaphyllum and on M. japonica were completely identical.

Discussion

Methods of obtaining rRNA gene sequences in the past have included cloning and sequencing of rRNA genes, and direct sequencing of the rRNA. The drawbacks to these methods are that they are time-consuming and require relatively large amounts of rRNA. PCR amplification of the rDNA followed by direct sequencing of PCR products, developed by White et al. (1990), seems to be the best technique available for phylogenetic studies of powdery mildew fungi. Its advantage is that only a small amount of starting material is needed, and a relatively crude DNA extract can be used to obtain the nucleotide sequences. However, several improvements to such procedures as DNA extraction, PCR amplification, and direct sequencing, were required in order to successfully obtain rDNA sequences from powdery mildew fungi. The present protocol including DNA extraction with Chelex, two PCR amplifications using a nested primer set, and DNA purification by electrophoresis made it possible to analyze rDNA sequences of powdery mildew fungi using a small amount of fungal materials, such as several hundred conidia or 20 cleistothecia.

The ITS sequences containing the 5.8S rRNA gene were identical in the DNAs derived from conidia and cleistothecia in all tested taxa except U. adunca var. adunca in which one nucleotide insertion was found in ITS2 region of the DNA extracted from cleistothecia. The insertion was not a misreading of the DNA sequences, because sequence

results using three kinds of primers all clearly supported the insertion. The one nucleotide difference between conidia and cleistothecia of U. adunca var. adunca may derive from intraspecies diversity, because the specimens were collected separately from two neighboring plant individuals. The ITS sequences of S. cucurbitae on G. pentaphyllum were identical in conidia and cleistothecia, even though the specimens were collected in different parts of Mie Prefecture. The above results show that the nucleotide sequences of the ITS region containing the 5.8S rRNA gene were highly conserved between the teleomorph and the anamorph. This indicates that the nucleotide sequence data obtained from either stage can be used for phylogenetic studies of powdery mildew fungi.

The nucleotide sequences of the 5.8S rRNA gene were highly conserved among the four taxa of powdery mildew fungi used in this study, but those of the two ITS regions were variable. In general, the coding regions of the rDNA, such as the 5.8S rRNA gene, are highly conserved and suited for phylogenetic studies among distantly related taxa (Bruns et al., 1991). On the other hand, the noncoding regions, such as the ITS regions or the intergenic region (IGR), are more variable and suited for phylogenetic studies among closely related taxa. The present result shows that this concept also applies to powdery mildew fungi. The four powdery mildew genera used in this study seem to be distantly related to one another on the basis of their morphology (Blumer, 1933; Braun, 1987). The nucleotide identities of ITS regions were relatively low (57.3-75.4%) among the four genera, and the sequences were ambiguously aligned. On the other hand, the alignment of the 5.8S rRNA showed that only 4 of 154 sites were variable, including 2 informative sites. This result suggests that neither region is suitable for phylogenetic studies of distantly related powdery mildew fungi, because too much sequence diversity exists in the ITS, and too little phylogenetic information is contained in the 5.8S rRNA gene. In this case, small-subunit or large-subunit rRNA genes may be suitable for phylogenetic studies. On the other hand, the ITS region will be useful for phylogenetic studies of closely related species or intraspecies.

In conclusion, we have established an efficient method for amplifying rDNA for sequence analysis from a small amount of fresh fungal material of powdery mildew fungi. We also tried to apply the techniques to herbarium specimens. Although amplified DNA products

were successfully obtained from dried specimens collected seven years ago, the amplification efficiency decreased as specimens became older. Further improvement will be required for DNA extraction from herbarium specimens.

Acknowledgement----We would like to thank Dr. J. W. Taylor, University of California, and Dr. T. Tsuge, Nagoya University, for their useful suggestions, and also Mr. G. Saenz, University of California, for reviewing the manuscript.

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FIGURE LEGENDS

Fig. 1. A map of the ribosomal DNA region containing ITS1, ITS2 and the 5.8S rRNA gene, and primers used for PCR and sequence analysis.

Fig. 2. Comparison of the nucleotide sequences of ITS1, 5.8S rRNA and ITS2 of the DNA extracted from conidia (upper line) and cleistothecia (lower line) of Uncinula adunca var. adunca. The arrow indicates insertion of guanine found in ITS2 of the DNA derived from cleistothecia.

Table 1. List of powdery mildew fungi and their host plants, and accession numbers of sequences submitted to DDBJ

Taxon	Host plant	Specimen no.	DNA extracted from:	DDBJ accession no.
Phyllactinia moricola	*Morus bombycis*	MUMH 35[a)]	Cleistothecia	D84385
		MUMH 79	Conidia	D84384
Pleochaeta shiraiana	*Celtis sinensis* var. *japonica*	MUMH 36	Cleistothecia	D84381
		MUMH 20	Conidia	D84380
Sphaerotheca cucurbitae	*Melothria japonica*	MUMH 68	Cleistothecia	D84387
		—[b)]	Conidia	D84386
Sphaerotheca cucurbitae	*Gynostemma pentaphyllum*	MUMH 111	Cleistothecia	D84378
		—[b)]	Conidia	D84377
Uncinula adunca var. *adunca*	*Salix vulpina*	MUMH 39	Cleistothecia	D84383
		MUMH 40	Conidia	D84382

a) DDBJ = DNA Data Bank of Japan, MUMH = Mie University Mycological Herbarium.
b) No specimen was deposited in the herbarium.

Table 2. Nucleotide length of the internal transcribed spacers and 5.8S rRNA

Taxon	Host plant	Nucleotide length (nt) [a]			
		ITS1	5.8S	ITS2	Total
Phyllactinia moricola	*Morus bombycis*	233	154	176	563
Pleochaeta shiraiana	*Celtis sinensis* var. *japonica*	214	154	166	534
Sphaerotheca cucurbitae	*Melothria japonica*	177	154	144	475
Sphaerotheca cucurbitae	*Gynostemma pentaphyllum*	177	154	144	475
Uncinula adunca var. *adunca*	*Salix vulpina*	221	154	182	557

a) Sequence data of DNA extracted from cleistothecia were used.

Table 3. Nucleotide sequence identities of the internal transcribed spacers and 5.8S rRNA

Taxon	*Pl. shiraiana*	*U. adunca*	*S. cucurbitae*
Ph. moricola	60.6/98.1/68.9/75.4 [a,b)]	60.4/98.1/66.2/72.1	60.2/96.8/72.1/75.4
Pl. shiraiana	60.9/100/74.6/73.9	63.9/98.7/67.7/71.1	
U. adunca	57.3/98.7/75.6/72.8		

a) The numerals indicate the identities (%) of ITS1/5.8S rRNA/ITS2/total, respectively.
b) Sequence data of DNA extracted from cleistothecia were used.

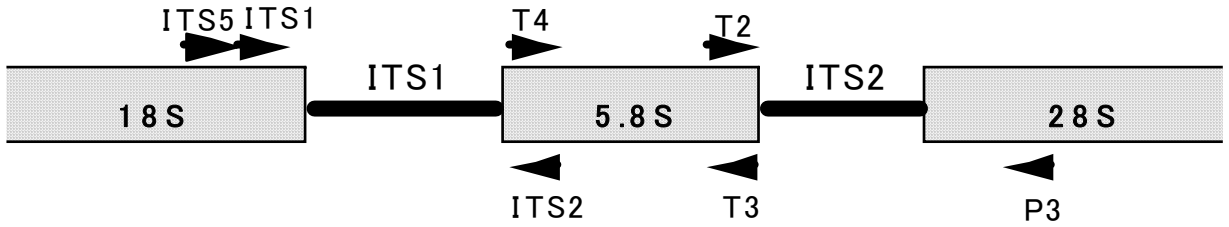

Fig. 1

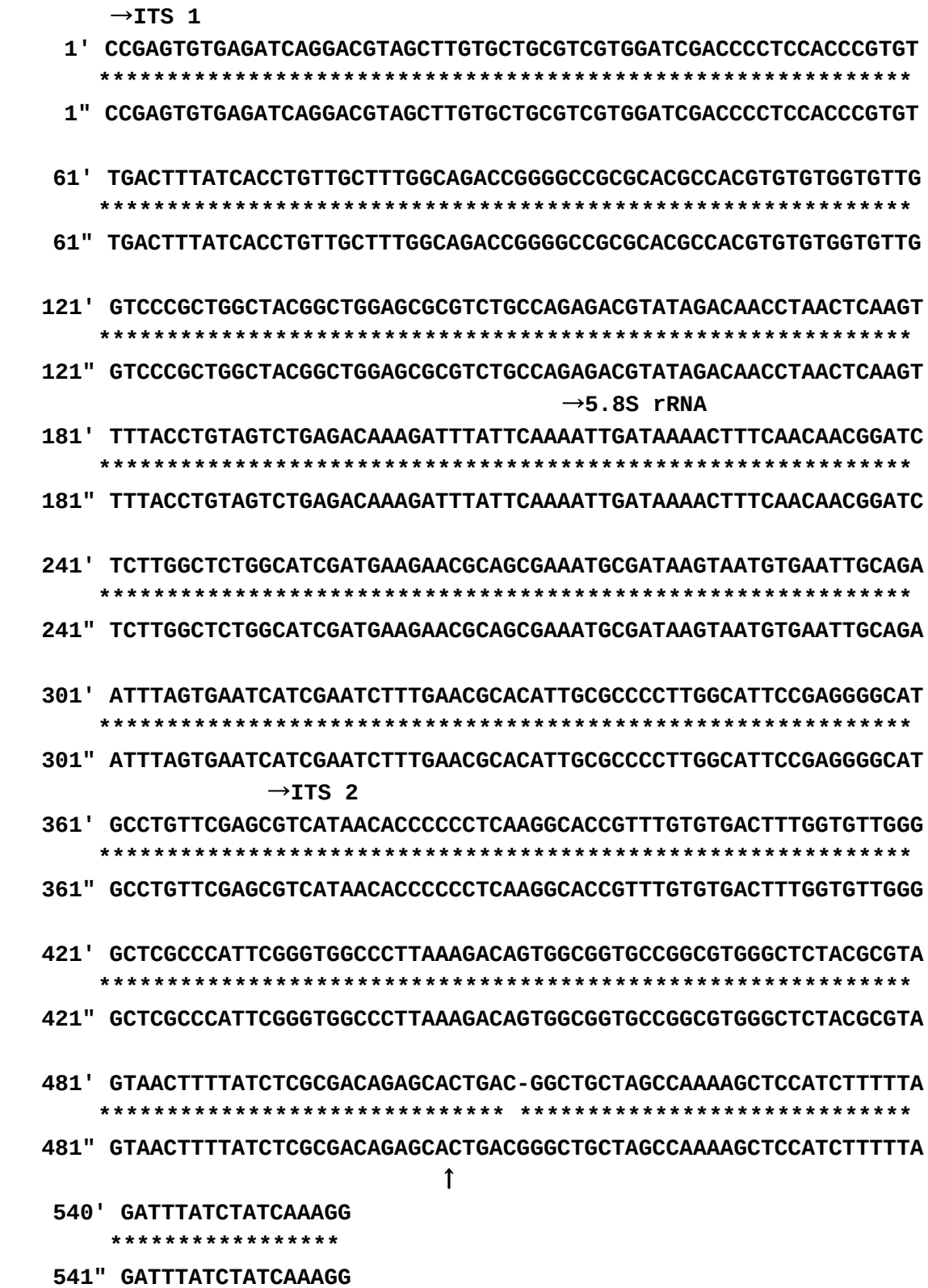
→ITS 1
1' CCGAGTGTGAGATCAGGACGTAGCTTGTGCTGCGTCGTGGATCGACCCCTCCACCCGTGT
1" CCGAGTGTGAGATCAGGACGTAGCTTGTGCTGCGTCGTGGATCGACCCCTCCACCCGTGT
61' TGACTTTATCACCTGTTGCTTTGGCAGACCGGGGCCGCGCACGCCACGTGTGTGGTGTTG
61" TGACTTTATCACCTGTTGCTTTGGCAGACCGGGGCCGCGCACGCCACGTGTGTGGTGTTG
121' GTCCCGCTGGCTACGGCTGGAGCGCGTCTGCCAGAGACGTATAGACAACCTAACTCAAGT
121" GTCCCGCTGGCTACGGCTGGAGCGCGTCTGCCAGAGACGTATAGACAACCTAACTCAAGT
→5.8S rRNA
181' TTTACCTGTAGTCTGAGACAAAGATTTATTCAAAATTGATAAAACTTTCAACAACGGATC
181" TTTACCTGTAGTCTGAGACAAAGATTTATTCAAAATTGATAAAACTTTCAACAACGGATC
241' TCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
241" TCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
301' ATTTAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGCATTCCGAGGGGCAT
301" ATTTAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGCATTCCGAGGGGCAT
→ITS 2
361' GCCTGTTCGAGCGTCATAACACCCCCCTCAAGGCACCGTTTGTGTGACTTTGGTGTTGGG
361" GCCTGTTCGAGCGTCATAACACCCCCCTCAAGGCACCGTTTGTGTGACTTTGGTGTTGGG
421' GCTCGCCCATTCGGGTGGCCCTTAAAGACAGTGGCGGTGCCGGCGTGGGCTCTACGCGTA
421" GCTCGCCCATTCGGGTGGCCCTTAAAGACAGTGGCGGTGCCGGCGTGGGCTCTACGCGTA
481' GTAACTTTTATCTCGCGACAGAGCACTGAC-GGCTGCTAGCCAAAAGCTCCATCTTTTTA
481" GTAACTTTTATCTCGCGACAGAGCACTGACGGGCTGCTAGCCAAAAGCTCCATCTTTTTA
↑
540' GATTTATCTATCAAAGG
541" GATTTATCTATCAAAGG

Fig. 2