Phylogenetic analysis and predicted secondary structures of the rDNA internal transcribed spacers of the powdery mildew fungi (Erysiphaceae)*

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The nucleotide sequences of the internal transcribed spacer (ITS) regions of the ribosomal DNA including the 5.8S rRNA gene and the 5′ end of the 28S rRNA gene have been determined for 19 species in 10 genera of the powdery mildew fungi in order to analyze their phylogenetic relationship. These fungi were divided into two large groups based on the nucleotide length of the ITS regions, and this grouping was in line with that based on the morphological characters of the anamorphic stage rather than the teleomorphic stage. Although the variable ITS sequences were often ambiguously aligned, conserved sites were also found. Thus, a neighbor-joining tree was constructed using the nucleotide sequence data of the conserved sites of the ITS regions, the 5.8S rRNA gene, and the 5′ end of the 28S rRNA gene. The phylogenetic tree displayed the presence of four groups in the powdery mildews, which were distinguished by their morphology and/or host ranges. In the ITS2 region, the presence of a common secondary structure having four hairpin domains was suggested, in spite of the highly variable nucleotide sequences of this region. The predicted secondary structure was supported by the compensatory mutations as well as compensatory conserved sequences and high G+C content in the predicted stem regions.

Key Words——Erysiphaceae; internal transcribed spacer; phylogeny; powdery mildew; secondary structure.

The powdery mildew fungi are important plant pathogens which are obligately parasitic on the surface of leaves, stems, fruits, and flowers of a wide range of Angiosperm plants. Host plants of the powdery mildews number up to 9838 species in 1617 genera, 169 families and 44 orders of Angiosperms (Amano, 1986). They belong to the order Erysiphales of the phylum Ascomycota in the Fungi (Hawksworth et al., 1995). Braun (1987) described 18 genera and 435 species of powdery mildews in his monograph. While most of them are entirely superficial except for haustoria that penetrate epidermal cells, 3 genera, i.e., *Leveillula*, *Phyllactinia*, and *Pleochaeta*, form endophytic mycelia and put haustoria into mesophyll cells.

The obligately parasitic nature of the powdery mildews limits the kinds of taxonomic and phylogenetic studies of the fungi that are possible. One reason is that the taxonomy of the fungi depends on the morphological characters preservable as herbarium specimens, because most taxonomic studies of the powdery mildews have been conducted based on herbarium specimens. The morphology of the cleistothecia, which is the teleomorph

of the powdery mildews, has been regarded as the most important taxonomic character, and the anamorph has been mostly neglected. Therefore, fungi having similar teleomorphs have been put into same genera, even when they have quite different anamorphs. Some of the problems have been solved recently by reevaluation of the anamorphic characters. However, many problems still remain.

The second problem is that cultural or physiological traits are difficult to use as taxonomic characters because these fungi cannot be cultured on artificial media. Zheng and Chen (1981) regarded host plant species as an important taxonomic character, and placed powdery mildews found on different plant families into different species. However, the questions whether the powdery mildews found on distantly related plants are usually distantly related to each other have not been answered yet. Therefore, more objective characters with which to determine the phylogenetic and taxonomic relationships of the powdery mildews are required to solve these problems.

Recently, molecular techniques such as isozyme analysis, DNA/DNA hybridization, electrophoretic karyotyping, RFLP, and DNA sequencing have been used for phylogenetic analysis of many kinds of organisms. Since powdery mildews are not culturable on artificial media, it is difficult to apply molecular techniques to

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them because only a small amount of starting material is available for analysis. Phylogenetic analysis of the powdery mildews using these techniques has been reported only for a few species such as *Blumeria graminis* (DC.) Speer f. sp. *hordei* (Saenz et al., 1994). On the other hand, the PCR direct sequencing method reported by White et al. (1990) is an excellent method applicable to the powdery mildews because it can be carried out using a small amount of starting material. Improving the method, we have made it possible to determine the rDNA sequences of the powdery mildews using tiny amounts of material (Hirata and Takamatsu, 1996).

Eukaryotic rDNA is composed of tandemly repeated clusters of 18S, 5.8S, and 28S rRNA genes, which are transcribed as a precursor molecule by RNA polymerase I (Raué and Planta, 1995). Then external and internal spacer molecules are removed. The nucleotide sequences of conservative rRNA coding regions have been used for phylogenetic analysis among families or distantly related genera (Carmean et al., 1992; Berbee and Taylor, 1993; Suh and Sugiyama, 1994; Nagahama et al., 1995). On the other hand, the variable internal transcribed spacer (ITS) regions have been used for the analysis of closely related genera, interspecies or intraspecies (Baldwin, 1992; Pleyte et al., 1992; Hsiao et al., 1994; Kusaba and Tsuge, 1995; Sang et al.,1995). We have determined the nucleotide sequences of the ITS regions including the 5.8S and the 5′ end of the 28S rRNA gene in order to analyse the phylogenetic relationships of the powdery mildews in this study. As expected, the nucleotide sequences of the ITS regions were highly variable among the genera of the powdery mildews and sometimes difficult to align. Therefore, only unambiguously aligned sites of the sequences were used for the current analysis, and the phylogenetic relationships among the powdery mildews are discussed on this basis.

The ITS regions have been regarded as nonfunctional sequences. However, analysis of the ITS regions in yeast revealed that these regions have some functions in processing of precursor molecules of rRNA: ITS1 in processing the 3′ end of 18S and the 5′ end of 5.8S, and ITS2 in processing the 3′ end of 5.8S and the 5′ end of 28S (Musters et al., 1990; Van der Sande et al., 1992; Raué and Planta, 1995). The secondary structures of the ITS regions were revealed to be important for the processing (Thweatt and Lee, 1990; Yeh and Lee, 1990; Van Nues et al., 1995). Therefore, the secondary structure, which will influence the variation of nucleotide sequences, is important for consideration of phylogenetic relationships of organisms by the nucleotide sequences of the ITS regions. The relationships between the nucleotide sequence mutations and the predicted secondary structures of the ITS regions of the powdery mildews are also discussed.

Materials and Methods

Sample sources Powdery mildew species used in this study, their original hosts, and accession numbers of the nucleotide sequence databases (DDBJ, EMBL, and GenBank) are given in Table 1. Species names of the taxa were identified by the morphological characters of the teleomorph according to the monographs of Nomura (1997) and Braun (1987). *Blumeria graminis* f. sp. *bromi*, *Cystotheca lanestris* (Harkn.) Miyabe and *Leveillula taurica* (Lév.) Arnaud were inferred from the morphology of the anamorph and their host plants, because no teleomorphic stage was found. *Blumeria graminis* f. sp. *hordei* race H1 was originally isolated by Hiura (1978) and has been maintained on the host plant in Mie University. Most of the specimens were preserved as herbarium specimens in Mie University Mycological Herbarium (MUMH).

DNA extraction and amplification of rDNA ITS sequences Whole-cell DNA was isolated from cleistothecia or conidia by the Chelex method (Walsh et al., 1991; Hirata and Takamatsu, 1996). The nuclear rDNA region including the ITS regions (ITS1 and ITS2), the 5.8S rRNA gene, and the 5′ end of the 28S rRNA gene were amplified by the polymerase chain reaction (PCR) using the primers ITS5 (White et al., 1990) and P3 (Kusaba and Tsuge, 1995). PCR reactions were conducted in 50 μl volumes as previously described (Hirata and Takamatsu, 1996). A negative control lacking template DNA was included for each set of reactions. One microliter of the first reaction mixture was used for the second amplification with the partial nested primer set ITS1 (White et al., 1990) and P3. The PCR product was subjected to preparative electrophoresis in 1.5% agarose gel in TAE buffer. The DNA product of each amplification was then excised from the ethidium bromide-stained gel and purified using the JETSORB kit (GENOMED) following the manufacturer's protocol.

DNA sequencing Nucleotide sequences of the PCR fragments were obtained for both strands using direct sequencing in an Applied Biosystems 373A sequencer. The sequence reactions were conducted using the PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems) following the manufacturer's protocol. Six primers, ITS1, ITS2 (White et al., 1990), P3, T2, T3, and T4 (Hirata and Takamatsu, 1996), were used for the sequencing in both directions.

Data analysis The obtained sequences were initially aligned using the Clustal V package (Higgins et al., 1992). The alignment was then refined visually with a word processing program with color coded nucleotides, and unalignable regions were excluded from the analysis. Phylogenetic trees were obtained from the data by both distance and parsimony methods. For distance analysis, DNADIST, in PHYLIP version 3.5 (Felsenstein, 1989) was used to obtain a matrix of Kimura's two-parameter distances (Kimura, 1980). The distance matrix was then analyzed by NEIGHBOR with algorithms based on Saitou and Nei's neighbor-joining method (Saitou and Nei, 1987). The strength of the internal branches of the resulting trees was statistically tested by bootstrap analysis (Felsenstein, 1985) from 1,000 bootstrap replications. For parsimony analysis, we used the maximum-parsimony method with heuristic search of the computer

Table 1. Sources of fungal materials and sequence database accession numbers.

Fungal species	Abbreviation	Host plants	Specimen no.[a]	Database accession no.[b]
Blumeria graminis f. sp. *hordei* race H1	BGRH	*Hordeum vulgare*	—	D84379
B. graminis f. sp. *bromi*	BGRB	*Bromus catharticus*	MUMH117	AB000935
Cystotheca lanestris	CYLA	*Quercus agrifolia*	MUMH114	AB000933
C. wrightii	CYWR	*Quercus glauca*	—	AB000932
Erysiphe aquilegiae var. *rununculi*	ERAQ	*Cimicifuga simplex*	MUMH12S	AB000944
E. cichoracearum var. *cichoracearum*	ERCI	*Eupatorium japonicum*	MUMH37	AB000934
E. heraclei sens. str.	ERHE	*Panax schin-seng*	MUMH73	AB000942
Leveillula taurica	LETA	*Caspicum annuum* var. *angulosum*	MUMH118	AB000940
Microsphaera friestii var. *dahurica*	MIFR	*Rhamnus japonica*	MUMH6	AB000939
M. pulchra var. *japonica*	MIPU	*Cornus controversa*	MUMH90	AB000941
Phyllactinia kakicola	PHKA	*Diospyros kaki*	MUMH19	AB000937
P. moricola	PHMO	*Morus bombycis*	MUMH35	D84385
Podosphaera longiseta	POLO	*Prunus grayana*	MUMH70	AB000945
P. tridactyla var. *tridactyla*	POTR	*Prunus japonica*	MUMH62S	AB000943
Sawadaea polyfida var. *japonica*	SAPO	*Acer palmatum* var. *palmatum*	MUMH47	AB000936
Sphaerotheca aphanis var. *aphanis*	SPAP	*Agrimonia pilosa*	MUMH49	AB000938
S. cucurbitae	SPCU	*Melothria japonica*	MUMH68	D84387
Uncinula adunca var. *adunca*	UNAD	*Salix vulpina*	MUMH39	D84383
U. mori	UNMO	*Morus bombycis*	MUMH77S	AB000946

a) MUMH=Mie University Mycological Herbarium; —=Specimens not preserved.
b) The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank Database under the respective accession number.

package PAUP version 3.1.1 (Swofford, 1993). This search was repeated several times from different random starting points using the stepwise addition option to make certain the most parsimonious tree was found. All nucleotide substitutions were equally weighted and unordered. Alignment gaps were treated as missing information.

The RNA secondary structure predicting program MulFold version 2.0 (Zuker, 1989) and LoopViewer (Gilbert, 1990) were used to predict the secondary structures of the ITS regions. At first, free energy of the folding structures of ITS sequences was calculated by Mul-Fold to generate suboptimal foldings. The output file was introduced into LoopViewer to draw the predicted secondary structures. The G+C contents distribution diagrams were obtained by the method of Bibb et al. (1984) using the program GENETYX (Software Development).

Results and Discussion

G+C contents and nucleotide length of rDNA ITS regions

The G+C contents and nucleotide length of ITS1, ITS2, 5.8S rRNA gene, and their total (ITS1-5.8S-ITS2) are shown in Table 2. Positive correlations in G+C contents and nucleotide length are found between ITS1 and ITS2 (Fig. 1), i.e., ITS2 of a taxon having GC-rich ITS1 is also GC- rich, and a taxon having a long ITS1 sequence also has a long ITS2 sequence, and vice versa. Torres et al. (1990) found similar phenomenom in the G+C contents of ITS regions in a wide range of organisms including filamentous fungi and called it "GC balance." The

total G+C contents of ITS1-5.8S-ITS2 ranged from 49.9% in *B. graminis* f. sp. *hordei* to 60.8% in *Sawadaea polyfida* (Wei) Zheng et Chen. Those of the 5.8S rRNA gene were stable (46.7–49.4%) among the 19 taxa investigated. On the other hand, the ITS regions showed relatively high G+C contents: 50.0–67.0% in ITS1 and 52.3–66.2% in ITS2. G+C contents of the two forma speciales of *B. graminis*, which are parasitic to monocots, are lower than those of dicots-parasitic powdery mildews. Bernardi et al. (1985) and Salinas et al. (1988) suggested that temperature is an important selection factor of GC bias in the genomes of plants and warm-blooded animals. For example, the genomes of warm-blooded vertebrates have higher G+C contents than those of cool-blooded vertebrates (Salinas et al., 1988). The optimum temperature for growth is 15–20°C in *B. graminis*, which is lower than those of the dicots-parasitic powdery mildews, 20–30°C (Yarwood et al., 1954; Lenkyn and Bainbridge, 1978). The relatively low G+C content of *B. graminis* might reflect the low optimum temperature of the fungus. The G+C content of *Uncinula adunca* (Wallr.: Fr.) Lév. on *Salix vulpina* Anders. was also lower than those of other dicots-parasitic taxa. However, the reason for this low G+C content is unclear because ecological and physiological information on the fungus is unavailable.

The shortest size of ITS1-5.8S-ITS2 was 475 nucleotides of *Sphaerotheca cucurbitae* (Jacz.) Z.Y. Zhao, and the longest was 563 nucleotides of *L. taurica* and *Microsphaera pulchra* Cook et Peck among 19 taxa sequenced in this study, a difference of 88 nucleotides (Table 2). This size variation was derived

Table 2.   The G+C contents and nucleotide length of the ITS1, ITS2, and 5.8S rRNA sequence.

Fungal species	ITS1		5.8S rRNA		ITS2		Total	
	GC content (%)	Length (nt)	GC content (%)	Length (nt)	GC content (%)	Length (nt)	GC content (%)	Length (nt)
Blumeria graminis f. sp. *hordei*	50.0	176	48.4	154	52.3	153	49.9	483
B. graminis f. sp. *bromi*	50.6	176	46.7	154	53.8	156	50.4	486
Cystotheca lanestris	64.7	184	49.4	154	59.1	149	58.1	487
C. wrightii	65.8	184	49.4	154	61.7	149	59.3	487
Erysiphe aquilegiae	58.3	218	46.8	154	58.3	180	55.1	552
E. cichoracearum	59.2	191	48.1	154	62.0	163	56.7	508
E. heraclei	59.3	221	46.8	154	61.6	185	56.6	560
Leveillula taurica	57.9	216	47.4	154	61.9	194	56.4	564
Microsphaera friestii	59.1	220	46.8	154	62.0	184	56.6	558
M. pulchra	56.8	227	46.8	154	61.0	182	55.4	563
Phyllactinia kakicola	56.6	235	47.4	154	62.6	182	56.0	571
P. moricola	59.7	233	46.8	154	61.4	176	56.7	563
Podosphaera longiseta	63.1	179	48.7	154	61.4	145	58.0	478
P. tridactyla	60.9	179	48.7	154	62.3	146	57.4	479
Sawadaea polyfida	67.0	185	48.1	154	66.2	145	60.8	484
Sphaerotheca aphanis	59.8	174	48.1	154	58.1	148	55.6	476
S. cucurbitae	64.4	177	48.7	154	64.6	144	59.4	475
Uncinula adunca	53.8	221	47.4	154	53.0	181	51.8	556
U. mori	57.7	222	46.8	154	56.8	185	54.4	561
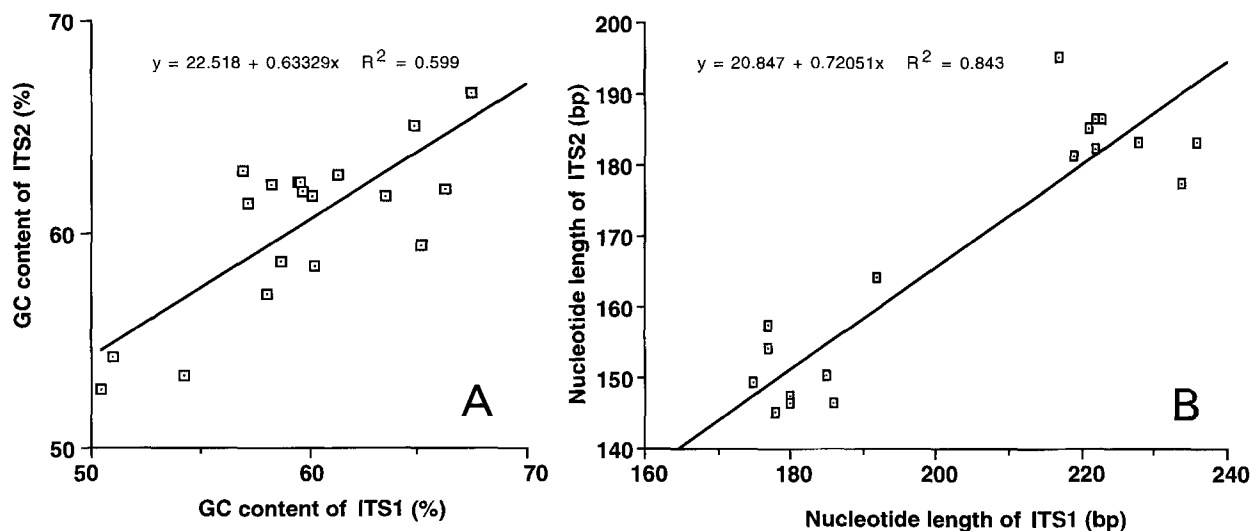
Fig. 1.   Positive corelations of G+C content (A) and nucleotide length (B) between ITS1 and ITS2.

from the variation of the ITS regions, because the size of the 5.8S rRNA gene was identical (154 nucleotides) among all the tested taxa.   Most of the tested taxa could be divided into two groups depending on the ITS length. The group with short ITS includes the genera *Blumeria*, *Cystotheca*, *Podosphaera*, *Sawadaea*, and *Sphaerotheca*, and the group with long ITS includes the genera *Erysiphe* section *Erysiphe*, *Leveillula*, *Microsphaera*, *Phyllactinia*, and *Uncinula*.   The length of ITS1-5.8S-ITS2 of the former group was between from 475 to 487 nucleotides, and that of the latter was from 552 to 563 nucleotides.   *Erysiphe cichoracearum* DC. which be-

longs to *Erysiphe* section *Golovinomyces* fell between the two groups (508 nucleotides).   Powdery mildews can be divided into two large groups by the number of asci in the cleistothecia, a feature of the teleomorphic stage.   Those having mono-ascal cleistothecia include the genera *Cystotheca*, *Podosphaera* and *Sphaerotheca*, and those with poly-ascal cleistothecia include *Blumeria*, *Erysiphe*, *Leveillula*, *Microsphaera*, *Phyllactinia*, *Sawadaea*, and *Uncinula*.   The grouping by this teleomorphic feature does not coincide with that by ITS length.   On the other hand, powdery midews are also divided into two groups by anamorphic features: the first group hav-

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         ↓ ITS 1                                                                                    100
BGRH  CAGAGCGTGA AGCTATGCGG AACTTCGTTT CGTGTA---- ---------- ---------- --------G TTGA-CCCTC C--ACCCGTG TCGATTATCT
BGRB  .......... T.......A. .....-..C T....---- ---------- ---------- ---------. C...-..... .--....... ..........
CYLA  .T........ G..C.C..T. GG.GC.TGGC GCC.GGCGTG ---------- ---------- ---------. C...-..... .--....... ..T..CT...
CYWR  .T........ G..C.C..T. GG.GC.TGGC GCCAGGCGTG ---------- ---------- ---------. C...-..... .--....... ..T..CT...
ERAG  .......... G...CA.TC. TGGCGTCAGC T.C..GCTGG ---------- ---------- ---------. CC..-..... .-C....... .......TCTA
ERCI  .......... GA..C...CC GGGC.T---- -------GCC CCGCGCGCAG A--------- ---------. ....-..... .--....... ..T..C.TATC
ERHE  .....T.C.. G...CA.TC. TGGCATC.GC T.C.CGCTGG ---------- ---------- ---------. CC..-..... .-C....... .....TGTA
LETA  ...G...... ..A-----CT CGG--.CC.C .ACA-GCGCA AGCTGGTGCG AGGGACACAT GC---CGGG. .C..-..... .-C....... ....C.CGTC
MIFR  .C...T.C.. G...CA.TC. TGGCGTC.GC T.C..GCTGG ---------- ---------- ---------. CC..-..... .-C....... .....TGTA
MIFU  .......... G...CA.TC. TGGCATT.GC ..C..GCTGG ---------- ---------- ---------. CC..-..... .-C....... .....TATA
PHKA  .......... .ACCCC.CT CGGGC.CC.C .AGCGGCGCA AGTCGGTGTG AGGGATACAT GC-CTCGGG. .C..-..... .-C....... ....AT.A.
PHMO  .T........ ..ACTCT... TC.CC..CCC .A.TGGTGCA AGCCAGTGCG AGGGGGGAGC ATGGCCGGA. .C..-..... .-C....... .....A.AAA
POLO  .C.....C.. G..C.C.... GG.GC.TG.C .TGCGTTG-- ---------- ---------- ---------. C...-..... .--....... .GA.C...A.
POTR  .C.....C.. G..C.C..A. GG.GC.TG.C .TGTGTTG-- ---------- ---------- ---------. C...-..... .--....... .GA...A.
SAPO  G......... CCGAGC...A GG.CG..CAG G.C.CCAGCC CCGCGCG--- ---------- ---------. CC..-..... .--....... CT...ATATC
SPAP  .T........ ...C.C..A. GG.GC.TG.C .TGCG-CG-- ---------- ---------- ---------. C...-..... .--....... .GA.C.GAT.
SPCU  .T.....C.. G..CCC..A. CG.GCACGCG .TGCGGCG-- ---------- ---------- ---------. ...-..... .--....... .GA.C.C.TA
UNAD  .C...T.... GATC.G.AC. T.GC.T..GC T.C..CGTGG ---------- ---------- --------A .C..C..... .--....... .T..C.T.A.
UNMO  .......... G.TCCA..C. TGGCAGT.G. ..C.CGCTGG ---------- ---------- --------A .C..-..... .AC....... ....C.TGTA
      ********** **                                                              * ********** ********** **********


                                                                                                    200
BGRH  CA---TGTTG CTTTGGCGGA TCGGGCTTGC CCGCG----- ---------- ---------- ---------- CCCACTTT-- --G-TGGGGG A-CATCCGCC
BGRB  ..---..... .......... .......... ..---..... ---------- ---------- ---------- ..--...... --..A. T-T.......
CYLA  ..---..... .......G C.....CGCG TGC.CTC--- ---------- ---------- ---------- .TGG.AC--- -CTGCT...A CGTGC.....
CYWR  ..---..... .......G C.....CCTG TGC.CTC--- ---------- ---------- ---------- ..GG.GC--- -CTGCT...A CG.GC.....
ERAG  TCT--..... .......G T..TCGCTGC CCGTACGG-- ACATGCGTCG GCCG--CCCA ..GGT..--- -C.ACT..A. CG.G.....
ERCI  TCA--..... .......G C.A..TGCCT .GCGCGCGG ---------- ---------- ---------- ..GG..C--- TGTGCT..CT CGTG......
ERHE  TCT--..... .......G C.....GCG T..TCGCTGT CCGCCTGG-- ACACGCGTCG GCCG--CCCA ..GGT..--- -C.ACT..A. CG.GC.....
LETA  TCC--..... .....GA.G C..ACTGCCT AGCG.TCCT- ---------- ---------- ---------- .TGG..C.C- -C.GCT..A. TG.GC.T...
MIFR  TCT--..... .......G C.....CGCG T..TCGCTGT CCGCATGG-- ACATGCGTCG GCCG--CCCA ..GGT..--- -C.ACT..A. CG.G......
MIFU  TCT--..... .......G C.....GCG ...ACGCTGT CCGCAAGGAA AGATGCGTCG GTCGCCCCCG ..GG...--- -C.GCT..A. CG.G......
PHKA  TCTCC..... .......A.G C....----- ---------- -.GCAA---- -------- ------CCCG .TGG.CC.T- -G.GCT..A. CGTGC.T...
PHMO  .GTC--.... ......TA.G C....----- -------GC CCGCCTGGCG GAT------- ------CCCG .TGG.C..TG AT.GCT..A. CGTGC.T...
POLO  .TAT-..... .......G C.....C.A ..TG------ ---------- ---------- ---------- ..GG..C--- -C.GCT.... .GTGC.....
POTR  .TAT-..... .......G C.....C.A ..TG------ ---------- ---------- ---------- ..GG..C--- -C.GCT.... .GTGC.....
SAPO  A----G.C.. ..C...T..G C.A....C.A ..TGA----- ---------- ---------- ---------- ..GG..C--- -C.GCC-... .G.GC.....
SPAP  A----..... .......G C.....C.A ..TA------ ---------- ---------- ---------- ..GG..C--- -C.GCT.... .GTGC.....
SPCU  TC---..... .......G C.....C.A ..TG------ ---------- ---------- ---------- ..GG..C--- -C.GCT..C. .GTGC...T.
UNAD  ..CC-..... ......A.. C...GCC.. G.A..CCACG TGTG-TGG-- -----TGTTG GT----CCCG .TGG..-A-- -C.GCT..A. CG.GG..T...
UNMO  TTTTT..... .......G C.....C.. ..AT.TTCGC TTGAGCATGG CGT------- GCTG--CCCA ..GG...--- -C.GCT..A. CG.G......
      ***** ********** *****                                                              ****** **********


                                                                              ↓ 5.8S           300
BGRH  AGGGAAAACC AAAA------ ---------- --CTCTTGTT ATTAG--TGA TGTCTGAGGA TGATATATAA TCAT------ ----GAAAAC TTTCAACAAC
BGRB  ......G... .......... ---------- --...C.. .C..--... ---------- .......... .......... A.. ---....... ..........
CYLA  ..A.CCCC.T T.------- ---------- --.G.G..C. G..T.--..T G...A...TG GT.GCA.A.T .AGAA----- ----T..... ..........
CYWR  ..A.CC.C.. T.------- ---------- --.G.G..C. G..T.--..T G...A...TG GT.GCAGA.T .AGAA----- ----T..... ..........
ERAG  .AA..CCTAA CC..AA---- ---------- --...A.... G.CTT--..T C....C...T .T..TAT-.G AAT.GA---- ----T..... ..........
ERCI  .AA..CCCAA CCT.A----- ---------- --...G.... G.C-.--..T A........ AA.CTAT.TG AAT.GT---- ----T..... ..........
ERHE  .AA..CCCAA CC..AAAA-- ---------- --...A.... G.CT.--..T C....T..CT .T...ATG.. AAT.GA---- ----T..... ..........
LETA  ..A..CT.TT C.------- ---------- --...G.... C.GGA--... A........C ATCA.GCA.T AA.AATGAAT AAGTT..... ..........
MIFR  .AA..CCCAA CC..AAA--- ---------- --...A.... G..T.--..T C....TT .....ATG.. AAT.GA---- ----T..... ..........
MIFU  .AA..CCCAA CC..AAAAAA ---------- --...A.... ..C.T--..C A....A..TC .TTAT...TG AAT.GA---- ----T..... ..........
PHKA  ..A...TGTA TTTTCAATGT GTTTTGGAAC AA...G.... ...G.--... A.......C. -C.ATGTGGG AA..TAGT-- ----T..... ..........
PHMO  ..A....GTT GG..CAA---- ---------- --...G..G ..GA--... A........C. ACCA.GTGGG AA..TAGT-- ----T..... ..........
POLO  ..A...GC.. C.------- ---------- --...G..C G.C..--..T G.......TG AATGTGGA.T .A.------- ----T..... ..........
POTR  ..A...GC.. C.------- ---------- --...G..C G.C..--..T ........TG AATGTGGA.T .A.------- ----T..... ..........
SAPO  ..A..GGC.. C.------- ---------- --...G..C G.C..--... ...TT GC.AT..AT. AAG------- ----T..... ..........
SPAP  ..A...GC.. C.------- ---------- --...A...A G....--..C A.......A. AC..T..AT. AG-------- ----T..... ..........
SPCU  ..A...GC.. C.------- ---------- --...G..C G.G..--..T .......... AATGTGGA.T .AG------- ----T..... ..........
UNAD  ..A..CGTAT .G.CAACCTA A--------- ---AA.... T.ACC--..T A........AC AA.G..T..T ...AAATTGA ----T..... ..........
UNMO  ..A..CCTTA C.CCCAAA-- ---------- --...A.... G.CCCC..T A....A..AT AAGAT.T..G AATGGAA--- ----T..... ..........
      ****                        ******** ********** ********                       ****** **********


                                                                                                    400
BGRH  GGATCTCTTG GCTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT AATGTGAATT GCAGAATTTA GTGAATCATC GAATCTTTGA ACGCACATTG
BGRB  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
CYLA  .......... ....C..... .......... .......... .......... .......... .......... .......... .......... ..........
CYWR  .......... ....C..... .......... .......... .......... .......... .......... .......... .......... ..........
ERAG  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
ERCI  .......... .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
ERHE  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
LETA  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
MIFR  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
MIFU  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
PHKA  .......... .......... .......... .......... .......... .......... .......C.. .......... .......... ..........
PHMO  .......... .......... .......... .......... .......... .......... .......C.. .......... .......... ..........
POLO  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
POTR  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
SAPO  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
SPAP  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
SPCU  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
UNAD  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
UNMO  .......... .......T.. .......... .......... .......... .......... .......C.. .......... .......... ..........
      ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
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                                          ↓ ITS 2                                                          500
BGRH  CGCCCCTGGG AATTCCCAGG GGCATGCCTG TTCGAGCGTC CGTAACAACC TC--TCAAGC C--------- ---TAGCT-T GGTATTGGGA CTCGCTGCCC
BGRB  .......... ......T... .......... .......... ..--...... ,--....... ,--------- ---....-. .C...... .AT....T.
CYLA  ......CC.. T.....G... .......... .C........ A...-..... C.--...... ,--------- ---....-. .C.....G .G...CCTGA
CYWR  ......CC.. T.....G... .......... .C........ A...-,.... C.--...... ,-------- ---.G...-, .C....AG .G...CC.G.
ERAG  .......T.. T.....G... .......... .......... A-........ C.C-..C... TGCC---TTT GTG.G.T.GC ...G.....G .C..TC..GT
ERCI  .......T.. C.....G... .......... .......... G-.C...-.. C.C-...... .GCGCCGTGT GTG.G.T-.. ...G.....G ....CCGT.
ERHE  .......T.. T.....G... ..G....... .......... A-....-... C.C-..C... TGCC---GTT GTG.G...GC ...G.....G ....TC..GA
LETA  .......... T.C...T... .......... .......... A-........ CG--..G... .GACT----- ---AG...-. .C.....G ....CCG.A
MIFR  .......T.. T.....G... .......... .......... A-....-... C.C-..C... TGCC---GTT GTG.G...GC ...G.....G ....TC..GA
MIPU  .......T.. T.....G... .......... .......... A-...AC... C.C-..C... TGCC---TTT GCG.G...GC ...G.....G .A..TC..GG
PHKA  .......T.. T.....G... .......... .......... A-........ C.--...... .GCTC----- ---.G...-. .C.....G ....CCG.A
PHMO  .......T.. T.....G... ..?....... .......... A-A....... C.--...T .GCTC----- ---.G...-. .C.....G .C...CCG.G
POLO  ......CC.. C.....G... .......... .......... A.-....-.. C.--...... ,--------- ---.T...-. .C.....G ....C.G.T
POTR  ......CC.. C.....G... .......... .......... A.-....-.. C.--...... ,--------- ---.....-. .C.....G ....C.G.T
SAPO  ......CC.. T.....G... .......... .......... A-........ C.--...... ,--------- ---....-. .CC....G .C...CCG..
SPAP  ......CC.. T.....G... ..?....... .......... A.-....T.. .--....... ,--------- ---.G...-. .C.....G .G...C.GTT
SPCU  ......CC.. C.....G... .......... .......... A.-....-.. C.--...... ,--------- ---.....-. .C.....G .....C.G.T
UNAD  .......T.. C.....G... .......... .......... A-....-.. C.CC.....G .ACCG--TTT GTG.GA..T. ...G.....G .....CCATT
UNMO  .......T.. T.....G... .......... .......... A-........ C.C-..G... TACCA-TTCT GTG.G...GC ...G.....G .....C..AT
      ********** ********** ********** ********** ********** ********** *         ******* ********** ****

                                                                                                          600
BGRH  TT----GTGG CGGTCCTCAA AAGCAGTGGC GGGACCATGT AA--CTCTCC GCGTAGTAA- -TACATCTCG CGACAGAGAA GCATT-GGG- ACTTGCCAAA
BGRB  .A-GTG.C.. .A........ ...A...... ...C...... .......... -.........,.......... ......G... ...GC-...-  .........
CYLA  -------.G.. .A.C..T.. .T........ .TG..G.CC GG--...A. .......T.- -.TTC..... .......TG .TGAC-...-  ..C.....G.
CYWR  -------.G.. ...CT..T.. .T........ .TG..G.CG GG--...A. .......T.- -.TTC..... ......G.TG .TGAC-...-  ..C.....G.
ERAG  .-----.C.. .A.CT..T.. .GA....... .TC.TGGCG TGGG....A. .........C T.G.T..... .......TG A.GAC-A.TG G.........
ERCI  -------G.. ...C...T.. .GA....... .TG..G..G TGGT....A. .........CG A---T..... .......CT ..TG.-..CC G.........T
ERHE  .-----.C.. ...C...T.. .GA....... .TC..GGCG TGGG....A. .........C T.G.T..... .......TG A.GCCT..TG G.......G.
LETA  ..TGGC.C.. ...CT..T.. .C........ .TG..GGTG GTG--.T... .......C.C --.TT..... .CG..G.C. .A..CC..-A C.CA....GC
MIFR  C-----.C.. ...C...T.. .GA.....C. .TC..GGCG TGGG....A. .........C T.G.T..... .......TG A.GAC-..TG G.......G.
MIPU  .-----.C.A ...C...G.. .GA....... .TC..GGCG TGGG....A. .........C T.G.T..... .......TG A.GACG.TTG G.........
PHKA  .CT-GC.C.. ...TC..T.. .CC....... ..A...GGTG GTG-...... .......C.C --GT-,.... ...G.TG ..-ACT..-A T.CA.....
PHMO  ACA-GC.... ...C...T.. .TCT...... .TG..GGTG GTG-...... .T.....C.C --GT-...... ...G.C. ..-ACT..-A C.CA.....
POLO  --------... ...C..CT.. .C........ .TG..G.TG TG--...... .T.....C.- -.GT...... ...CG ..GAC-...- ..C.....G.
POTR  --------.. ...C..CT.. .C........ .TG..G.TG TG--...... .T.....C.- -.GT...... ...CG ..GAC-...- ..C.....G.
SAPO  ------G.. ...C...T.. .CA....... .CG..G.CG TG--....A. .......... -CT.T..... .......G.CG CTGAC-...- G.C....G..
SPAP  -------C.. ...TC..CT.. .CAT...... ..T...G.TG TG--...... .......C.- -CGT...... .......TG ..GA.-...- ..C.......
SPCU  -------C.. ...C..CT.. .C........ .TG.TGGTG TG--...... .......C.- -.GT...... .......TG ..GAC-..C- ..CC....G.
UNAD  C------G.. T..C..T.. .GA....... .TG..GCGG TGGG....A. .........C T.TT...... .......C. CTGACG.CTG -..A......
UNMO  .------... ...C...T.. .GA....... ..T...GGCG CGGG....A. .........- --.AGA.... .......TG ..GA.G..CG ...A....G.
      ** ********** ********** *******    ****** *******     ***** ********** ********** **********

                                          ↓ 28S                                                           700
BGRH  ACTCCTTAAT TGCTCA---- ---------- ---------- --------GG TTGAACTCGA ATCAGGTAGG GATACCCGCT GAACTTAAGC ATATCAATAA
BGRB  .......... ......---- ---------- ---------,. .......... .......... .......... .......... .......... ..........
CYLA  .TA..CATC. .AACA----- ----------- ---------- ---------,. .........G .......... .......... .......... ..........
CYWR  .GC.TCATCA .A..A----- ----------- ---------- ---------,. .........G .......... .......... .......... ..........
ERAG  .--G.CC--G .TTGTTCC-A GTCACATGGA TCACA----- --------,. .......... .......... .......A.. .......... ..........
ERCI  CAAT.CATCA .CTCA.---- ----------- ----------- --------,. .......... .......... .......... .......... ..........
ERHE  ..AA.CCCTA .TGGGTCC-A GTCACATGGA TCACA----- --------,. .......... .......... .......A.. .......... ..........
LETA  .AC.ACA..G .C.C..GCGC TCTGCGCGGC GACTTTTGTA CTTCTTCT.. .......... .......... .......... .......... ..........
MIFR  ..AA.CCCC. .TTG.TCC-A GTCACATGGA TCACA----- --------,. .......... .......... .......A.. ....??????  ??????????
MIPU  .--G.CC--G .TTGTTCC-A GTCACATGGA TCACA----- --------,. .......... .......... .......A.. .......... ..........
PHKA  .AGA.CC.CG GCG..GTTGC GCGTCGTGTC TCTCTCAAT- --------,. .......... .......... .......... .......... ..........
PHMO  .GA.AACCTG ...GTCTGTC GCACGCT--A TCTAT----- --------,. .......... .......... .......... .......... ..........
POLO  .GC.TCA.T. .T..A----- ----------- ----------- --------,. A........G .......... .......... .......... ..........
POTR  ..C..CC... .TTCTT---- ----------- ----------- --------,. A........G .......... .......... .......... ..........
SAPO  G.....CTC .C-------- ----------- ----------- --------,. A........G .......... .......... .......... ..........
SPAP  ..C.ACAT.. .CT.T----- ----------- ----------- --------,. A........G .......... .......... .......... ..........
SPCU  ..C..AGTC. .T------- ----------- ----------- -------,. A........G .......... .......... .......... ..........
UNAD  .GCT.CATC. .TT.AGATTT ATC---T--A TCAAA----- --------,. .......... .......... .......... .......... ..........
UNMO  .AA.AAA..G CA.CTTGTGT GCCTTCTACA TCATA----- --------,. .......... .......... .......A.. .......... .........,
      *         **  ********** ********** ********** ********** ********** **********

                                          753
BGRH  GCGGAGGAAA AGAAACCAAC AGGGATTACC ?CAGTAACGG CGAGTGAAGC GGC
BGRB  .......... .......... .......... T......... .......... ...
CYLA  .......... .......C. .......... T......... .......... ...
CYWR  .......... .......C. .......... T......... .......... ...
ERAG  .......... .......... C......... T......... .......... ..?
ERCI  .......... ........?. C......... T......... .......... ...
ERHE  .......... .......... C......... T......... .......... ...
LETA  .......... ........C. ?????????? ?????????? ?????????? ???
MIFR  ?????????? ?????????? ?????????? ?????????? ?????????? ???
MIPU  .......... .......... C......... T........? ?????????? ???
PHKA  .......... .......... C......... CT........ .......... ...
PHMO  .......... .......... C......... CT........ ......??? ???
POLO  .......... .......... .......... T......... ?......... ..?
POTR  .......... .......... .......... T......... .......... ...
SAPO  .......... .......... .......... T......... .......... ...
SPAP  .......... .......... .......... T......... .......... ..?
SPCU  .......... .......... .......... T......... .......? ???
UNAD  .......... .......... C......... T.....?..? ?......??? ???
UNMO  .......... .......... C......... T......... .......... ...
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ing conidia produced in chains and club-shaped germ tube appressoria, and the second having conidia produced singly and lobed appressoria. The first group includes the genera *Blumeria, Cystotheca, Erysiphe* section *Golovinomyces, Podosphaera, Sawadaea,* and *Sphaerotheca,* and the second includes the genera *Erysiphe* section *Erysiphe, Leveillula, Microsphaera, Phyllactinia,* and *Uncinula.* When *E. cichoracearum* is placed in the short-ITS group, all of the taxa having conidia produced in chains and club-shaped appressoria are included in the short-ITS group, and the taxa having conidia produced singly and lobed appressoria in the long-ITS group. Thus, the grouping by ITS length corresponds with the grouping by the anamorphic features of conidia formation process or appressorial shape rather than by the teleomorphic feature of ascal number.

Phylogeny of the powdery mildew fungi The nucleotide sequence data set obtained from the 19 taxa in Table 1 gave a 753-nucleotide aligned sequence, including many ambiguously aligned sites due to the variable nucleotide sequence of the ITS regions (Fig. 2). However, since some conserved sites were found in the ITS regions, the conserved sites of the ITS regions, the 5.8S rRNA gene, and the 5' end of the 28S rRNA gene were used for the current analysis. After excluding the ambiguously aligned data, 479 aligned sites remained, of which 319 sites were variable. Figure 3 shows a neighbor-joining tree obtained by use of PHYLIP 3.5. A similar tree topology was obtained by the maximum-parsimony method using PAUP 3.1.1 (data not shown). The 19 tested taxa were divided into four groups. Although groups 2 and 4 were strongly supported by the bootstrap analysis (100% and 98%), the bootstrap values of the groups 1 (89%) and 3 (83%) were relatively low. All taxa included in groups 1 and 2 except *E. cichoracearum* belonged to the long-ITS group. On the other hand, those included in groups 3 and 4 belonged to the short-ITS group. Thus, the grouping based on the ITS length mostly coincided with grouping by the phylogenetic tree inferred from the nucleotide sequences of rDNA. Taxonomic and phylogenetic interpretation of these results is discussed in the context of groups 1–4.

GROUP 1: The genera *Erysiphe, Microsphaera,* and *Uncinula* are included in this group. These genera are ectophytically parasitic to plants and characterized by poly-ascal cleistothecia and conidia produced singly, except for *E. cichoracearum,* which has conidia produced in chains.

Braun (1981) divided the genus *Erysiphe* into three sections based on the anamorphic features, i.e., section *Erysiphe* (conidia produced singly and lobed appressoria, Pseudoidium-type), section *Golovinomyces* (conidia

produced in chains and club-shaped appressoria, Euoidium-type), and section *Galeopsidis* (conidia produced in chains and lobed appressoria, Euoidium-type). Some authors proposed dividing the genus *Erysiphe* into separate two different genera based on the distinct anamorphic states (reviewed by Braun, 1987). However, Braun (1987) disagreed, because he regarded the section *Galeopsidis* as an intermediate between section *Erysiphe* and section *Golovinomyces.* Of the three *Erysiphe* species used in this study, *E. cichoracearum* belongs to the section *Golovinomyces,* and *Erysiphe heraclei* DC. s. str. and *Erysiphe aquilegiae* DC. belong to the section *Erysiphe.* In the present phylogenetic tree (Fig. 3), the three *Erysiphe* species did not form a monophyletic cluster, suggesting that the section *Golovinomyces* and the section *Erysiphe* could be divided into different phylogenetic linages. Further nucleotide sequence data including section *Galeopsidis* are required to understand the phylogenetic relationships of *Erysiphe* species more precisely.

The genera *Microsphaera* and *Erysiphe* are clearly distinguished from each other by their teleomorphic states, especially by the morphology of appendages: dichotomously branched appendages in *Microsphaera* and hypha-like simple appendages in *Erysiphe.* However, these genera have identical anamorphs (singly-produced conidia and lobed appressoria), and they cannot be distinguished at this stage. In the present phylogenetic tree (Fig. 3), *Erysiphe* section *Erysiphe* and *Microsphaera* species form a distinct monophyletic cluster with a high bootstrap value (97%). However, *Microsphaera friestii* Lév. formed a subcluster with *E. heraclei* (bootstrap value 100%), and *M. pulchra* formed another subcluster with *E. aquilegiae* (bootstrap value 61%). The result suggests that the genera *Microsphaera* and *Erysiphe* section *Erysiphe* cannot be phylogenetically distinguished from each other, and that the phylogeny of the two genera coincides with the anamorph rather than the teleomorph.

Erysiphe cichoracearum can be regarded as the ancestor of the ectophytic fungi having singly-produced conidia, because it stands at the base of group 1 in the phylogenetic tree. However, the morphological character of *E. cichoracearum* of having 2–3 ascospores in an ascus is shared by *Phyllactinia* and *Leveillula.* This might indicate a direct phylogenetic relations between the endophytic genera and *E. cichoracearum.* The phylogenetic situation of *E. cichoracearum* may require further investigation.

GROUP 2: This group includes *Phyllactinia* spp. (*P. moricola* (P.Henn.) Homma and *P. kakicola* Sawada) and *L. taurica,* which is strongly supported (98%) by boot-

Fig. 2. Aligned sequence of the two internal transcribed spacers, the 5.8S rRNA gene and 103 sites from the 5' end of the 28S rRNA gene.
Asterisks mark aligned sites included in the phylogenetic analysis. The sites without asterisks mark nucleotides excluded from the phylogenetic analysis due to ambiguities in alignment. Much of the data from the spacers was excluded because of ambiguities resulting from numerous deletions and insertions. Positions identical to the reference sequence (*Blumeria graminis* f. sp. *hordei*) are indicated by dots. Gaps, representing putative insertion-deletion sites, are indicated by dashes. Ambiguous sites are indicated by question marks.
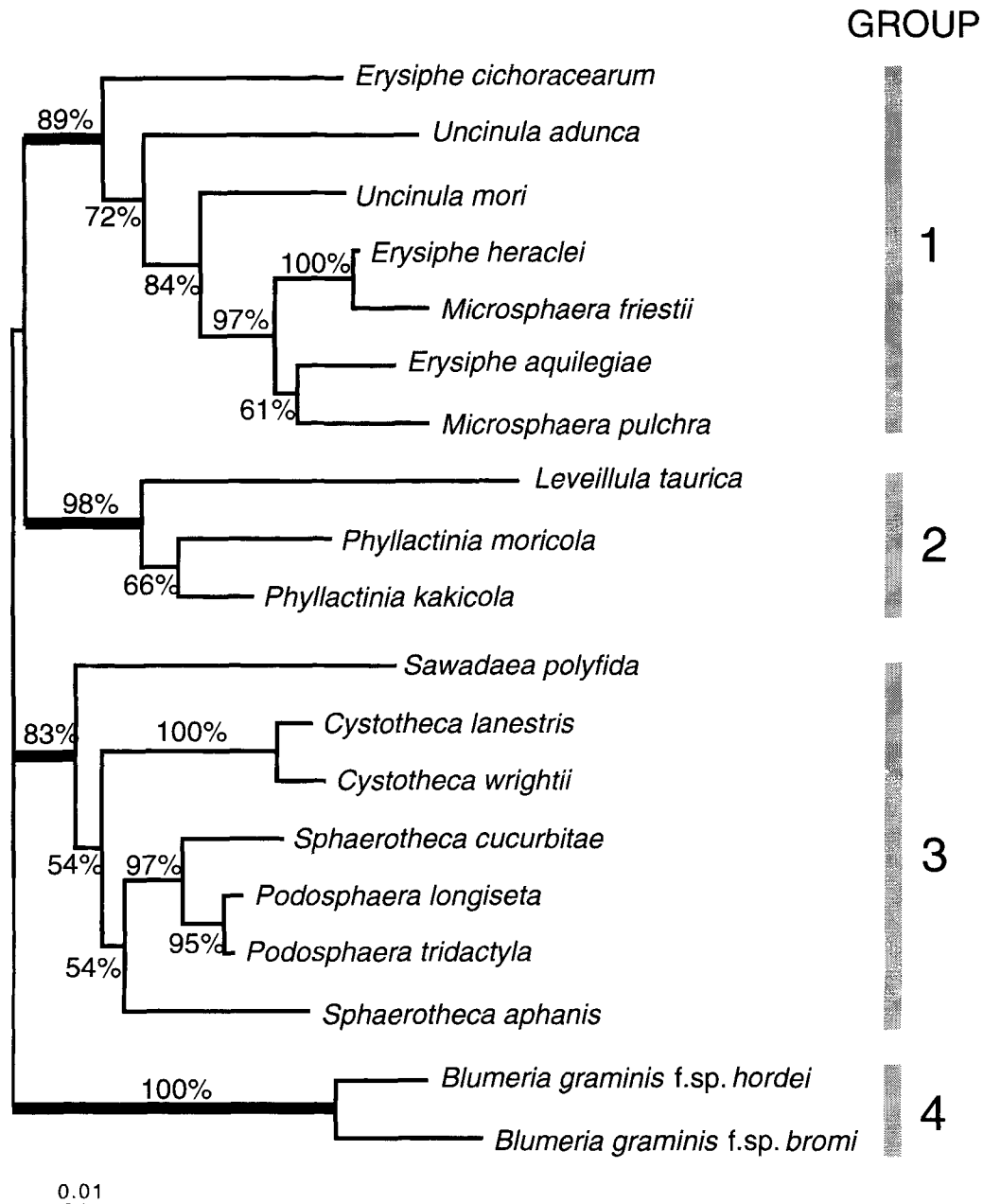
GROUP

Fig. 3.   Unrooted neighbor-joining tree on distances derived from sequences of the ITS1, ITS 2, the 5.8S rRNA gene, and the 5′ end of the 28S rRNA gene of the powdery mildews.
Distances were determined by Kimura's two-parameter method (Kimura, 1980), using the aligned sequences shown in Fig. 2.   The bar indicates a distance of 0.01 (one base change per 100 nucleotide positions).   The percentages represent the proportion of 1,000 bootstrap replications in which the groups to the right were placed together.   The phylogenetic tree shows that the powdery mildews investigated in this study are divided into four distinct groups indicated at the right of the tree.

strap analysis. These taxa are characterized morphologically by the presence of endophytic or partially endophytic mycelia. Although Blumer (1933) considered that *Leveillula* was phylogenetically distantly related to *Phyllactinia*, Braun (1987) considered that the two genera were closely related and placed them into the subfamily Phyllactinioideae. The present phylogenetic tree confirms a close relationship between *Leveillula* and *Phyllactinia*.

There are contrary hypotheses on the phylogenetical situation of these endophytic genera. Arnaud (1921) and Katumoto (1973) placed the endophytic genera at the primitive bases in their phylogenetic trees. On the other hand, most other authors including Blumer (1933) and Braun (1987), regard the genera as derived. The present result suggests that these endophytic and ectophytic genera divided each other at early stage of evolution, but it dose not answer the question whether

the endophyric genera are primitive or derived, because of the large variation of the ITS nucleotide sequences, and also because no appropriate outgroup was found. Analysis of a more conserved region will be required to answer the question.

GROUP 3: The genera *Sawadaea*, *Cystotheca*, *Podosphaera*, and *Sphaerotheca* are included in this group. Although group 3 had relatively low bootstrap support (83%), the constituent fungi can be distinquished by the presence of fibrosin bodies in the conidia and conidiophores. Most taxa belonging to the group have a single ascus in their cleistothecia, except the genus *Sawadaea*, which has many asci. *Sawadaea* may be regarded as an ancestral taxon of this group because it is situated at the primitive base of the group in the phylogenetic tree (Fig. 3). However, it would not be a direct ancestor because the host ranges of the group 3 fungi are quite different and dependent on the fungal genera, e.g., the host range of *Sawadaea* is restricted to the Family Aceraceae, that of *Cystotheca* to Fagaceae, and most host plants of *Podosphaera* and *Sphaerotheca* section *Sphaerotheca* belong to Rosaceae.

Sawadaea species have been historically placed in the genus *Uncinula*, because the teleomorphic feature of *Sawadaea* is similar to that of *Uncinula*, i.e., uncinate-circinate tips of appendages. However, *Sawadaea* is quite different from the *Uncinula* anamorph, in that conidia are produced in chains and contain distinct fibrosin bodies, and a second type of conidia is also found. The present phylogenetic tree confirmed that *Sawadaea* is distantly related to *Uncinula* and closely related to mono-ascal type genera having fibrosin bodies in conidia.

GROUP 4: Group 4 includes a single species, *B. graminis*. This species is characterized by being parasitic to monocots, especially to cereal plants, in contrast to parasitism in dicots by all other powdery mildews. Although *B. graminis* was historically regarded as a species of *Erysiphe*, Speer (1973) revised it to the new genus *Blumeria* because it is distinguished from other *Erysiphe* species by the unique features of its anamorph as well as the cleistothecium structure, e.g., digitate haustoria, secondary mycelium with bristle-like hyphae, bulbous swellings of the conidiophores, and formation of primary germ tubes in addition to appressorial germination (Kunoh et al., 1979). The present phylogenetic tree with a strong support of bootstrap (100%) demonstrates that *B. graminis* is distantly related to other powdery mildews. Braun (1987) considered that *Blumeria* is phylogenetically closely related to *Erysiphe*, and he placed it into the subtribe Erysiphinae. However, the present result suggests that the genus *Blumeria* is quite special among powdery mildews, and that it diverged from dicot-parasitic genera at an early stage of evolution.

Secondary structure As described previously, the nucleotide sequences of the ITS regions were highly variable among powdery mildew species, while relatively conserved regions were also present. It has been reported that the ITS regions as well as rRNA coding regions form secondary structures, which function in the maturation of rRNA precursors in yeast (Raué and Planta, 1995).

We therefore calculated the secondary structures using the computer programs MulFold and LoopViewer. The structures found for the different taxa were compared with each other.

ITS1 SECONDARY STRUCTURE: Many different folding structures with a similar energy were obtained from the nucleotide sequences of the ITS1. Therefore, we could not detect any stable structures in this region.

ITS2 SECONDARY STRUCTURE: Several different secondary structures with a similar energy were also obtained in the ITS2. However, the structures were relatively consistent compared with those obtained in ITS1, and a certain structure was commonly found in all investigated taxa. The predicted secondary structures of several taxa belonging to each different group are shown in Fig. 4. It was suggested that the 3' end of the 5.8S rRNA and the 5' end of the 28S rRNA can pair with each other in yeast (Van der Sande et al., 1992; Van Nues et al., 1995). Similar stem structures were also found in all of the powdery mildews investigated in this study. Four domains having distinctive hairpin structures were found in the ITS2 region. The nucleotide sequences of domain 1 were highly conserved among taxa belonging the short-ITS group, and a highly conserved sequence, 5'-CUCAAGCCURGCUUGGU-3' (nucleotide positions 452–483 in Fig. 2), was found in all taxa of this group. Although the stem structure of domain 1 was also conserved in the long-ITS group, an insertion of several nucleotides was observed at the apical part of the hairpin structure compared with the short-ITS group. No length mutation was detected in the hairpin structures of domains 2 and 3 between the long- and short-ITS groups, and the hairpin structures seemed to be stable among powdery mildews. A conserved sequence motif, 5'-GGGG-3' (positions 487–490), was found at the stem base of domain 2 and formed stable pairs with C or U at the opposite side of the stem (positons 514–517). In the conserved motif, mutations from G to A were observed at the nucleotide position 489 in *Cystotheca wrightii* Berk. et Curt. and at position 490 in *B. graminis* f. sp. *hordei* and f. sp. *bromi*. In these cases, compensatory mutations from C to U were commonly found in the opposite chain, producing A-U base pairs instead of G-C pairs. These compensatory mutations support the predicted folding structure of domain 2.

A conserved sequence motif, 5'-CTYTMCGYG-3' (positions 545–553), was found near the apical part of the stem of domain 3, and formed stable base pairs with another conserved motif, 5'-CGCGMSAGRG-3' (positions 569–578). A further conserved sequence, 5'-AGUGGCGG-3' (5'-AGUGCCGG-3' in *E. aquilegiae*, positions 525–532) was also found at the stem base of domain 3, and formed partial stable base pairs with the conserved sequence, 5'-GCCA-3' (5'-GCCG-3' in *S. polyfida*, positions 595–598). These results may support the folding structure of domain 3. Nucleotide sequences in the apical loop of domains 2 and 3 were diverged compared with those in the stem regions of the domains. A similar result was also obtained in ITS1 for yeast. On the other hand, it is known that the loops of
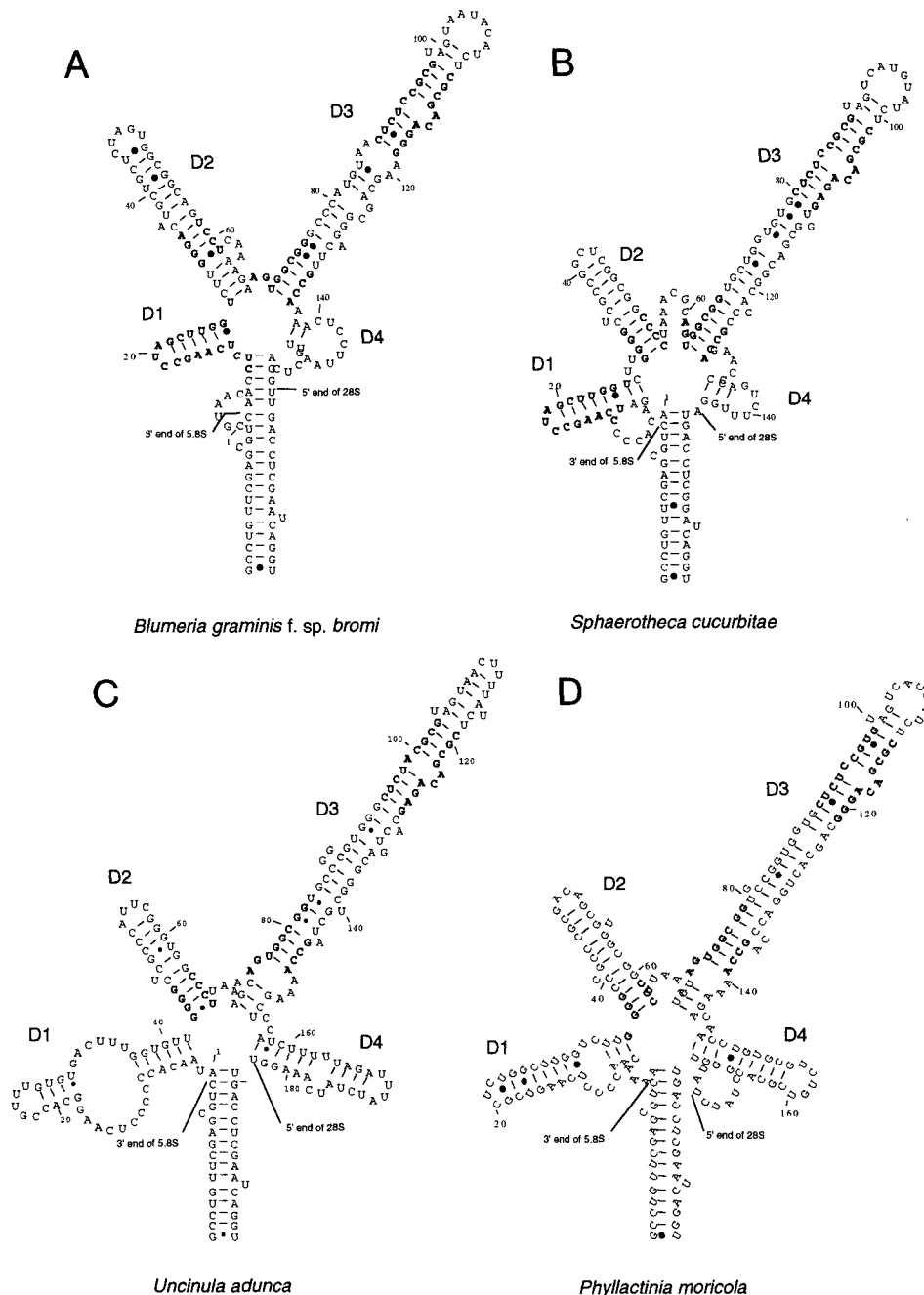
Fig. 4.   Secondary structures calculated for the ITS2 sequences of the powdery mildews.
   The four domains commonly found in these structures are numbered D1–D4.   The characters represented in boldface are higly conserved sequences.   *Blumeria graminis* f. sp. *bromi* (A) and *Sphaerotheca cucurbitae* (B) belong to the short-ITS group, and *Uncinula adunca* (C) and *Phyllactinia moricola* (D) to the long-ITS group.

the coding region of rRNA are more conserved than the stem region.   This inconsistency between spacer regions and coding regions may be derived from functional difference between the regions.

   Domain 4 was also observed in all the tested taxa. However, the nucleotide sequences and stem length of domain 4 were extremely variable.   Stem structure was sometimes obscured in the fungi belonging to the short-ITS group (Figs. 4A, B).   The difference in nucleotide

length of ITS2 between the short- and long-ITS groups derived from the differences in domain 4 as well as domain 1.   This result may indicate that domain 4 is not important for the function of ITS2.

   The G+C contents distribution in the ITS2 region of four taxa are shown in Fig. 5.   The predicted stem regions of domains 2 and 3 were extremely GC-rich, indicating the presence of stable stem structures in the domains.   On the other hand, the apical loop of domain 3

and intermediate regions between each domain were relatively AT-rich. The G+C contents in domains 1 and 4 were inconsistent among taxa. In particular, domain 4 of *U. adunca* was highly AT-rich, resulting in relative low G+C content in ITS2 of the fungus. These results may support the reliability of the predicted secondary structures.

Conclusion

The nucleotide sequences of rDNA ITS regions including the 5.8S rRNA and the 5' end of the 28S rRNA gene were obtained for 19 species in 10 genera of the powdery mildews. The nucleotide length of this region varied from 475 bp to 563 bp depending on the taxa. The taxa investigated were divided into two large groups based on the ITS length, i.e., a long-ITS group and a short-ITS group. The phylogenetic tree constructed from the nucleotide sequences supported this grouping. This grouping was also in line with grouping based on anamorphic rather than teleomorphic characters. The phylogenetic relationship between *Erysiphe* and *Microsphaera*, and the phylogenetic situation of the genus *Sawadaea* shown in Fig. 3, also reflected the anamorphic rather than teleomorphic features. Taxonomy of the powdery mildews has usually been based on the morphological characters of the teleomorphic stage, and those of the anamorphic stage have mostly been neglected. However, the present results indicate that the anamorphic features, especially conidia formation and appressorial shape, should be regarded as more important for taxonomy of the powdery mildews.
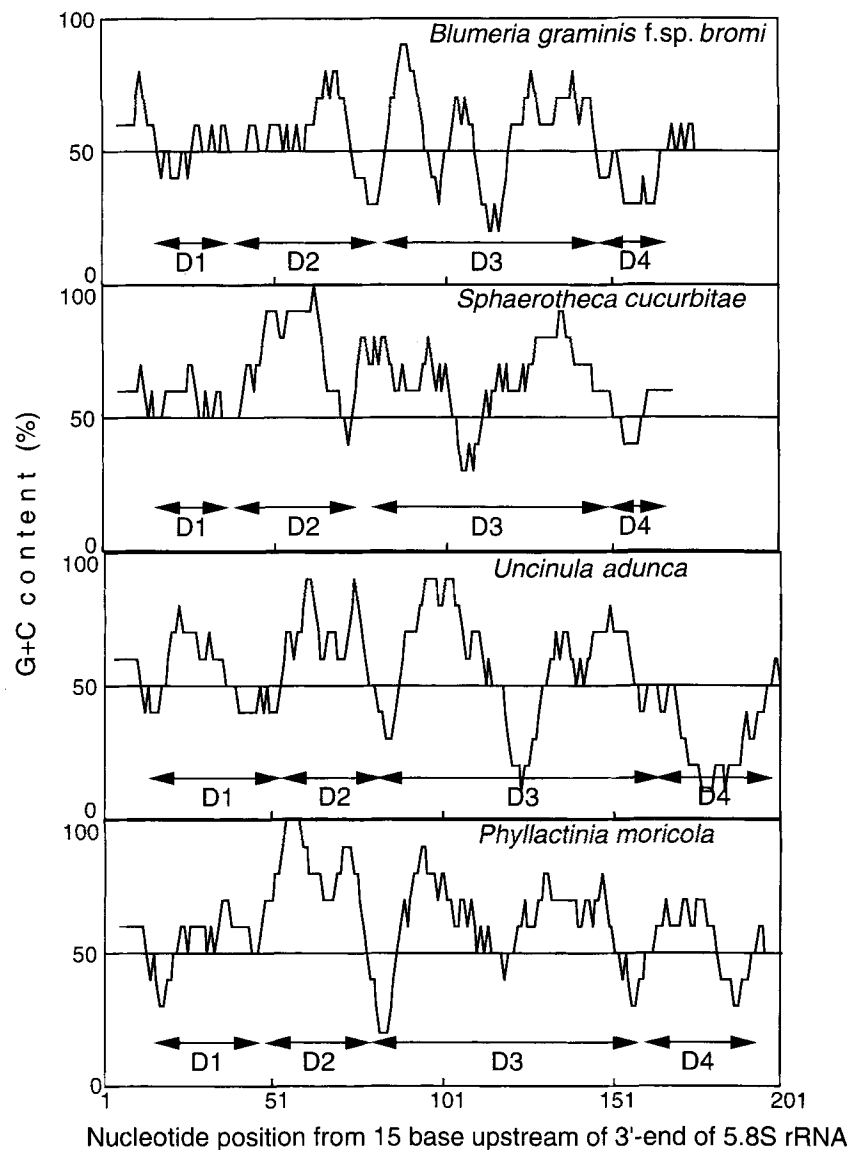
Fig. 5. G+C content distribution diagram of the ITS2 sequences of four powdery mildew species. D1–D4 indicate the domains found in the secondary structures shown in Fig. 4.

The variable spacer regions of rDNA have been considered to be useful for phylogenetic analysis of closely related genera, interspecies or intraspecies (Bruns et al., 1991). This is also true of the powdery mildews, and the sequences obtained were sometimes difficult to align between distantly related taxa. However, some conserved sequences were found in the spacer regions, which allowed the phylogenetic analysis of the powdery mildews using the conserved sequences of the spacers and the coding regions.

The phylogenetic tree obtained in the current analysis showed that the powdery mildews investigated can be divided into four groups. Each group was distinguished by its morphology and/or host range. Group 2, composed of the genera *Phyllactinia* and *Leveillula*, and group 4, composed of only one species, *B. graminis*, were strongly supported by the bootstrap analysis, indicating the monophyly of the groups 2 and 4. Although the bootstrap values were more than 80% in groups 1 and 3, they were lower than those of groups 2 and 4. Therefore, monophyly of the former groups should be reevaluated using another DNA region.

Although the present phylogenetic tree showed the presence of four groups in the powdery mildews, it did not show the branching order among the groups, because the nucleotide sequences of the ITS regions were highly variable, and also because no appropriate outgroup was found. Analysis of more conserved regions will be required to show the branching order among the groups. Nucleotide sequence analysis of the 18S rRNA gene is now progressing in our laboratory. The data will provide us more precise knowledge on the evolutional process of the powdery mildews.

The presence of a common secondary structure in the ITS2 region of the powdery mildews investigated was suggested in spite of highly variable nucleotide sequences of this region. The predicted secondary structure was supported by the compensatory mutations as well as conserved sequences and high G+C content in the predicted stem regions. It is known that the secondary structure of ITS2 is necessary for processing of the precursor molecule in *Saccharomyces* (Van der Sande et al., 1992; Van Nues et al., 1995). The present result suggests that the ITS of the powdery mildews have a similar function, and also that the secondary structure is more important than the nucleotide sequences in this region. Consideration of the secondary structure will be important for phylogenetic analysis based on the nucleotide sequences of rDNA.

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