Genetic Constitution and Pathogenicity of *Lolium* Isolates of *Magnaporthe oryzae* in Comparison with Host Species-Specific Pathotypes of the Blast Fungus

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ABSTRACT

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Fungal isolates from gray leaf spot on perennial ryegrass (prg isolates) were characterized by DNA analyses, mating tests, and pathogenicity assays. All of the prg isolates were interfertile with *Triticum* isolates and clustered into the crop isolate group (CC group) on a dendrogram constructed from rDNA-internal transcribed spacer 2 sequences. Since the CC group corresponded to a newly proposed species, *Magnaporthe oryzae*, all of the prg isolates were designated *M. oryzae*. However, DNA fingerprinting with MGR586, MGR583, and Pot2 showed that the prg isolates are divided into two distinct populations, i.e., TALF isolates and WK isolates. The TALF isolates were virulent only on *Lolium* species, whereas the WK isolates were less specific, suggesting that gray leaf spot can be caused not only by *Lolium*-specific isolates but also by less specific isolates. We designated the TALF isolates as *Lolium* pathotype. The TALF isolates showed diverse karyotypes in spite of being uniform in DNA fingerprints, suggesting that they are unstable in genome organization.

Additional keywords: *Pyricularia grisea*, *P. oryzae*.

Magnaporthe grisea (Hebert) Barr. [anamorph, *Pyricularia grisea* (Cooke) Sacc. (19)] is the causal agent of blast disease of many gramineous plants. This fungal species includes several subgroups, each of which has a restricted range of host species (14). Representative subgroups are *Oryza* pathotype pathogenic on rice (*Oryza sativa*), *Setaria* pathotype pathogenic on foxtail millet (*Setaria italica*), *Panicum* pathotype pathogenic on common millet (*Panicum miliaceum*), *Eleusine* pathotype pathogenic on finger millet (*Eleusine coracana*), and *Digitaria* pathotype pathogenic on crabgrass (*Digitaria sanguinalis*) (14). Kato et al. (14) found that the *Oryza*, *Setaria*, *Panicum*, and *Eleusine* pathotypes form a genetically close, interfertile group and designated it CC group.

The blast fungus can potentially cause destructive epidemics on plant species that have not been known as primary hosts. In the 1980s, severe blast disease occurred on wheat (*Triticum aestivum*) in Brazil and then spread to other countries in South America (31). Studies on host range and sexual fertility suggested that isolates from wheat (*Triticum* isolates) are similar to *Eleusine* isolates and distinct from the other host-specific pathotypes (14,31). Subsequent molecular analysis showed that the *Triticum* isolates also are distinct from the *Eleusine* isolates and belong to a separate, single lineage of their own (30). Furthermore, pathogenicity tests at low temperature (20°C) showed that the lineage of *Triticum* isolates constitutes a wheat-specific pathotype, i.e., *Triticum* pathotype (*unpublished data*).

Another example of recent destructive epidemics of blast disease may be found on perennial ryegrass ([prg] *Lolium perenne*) in golf courses. In 1991, a severe disease occurred on prg fairways in southeastern Pennsylvania (16). This disease was designated gray leaf spot, and its causal agent was identified as *Pyricularia grisea* (16). A more severe and widespread epidemic occurred in the mid-Atlantic region in 1995 and then expanded to other regions in the United States (7,21,29). The outbreak of gray leaf spot on prg turf also was reported in Japan (25). The first occurrence was recorded in west Japan (Yamaguchi prefecture) in 1996. Subsequently, this disease occurred in east Japan (Tochigi prefecture) in 1997 and in north Japan (Akita prefecture) in 1998. By 1999, this disease expanded to at least six prefectures (24).

Isolates from prg collected in the United States were characterized in two laboratories. Viji et al. (33) showed that prg isolates are genetically close to isolates from wheat and triticale and that wheat and prg are cross infected with those isolates under an artificial condition. Farman (4) showed that, while these two groups of isolates share high similarity, they are still distinguishable on the basis of DNA fingerprint profiles. He also showed that prg isolates collected in the United States have very similar fingerprint profiles with all probes tested. This result led him to a hypothesis that gray leaf spot was caused by a specific *P. grisea* population that constitutes a host-specific form of the fungus. This hypothesis involves three points that must be checked. First, the scientific name of prg isolates should be confirmed. A new species name, *M. oryzae* Couch, was recently proposed by Couch

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and Kohn (2). Second, the host range of the prg isolate population must be confirmed before it is called a host-specific form. Third, it should be checked whether a single population causes gray leaf spot. Farman (4) noted that U.S. gray leaf spot isolates were quite unlike *P. grisea* isolates found on prg in Japan. This information suggests that different populations can be involved in the gray leaf spot epidemics on prg. To address these three points, we examined DNA profiles and host ranges of Japanese prg isolates.

MATERIALS AND METHODS

Fungal materials. *Pyricularia* strains used were 24 isolates from prg (*L. perenne*) collected in five prefectures in Japan, eight isolates from wheat (*T. aestivum*) collected in Brazil, and 13 isolates from other gramineous plants collected in Japan or Brazil (Table 1). They were maintained in the Laboratory of Plant Pathology, Kobe University, at 4°C as dried-down cultures in barley

TABLE 1. Summary of the characteristics of *Pyricularia* isolates tested

Isolate	Original host	Locality	Year	Collector	Group[a]	Mating characteristics						PCR-RFLP type[e]
						Testers	Perithecium[b]	Ascus[c]	Ascospore[c]	Mating type[d]	Sexuality[d]	
AK1	*Lolium perenne*	Akita, Japan	1998	A. Tanaka, T. Tani	CC	Br48/Br116.2	2	+	+	*MAT1-2*	♂	+
AK2	*L. perenne*	Akita, Japan	1998	A. Tanaka, T. Tani	CC	Br48/Br116.2	2	+	+	*MAT1-2*	♂	+
AK3	*L. perenne*	Akita, Japan	1998	A. Tanaka, T. Tani	CC	Br48/Br116.2	2	+	+	*MAT1-2*	♂	+
AK4	*L. perenne*	Akita, Japan	1998	A. Tanaka, T. Tani	CC	Br48/Br116.2	2	+	+	*MAT1-2*	♂	+
AK5	*L. perenne*	Akita, Japan	1998	A. Tanaka, T. Tani	CC	Br48/Br116.2	3	+	+	*MAT1-2*	♂	+
LW1	*L. perenne*	Yamanashi, Japan	1999	A. Tanaka, T. Tani	CC	Br48/Br116.2	3	+	+	*MAT1-2*	♂	+
LW2	*L. perenne*	Yamanashi, Japan	1999	A. Tanaka, T. Tani	CC	Br48/Br116.2	3	+	+	*MAT1-2*	♂	+
LW3	*L. perenne*	Yamanashi, Japan	1999	A. Tanaka, T. Tani	CC	Br48/Br116.2	3	+	+	*MAT1-2*	♂	+
FI2	*L. perenne*	Chiba, Japan	1998	A. Tanaka, T. Tani	CC	Br48/Br116.2	1	+	+	*MAT1-2*	♂	+
FI3	*L. perenne*	Chiba, Japan	1998	A. Tanaka, T. Tani	CC	Br48/Br116.2	1	+	+	*MAT1-2*	♂	+
FI4	*L. perenne*	Chiba, Japan	1998	A. Tanaka, T. Tani	CC	Br48/Br116.2	3	+	+	*MAT1-2*	♂	+
FI5	*L. perenne*	Chiba, Japan	1998	A. Tanaka, T. Tani	CC	Br48/Br116.2	3	+	+	*MAT1-2*	♂	+
TP1	*L. perenne*	Tochigi, Japan	1997	A. Tanaka, T. Tani	CC	Br48/Br116.5	2	+	+	*MAT1-2*	♂	+
TP2	*L. perenne*	Tochigi, Japan	1997	A. Tanaka, T. Tani	CC	Br48/Br116.5	2	+	+	*MAT1-2*	♂	+
TP3	*L. perenne*	Tochigi, Japan	1997	A. Tanaka, T. Tani	CC	Br48/Br116.5	2	+	+	*MAT1-2*	♂	+
TP4	*L. perenne*	Tochigi, Japan	1997	A. Tanaka, T. Tani	CC	Br48/Br116.5	1	+	+	*MAT1-2*	♂	+
TP5	*L. perenne*	Tochigi, Japan	1997	A. Tanaka, T. Tani	CC	Br48/Br116.5	1	+	+	*MAT1-2*	♂	+
TP6	*L. perenne*	Tochigi, Japan	1997	A. Tanaka, T. Tani	CC	Br48/Br116.5	2	+	+	*MAT1-2*	♂	+
WK3-1	*L. perenne*	Yamaguchi, Japan	1996	A. Tanaka, T. Tani	CC	Br48/Br116.5	3	+	+	*MAT1-2*	♂♀	+
WK3-2	*L. perenne*	Yamaguchi, Japan	1996	A. Tanaka, T. Tani	CC	Br48/Br116.5	3	+	+	*MAT1-2*	♂♀	+
WK4-1	*L. perenne*	Yamaguchi, Japan	1996	A. Tanaka, T. Tani	CC	Br48/Br116.5	3	+	+	*MAT1-2*	♂♀	+
WK4-2	*L. perenne*	Yamaguchi, Japan	1996	A. Tanaka, T. Tani	CC	Br48/Br116.5	3	+	+	*MAT1-2*	♂♀	+
WK4-3	*L. perenne*	Yamaguchi, Japan	1996	A. Tanaka, T. Tani	CC	Br48/Br116.5	3	+	+	*MAT1-2*	♂♀	+
WK4-4	*L. perenne*	Yamaguchi, Japan	1996	A. Tanaka, T. Tani	CC	Br48/Br116.5	3	+	+	*MAT1-2*	♂	+
Br3	*Triticum aestivum*	Londrina, Brazil	1990	S. Igarashi	CC	Br48/Br116.2	3	+	+	*MAT1-1*	♂♀	+
Br8	*T. aestivum*	B.V. Paraiso, Brazil	1990	S. Igarashi	CC	G10-1/Z2-1[f]	3	+	+	*MAT1-1*	♂♀	+
Br48	*T. aestivum*	Itapora, Brazil	1990	S. Igarashi	CC	Br48/Br116.5	3	+	+	*MAT1-1*	♂♀	+
Br49	*T. aestivum*	Dourados, Brazil	1990	S. Igarashi	CC	G10-1/Z2-1	3	+	+	*MAT1-1*	♂♀	+
Br115.7	*T. aestivum*	Cornelio, Brazil	1992	A. S. Urashima	CC	G10-1/Z2-1	3	+	+	*MAT1-1*	♂♀	+
Br116.2	*T. aestivum*	Santa Mariana, Brazil	1992	A. S. Urashima	CC	Br48/Br116.5	3	+	+	*MAT1-2*	♂♀	+
Br116.5	*T. aestivum*	Santa Mariana, Brazil	1992	A. S. Urashima	CC	Br48/Br116.2	3	+	+	*MAT1-2*	♂♀	+
Br118.2D	*T. aestivum*	Panema, Brazil	1992	A. S. Urashima	CC	G10-1/Z2-1	3	+	+	*MAT1-2*	♂	+
MZ5-1-6	*Eleusine coracana*	Miyazaki, Japan	1976	H. Kato	CC	Br48/Br116.2	3	+	+	*MAT1-2*	♂	+
Ken15-15-1	*E. coracana*	Tokyo, Japan	1975	H. Kato	CC	Br48/Br116.2	2	+	+	*MAT1-2*	♂	+
GFSI1-7-2	*Setaria italica*	Gifu, Japan	1977	H. Kato	CC	G10-1/Z2-1[f]	3	+	+	*MAT1-2*	♂	+
NRSI2-2-2	*S. italica*	Nara, Japan	1977	H. Kato	CC	G10-1/Z2-1[f]	3	+	+	*MAT1-2*	♂	+
NNPM1-2-1	*Panicum miliaceum*	Nagano, Japan	1983	N. Hayashi	CC	G10-1/Z2-1[f]	3	+	+	*MAT1-2*	♂	+
STPM1-3-2	*P. miliaceum*	Saitama, Japan	1981	N. Hayashi	CC	G10-1/Z2-1[f]	3	+	+	*MAT1-2*	♂	+
Ken53-33	*Oryza sativa*	Aichi, Japan	1953	Y. Yamasaki et al.	CC	Br48/Br116.2	1	+	+	*MAT1-2*	♂	+
1836-3	*O. sativa*	Niigata, Japan	1976	M. Yamada et al.	CC	G10-1/Z2-1[f]	±	+	+	*MAT1-2*	ND	+
Br58	*Avena sativa*	Itambe, Brazil	1990	S. Igarashi	CC	Br48/Br116.5	3	+	+	*MAT1-2*	♂♀	+
NI919	*Leersia oryzoides*	Chiba, Japan	1974	N. Nishihara	L&S	G10-1/Z2-1[f]	0	–	–	ND	ND	–
NI981	*Cenchrus ciliaris*	Kumamoto, Japan	1975	N. Nishihara	C&E	G10-1/Z2-1[f]	0	–	–	ND	ND	–
Dig4-1	*Digitaria sanguinalis*	Hyogo, Japan	1991	Y. Iwamoto	DS	G10-1/Z2-1[f]	0	–	–	ND	ND	–
INA-B-92-45	*Sasa* sp.	Aichi, Japan	1993	S. Koizumi	S&P	G10-1/Z2-1[f]	0	–	–	ND	ND	–

[a] According to the designation proposed by Kato et al. (14).
[b] 0, not produced; ±, occasionally produced; 1, 1 to 10 perithecia in a petri dish; 2, 11 to 50 perithecia in a petri dish; and 3, more than 50 perithecia in a petri dish.
[c] +, produced ; –, not produced.
[d] ND, not determined.
[e] Presence (+) or absence (–) of the *Hpa*II site (Fig. 6).
[f] Data from Kato et al. (14). G10-1 and Z2-1 are highly fertile *Eleusine* isolates with *MAT1-1* and *MAT1-2*, respectively.

seed media (8). Briefly, barley seeds with husks were soaked in 1% sucrose solution in test tubes (13 by 75 mm) at room temperature overnight. After the sucrose solution was removed, the test tubes were capped with cotton plugs and autoclaved. Isolates were transferred to the barley seeds and incubated at room temperature for 7 to 10 days. After the barley seeds in the test tubes were covered with mycelia, they were transferred to a container with silica gel and preserved at 4°C. They were transferred to a potato dextrose agar slant just before use and grown at room temperature. One-week to three-month-old cultures were used for experiments.

Construction of a dendrogram based on rDNA sequences. Mycelial plugs were transferred to 50 ml of complete medium ([CM] yeast extract, 3 g; Casamino acid, 3 g; and sucrose, 5 g per liter) and grown at 25°C for 4 days. Total DNA was extracted from the resulting mycelia as described previously (18). The internal transcribed spacer 1 (ITS1)-5.8S-ITS2 region of the nuclear ribosomal RNA gene (rDNA) was amplified with the ITS4 and ITS5 primers (35). The amplification was performed in a 50-µl reaction containing 1.5 units of HotStar*Taq* DNA polymerase (Qiagen, Hilden, Germany), 1× polymerase chain reaction (PCR) buffer provided by the manufacturer, 200 µM each dNTP, 0.2 µM each primer, and 100 ng of template DNA. Reactions were heated to 95°C for 15 min and then amplified for 30 cycles (0.5 min at 94°C, 0.5 min at 56°C, and 2 min at 72°C) with the final step at 72°C for 7 min. The PCR product was electrophoresed in a 0.8% agarose gel, cut from the gel, and purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, England). The purified DNA was directly sequenced with primers ITS2, ITS3, ITS4, and ITS5 (35) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Nucleotide sequences of the ITS2 region were aligned using the CLUSTAL W program (27) and finally optimized manually. Aligned sequences were analyzed by a neighbor-joining (NJ) program in PAUP 4.0 (22). Evolutionary distances between nucleotide sequences were estimated by the method of Tajima and Nei (23). An unrooted tree was constructed using the NJ method of Saitou and Nei (20), which was then rooted using an isolate from *Sasa* sp. (INA-B-92-45) as an outgroup. The robustness of clusters was assessed by bootstrap analysis (5).

DNA fingerprinting. DNA fingerprinting was performed using the ECL Direct Nucleic Acid Labeling and Detection System (Amersham Pharmacia Biotech). Probes were pCB586 containing a fragment of MGR586 (6), pEBA18 (30) containing a fragment of MGR583 (6,11,32), and pPO-H (3) containing a fragment of Pot2 (12). These plasmids were labeled following the manufacturer's instructions.

Total DNA was digested with *Eco*RI (Takara, Tokyo, Japan) and fractionated on a 0.8% agarose gel (Nacalai Tesque, Kyoto, Japan) in 1× Tris-acetate-EDTA buffer. The fractionated DNA was transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then fixed by UV irradiation. The membrane was hybridized overnight with labeled probes in Gold hybridization buffer (Amersham Pharmacia Biotech) containing 0.5 M NaCl at 42°C. After hybridization, the membrane was washed twice in the primary wash buffer (6 M urea, 0.4% sodium dodecyl sulfate (SDS), and 0.5× SSC) for 20 min at 42°C followed by two washes in 2× SSC for 5 min at room temperature. Detection of target DNA was performed according to the manufacturer's instructions.

Electrophoretic karyotyping. Mycelial plugs were grown in 50 ml of CM at 25°C in the dark. Three-day-old cultures were homogenized briefly at full speed in a blender and transferred to 100 ml of fresh CM. After further incubation at 28°C for 24 h, the mycelia were harvested by filtration through two layers of gauze and washed with osmotic medium ([OM] 10 mM Na_2HPO_4 and 1.2 M $MgSO_4$, pH 5.8). They were suspended in 50 ml of filter-sterilized enzyme solution (10 mg of lysing enzymes per ml [Sigma-Aldrich, St. Louis] in OM) and incubated at 25°C for 3 h with vigorous shaking. The protoplast suspension was mixed with a half volume of 1 M sorbitol and centrifuged briefly at 1,600 × *g*. The supernatant containing protoplasts was transferred to a fresh centrifuge tube and centrifuged again at 1,600 × *g* for 10 min. The pellet was washed twice with 10 ml of SE (1 M sorbitol and 50 mM EDTA) and finally suspended in SE at 1×10^9 protoplasts per ml.

Gel plugs for pulsed-field gel electrophoresis (PFGE) were prepared following Akamatsu et al. (1) with a slight modification. The protoplast suspension was mixed with an equal volume of 1.5% low melting agarose (Nakarai Tesque) in SE at 50°C, pipetted into plug molds, and solidified at 4°C for 30 min. The plugs were incubated in washing solution I (2% SDS and 250 mM EDTA) at 50°C for 24 h and then treated with washing solution II (1% *N*-lauroylsarcosine sodium salt, 0.5 M EDTA, 10 mM Tris-HCl, pH 8.0, and 1 mg of proteinase K per ml) at 50°C for 24 h. After two washes with ET (50 mM EDTA and 1 mM Tris-HCl, pH 8.0), the plugs were stored in ET at 4°C.

The agarose plugs were loaded on a 0.6% agarose gel (agarose NA, Amersham Pharmacia Biotech) and electrophoresed in 0.5× Tris-borate-EDTA buffer at 11°C with the Gene Navigator System (Amersham Pharmacia Biotech). Voltage, switch time, and running time were 2 V/cm, 100 min, and 190 h, respectively. Separated chromosomes were stained with 1.0 µg of ethidium bromide per ml for 30 min and visualized under UV light. Chromosome sizes were estimated by comparison with commercially prepared chromosomes of *Schizosaccharomyces pombe* (Bio-Rad Laboratories, Hercules, CA).

Pathogenicity assay. The first experiment (experiment I) was performed to confirm the pathogenicity of the prg isolates on their original host, prg (*L. perenne*) and its relative, Italian ryegrass (*L. multiflorum*). Prg cvs. Friend and Yatsunami, and Italian ryegrass cvs. Mammoth B and Ace were grown in plastic pots (15 by 6 by 9 cm) filled with a mixture of Sakata Supermix soil (Sakata Co., Yokohama, Japan) and peat moss (3:1) in a greenhouse (25 to 30°C) for 3 weeks. Conidial suspension (2×10^5 per ml) of the prg isolates was prepared as described previously (17) and sprayed on the seedlings with 0.01% Tween 20. Inoculated seedlings were incubated in a dark moistened box for 24 h at 25°C and then returned to the greenhouse. At 5 to 7 days after inoculation, infection was evaluated using five grades from 0 to 5: 0, no visible reaction; 1, brown pinpoint spots; 2, small brown lesions; 3, intermediate lesions; 4, large lesions; and 5, complete blighting of leaf blades. Experiment I was repeated twice.

The second experiment (experiment II) was performed to determine the host range of the prg isolates. Test plants were the two cultivars of prg, the two cultivars of Italian ryegrass, common wheat (*T. aestivum*) cv. Norin 4 and cv. Chinese Spring, and the five differential plant species (specific suscepts) proposed by Kato et al. (14), i.e., finger millet (*E. coracana*) accessions EC19 and EC28, foxtail millet (*S. italica*) cv. Mosho-awa (accession SI12) and cv. Kariwano-zairai (accession SI17), common millet (*P. miliaceum*) accessions PM16 and PM18, crabgrass (*D. sanguinalis*) strain 136-102, and rice (*O. sativa*) cv. Shin 2 (*Pik-s*; *Pish*), Aichi-asahi (*Pia*), Ishikari-shiroke (*Pii*; *Pik-s*), Kanto 51 (*Pik*), Tsuyuake (*Pik-m*), Fukunishiki (*Piz*; *Pish*), Yashiromochi (*Pita*), Pi No.4 (*Pita-2*; *Pish*), Toride 1 (*Piz-t*; *Pish*), and CO-39. The rice cultivars from Shin 2 through Toride 1 are Japanese differentials for races of *Oryza* isolates (36). Wheat seedlings were grown in the plastic pots filled with vermiculite (supplied with liquid fertilizer) in a controlled environment room with a 12-h photoperiod of fluorescent lighting at 20°C for 9 to 10 days. The other plants were grown in the plastic pots filled with a mixture of Sakata Supermix soil and peat moss

(3:1) in a controlled environment room with natural light at 20 to 25°C for 25 days (prg, Italian ryegrass, finger millet, foxtail millet, common millet, and rice) or 30 days (crabgrass). These seedlings were inoculated with conidial suspensions (2×10^5 per ml) as described previously, kept in dark moistened boxes for 24 h at 20°C, and returned to the controlled environment room regulated at 20°C. At 5 to 7 days after inoculation, infection was evaluated as described previously. Experiment II was repeated three to five times.

Evaluation of mating ability. Mating ability of prg isolates was evaluated by incubating them with highly fertile tester isolates, Br48 (*MAT1-1*, hermaphroditic) and Br116.2 (*MAT1-2*, hermaphroditic). In some cases, Br116.5 was used in place of Br116.2 because it was also a hermaphrodite with the *MAT1-2* mating type and comparable to Br116.2 in fertility. They were isolated from wheat in Brazil in 1990 to 1992 (Table 1). The mating types of these testers had been determined by crossing with traditional testers, G10-1 (mating type A or *MAT1-1*) and Z2-1 (mating type a or *MAT1-2*), isolated from finger millet (9,10) beforehand. Mycelial fragments of the two testers (Br48/Br116.2 or Br48/Br116.5) and an isolate to be tested were placed about 4 cm apart on an oatmeal agar medium (20 g of oatmeal, 10 g of agar, and 2.5 g of sucrose in 500 ml of water) in a 9-cm-diameter petri dish, and incubated at 22°C under continuous fluorescent illumination. When the test isolate was interfertile with one of the testers, perithecia were produced along the borderline of their mycelial mats within 1 month. The presence and absence of perithecia was determined with a stereomicroscope. Perithecia produced were transferred into a drop of water on a slide grass, crushed by pressing the cover glass, and observed under a microscope to determine the quantity of asci and ascospores. These mating tests were repeated twice.

PCR-restriction fragment length polymorphism diagnostic for species identification. A region of the beta-tubulin gene was amplified as described by Couch and Kohn (2). The amplicon was digested with *Hpa*II (Takara) and separated on a 1.5% agarose gel (Nacalai Tesque). After staining with ethidium bromide, the digested fragments were visualized under UV light.

RESULTS

Phylogenetic analysis with rDNA-ITS2 sequences. The ITS2 region of rDNA was amplified and sequenced. All 24 prg isolates had the same ITS2 sequence, which was 100% identical to those of *Triticum* isolates (Br3, Br48, and Br116.5) and *Eleusine* isolates (MZ5-1-6 and Ken 15-15-1) (refer to DDBJ/EMBL/GenBank Accession No. AB031340). In an NJ tree constructed from the ITS2 sequences, the prg isolates were clustered into the CC group (14) with *Oryza*, *Setaria*, *Panicum*, *Triticum*, *Eleusine*, and *Avena* isolates (Fig. 1). A bootstrap analysis supported this cluster with more than 90% probability (Fig. 1).

DNA fingerprint profile. Total DNA of the prg isolates was digested with *Eco*RI and subjected to Southern analysis. The MGR583 probe produced two patterns of DNA profiles (fingerprints) among the prg isolates (Fig. 2A). One profile, composed of a few bands, was shared by isolates from west Japan (WK isolates), while the other profile, composed of more than 40 bands, was shared by isolates from east or north Japan (TP, AK, LW, and FI isolates). The latter group was tentatively designated as TALF isolates. Both groups of prg isolates were distinct not only from *Digitaria*, *Oryza*, *Panicum*, and *Setaria* isolates but also from *Eleusine* and *Triticum* isolates in the MGR583 profiles (Fig. 2B). Similar results were obtained with the MGR586 (Fig. 2) and Pot2 (data not shown) probes. These results suggest that our

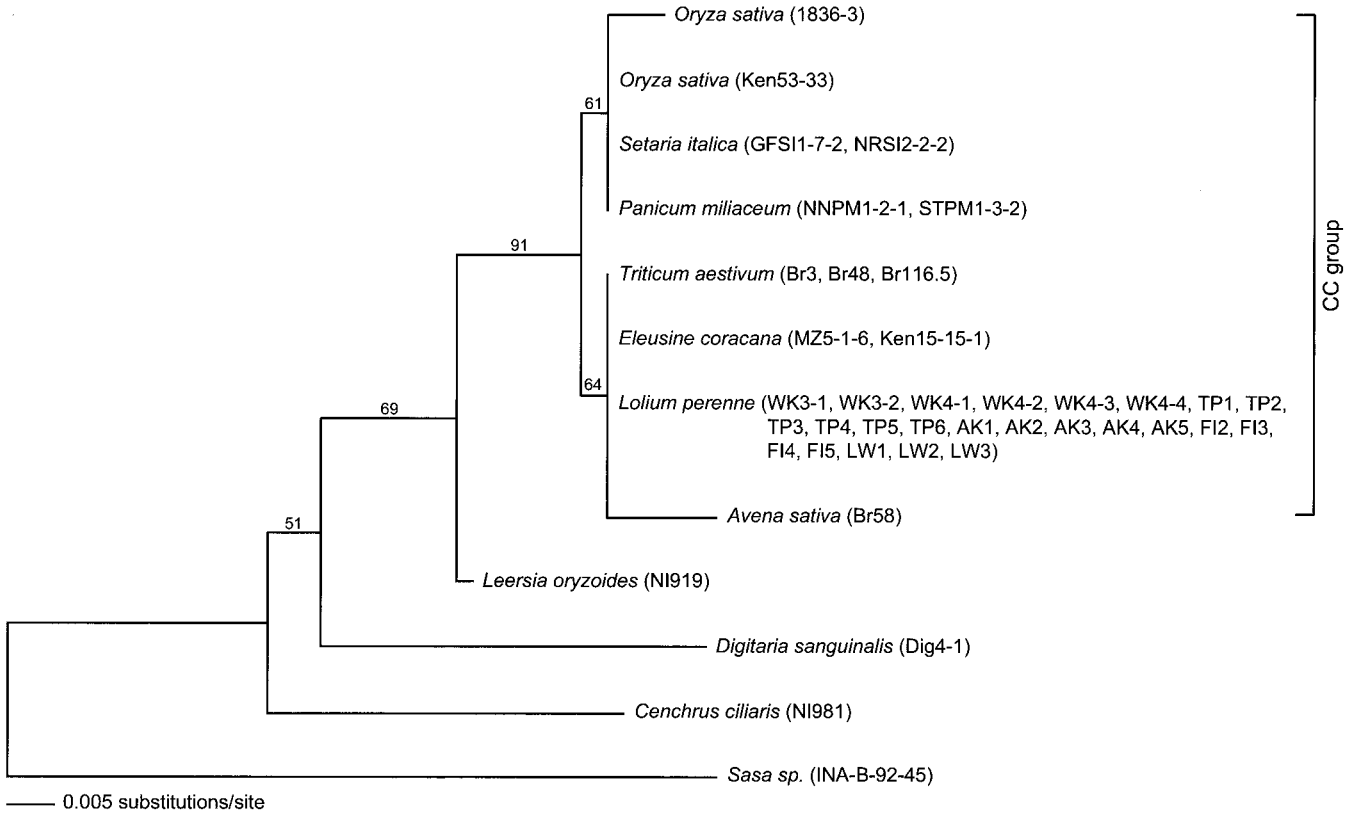

Fig. 1. Neighbor-joining dendrogram of *Pyricularia* isolates constructed from nucleotide sequences of the rDNA-internal transcribed spacer 2 region. Isolate names (in parentheses) are preceded by scientific names of their original hosts. Bootstrap values are indicated on the corresponding node for each cluster. Refer to DDBJ/EMBL/GenBank nucleotide sequence databases accession nos. AB031336 for GFSI1-7-2, NRSI2-2-2, NNPM1-2-1, STPM1-3-2, and Ken53-33; AB031340 for Br3, Br48, Br116.5, MZ5-1-6, and Ken15-15-1; AB031342 for NI981; AB031343 for NI919; AB031345 for Dig4-1; and AB031347 for INA-B-92-45.

prg isolates are divided into two distinct populations and that both are genetically different from the other host-specific pathotypes.

Electrophoretic karyotype. When run on a PFGE gel, the prg isolates showed various karyotypes. The variation was prominent even within the TALF isolates (Fig. 3) that showed similar DNA fingerprints (Fig. 2). On the other hand, *Triticum* isolates were uniform in the karyotype (Fig. 3) although they represented almost entire variation in the 63 *Triticum* isolates tested by Urashima et al. (30). These results suggest that the prg isolates are unstable in chromosomal structure compared with *Triticum* isolates.

Pathogenicity. First, we checked the pathogenicity of the 24 prg isolates on prg and Italian ryegrass in the greenhouse (experiment I). At the high temperature in the greenhouse (25 to 30°C) all but one prg isolates were highly virulent on both *Lolium* species. Inoculated seedlings were shriveled with its color remaining green by 5 to 7 days after inoculation (Fig. 4), resulting in infection type 5. The exceptional isolate was TP4, which produced no symptom on either plant species. This isolate was considered to have lost the basic capacity of infection during the subculture and, therefore, was excluded from further analysis.

Second, host ranges of the prg isolates were determined in a controlled environment room regulated at 20°C (experiment II). At this low temperature, symptoms on the *Lolium* species developed slowly. On Italian ryegrass, several prg isolates induced shriveling (infection type 5) while others did not (infection type 4) (Table 2). Nevertheless, all of the prg isolates formed a lot of typical blast lesions and, therefore, were concluded to be highly

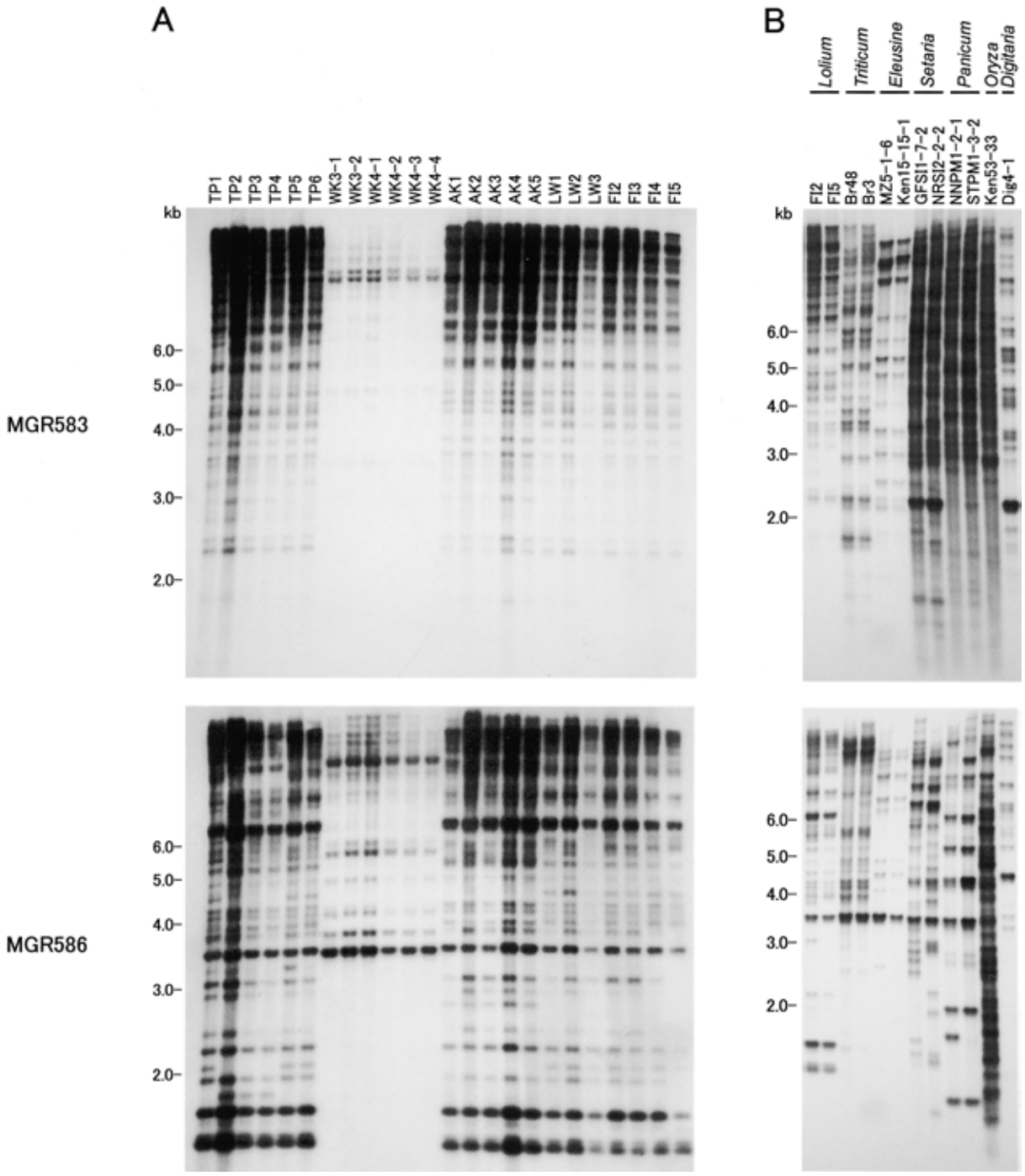

Fig. 2. Southern blot analysis of *Pyricularia* isolates from **A,** perennial ryegrass and **B,** various hosts. Genomic DNA was digested with *Eco*RI, run on a 0.8% gel, and probed with MGR583 and MGR586.

virulent on Italian ryegrass. On prg, most of the prg isolates also produced typical type 4 or type 5 infection, but four WK isolates and one TP isolate produced sporadic lesions of type 4 (4*) or type 3 (3*) (Table 2). Roughly speaking, the TALF isolates were highly virulent on prg, but the WK isolates were less virulent. Interestingly, their pathogenicity on wheat was the reverse; the WK isolates caused some infection up to type 3, while the TALF isolates were completely avirulent (type 0 or 1). On the other test plants, all of the prg isolates were avirulent as a whole, although a few isolates caused type 3 infection on crabgrass (Table 2).

Triticum isolates (Br48 and Br3) were virulent on wheat but avirulent on millets, rice, and crabgrass (Table 2). Interestingly, they produced sporadic lesions of type 4 on Italian ryegrass and prg (Table 2). The other isolates from millets, rice, and crabgrass were exclusively virulent on their original host plants (Table 2).

Mating characteristics. The CC group consists of interfertile isolates (14). Therefore, if the prg isolates are actually members of the CC group, they should be interfertile with other members of the CC group. To check if this is the case, the prg isolates were crossed with highly fertile, hermaphroditic *Triticum* isolates, Br48 (*MAT1-1*) and Br116.2/Br116.5 (*MAT1-2*). All of the prg isolates produced perithecia with mature asci and ascospores along the borderline with Br48 (*MAT1-1*) (Fig. 5; Table 1). This result indicates that the prg isolates are members of the CC group with *MAT1-2* (Table 1). Most of the WK isolates produced perithecia in two rows, indicating that they are hermaphrodites (Table 1). By contrast, the TALF isolates produced them in a single row in the tester (Br48) side of the borderline (Fig. 5), indicating that they are males (Table 1). Furthermore, the WK isolates were all highly fertile (score 3 for perithecium formation), while the TALF isolates varied in the degree of fertility (score 1 to 3) (Table 1).

Species name. Couch and Kohn (2) proposed a new species name, *M. oryzae*, and a PCR-restriction fragment length polymorphism (RFLP)-based method for its identification, i.e., PCR amplification of a region of the beta-tubulin gene followed by restriction digestion with *Hpa*II. When this method was applied to our isolates, all of the prg isolates and the other members of the CC group showed two fragments (188 and 362 bp) that are diagnostic characteristics of *M. oryzae* (Fig. 6; Table 1). On the other hand, amplicons from isolates other than the CC group (NI919, NI981, Dig4-1, and INA-B-92-45) were not cleaved into the two fragments (Fig. 6; Table 1). These results suggest that the CC group corresponds to *M. oryzae* and that the prg isolates should be designated *M. oryzae*.

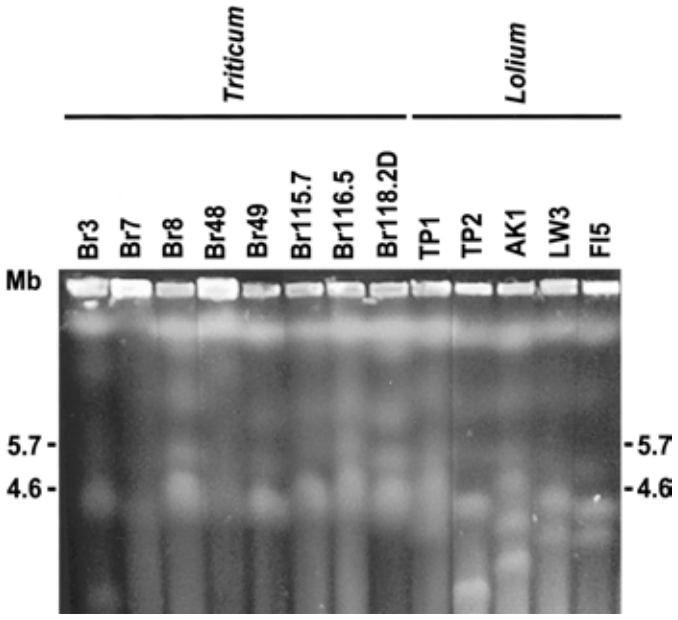

Fig. 3. Pulsed-field gel electrophoresis (PFGE) analysis of *Pyricularia* isolates from perennial ryegrass and wheat. Chromosomal DNA was run on a PFGE gel and stained with ethidium bromide.

DISCUSSION

Species designation of *Pyricularia/Magnaporthe* isolates is a tough job with a lot of debates. Rossman et al. (19) argued that the rice blast fungus, which had been designated as *P. oryzae*, should be referred to as *P. grisea*, which had first been assigned to an isolate from crabgrass. On the other hand, Kato et al. (14) showed that the rice blast fungus belongs to the CC group, which is distinct from isolates from crabgrass. The CC group consists of isolates from *Oryza*, *Setaria*, *Panicum*, *Eleusine*, *Triticum*, and *Eragrostis*, etc. (14), and meets any of the three criteria for species recognition, i.e., biological, morphological, and phylogenetic species recognition (26). First, members of the CC group are interfertile but reproductively isolated from other isolates including *Digitaria* isolates (14) (biological species recognition). Second, they are morphologically indistinguishable from one another but distinguishable from *Digitaria* isolates; macroconidia of *Digitaria* isolates are slightly slender compared with those of the CC group (14) (morphological species recognition). Third, they form a single clade on dendrograms constructed from single copy RFLP data (14) and rDNA data (15) (phylogenetic species recognition). Based on these data, Kato et al. (14) suggested that the CC group should be referred to as *P. oryzae*. Subsequently, Couch and Kohn (2) proposed a new species name, *M. oryzae*, for isolates from *Oryza*, *Setaria*, and *Eleusine*, etc., including two prg isolates collected in the United States. The present study showed that (i) the Japanese prg isolates belong to the CC group (Fig. 1), and (ii) the CC group corresponds to *M. oryzae* (Table 1; Fig. 6). These results suggest that prg isolates should be designated *P. oryzae* in their anamorph and *M. oryzae* in their teleomorph or holomorph.

Fig. 4. Symptoms on perennial ryegrass cvs. Friend (left) and Yatsunami (right) produced by inoculation with a *Pyricularia* isolate from perennial ryegrass (TP5). Inoculated leaves were incubated at 25 to 30°C for 7 days. Note that TP5 causes shriveling of the leaves (the type 5 infection) at this high temperature although it only produces sporadic lesions of type 4 (4*) at 20°C (Table 2).

Farman (4) reported that U.S. prg isolates show very similar fingerprint patterns with all probes tested. However, the Japanese prg isolates tested in the present study were divided into two populations (WK isolates and TALF isolates) based on the fingerprint patterns with MGR586, MGR583, and Pot2 (Fig. 2). These results suggest that more than a single population has caused the epidemics of gray leaf spot.

The two populations also were distinctive in intrinsic pathogenicity. When their pathogenicity was tested at the low temperature condition (20°C), the TALF isolates were virulent only on the *Lolium* species, whereas the WK isolates were relatively less virulent on and less specific to them (Table 2). The WK isolates appeared to be an intermediate between the TALF isolates and the *Triticum* isolates in pathogenicity (Table 2). Why could less virulent and less specific isolates such as the WK isolates cause gray leaf spot on prg in the field? Uddin et al. (28) showed that 28°C is the most favorable to gray leaf spot development. The present study showed that the WK isolates and TP5, which produced only sporadic lesions of type 4 at 20°C, caused shriveling of prg leaves (the type 5 infection) at 25 to 30°C (Fig. 4). This temperature is comparable to that in late summer to early fall when gray leaf spot epidemics have been recognized in the field (7,16,21,24, 25,29). Therefore, the natural condition in the gray leaf spot season is basically conducive for such isolates to cause the disease in the field. Prg is known to be susceptible to a wide range of *Magnaporthe* isolates in artificial inoculation tests (13). Although caution should be exercised when data under artificial condition are extrapolated to what actually might occur in the field, there seems to be a possibility that *Magnaporthe* strains other than the present prg isolates will cause gray leaf spot epidemics in the future. In summary, gray leaf spot of prg can be caused by not only a *Lolium*-specific form but also less specific forms. We suggest that the terms "*Lolium*-specific form" or "*Lolium* pathotype" be restrictively applied to the TALF isolates and their relatives.

It should be noted that the TALF isolates showed diverse karyotypes (Fig. 3) in spite of being uniform in fingerprints (Fig. 2). These results suggest that the TALF isolates are derived from a single origin but are unstable in genome organization. This may be a situation of an early stage of evolution of a new pathotype.

TABLE 2. Pathogenicity of *Pyricularia* isolates from various gramineous plants

	Infection type[a]																																			
	Lolium[b]																								*Triticum*		*Eleusine*		*Setaria*		*Panicum*		*Oryza*		*Digitaria*
Species cultivar/ accession	AK1	AK2	AK3	AK4	AK5	FI2	FI3	FI4	FI5	LW1	LW2	LW3	TP1	TP2	TP3	TP5	TP6	WK3-1	WK3-2	WK4-1	WK4-2	WK4-3	WK4-4	Br48	Br3	MZ5-1-6	Ken15-15-1	GFSI1-7-2	NRSI2-2-2	NNPM1-2-1	STPM1-3-2	Ken53-33	1836-3	Dig4-1
Lolium perenne (perennial ryegrass)																																				
Friend	5	4	5	5	4	4	4	4	5	4	4	4	5	4	5	4*	4	4	4	4*	4*	3*	4*	4*	4*	0	0	0	0	0	0	0	0	0
Yatsunami	5	4	5	5	5	5	4	4	5	4	4	5	5	4	5	4*	4	4	4	4*	4*	4*	4*	4*	4*	0	1	0	0	0	0	0	0	0
Lolium multiflorum (Italian ryegrass)																																				
Mammoth B	5	4	4	5	5	5	5	5	4	5	5	5	5	5	5	5	4	5	4	5	4	4	4	4*	4*	0	0	0	0	0	0	2*	1	0
Ace	5	4	4	5	5	4	5	5	5	4	4	4	5	5	5	5	4	5	4	5	4	4	4	4*	4*	0	1	0	0	0	0	3*	1	0
Triticum aestivum (wheat)																																				
Chinese Spring	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	3	1	2	2	1	4	4	0	0	0	0	0	0	0	0	0
Norin 4	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	2	3	1	3	3	2	5	5	0	0	0	0	0	0	0	0	0
Eleusine coracana (finger millet)																																				
EC19	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0	1	1	0	0	0	1	0	0	1	1	5	4	1	0	0	0	1	1	0
EC28	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	0	1	0	1	1	1	1	1	1	5	4	1	0	1	0	2	1	1
Setaria italica (foxtail millet)																																				
SI12	1	1	2	0	1	1	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	2	0	0
SI17	1	1	2	1	1	1	1	2	1	1	1	2	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	4	4	1	0	2	0	0
Panicum miliaceum (common millet)																																				
PM16	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	0	0	1	1	0	0	4	5	1	1	1
PM18	1	1	0	1	1	0	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	0	1	1	0	0	4	5	1	1	1
Oryza sativa (rice)																																				
Shin 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	4	0
Aichi-asahi	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	4	5	0
Ishikari-shiroke	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	4	1	0
Kanto 51	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	4	5	0
Tsuyuake	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	3	4	0
Fukunishiki	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0
Yashiromochi	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0
Pi No.4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
Toride 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CO-39	1	0	0	1	0	0	1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	3	5	0
Digitaria sanguinalis (crabgrass)																																				
DS136-102	1	1	2	1	2	0	1	1	1	1	1	1	3	2	1	1	0	1	3	1	3	2	0	1	2	0	1	0	0	1	1	0	3*	5

[a] 0, no visible reaction; 1, minute, pinhead-sized spots; 2, small brown lesions with no distinguishable center; 3, intermediate lesions with a gray-green center; 4, large lesions with a gray-green center; 5, complete blighting of leaf blades; and *, sporadic lesions. Interactions between isolates and their original hosts (genera) are boxed. Inoculated seedlings were incubated for 5 to 7 days at 20°C.

[b] Original host.

What is the geographical origin of the prg isolates collected in Japan? They are very similar to U.S. isolates in that they have the same ITS sequence as that of *Triticum* isolates (Fig. 1; Viji et al. [33]) and that they belong to the *MAT1-2* mating type (Table 1; Viji and Uddin [34]). Furthermore, the DNA fingerprints with MGR586 of the TALF isolates (Fig. 2) look very similar to those of the U.S. isolates (Fig. 4 in Viji et al. [33]). A direct comparison on the same gel is needed to elucidate the genetic relationship between the Japanese and U.S. isolates.

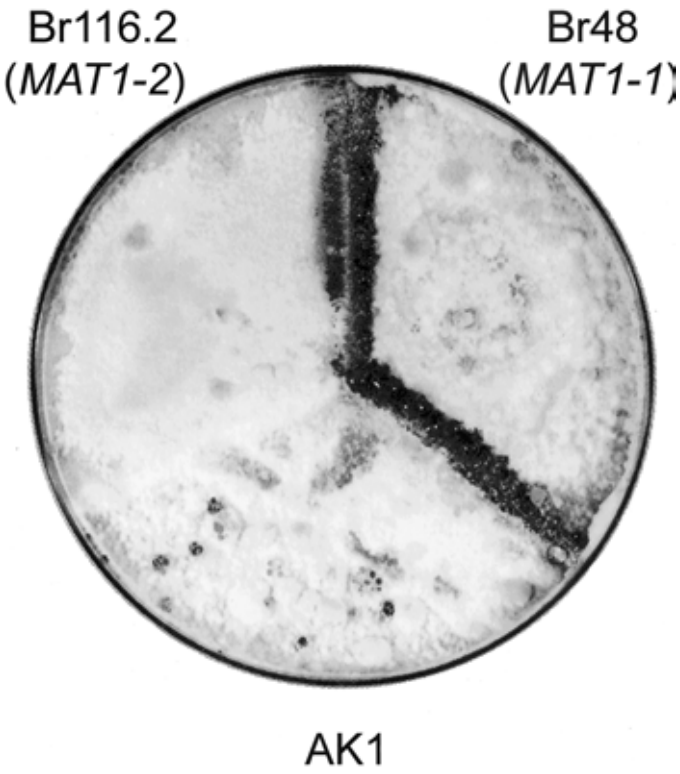

Fig. 5. Mating test of a *Pyricularia* isolate from perennial ryegrass (AK1) on an oatmeal agar medium. AK1 was co-cultured with two testers, Br48 (hermaphrodite with *MAT1-1*) and Br116.2 (hermaphrodite with *MAT1-2*). Note that cleistothecia were produced in double rows between the two testers, but in a single row between AK1 and a tester.

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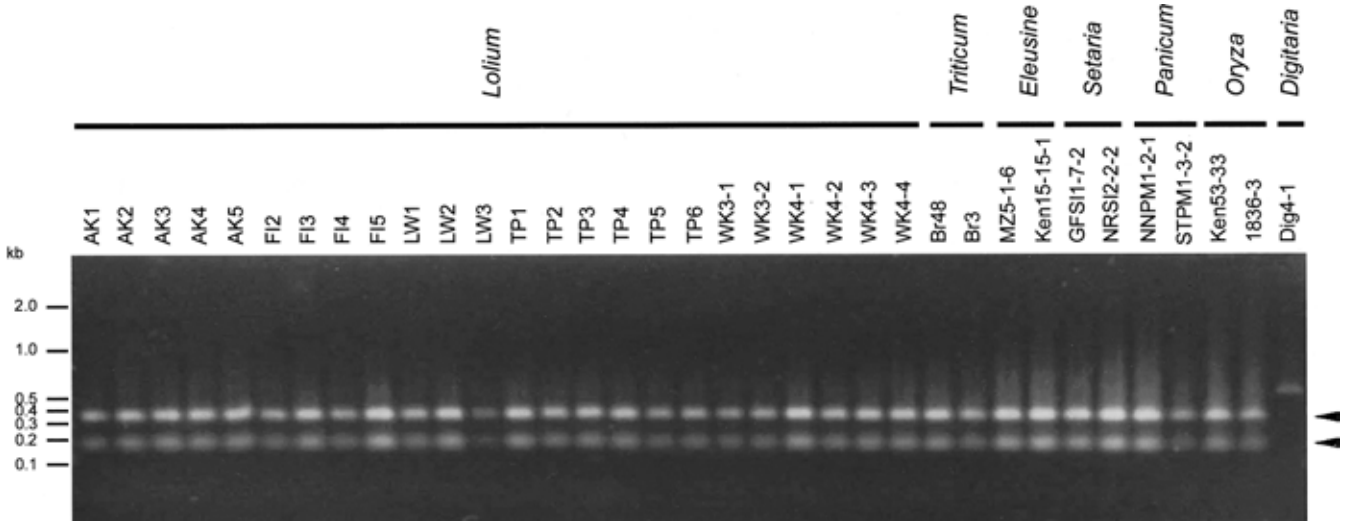

Fig. 6. Polymerase chain reaction-restriction fragment length polymorphism diagnosis of *Pyricularia* isolates from various hosts. A region of the β-tubulin gene was amplified, digested with *Hpa*II, and separated on a 1.5% agarose gel. The arrows represent the two fragments (188 and 362 bp) that are characteristic of *Magnaporthe oryzae*.

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