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Molecule Genetic Characterization and Host Specificity of *Pyricularia* Isolates from Annual Ryegrass in Japan

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Abstract: A total of 89 isolates from blast disease of annual ryegrass (*Lolium multiflorum*) in Japan were characterized by DNA analyses and pathogenicity assay. On the basis of sequence variation in the ITS 2 region, isolates from annual ryegrass could not be distinguished from those from *Triticum* and *Eleusine*. All of the annual ryegrass isolates, however, possessed *Grasshopper*, a retrotransposon specific to *Eleusine* isolates, indicating that the annual ryegrass isolates are closely related to those from *Eleusine* but still distinct from those from *Triticum*. Inoculation test showed that, despite the close relationship between these ryegrass isolates and those from *Eleusine,* all of the annual ryegrass isolates were pathogenic to annual ryegrass but failed to produce susceptibility reactions on finger millet (*Eleusine coracana*). The results suggested that blast disease in annual ryegrass in Japan is caused by blast fungal isolates that are relatives of the *Eleusine* isolates but specialized to annual ryegrass in their pathogenicity. To determine an appropriate scientific name for the annual ryegrass pathogen, we used a diagnostic method for species identification, namely PCR amplification of a region of the beta-tubulin gene followed by restriction digestion with *Hpa*II. The study concluded that the annual ryegrass pathogen should be designated *Pyricularia oryzae* in the anamorph form and *Magnaporthe oryzae* in the teleomorph and holomorph forms.

Key words: Annual ryegrass, blast disease, *Magnaporthe oryzae, Pyricularia oryzae*

INTRODUCTION

Annual ryegrass (*Lolium multiflorum*) and perennial ryegrass (*Lolium perenne*) are the most economically important species in the genus *Lolium*. These *Lolium* species are widely used as cool-season forage crops in temperate regions in such countries as Japan and the southern United States. Blast disease occurs on both economically important *Lolium* species and causes severe damage to the commercial production of these plants[1].

Magnaporthe grisea (Hebert) Barr. [anamorph *Pyricularia grisea* (Cooke) Sacc.][2] is the causal agent of blast disease of many gramineous plants, including *Lolium* species. This fungal species includes several subgroups, each of which has a restricted range of host species[3]. Artificial inoculation tests have shown that *Lolium* species, including annual ryegrass and perennial ryegrass, are susceptible to blast isolates belonging to almost of all of the subgroups[4]. From these findings, it was suspected that blast isolates from other plant species could potentially cause blast disease on *Lolium* species in nature[4]. However, recent studies have provided a new concept of the population structure of blast isolates on perennial ryegrass. Viji *et al.*[5] characterized isolates from perennial ryegrass collected in the United States and showed that these isolates are genetically close to isolates from wheat and triticale. Farman[6] used DNA fingerprint profiles to show that perennial ryegrass isolates are still distinguishable from wheat and triticale and concluded that blast disease in the United States is caused by a population of blast fungus that is specific to perennial ryegrass. Tosa *et al.*[7] characterized Japanese isolates from perennial ryegrass and found that these isolates can be classified into two groups, a *Lolium*-specific isolate group and a less *Lolium* -specific isolate group. They proposed that the former group should be designated as a unique host-specific subgroup, the *Lolium* pathotype. In contrast to the perennial ryegrass pathogen, however, little is known about the annual ryegrass blast fungus.

The first objective of the study was to determine whether annual ryegrass isolates are related to those from other host species by analyzing the nucleotide sequences of the Internal Transcribed Spacer 2 region (ITS 2) of the

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ribosomal RNA gene (rDNA), the distribution of transposable elements, *Grasshopper*[8] and MAGGY[9-11] and the host range. *Grasshopper* and MAGGY are retrotransposons with long terminal repeats found in the genome of *M. grisea*[8-11]. Both elements have been demonstrated to be exclusively distributed in host-specific subgroups of *M. grisea* populations: *Grasshopper* is distributed only in the *Eleusine* subgroup[8,12], whereas MAGGY is specific to the *Oryzae* and *Setaria* subgroups[9,11,12]. The second objective was to determine the most appropriate scientific name for annual ryegrass isolates by using a diagnostic polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)-based method[13]. Recently, Couch and Kohn[13] proposed a new species name, *Magnaporthe oryzae* B. Couch [anamorph *Pyricularia oryzae* Cavara] and a diagnostic PCR-RFLP-based method for the identification of this species.

MATERIALS AND METHODS

Fungal materials: A total of 91 *Pyricularia* isolates, including 89 isolates from annual ryegrass and two isolates from rice, was used in this study (Table 1). Single spore cultures were obtained from infected plant material as follows. Infected leaves were cut into 5 cm long, placed on U-type grass tube in petri dishes with a moistened filter paper. After incubation for 24 h at 28°C, infected leaf dried up and conidia produced on a single lesion was attached onto the surface of 3% water agar in a petri dish. A single spore was picked up from the water agar surface under microscope and transferred to a potato-sucrose agar slant.

PCR and DNA sequencing: The ITS 1 -5.8S- ITS 2 region of the nuclear rDNA was amplified with ITS 4 and ITS 5 primers[14]. Extraction of total DNA from each isolate was performed as described previously[15]. The extracted DNA sample was used as a template DNA for the PCR amplification. The amplification was performed following Tosa *et al*.[7]. The PCR product was electrophoresed in a 0.8% agarose gel, cut from the gel and purified with QIA quick Gel Extraction Kit. The purified DNA was directly sequenced with ITS 4 and ITS 5 primers using Big Dye Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 310 Genetic Analyzer following the manufacturer's instructions. Nucleotide sequences of the ITS 2 region were aligned using CLUSTAL W program[16]. Aligned sequences were analyzed by using PHYLIP V3.6[17]. The evolutionary distance was calculated from the Jukes and Cantor one-parameter method[18]. A phylogenic tree was constructed by using the Neighbor-joining (NJ) method[19].

Hybridization: The total DNA sample described above was digested overnight with *Eco*RI and fractionated on a 0.8% agarose gel in 0.5 × TBE buffer at 20V for 18h. The fractionated DNA was transferred to a MSI nylon membrane (Osmonics, Westborough, MA) and fixed by UV radiation following the manufacturer's instruction.

pGH-H (*Grasshopper*)[12] and pMGY23 (MAGGY)[11] were used for hybridization probes. Each of the plasmid clones was labeled with biotin by using NEBlot Kit (New England Biolabs Inc, Beverly, MA). Hybridization with the biotin-labeled probes and detection of target DNA were performed as described previously[15].

Pathogenicity assay: Four plant species, annual ryegrass (*L. multiflorum*) cv. Tachiwase, crabgrass (*D. sanguinalis*), rice (*O. sativa*) cv. Jikoku and finger millet (*E. coracana*) cv. Yukijirushi-kei, were used for pathogenicity assay in this study. At least four plants of each species were grown in a greenhouse in seedling case (5×15×10 cm) for three weeks. Conidial suspension (2×10^5 mL^{-1}) was prepared as described previously[20] and sprayed on the seedlings with 0.01% Tween 20. Inoculated seedlings were incubated in a dark moistened box for 20 h at 26°C and then returned to the greenhouse. Five day after inoculation, infection was evaluated using five grades from 0 to 4: 0, no visible lesions; 1, brown pinpoint lesions; 2, less than 2 mm brown lesions, sometimes with gray centers; 3, circular lesions with gray centers, lesion size quite variable; 4, necrotic eyespot lesions with gray centers (Table 1). Lesions with infection types 0, 1 and 2 were considered avirulence reactions, whereas lesions with infection types 3 and 4 were considered virulence reactions. Inoculation test was repeated twice.

PCR-RFLP diagnostic for species identification: A region of the beta-tubulin gene was amplified as described by Couch and Kohn[13]. The amplicon was digested with *Hpa*II and separated on a 1.5% agarose gel. After staining with ethidium bromide, the digested fragments were visualized under UV light.

Experimental period: Collection of the infected leaves and the conidial isolation from the collected samples were performed in 1999. All of the molecule analyses described above had been performed from 2000 to 2003. The pathogenicity assay was performed in a period during April to September in 2000.

RESULTS

Phylogenetic analysis with rDNA-ITS 2 sequences: A total of 41 *Pyricularia* isolates from annual ryegrass was

Table 1: *Pyricularia* isolates used in this study

Isolate	Host[a]	Locality	Source[b]	Sampling date	Distribution of transposable elements[c]		Infection type on[d]:			
					G	M	LM	DS	OS	EC
T-9809[e]	LM	Tochigi, Japan	1	Unknown[f]	+	-	4	0	0	0
I-9802	LM	Ibaraki, Japan	1	Unknown	+	+	4	0	0	0(2)
I-9807[e]	LM	Ibaraki, Japan	1	Unknown	+	-	4	0	0	0
Y99I-2[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	++	++	4	0	0	0
Y99I-3[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99I-5[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99I-6	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99I-7	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0(1)
Y99I-8	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99I-9	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99I-11	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99I-12	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99I-14	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99I-15[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99II-2	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	++	4	0	0	0
Y99II-3	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99II-4[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99II-5	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99II-6[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99II-7	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99II-8[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99II-9[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99II-11[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99II-12[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99II-13	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99II-14	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99II-15[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99III-2[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99III-3	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99III-4[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99III-5	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	++	4	0	0	0
Y99III-7[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99III-8	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99III-9[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	++	4	0	0	0
Y99III-10[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	++	4	0	0	0
Y99AII-4-1	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	-	4	0	0	0
Y99A-b-1[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+++	4	0	0	0
Y99A-b-2	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99A-b-3[e]	LM	Yamaguchi, Yamaguchi, Japan	2	September 1999	+	+	4	0	0	0
Y99IV-2[e]	LM	Abu, Yamaguchi, Japan	2	October 1999	+	-	4	0	0	0
Y99IV-4	LM	Abu, Yamaguchi, Japan	2	October 1999	+	-	4	0	0	0
Y99IV-5	LM	Abu, Yamaguchi, Japan	2	October 1999	+	++	4	0	0	0(1)
Y99IV-6[e]	LM	Abu, Yamaguchi, Japan	2	October 1999	+	-	4	0	0	0
Y99IV-10	LM	Abu, Yamaguchi, Japan	2	October 1999	+	-	4	0	0	0
Y99IV-11[e]	LM	Abu, Yamaguchi, Japan	2	October 1999	+	+	4	0	0	0
Y99IV-12	LM	Abu, Yamaguchi, Japan	2	October 1999	+	-	4	0	0	0
Y99IV-13[e]	LM	Abu, Yamaguchi, Japan	2	October 1999	+	-	4	0	0	0
Y99IV-14	LM	Abu, Yamaguchi, Japan	2	October 1999	+	+	4	0	0	0
Y99IV-16	LM	Abu, Yamaguchi, Japan	2	October 1999	+	-	4	0	0	0
Y99IV-18	LM	Abu, Yamaguchi, Japan	2	October 1999	+	++	4	0	0	0
Y99IV-20	LM	Abu, Yamaguchi, Japan	2	October 1999	+	++	4	0	0	0
Y99IV-21[e]	LM	Abu, Yamaguchi, Japan	2	October 1999	+	++	4	0	0	0
Y99IV-22	LM	Abu, Yamaguchi, Japan	2	October 1999	+	-	4	0	0	0
Y99IV-23	LM	Abu, Yamaguchi, Japan	2	October 1999	+	+	4	0	0	0
YA89-1[e]	LM	Yamaguchi, Japan	1	1988	+	+	4	0	0	0
Y-9806[e]	LM	Yamaguchi, Japan	1	Unknown	+	+	4	0	0	0
KU97-1[e]	LM	Kumamoto, Japan	1	Unknown	++	++	4(3)	0	0	0
K-9701[e]	LM	Kumamoto, Japan	1	1996	++	++	4(3)	0	0	0
M99A1[e]	LM	Koyu, Miyazaki, Japan	2	October 1999	+	-	4	0	0	0
M99A4	LM	Koyu, Miyazaki, Japan	2	October 1999	+	++	4	0	0	0
M99A7	LM	Koyu, Miyazaki, Japan	2	October 1999	+	-	4	0	0	0
M99A8[e]	LM	Koyu, Miyazaki, Japan	2	October 1999	+	++	4(3)	0	0	0(2)

Table 1: *Pyricularia* isolates used in this study

Isolate	Host[a]	Locality	Source[b]	Sampling date	Distribution of transposable elements[c]		Infection type on[d]:			
					G	M	LM	DS	OS	EC
M99A9	LM	Koyu, Miyazaki, Japan	2	October 1999	++	++	4	0	0	0
M99A10	LM	Koyu, Miyazaki, Japan	2	October 1999	+	+	4	0	0	0(1)
M99A11	LM	Koyu, Miyazaki, Japan	2	October 1999	+	-	4	0	0	0
M99A13	LM	Koyu, Miyazaki, Japan	2	October 1999	+	-	4	0	0	0
M99A15[e]	LM	Koyu, Miyazaki, Japan	2	October 1999	+	-	4	0	0	0
M99A17	LM	Koyu, Miyazaki, Japan	2	October 1999	+	++	4	0	0	0
M99A19	LM	Koyu, Miyazaki, Japan	2	October 1999	+	++	4	0	0	0
M99A20	LM	Koyu, Miyazaki, Japan	2	October 1999	+	-	4(3)	0	0	0
M99A21	LM	Koyu, Miyazaki, Japan	2	October 1999	+	++	4	0	0	0
M99B1[e]	LM	Koyu, Miyazaki, Japan	2	October 1999	++	+	4	0	0	0
M99B2[e]	LM	Koyu, Miyazaki, Japan	2	October 1999	+	-	4	0	0	0
M99B4	LM	Koyu, Miyazaki, Japan	2	October 1999	+	+	4	0	0	0
M99B5	LM	Koyu, Miyazaki, Japan	2	October 1999	+	-	4	0	0	0
M99B6	LM	Koyu, Miyazaki, Japan	2	October 1999	+	++	4	0	0	0
M99B7[e]	LM	Koyu, Miyazaki, Japan	2	October 1999	++	+	4	0	0	0
M99B8	LM	Koyu, Miyazaki, Japan	2	October 1999	++	++	4	0	0	0
M99B10	LM	Koyu, Miyazaki, Japan	2	October 1999	+	-	4	0(1)	0	0
M99B11	LM	Koyu, Miyazaki, Japan	2	October 1999	+	+	4(3)	0	0	0
M99B12[e]	LM	Koyu, Miyazaki, Japan	2	October 1999	+	+	4	0	0	0
M99B16	LM	Koyu, Miyazaki, Japan	2	October 1999	+	+	4	0	0	0
M99B18	LM	Koyu, Miyazaki, Japan	2	October 1999	+	+	4	0	0	0(2)
M99B20	LM	Koyu, Miyazaki, Japan	2	October 1999	++	++	4	0	0	0(2)
MI92-1[e]	LM	Miyazaki, Japan	1	1991	+	+	4	0	0	0
M-9803[e]	LM	Miyazaki, Japan	1	Unknown	++	++	4	0	0	0
OK92-1[e]	LM	Okinawa, Japan	1	1991	+	-	4	0	0	0
O-9804[e]	LM	Okinawa, Japan	1	Unknown	++	-	4(3)	0	0	0(1)
O-9808[e]	LM	Okinawa, Japan	1	Unknown	+	-	4	0	0	0
Y93-245c-2	OS	Yunnan, China	3	1993	NT	NT	4	0	0	0
84R-62B	OS	Aichi, Japan	4	Unknown	NT	NT	4	0	0	0

[a]LM, *Lolium multiflorum*; OS, *Oryza sativa*.
[b]1,Yamaguchi Agricultural Experiment Station, Yamaguchi, Japan; 2, present study; 3, National Agricultural Research Center, Hokuriku Research Center, Niigata, Japan; 4, National Agricultural Research Center for Kyushu Okinawa Region, Kumamoto, Japan.
[c]G, *Grasshopper*; M, MAGGY. The number of hybridizing bands (signals) is indicated by a four-point scale: -, no signal; +, 1-4 signals; ++, 6-9 signals; +++, more than 9 signals. NT, not tested.
[d]LM, *Lolium multiflorum*; DS, *Digitaria sanguinalis*; OS, *Oryzae sativa*; EC, *Ekusine coracana*. Infection types: 0, no visible lesions; 1, brown pinpoint lesions; 2, less than 2 mm brown lesions, sometimes with gray centers; 3, circular lesions with gray centers, lesion size quite variable; 4, necrotic eyespot lesions with gray centers. Minor infection type is described in a parenthesis.
[e]Isolates are used for analyzing the nucleotide sequences of the internal transcribed spacer 2 region of the ribosomal RNA gene.
[f]Unknown, but before 1999.

selected as representatives and was used for rDNA-ITS 2 sequence analysis (Table 1). All of the isolates analyzed had the same ITS 2 sequence, which was 100% identical to those of isolates from *Triticum* and *Eleusine*. In an NJ tree constructed from the ITS 2 sequences, the 41 isolates from annual ryegrass were clustered into a single group which contains 12 isolates from six plant species, *Oryza, Setaria*, *Panicum, Triticum*, *Eleusine* and *Avena* (Fig. 1). Isolates from other host species, namely *Leersia*, *Digitaria*, *Cenchrus* and *Sasa*, were separated into distinct blanches, respectively and were remote from those six plants and annual ryegrass in the tree.

Distribution of transposable elements: To examine the distribution of *Grasshopper* and MAGGY, total DNA from each isolate was digested with *Eco*RI and hybridized with pGH-H (*Grasshopper*) or pMGY23 (MAGGY). The hybridization profiles of 17 isolates are shown in Fig. 1A(*Grasshopper*) and B (MAGGY) as examples. The numbers of hybridization signals for each element are given in Table 1, using a four-point scale: –, no signal; +, 1-4 signals; ++, 6-9 signals; +++ ; more than 9 signals.

As shown in Fig. 2A and Table 1, *Grasshopper* was detected in all of the 89 annual ryegrass isolates. The number of hybridization signals of *Grasshopper* ranged from + to ++. In contrast to *Grasshopper*, MAGGY was not detected in all of the isolates used (Fig. 2B and Table 1). This element was present in 52 out of the 89 isolates. Among the 52 isolates carrying MAGGY, the number of hybridization signals ranged from + to +++.

Host ranges of *Pyricularia* isolates from annual ryegrass: Host ranges of annual ryegrass isolates were determined using four differential plant species, annual

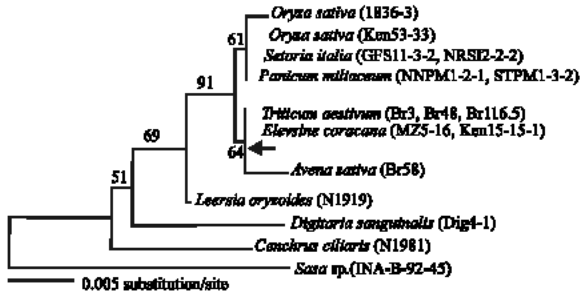

Fig. 1: Neighbor-joining dendrogram of *Pyricularia* isolates constructed from nucleotide sequences of the rDNA-internal transcribed spacer 2 (ITS 2) region. Bootstrap values are indicated on the corresponding node for each cluster. Isolate names (in parentheses) are preceded by scientific names of their original hosts. *Pyricularia* isolates from *Lolium multiflorum* used in the phylogenic analysis are described in Table1. All of these *L. multiflorum* isolates possessed identical ITS 2 sequences and their position in the dendrogram is indicated by the arrow. *Pyricularia* isolates from other hosts are referred to in references 12 and 25. 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.

ryegrass, crabgrass, rice and finger millet (Table 1). All of the 89 isolates produced lesions with infection types 3 or 4 on annual ryegrass but failed to produce susceptibility reactions on the other plant species. Thus, all of the isolates used were pathogenic only to annual ryegrass.

PCR-RFLP analysis for species identification: Couch and Kohn[13] found that *M. grisea* isolates could be distinguished into two phylogenetically distinct groups: one group composed of isolates from rice and other cultivated grass species and another composed of isolates from *Digitaria*. They proposed a new species name, *M. oryzae* for the former group and a diagnostic PCR-RFLP-based method for its identification. This method involved PCR amplification of a region of the beta-tubulin gene, followed by restriction digestion with *Hpa*II[13]. For species identification, we applied this diagnostic method to all of the isolates listed in Table 1, including standard isolates for *M. oryzae* i.e., rice isolates, Y93-245c-2 and 84R-62B. Amplicons from all of annual

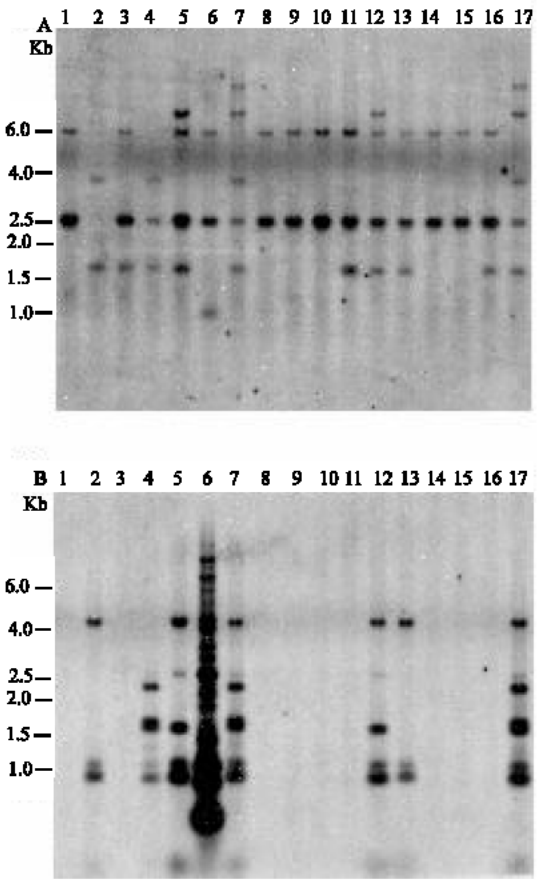

Fig. 2: Hybridization analysis of *Pyricularia* isolates from *Lolium multiflorum*. Genomic DNA was digested with *Eco*RI, separated in a 0.8% Agarose and probed with *Grasshopper* (A) and MAGGY(B). Hybridization patterns of 17 isolates are shown here as examples. Isolates are as follows: lane 1, M99B2; 2, M99B4; 3, M99B5; 4, M99B6; 5, M99B20; 6, Y99A-b-1; 7, Y99I-2; 8, Y99II-4; 9, Y99II-7; 10, Y99II-15; 11, Y99IV-2; 12, Y99IV-18; 13, Y99IV-23; 14, I-9807; 15, T-9809; 16, OK92-1; 17, K-9701. Molecular sizes are shown in Kb.

ryegrass isolates and the two rice isolates were cleaved into two fragments (188 and 362 bp) by *Hpa*II-digestion (Fig. 3). These restriction patterns were identical to those seen in the diagnostic PCR-RFLP phenotype for *M. oryzae*[13].

DISCUSSION

Blast disease of annual ryegrass was first reported in 1972 in Louisiana[21] and Mississippi[22] in the USA.

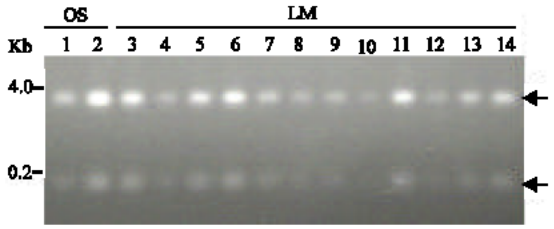

Fig. 3: Polymerase chain reaction-restriction fragment length polymorphism of the beta-tubulin gene. A region of the beta-tubulin gene was amplified, digested with *Hpa*II and separated in a 1.5% agarose gel. The abbreviations above lane numbers indicate the hosts from which isolates were collected: OR, *Oryza sativa*; LM, *Lolium multiflorum*. 12 isolates from *L. multiflorum* are shown here as examples (3 to 14). Isolates are as follows: lane 1, Y93-245c-2; 2, 84R-62B; 3,Y99II-5; 4, Y99II-7; 5, Y99A-b-1; 6, Y99IV-2; 7, Y99IV-4; 8, Y99IV-5; 9, Y99IV-18; 10, Y99IV-23; 11, OK92-1; 12, K-9701; 13, T-9809; 14, I-9807. The arrows indicate the two fragments (188 and 362 bp) that are characteristic of *Magnaporthe oryzae*. Molecular sizes are shown in Kb.

Makino and Hisanaga[23] reported that blast disease occurred on annual ryegrass in 1970 in Shizuoka Prefecture in Japan. Although their description was the first of the natural occurrence of blast disease on annual ryegrass in Japan, this disease has been recognized since 1969 in Japan[23].

It has been emphasized that *Pyricularia* sp. from wild grasses and other hosts could be reservoirs of inoculum for infection of annual ryegrass[1,3,4,24]. Kato and Yamaguchi[4] performed an artificial inoculation test using 30 *Pyricularia* isolates from 23 plant species of the Gramineae and Zingiberaceae. In their report, 28 out of the 30 isolates produced susceptible lesions on annual ryegrass. The exceptions were those from *Zizania latifolia* and *Zingiber mioga*. Furthermore, natural infection of annual ryegrass with rice blast fungus has been reported by several researchers[3,4,24]. On the bases of the ITS 2 sequence variation, isolates from annual ryegrass were indistinguishable from those from *Eleusine* and *Triticum*. The fact that all of our annual ryegrass isolates possessed *Grasshopper*, a retrotransposon specific to *Eleusine* isolates[8,12], makes it evident that the annual ryegrass isolates are more closely related to those from *Eleusine* than to those from *Triticum*. Our inoculation test showed that all of the isolates from annual ryegrass were pathogenic to annual ryegrass but failed to produce susceptibility reactions on finger millet, crabgrass and rice. Surprisingly, despite the close relationship between these ryegrass isolates and those from *Eleusine*, none of the ryegrass isolates was pathogenic to finger millet. These results lead us a hypothesis that blast disease in annual ryegrass in Japan is caused by blast fungal isolates that are relatives of the *Eleusine* isolates but specialized to annual ryegrass in their pathogenicity. We, therefore, consider that the natural infections with rice blast fungus reported previously [3,4,24] should be exceptional cases. Tosa *et al.*[7] found *Lolium* specific isolates in their perennial ryegrass isolates and designated the *Lolium* pathotype as a unique host-specific subgroup for these *Lolium* specific isolates. They performed inoculation testing on eight gramineous species, including two *Lolium* species, perennial and annual ryegrass and showed that the *Lolium* specific isolates were pathogenic only the two *Lolium* species. It is not clear whether our annual ryegrass isolates are classified into the *Lolium* pathotype or not. Further inoculation test by using more plant species, particularly perennial ryegrass, is needed to clarify host-specificity of the annual ryegrass isolates.

Although the annual ryegrass isolates used here could be considered to be relatives of the *Eleusine* isolates, more than half of these isolates also possessed MAGGY, a retrotransposon specific to *Oryza* and *Setaria* isolates. We suggest that the annual ryegrass isolates may have acquired MAGGY by horizontal transfer. A similar phenomenon has also been reported in NI981, a *Pyricularia* isolate from buffelgrass (*Cenchrus ciliaris*) that carries multiple copies of MAGGY[25]. This isolate is phylogenetically distant from other MAGGY carriers (isolates from *Oryza* and *Setaria*) in the *Pyricularia* population, suggesting that MAGGY invaded this isolate through a horizontal transfer event.

M. grisea has been established as the causal agent of ryegrass blast, including in annual and perennial ryegrass[1]. However, *M. oryzae* was recently described by Couch and Kohn[13] as a new scientific name distinct from *M. grisea*, i.e., isolates from *Digitaria*. They proposed the new name for isolates from such genera as *Oryza*, *Setaria* and *Eleusine*, including two isolates from perennial ryegrass. Application of the PCR-RFLP based method proposed by Couch and Kohn to our samples produced the diagnostic phenotype for *M. oryzae* from all of the annual ryegrass isolates. Furthermore, the annual ryegrass isolates were clustered into a single group with isolates from *Oryza* and other cultivated hosts, including *Setaria* and *Eleusine* and were clearly distinct from isolates from *Digitaria*. Tosa *et al.*[7] confirmed the scientific name of 24 isolates from perennial ryegrass on

the basis of evaluation of fertility with *Triticum* isolates, phylogenetic analysis inferred from the ITS 2 sequence and the results of application of the diagnostic PCR-RFLP-based method; they concluded by designating their isolates *M. oryzae* (anamorph *P. oryzae*). Although we have not yet evaluated fertility in our samples, we consider that annual ryegrass isolates should be designated *P. oryzae* in the anamorph form and *M. oryzae* in the teleomorph and holomorph forms.

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