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Genetic Variation Analysis of Mold (*Magnaporthe oryzae* B.Couch) Using Random Amplified Polymorphic DNA

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Abstract

Magnaporthe oryzae B.Couch is a host-specific fungi, certain strain only infect certain host plant species. Genetic variety among *M. oryzae* isolates was explained by dendogram which was constructed using similarity data of Random Amplified Polymorphic DNA (RAPD). Dendogram construction was achieved by computer software, Numerical Taxonomy System (NTSYS). The aim of the research were to study the genetic variation among *M. Oryzae* using RAPD and to construct a dendogram of genetic similarities among the ten isolates from green foxtail (*Setaria viridis* L.), finger millet (*Eleusine coracana* L.) and rice (*Oryza sativa* L.).RAPD was performed in 30 cycles using 5 primers (OPA-02, OPA-03, OPA-04, OPA-05, OPA-07). Polymorphism data was used to constructed dendogram using Dice index and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in NTSYS software. There were 68 polymorphism fragments from 74 amplified fragments. Three clusters were formed in the dendrogram, based on host pathotype: foxtail millet type, finger millet type and rice type. There were two subclusters in foxtail millet type based on mating type, *MAT1-1* dan *MAT1-2*. Thus, RAPD could be used as a method for genetic variation analysis of *Magnaporthe oryzae* to show host-specific specificity.

Key words: *Magnaporthe oryzae*, RAPD, mating type

Introduction

Blast is one of the most devastating rice's diseases because of its vast distribution across the world, expanding more than 85 countries (Kato, 2001). Blast disease is caused by filamentous fungi from Ascomycetes, which bears appresoria to invade the host plant. The fungi first described in 1879 by Cooke and Ellis as *Trichothecium griseum* (Rossman *et al*., 1990). For years, the name *Magnaporthe grisea* (Hebert) Barr had been commonly adopted as the scientific name of *Pyricularia* isolates from many grasses. Two definite species names had been applied to the anamorph of *M. grisea*, i.e., while *Pyricularia grisea* (Cooke) Sacc. was described for crabgrass [*Digitaria sanguinalis* (L.) Scop.], and *P. oryzae* Cavara was described for rice (*Oryza sativa* L.). Rossman *et al.* (1990) confirmed the morphological similarity between type specimens of the two species and argued that *P. oryzae* should be a synonymy of *P. grisea*. However, Kato *et al.* (2000) identify that isolates from crabgrass were genetically differ from those of rice, finger millet [*Eleusine coracana* (L.) Gaertn.], and other crop species. In 2002, B.C. Couch and L.M.Kohn proposed that isolates from the later host should be reffered as new *Magnaporthe oryzae* (*P. oryzae*), based on multilocus gene genealogy concordant (Couch and Kohn, 2002). Currently, *M.oryzae* is accepted widely as a new scientific name distinct from *M. grisea* (*P. grisea*).

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M. oryzae exhibit host-specific characteristic, which means certain isolates of the fungi only infect certain specific host plant (Couch *et al*., 2005). Therefore, each isolate has different genetic variation which could be detected by random amplified polymorphic DNA (RAPD) method. RAPD is a simple method because only one set of short primer is needed without specifically designed beforehand. DNA bands data from RAPD is further processed into binary form as phylogenetic software data input. Finally, the data is visualized as a dendrogram to reveal phenetic relationship among them.

Materials and Methods

Fungal materials

Ten *Magnaporthe oryzae* isolates, including two isolates from green foxtail (*Setaria viridis* L.), six isolates from finger millet (*Eleusine coracana* L.), both from Japan, and two isolates from rice (*Oryza sativa* L.) from Yunan Province, China, were used in this study. The green foxtail and rice isolates were provided by Dr. Motoaki Kusaba, Plant Pathology Laboratory, Saga University, Japan, while finger millet isolates were obtained from culture collection of Kobe University, Japan. All of the isolates had been tested for its mating type based on Yamagashira *et al* (2008).

Table 1. Host, strain dan *mating type* of *Magnaporthe oryzae* isolates

Host	Strain	Mating Type
green foxtail	SA 05-40	*MAT1-2*
	SA 05-63	*MAT1-1*
finger millet	G10-1	*MAT1-1*
	z2-1	*MAT1-2*
	MZ5-1-6	*MAT1-2*
	Ken 15-10-1	*MAT1-2*
	SZEC 1-1-1	*MAT1-1*
	GFEC 1-5-1	*MAT1-1*
rice	Y93-1642-1	*MAT1-1*
	Y93-2452-2	*MAT1-2*

Genomic DNA extraction

Ten days old mycelia from slants culture in potato dextrose agar (PDA) were transferred into potato broth in erlenmeyer flask, and then placed on the shaker for five days. The mycelia were frozen and thawed with liquid nitrogen, resulting mycelia powder. Next, the powder was purified with RNAse, followed by DNA extraction (Yoder, 1988).

RAPD-PCR analysis

RAPD-PCR analysis was performed to all the isolates. All the chemicals were purchased from Nippon Gene. Five DNA primers, 10 nucleotides long (Table 2) were used for the experiment (OPA-02, OPA-03, OPA-04, OPA-05, OPA-07). The concentration of each DNA sample were 10 ng/µl. Amplifications were performed in a 20 µl reaction mixture consisting of genomic DNA (2 µl) and PCR mixture (18 µl). PCR mixture for 12 portions were made by mixing these chemicals in sequence: DDW (147.6 µl), PCR kit buffer 10X (24 µl), dNTP *mixture* (18 µl; 2.5 µmol/µl), primer (12 µl; 10 pmol/µl), DDW (12 µl) dan *gene Taq NT* (2.4 µl; 5U/µl). A single primer was used in each reaction. Amplification was performed in a Progene Thermal Cycler PCR Machine for 30 cycles divided into three programes. The first program (1 cycle) was divided into three segments (i) 94°C for 3 min; (ii) 36°C for 1 min; (iii) 72°C for 2 min. The second program (28 cycles) was divided into three segments : 94°C for 1 min, 36°C for 1 min and 72°C for 2 min. The third program (1 cycle) was also divided into three segments : 94°C for 1 min, 36°C for 1 min and 72°C for 5 min. The amplification products were resolved by electrophoresis in a 0.8% agarose gel (250 ml; containing 12.5 µl (10 mg/ml) ethidium bromide) using TBE buffer 0.5X at 400 Volt for 15 min, then at 20 Volt for 19 h. A SmartLadder (720 ng/5µl) Nippongene was included as a molecular size marker. Gels were visualized and photographed by iluminator UV which was connected with FAS III Toyobo.

Phenetic analysis

Positions of RAPD bands were transformed into a binary character matrix ("1" for the presence and "0" for the absence

of a band at a particular position). Distance matrix was calculated by the NTSYS-pc 2.02 software (Rohlf, 1998) using similarity (SimQual) and DICE coefficient. Finally, dendrogram was construct by SAHN/ UPGMA clustering.

Results

RAPD-PCR analysis

All of five primers showed polymorphism among individual isolates. The amplification reactions with the five primers generated 74 bands, 68 of them being polymorphic (Table 2) with sizes ranging between 400 and 3,000 base pairs (Figure 1, 2 and 3). OPA-02 primer produced the most number of polymorphic, 18 polymorphic bands out of 19 total bands. OPA-03 primer produced the least number of polymorphic bands, 10 polymorphic bands out of 12 total bands. SZEC 1-1-1 (finger millet) produced the most polymorphic amplicons, while Y93-2452-2 (rice) produced the least amplicons.

Phenetic analysis

At 81.01% similarity, four clusters were formed (Figure 4). Genetic similarities were ranging from 67% to 99%. The first cluster was formed by isolates from green foxtail (SA05-40, SA05-63), while the second cluster contains one isolate from finger millet (G10-1). The third cluster was formed by

Table 2. Amplification performance of oligonucleotide on the isolates from *Magnaporthe oryzae*

No.	Primer	Oligonucleotide Sequence	Amplified Fragment	Polymorphism Fragment
1.	OPA-02	5-TGCCGAGCTG-3	19	18
2.	OPA-03	5-AGTCAGCCAC-3	12	10
3.	OPA-04	5-AATCGGGCTG-3	16	15
4.	OPA-05	5-AGGGGTCTTG-3	13	11
5.	OPA-07	5-GAAACGGGTG-3	14	14
		Total	74	68

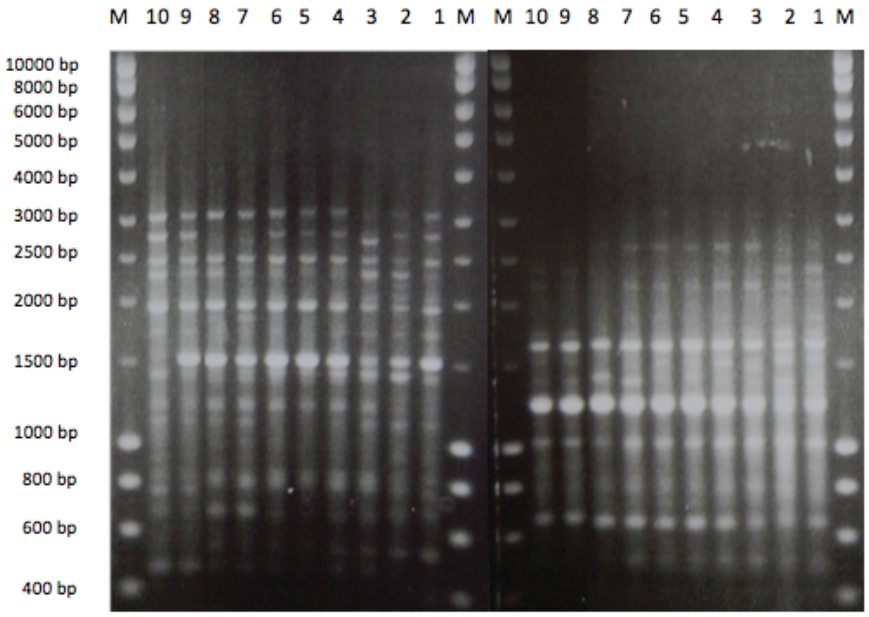

Figure 1. RAPD profile from OPA-02 (left) dan OPA-03 (right); SA05-40 (1), SA 05-63 (2), G10-1 (3), z2-1 (4), MZ5-1-6 (5), Ken 15-10-1 (6), SZEC 1-1-1 (7), GFEC 1-5-1 (8), Y93-1642-1 (9), Y93-2452-2 (10), SmartLadder Marker (M).

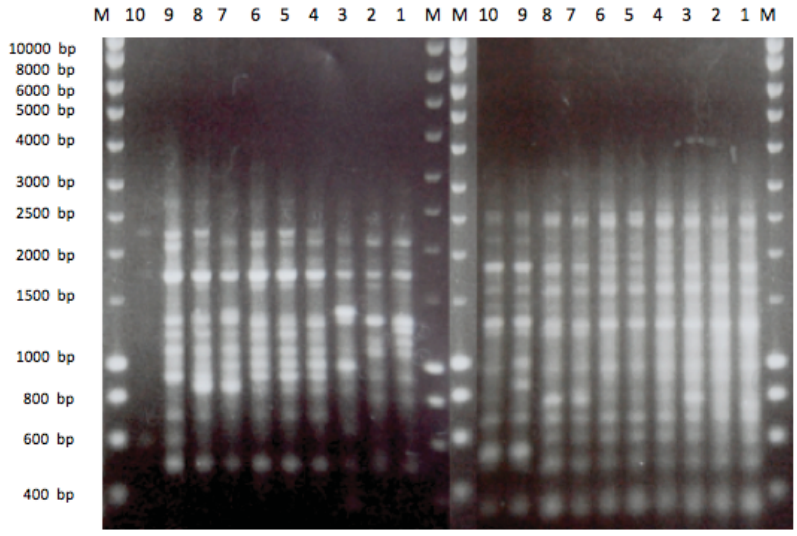

Figure 2. RAPD profile from OPA-04 (left) and OPA-05 (right); SA05-40 (1), SA 05-63 (2), G10-1 (3), z2-1 (4), MZ5-1-6 (5), Ken 15-10-1 (6), SZEC 1-1-1 (7), GFEC 1-5-1 (8), Y93-1642-1 (9), Y93-2452-2 (10), SmartLadder Marker (M).

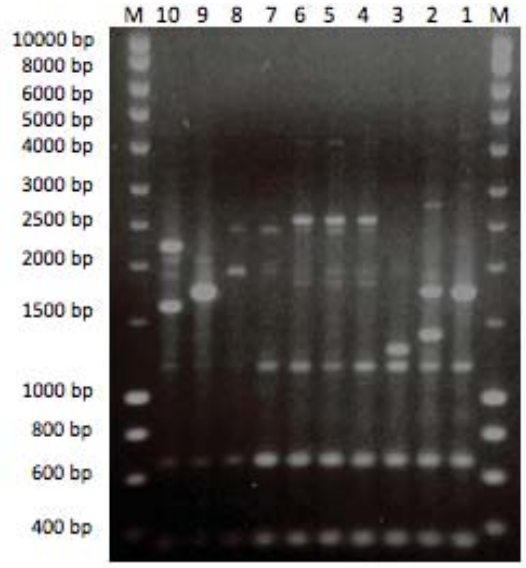

Figure 3. RAPD profile from OPA-07; SA05-40 (1), SA 05-63 (2), G10-1 (3), z2-1 (4), MZ5-1-6 (5), Ken 15-10-1 (6), SZEC 1-1-1 (7), GFEC 1-5-1 (8), Y93-1642-1 (9), Y932452-2 (10), SmartLadder Marker (M).

another five isolates from finger millet (Z2-1, MZ5-1-6, Ken 15-10-1, SZEC 1-1-1, GFEC 1-5-1). The fourth cluster was formed by isolates from rice (Y93-1642-1, Y93-2452-2). Two sub-clusters were formed in the third group, which was the largest cluster. The first sub-cluster was formed by isolates from Z2-1, MZ5-1-6, and Ken15-10-1, then the second sub-cluster was formed by isolates from SZEC1-1-1 and GFEC1-5-1.

Discussion

Magnaporthe oryzae invades limited range of host. It indicates host-specific phenomenon that correspondent to the pathotype. Previously known from Kato *et*

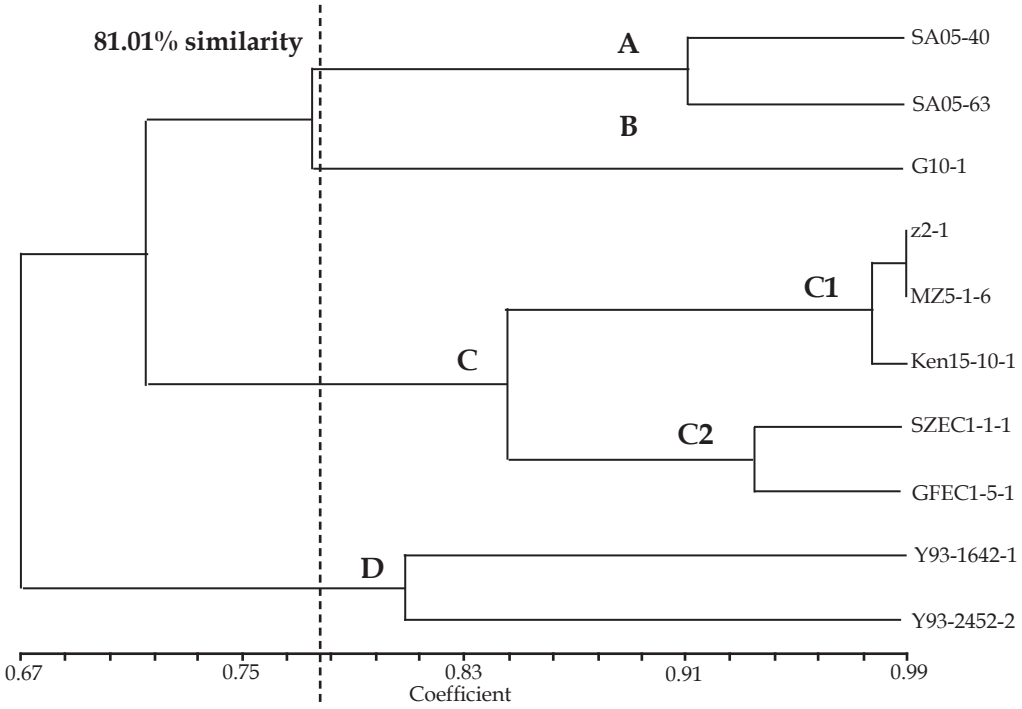

Figure 4. Similarity dendrogram of 10 strains of *Magnaporthe oryzae* from five primers formed by Dice index and UPGMA/ SAHN clustering

al. (2000), there were at least six pathotype differentiated from *M.oryzae*, e.g. rice (*Oryza sativa* L.), foxtail millet [*Setaria italica* (L.) P. Beauv.], common millet (*Panicum miliacum* L.), finger millet, (*Eleusine* sp.), wheat (*Triticum aestivum* L.), and perennial ryegrass (*Lolium perenne* L.). In our study, the dendrogram showed three clusters based on pathotype pattern. They were foxtail millet type (Figure 4, cluster A), finger millet type (Figure 4, cluster C) and rice type (Figure 4, cluster D). Meanwhile, G10-1 isolates (foxtail millet) formed single member cluster apart from finger millet main cluster, surprisingly linked with foxtail millet cluster at 77.85% similarity. This might happen because the isolates bear more resemblances to foxtail millet isolates. The foxtail millet isolates used in our experiment, were special isolates from green foxtail, SA05-40 and SA05-63. These isolates were uncommon fertil isolates while generally green foxtail exhibit low fertility. In addition, *M.oryzae* population from green foxtail alledgedly consist of more than one type of host-specific specificity (Yamagashira *et al*., 2008). It could be presumed that those two isolates at some point might invade the same G10-1 isolate host plant, so these three isolates shared more similarities than to others.

Two sub-clusters were formed in the finger millet cluster. The first sub-cluster consists of isolates from Z2-1, MZ5-1-6, and Ken15-10-1. The second sub-cluster consists of isolates from SZEC1-1-1 and GFEC1-5-1. These arrangement coincidence with mating type difference. All of the first sub-cluster isolates have mating gene *MAT 1-2*. Otherwise, the second sub-cluster isolates have mating gene *MAT 1-1*.

Previous researches in *Magnaporthe oryzae* classification were mainly conducted using RFLP method with MGR-586 probe. The probe accurately determined the presence of certain genes. However, RFLP is expensive and time consuming, then RAPD was used. RAPD is simple yet powerful method to perform. RAPD was able to show relationship among host origins, mutation and genetic varieties of tested isolates (Sere

et al., 2007). Another experiment showed that RAPD allowed clear and simple description of *M.grisea* population in India as described by Chadha and Gopalakrishna (2005). In correlation to those, our experiment showed that RAPD could be used to classify *Magnaporthe oryzae* isolates according to its host, pathotype and mating type. Above all, further study should be conducted to explore RAPD potential as a reliable diagnostic and fingerprinting method using more primers and isolates.

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