DNA Fingerprinting and Electrophoretic Karyotyping of Japanese Isolates of Rice Blast Fungus*

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Abstract

We characterized 24 Japanese isolates of *Magnaporthe grisea* by DNA fingerprinting and electrophoretic karyotyping. These isolates included seven Japanese differential strains as well as field isolates belonging to various pathogenic races which were collected from all over Japan. We used previously cloned repetitive DNAs, MGR586 and pMG6015, as DNA fingerprinting probes. Bootstrapping analysis revealed that pMG6015 is effective for constructing a robust phylogenetic tree. Five clonal lineages were found in the phylogenetic analysis. All field isolates belonged to one particular clonal lineage JBLA-K04. Electrophoretic karyotypes were polymorphic among isolates in the lineage JBLA-K04, but some similarities of karyotype were observed in the isolates of similar pathogenic race. These results indicate that field isolates of *M. grisea* were derived from a limited number of clonal lineages and diverged in their host-cultivar specificity, and that variation at the chromosomal level is correlated to race differentiation in the clonal lineage JBLA-K04.

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Key words: *Magnaporthe grisea*, DNA fingerprinting, electrophoretic karyotyping, Japanese differential strains, clonal lineage.

INTRODUCTION

Rice blast is one of the most damaging diseases of rice (*Oryza sativa*), occurring in all rice-growing areas in the world. Particularly in 1993, it caused extensive losses to rice crops in Japan. The pathogen of the disease is the heterothallic ascomycete *Magnaporthe grisea* (Hebert) Barr (anamorph: *Pyricularia grisea* (Cooke) Sacc.)19). In order to defend the crop from the disease, fungicides or rice cultivars with blast resistance have been developed. However, the frequent appearance of fungicide-resistant isolates and new pathogenic races overcoming blast resistance genes in rice have made those efforts less effective. Although variability of the rice blast fungus has been known for over 40 years, its genetic mechanisms are not understood well40).

In order to develop a novel method to control the disease, we have to elucidate the genetic mechanism of instability of the fungal genome which results in the appearance of new pathogenic races and fungicide-resistant isolates. Since the discovery of the perfect stage of the rice blast fungus39), genetic analyses have been performed to construct genetic maps. However, the fertility of rice-pathogenic field isolates is usually too low to be suitable for genetic analyses by sexual crossing40). Molecular genetic techniques are widely used for analyzing these field isolates. For instance, MGR-DNA fingerprinting6) has resolved genomic variability of field isolates1,3,12,13,29,32) and has contributed to blast resistance breeding40). Electrophoretic karyotyping has revealed chromosomal length polymorphisms in populations of field isolates15,35). Analyses of transposable elements and transposon-like elements have provided valuable information about variation of its genome4,8,9,11,22,23,27)

These studies, however, mainly dealt with isolates outside Japan; Japanese isolates, including Japanese differential strains used in most rice blast research, have not yet been well characterized genetically. Because variability of the fungus depends on the environment of rice cultivation39), and because rice cultivation in Japan is highly intensive and encompasses many blast-resistant cultivars and fungicides, we expected that the population of field isolates in Japan would be an appropriate
target for analyzing the variability and instability of the genome of this fungus.

In this paper, we examine the molecular genetic aspects of pathogenic race variation of *M. grisea* field isolates pathogenic to rice in Japan, including seven Japanese differential strains which have been used in the study of blast-resistance genes. DNA fingerprinting with repetitive DNAs and electrophoretic karyotyping were performed to pursue the purpose.

**MATERIALS AND METHODS**

**Fungal isolates** *M. grisea* rice-pathogenic field isolates which were used in this study are listed in Table 1. Except for Ken 54-04, these isolates were kindly provided by Dr. S. Kiyosawa. Ken 54-04 was a gift from Dr. Y. Fujita. These isolates were stored at −20°C on small pieces of filter paper as described by Valent *et al.*

**Preparation of genomic DNA** Genomic DNA of *M. grisea* was prepared by the procedure described by Raeder and Broda with some modifications. Each isolate was grown in 100 ml of Czapek-Dox liquid medium (OXOID, Hampshire, UK) supplemented with 0.2% yeast extract (Oriental Yeast Co., Ltd., Tokyo) in shake flasks at 27°C and 130 rpm for 3 to 5 days. Mycelia were collected on a filter paper under vacuum, then lyophilized. The lyophilized mycelia (50 mg) were ground to a fine powder with a mortar and pestle, then suspended in 500 μl of extraction buffer (0.2 M Tris-HCl pH 8.5, 0.25 M NaCl, 25 mM EDTA, 0.5% SDS) in a 1.5 ml microcentrifuge tube. Phenol saturated with TE (350 μl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and chloroform-isoamyl alcohol (150 μl; 24:1, v/v) were added to the slurry and mixed thoroughly. After the suspension was centrifuged in a microcentrifuge at 15,000 rpm for 1 hr, the upper aqueous phase was transferred to a microcentrifuge tube containing 25 μl RNase A solution (Sigma, St. Louis, USA; 20 mg/ml in 10 mM Tris-HCl pH 7.5, 15 mM NaCl) and incubated at 37°C for 10 min. The solution was mixed with phenol, centrifuged and the upper aqueous phase was transferred to a new tube. Similar extractions were performed twice with phenol-chloroform and twice with chloroform. Finally, DNA was precipitated with isopropanol.

**Probes for DNA fingerprinting** We used pMG6015 and MGR586 as DNA fingerprinting probes. pMG6015 was cloned by us from rice-pathogenic field isolate Ina 72. MGR586 is one of the MGR fingerprinting probes kindly provided by Dr. B. Valent. These DNA probes were propagated in *Escherichia coli* JM109 and isolated by the alkaline lysis method. For hybridization, inserts were purified from restriction endonuclease-digested plasmid DNA by recovery from agarose gel.

**DNA fingerprinting** Genomic DNA (2 μg) was digested completely with EcoRI (Takara Shuzo Co. Ltd., Otsu) as described by Sambrook *et al.* and electrophoresed in 0.6% agarose gel (21 cm × 12.5 cm, Seakem GTG Agarose, FMC Bioproducts, ME, USA) in 0.5 x TBE (45 mM Tris-borate, 1 mM EDTA) at 25 V for approximately 40 hr. Capillary transfer of DNA to a nylon membrane (Hybond-N*, Amersham, Buckinghamshire, UK) was done overnight. Labeling of probe DNA and hybridization were performed with an ECL direct nucleic acid labeling/detection system (Amersham, Buckinghamshire, UK) according to the manufacturer’s instructions.

**Phylogenetic analysis** For the fingerprint pattern ranging from 1.6 to 19.0 kbp, a binary data matrix was made, using “1” for the presence of a particular band and “0” for its absence with image analysis software (Advanced Quantifier 1-D match, Biolimage Co., MI, USA). This data matrix was then used for clustering analysis of the isolates. A similarity data matrix was produced with the software WINDIST, using Dice’s coefficient $F = 2N_{xy}/(N_x + N_y)$, where $N_{xy} = the number of the bands shared by a given pair of isolates and $(N_x + N_y) = the total number of bands observed for the pair of isolates$. A phylogram was constructed by the “unweighted pair group using arithmetic mean” (UPGMA) with the SAHN programs of the NTSYS-pc package.

Bootstrap analysis was conducted with the program...
Preparation of chromosome-sized DNA samples

Chromosomal DNA samples for pulsed-field gel electrophoresis were prepared by the method described by Talbot et al. with some modifications. Each isolate was grown in 10 ml of 2YEG (2 g/l yeast extract, 10 g/l glucose) in a test tube (20 mm × 200 mm) shaking at 27°C and 326 rpm for 3 to 4 days. Mycelia were harvested by centrifugation in a 15 ml-polypropylene conical tube (Falcon 2096, Becton Dickinson and Co., NJ, USA) at 800 × g for 5 min. The mycelia were then suspended in 5 ml of enzyme solution (2 g/l Novozym 234 (Novo Nordisk, Bagsvaerd, Denmark) in 0.7 M NaCl), followed by incubation for 3 hr at room temperature with gentle shaking. After the digestion of cell walls, mycelial debris was removed by filtration through a JK wiper (Jujo Kimberly Co., Tokyo). Protoplasts were pelleted by centrifugation at 1700 × g for 10 min at room temperature, then resuspended into 100 µl of isotonic buffer (1.2 M sorbitol, 20 mM EDTA). After calculating the concentration of protoplasts with a hemacytometer, the suspension was diluted with molten low-melting-point agarose solution poured into a sample mold and solidified at 4°C for 30 min. Sample blocks were incubated in ESP (1% lauroyl sarcosine, 1 g/l proteinase K (Wako Pure Chemical Industries, Ltd., Osaka) in 0.5 M EDTA (pH 9.2)) at 50°C for 2 days with gentle shaking and then stored at 4°C.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed with a contour-clamped homogeneous electric field (CHEF)-type apparatus (CHEF-DRII, Bio-Rad Laboratories, CA, USA) under conditions described by Budde et al., except for the agarose concentration of gels. We used 0.7% agarose (FastLane, FMC Bioproducts, ME, USA) molten in 0.5 × modified TBE (25 mM Tris, 22.5 mM boric acid, 50 mM EDTA). Electrophoresis was performed at 14°C in 0.5 × modified TBE, 35 V for 5 days with a 90 min-pulse, followed by 2 days on a 60 min-pulse. After electrophoresis, gels were stained with ethidium bromide (1 µg/ml) for 30 min, destained in distilled water for 30 min to 1 day, then photographed. Schizosaccharomyces pombe chromosomal DNAs (FMC Bioproducts, ME, USA) were used as size markers.

RESULTS

Experimental design

We used two DNA fingerprinting probes, pMG6015 and MGR586. A restriction map of pMG6015 is shown in Fig. 1. It has a SINEs-like element MGSR1 in the M. grisea genomic insert. MGR586 is widely used in DNA fingerprinting of M. grisea. We used these two probes to obtain more detailed data and to efficiently analyze Japanese isolates using pMG6015, derived from a Japanese strain Ina 72. The homology between these two probes was investigated with cross-hybridization analysis (data not shown). Because pMG6015 weakly hybridized to MGR586, we considered that this homologous sequence was not important in DNA fingerprinting; fingerprint patterns provided with these probes are easily distinguishable. pMG6015 revealed approximately 30 bands ranging from 20 kb to 1 kb. MGR586 showed approximate 40 bands ranging from 20 kb to 0.5 kb.

As shown in Table 1, we used 24 Japanese isolates, including 17 field isolates. Except Ina72 and Ina168, these isolates were classified into six groups based on their pathogenicity. Each group consisted of isolates with exactly identical or almost identical cultivar specificity which differs in reaction with only one or two cultivars in 12 differential lines of rice.

To investigate the applicability of the procedure, preliminary analyses were made with a limited number of isolates. We analyzed seven Japanese differential strains and two field isolates (Fuku 5-2 and Nakayama 3-3). Through these preliminary analyses, we could make a phylogenetic tree and investigate electrophoretic karyotypes. Then we analyzed 22 isolates except Ina 72 and Ina 168 in order to investigate the relationship between pathogenic race and clonal lineage or electrophoretic karyotype on the basis of pathogenic race groups. Data of these two analyses were analyzed together as described below.

DNA fingerprinting

In the preliminary analysis with both probes, similarity of the fingerprint was detected in three groups of isolates: Ina 72 and Ina 168, Ken 53-33 and Ken 54-20, and Ken 54-04 and two field isolates (Fig. 2(a)). From these fingerprints, we made a UPGMA phylogenetic tree and found some isolate clusters (data not shown).

We then analyzed the pathogenic race groups of the isolates by DNA fingerprinting using both probes. Fingerprints of those groups with pMG6015 are shown in Fig. 2(b). Using data from both preliminary and pathogenic race group analyses, phylogenetic trees were produced.
Fig. 2. DNA fingerprinting of Japanese isolates. (a) preliminary analysis with limited number of isolates using MGR586 as a probe. 1, Ina 72; 2, Ina 168; 3, Ken 53-33; 4, Ken 54-20; 5, Hoku 1; 6, P-2b; 7, Ken 54-04; 8, Fuku 5-2; 9, Nakayama 3-3. (b) pMG6015-fingerprint of isolates grouped on the basis of pathogenicity. Numbers above lanes represent pathogenicity groups. 1, Kitaibaraki 4; 2, Nakayama 1-9; 3, Hitachiohta II-9; 4, Nakayama 3-3; 5, Shimone 7; 6, Hanawa 2; 7, Ken 54-20; 8, Ken 54-04; 9, Hanawa 4; 10, Dai 74-03; 11, Ena 3; 12, Hoku 1; 13, Nakayama 3; 14, Nakayama 10; 15, Ken 53-33; 16, Kitaibaraki 9; 17, Shimomurogashima 3; 18, P-2b; 19, Ena 9; 20, Kamisato 13; 21, Fuku 5-2; 22, Ao 14-20.

Figure 3 shows the UPGMA phylograms based on four DNA fingerprints (two preliminary and two pathogenic race group analyses) obtained with the two probes. In general, the pMG6015 tree (Fig. 3-(a)) showed greater similarity than MGR586 tree (Fig. 3-(b)). In these two phylogenetic trees, two clusters similarly appeared; one group of Ina 72 and Ina 168 and the other of field isolates. In addition, a large cluster including these two groups and isolate Ken 54-04 was found in both fingerprints.

On the other hand, there were some differences between the two trees. Isolates Ken 53-33, Ken 54-20, Hoku 1 and P-2b clustered differently in the two trees. In the phylogenetic tree of pMG6015, these four isolates were clustered into three groups, one of Ken 53-33 and Ken 54-20, Hoku 1 and P-2b at similarity level 0.8, whereas in the tree of MGR586 these isolates clustered differently into two groups, Ken 53-33 and P-2b, and Ken 54-20 and Hoku 1. Ken 54-04 also distinctly clustered in the two phylogenetic trees. In the pMG6015 tree Ken 54-04 did not belong to any cluster at 0.8 similarity, but in MGR586 tree it clustered with field isolates at 0.75 similarity. Although all field isolates were clustered in one large group in both trees, they sub-clustered differently between the two trees, except for the cluster of Ena 3, Ena 9 and Kamisato 13.

Bootstrapping analysis was useful to anticipate the robustness of each cluster. Clustering of Ken 53-33, Ken 54-20, Hoku 1 and P-2b in the pMG6015 tree exhibited much higher bootstrapping probability than that in MGR586 tree. Clustering of Ken 54-04 in the MGR586 tree also exhibited higher probability than that in the pMG6015 tree. Regarding field isolates, bootstrapping probabilities were higher in the pMG6015 tree than the MGR586 tree in general.

In addition to the two phylograms made with each probe, a double-probe phylogram was constructed by combining the data from the two probes [Fig. 3-(c)]. In this tree, the clusters having the higher bootstrapping probability were conserved. Since the phylogenetic tree which has the most information is expected to be more reliable, the double-probe tree was the most suitable for the clonal lineage assignment.

At 0.68 similarity, we assigned the clonal lineages JBLA, JBLB and JBLC (for Japanese Blast Lineage A, B, and C) to the two robust clusters and P-2b. However, subclusters with the high 0.74 similarity level remained, for which we detected a similar pattern in the prelimi-
Fig. 3. UPGMA dendrograms of Japanese isolates based upon (a) pMG6015, (b) MGR586 and (c) both DNA fingerprint(s). Values at cluster branches indicate the results of the bootstrap analysis. A criterion for the clonal lineage assignment (a point of 0.65 similarity) is indicated with a broken line (-----). Clonal lineages are indicated on the right side of the isolate name. Clusters A, B and a subcluster which contains isolates of race 001 are indicated.
nary analysis. These subclusters were not negligible because the clonal lineage assignment described above was made at lower similarity level than generally done. When we assigned clonal lineages to these subclusters in addition to the three clusters at 0.68 similarity, we could detail a phylogenetic relationship of isolates. Thus we proposed five clonal lineages of M. grisea field isolates in Japan: JBLA-INA (Ina 72 & Ina 168), JBLB-K33 (Ken 53-33 and Ken 54-20), JBLC-P2B (P-2b), JBLB-HK1 (Hoku 1) and JBLA-K04 (Ken 54-04 and all field isolates).

We then investigated the relationship between clonal lineage and pathogenic groups. All isolates of group 001 and 337 belonged to lineage JBLA-K04. The rest of the groups corresponded to both the lineage of a differential isolate and the lineage JBLA-K04. In other words, lineage JBLA-K04 included all field isolates and one reference isolate Ken54-04. These aspects indicated the predominance of lineage JBLA-K04 in the rice field and the variability of host specificity within the clonal lineage. Further, field isolates were clustered into two groups (clusters A and B, Fig. 3-(c)), consisting of various pathogenic races. This result denies a direct relationship between clonal lineage and host specificity and reveals the variability of host specificity within a clonal lineage.

However, a subcluster in cluster A which consisted of isolates of race 001.0 [Fig. 3-(c)], does indicate a relationship between fingerprint pattern and host specificity.

**Electrophoretic karyotyping**

Talbot et al.25) reported that karyotype variation in M. grisea was often observed in a population, even within a clonal lineage. We investigated karyotype polymorphism among isolates by CHEF-electrophoresis. Figure 4-(a) shows the electrophoretic karyotype of isolates used in a preliminary analysis and indicates that each isolate had a distinct karyotype. The chromosome number of each isolate was estimated to be six or seven, but the frequent appearance of co-migrating bands limited the accuracy of the analysis. The karyotypes of nine isolates were quite different from each other; no two isolates had an identical karyotype. Karyotype polymorphism was detected even among isolates belonging to the same clonal lineage. A "mini chromosome"15), a chromosome less than 2 megabase pairs (Mb), was detected in all isolates. These results agreed with the observations by Valent et al.27) and Talbot et al.25).

In our variability analysis of the electrophoretic karyotypes of the field isolates of lineage JBLA-K04 [Fig. 4-(b)], the karyotypes were highly polymorphic in all

![Fig. 4. Electrophoretic karyotyping of Japanese isolates. (a) preliminary analysis with limited number of isolates. 1, Ina72; 2, Ina 168; 3, Ken 53-33; 4, Ken 54-20; 5, Hoku 1; 6, Ken 54-04; 7, Fuku 5-2; 8, Nakayama 3-3; 9, P-2b. (b) Electrophoretic karyotyping of field isolates of lineage JBLA-K04. Pathogenicity groups are indicated. M, size marker S. pombe (FMC Bioproducts, ME, USA); 1, Kitaibaraki 4; 2, Nakayama 1-9; 3, Hitachihoita II-9; 4, Nakayama 3-3; 5, Shimone 7; 6, Hanawa 2; 7, Ken 54-04; 8, Hanawa 4; 9, Dai 74-03; 10, Ene 3; 11, Nakayama 3; 12, Nakayama 10; 13, Kitaibaraki 9; 14, Shimomurogashima 3; 15, Kamisato 13; 16, Ene 9; 17, Fuku 5-2; 18, Ao 14-20.](image)
pathogenicity groups, as well as in the Japanese differential strains. Interestingly, however, some similarities existed among isolates in the same group. For instance, two isolates in group 007 lacked a 4.7 Mb band. The two isolates of group 303 did not have any minichromosome band. Isolates in group 337 lacked a band of 3.5 Mb. Each of these pairs were closely related in phylogenetic analysis (Fig. 3), suggesting a relationship between karyotype, DNA-fingerprint and pathogenic race.

**DISCUSSION**

In DNA fingerprinting of *M. grisea*, MGR586 is widely used. Although we also used MGR586 in this study, its sole usage was not sufficient for accurate analysis of all the isolates used here. In our results, we could construct the most robust phylogenetic tree by combining DNA fingerprints by pMG6015 and MGR586, probably due to the merit of complementation of two distinct probes. The most remarkable difference between the two probes was the similarity level of the fingerprints among Japanese isolates. In pMG6015 fingerprinting, the similarity level of the fingerprint patterns was too high to determine genetic differences at an individual isolate level. Contrarily, the similarity level with MGR586 was too low for general analysis of isolates collected over a long period. This difference may indicate that the repetitive sequence in each probe was dispersed individually in the genome of *M. grisea*. The higher similarity of pMG6015 fingerprints would indicate that its amplification was initiated earlier than MGR586.

This study, the first proposal of clonal lineages of the Japanese blast fungus, has enabled us to analyze genetic relationships in a collection of isolates, including reference and field isolates. The Japanese differential strains, first used in the 1960's, were selected from culture collections for stability, distinctness of pathogenicity and host cultivar specificity. Our results revealed that seven Japanese differential strains were derived from five distinct clonal lineages. In these Japanese differential strains, distinctness of pathogenicity and genetic source might relate to each other.

We also found that our field isolates may have originated from a limited number of clonal lineages and diverged in their host specificity. Pathogenic race variation in *M. grisea* in Japan, therefore, may be mainly due to mutations in genes specifying host specificity, because sexual recombinations seldom occur in rice pathogenic isolates. Parasexual recombination between two isolates of the same clonal lineage is another candidate for the cause of diversification of host specificity, but it is almost impossible to discriminate it from mutation in a natural population.

It will be interesting to analyze the relationships between pathogenic race diversity in the lineage JBLAK04, the predominance of the lineage and the breakdown of blast resistance. From the results of this report, pathogenic race diversity and the simplification of clonal lineage occurred in between the 1960's and the 1980's, because the field isolates were collected mainly in the 1980's (Table 1). Before the 1960's, when Japanese differential strains were collected from rice fields, there would be at least five clonal lineages with distinct pathogenic races. During that period, breakdown of some resistant cultivars also occurred in rice fields in Japan. Analysis of a collection of isolates from that period will enable a more detailed understanding of the relationship.

Comparing clonal lineages in this report with those reported elsewhere is difficult; the collection of isolates, the most important factor in phylogenetic analysis, is specific to each report. In an analysis similar to ours, Levy et al. found clonal lineages in a USA fungal collection spanning a 30-year period, as well as a relationship between clonal lineage and host specificity. Their report suggested that host specificity of *M. grisea* did not diverge frequently in the United States, which is in contrast to our results. In addition, Levy et al. discussed in a recent report that variability of *M. grisea* in a field population depends on the cultivation environment. The agricultural environment in Japan would also be responsible for frequent variation in host specificity of the Japanese blast fungus. Japanese isolates would be appropriate materials to analyze variability of host specificity.

Karyotypes of Japanese isolates were highly polymorphic. However, some similarities related to pathogenic race were observed within isolates belonging to the same clonal lineage. These results indicate that variation at the chromosomal level (chromosomal rearrangements) would be an important process in genetic diversity in this fungus. Talbot et al. also reported karyotype diversity in a clonal lineage, mentioning that chromosomal rearrangement will be a main system in neutral genetic variation. Recently, Sone et al. found a chromosomal length mutation in isolate Ina168. The mutation was neutral as far as pathogenic race, but occurred at a high rate (12.5%). Thus, chromosomal rearrangement may be a major factor responsible for genetic instability in *M. grisea* and electrophoretic karyotype may be a key feature in the analyses of pathogenic race variability in a clonal lineage.

Although derived from a limited number of isolates, the data reported here uncovered aspects of pathogenic race variability in field isolates of the Japanese rice blast fungus. Japanese isolates of this fungus were also revealed to be interesting materials to analyze the genomic variability and instability. As shown by the recent proposal of Ziegler et al. for a novel blast control system using the knowledge of clonal lineage of the fungus, further analysis of clonal lineage, chromosomal rearrangement and pathogenic race variation will help disclose the genetic basis of pathogenic race variation and establish a new, effective control system for the disease.
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Literature cited


和文摘要

長根輝雄・阿部卓己・吉田奈美・須藤 学・富田房男：日本産いもち病菌菌株のDNAフィンガープリントと電気泳動解析