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Study on Parasexual Recombination between *Pyricularia oryzae* and *Pyricularia grisea*

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Abstract

Rice blast fungus is notorious for changeability in pathogenicity, but it lacks sexual life cycle. It can be postulated that such an imperfect fungus has another mechanism for generating genetic variations. Recent studies concerning comparative genomics reveal that parasexual recombination may play important role in the evolution of rice blast fungus. To observe the parasexualism of rice blast (*Pyricularia oryzae*) and crabgrass blast (*Pyricularia grisea*) fungus double inoculation and punch method were applied in this experiment. A total of 520 isolates collected from the double inoculated lesions was subjected to PCR-RFLP analysis of the ITS region to identify subcultures of the inoculated rice blast isolates. As a result, four isolates from the three double inoculated lesions with SA13-1ME and TP106 were identified as subcultures of TP106. To access the recombination genotypes, a total of 17 isolates from the three lesions was subjected to MAGGY-DNA fingerprint analysis. However, recombinant DNA fingerprint patterns between TP106 and SA13-1ME were not detected among the 17 isolates. Although TP022 was not recovered from the double inoculated lesions, the fact that TP106 was recovered from the double inoculated lesion indicates that rice blast fungus can invade and colonized in blast lesion on crabgrass. The opportunistic infection on the double inoculated lesions observed in this study potentially provides new insight into the life cycle of rice blast pathogen.

Keywords

Rice Blast, Parasexual, Double Inoculation, Punch

1. Introduction

Magnaporthe oryzae (*teleomorph*) (*Herbert*) *Barr* (anamorph: *Pyricularia oryzae*) [1] is one of the most important plant pathogenic fungi having an excep-

tional capacity of rapidly changing its genetic make-up resulting in new pathogenic variants (races) [2]. It is the causal agent of rice blast, one of most devastating diseases of rice (*Oryzae sativa* L.) observed in most of the rice growing-countries across the world [3]. Although the perfect stage of the fungus was observed in cross experiments in which highly fertile laboratory strains were used as tester isolates [4]. However, crosses between field isolates of this pathogen usually result in infertility [5]. Because of the infertility of the field isolates, this pathogen is considered to be limited to asexual reproduction in nature. Therefore, sexual recombination, if any, contributes little to the genetic variations in the pathogen.

It was pointed out in several previous findings that the genetic variations in rice blast fungus were induced by parasexual recombination following hyphal anastomosis among different blast isolates [6] [7] [8]. Again, reported that mutations in MGR586-DNA fingerprint profiles were frequently observed in isolates from tufted zones in two separate vegetative parings involving isolates with dissimilar MGR586-DNA fingerprint patterns [8]. These mutated fingerprint patterns were characterized by addition of 1 to 10 MGR586 bands from one parent into the background the other. *P. oryzae* and its relatives also cause blast disease on more than 50 Gramineous plant species [9]. Blast fungi from the Gramineous plants could be differentiated into several genetically distinct host-specific groups [10]. However, in case of wheat blast disease while leaf infection, varietal characteristics may play important roles epidemiologically [11]. Now, these groups are designated as pathotypes of *P. oryzae* or distinct species from *P. oryzae*.

It has already been mentioned that *P. oryzae* exhibits a high degree of genetic variability. However, the central question that how this fungus so rapidly changes its genetic makeup is still unanswered.

There are several hypotheses in relation to the sources of genetic variation in *P. oryzae*. Generation of genetic variability in any organism generally involves sexual genetic recombination. However, sexual reproduction of *P. oryzae,* which facilitates meiotic recombination, is only observed under experimental conditions [12]. Therefore, sexual recombination, if any, contributes little to the genetic variations in the rice blast. Another possible source of genetic variability in imperfect fungi is parasexual recombination followed by vegetative hyphal fusion [13], which allows horizontal gene or chromosome transfer among different isolates. Such possibility in *P. oryzae* was first suggested by [14] who observed nuclear behavior in anastomosis and obtained variants by pairing two different autotrophic strains rice blast pathogen. Experimentally demonstrated that auxotropihic recombinants could be produced by pairing of *P. oryzae* different auxotrophic parental isolates [15]. Paired different isolates and recovered recombinants [16]. There are several strains of *M. oryzae* which tend to display a degree of host specificity and they have been divided into pathotypes based on their host preference [17]. Presumed that pathogenicity changes in rice blast fungus

were caused by parasexual recombination [18]. Reported heterokaryosis and parasexual cycle of *P. oryzae* [19]. In Japanese rice blast fungal population, parasexual recombination is possibly the only mechanism of genetic recombination between isolates as ascospore (sexual spore of *P. oryzae*) production from any cross between Japanese field isolates has not been reported to date.

This study investigated whether parasexual recombination occurs between the blast fungi sourced from different host plants. If so, then the hypothesis of parasexual recombination in rice blast fungus would get a significant momentum. To do so, crosses were made between rice blast fungus (*P. oryzae*) and crabgrass (*Digitaria ciliaris*) blast fungus (*P. grisea*), both of which are well-distributed in rice production areas in the world.

2. Materials and Methods

2.1. Experimental Site, Period and Design

The study was undertaken in green house and plant pathology laboratory of Saga University, Japan from 2012 to 2014. Experiments were conducted under controlled conditions following completely randomized design.

2.2. Fungal Isolates

Two rice blast (*P. oryzae*) isolates TP106 and TP022, and one crabgrass blast (*P. grisea*) isolate SA13-1ME were used in this study. The isolates were collected from Akita and Kumamoto prefecture in Japan.

2.3. Growing Host Plants

The crab grass accession SACB-1 (Saga Crab Grass, Available in Saga University, Japan Plant Pathology field) was used as the host plant where inoculation was conducted (**Table 2**). Seeds treated with 0.2% Techlead C were sowed in a plastic pot (17 cm × 22 cm) containing sterile soil. Young 8 - 10-days-old seedlings were transplanted in small plastic packets (8 cm × 9 cm) and placed in a greenhouse operating at 28˚C. To ensure growth, the seedlings were regularly watered and monitored for insect infestation. No additional fertilizer was applied during the course of this study.

2.4. Inocula Preparation

The blast isolates SA13-1ME, TP106 and TP022 were cultured on oatmeal agar medium (1 liter distilled water, 50 g grinding oat powder, 20 g sugar, 20 g agar) for 17 days at 26˚C. The surface of the oat meal agar media in Petridish was gently rubbed with a sterile water soaked paint brush to remove aerial mycelia. To induce sporulation, the cultures were then exposed to fluorescent light for 3 days in a chamber covered with perforated polythene sheet and operating at 26˚C. For punch inoculation, conidia of the test-isolates were picked up from sporulated oat meal agar culture by gently rubbing with a sterile cotton plug and

then transferred on to the surface of water agar medium (1% water agar medium). Presence of conidia on water agar was confirmed by microscopic observation. For foliar spray inoculation, conidia from surface of sporulated oat meal culture were scraped gently with sterile paint brush and suspended in sterile water containing 0.01% Tween 20. The suspension was filtered through 2 layer of Kimwiper® S-200 filter paper (Nippon Seishi Crecia Co., Ltd) and conidial concentration was adjusted to 1×10^5 conidia per milliliter.

2.5. Punch Inoculation

Leaves of the 21-day-old crabgrass seedlings were gently punched by a sterile needle (1 mm diameter) to make a circular scraped area. Single agar plug (1 mm diameter) containing conidia (approximately 20 conidia/plug) were placed on each of the scraped areas on leaves. The inoculated seedlings were incubated at 26˚C for 18 - 20 hrs in a moist plastic chamber covered with thick black plastic sheet, and then subsequently transferred to a greenhouse. Plants inoculated with blank agar plug served as control treatment.

2.6. Spray Inoculation

The spore suspension was sprayed on 21 days old seedlings (3 - 4 leaf stage) of the plants tested. The inoculated plants were incubated at 26˚C for 18 - 20 hrs in a moist plastic chamber above 80% relative humidity covered with thick black plastic sheet and then transferred to a green house operating at 28˚C. Disease reactions on the seedlings were assessed at 7 days after inoculation. Plants sprayed with sterile water served as control treatment.

2.7. Double Inoculation

The crabgrass blast (*P. grisea*) isolate SA13-1ME were sprayed on 21-day-old seedlings (3 - 4 leaf stage) of crabgrass (Accession SACB-1) by using Conidial suspension (10^5 conidia/ml) as the first inoculation. The inoculated seedlings were placed in a moist plastic chamber above 80% relative humidity for 18 - 20 hours covering with thick black plastic sheet at 26˚C temperature and subsequently transferred to a greenhouse. Blast lesions were appeared on crabgrass leaves 7 days after first inoculation. Distinct isolated blast lesions with gray center were selected, and the agar plug of Rice blast (*P. oryzae*) isolates TP106 and TP022 were placed on the selected lesion that were developed by first inoculation. Incubation and growing of the inoculated seedlings were conducted following the same techniques described above. The double inoculated lesions were collected at 7 days after second inoculation and kept in envelop for drying until they were used for single conidial isolation.

2.8. Single Conidia Isolation and Culture

The double inoculated leaves were incubated on water soaked paper (Comfort-200, Crecia Co., Japan.) using small triangle glass stick in a Petridish and

kept at 26˚C temperature for less than 14 hours to induce sporulation. Conidia on the double inoculated lesion were transferred to the surface of water agar, and single conidia was picked up under microscope and transferred to PSA medium for mycelial growth. From each double inoculated lesion, maximum 10 conidia were collected and transferred to PSA plate.

2.9. PCR-RFLP Analysis of ITS Region

Protocol for isolation of fungal genomic DNA was followed to extract the template DNA for PCR reaction [20]. Mycelial biomass grown on PSA plate were scraped with sterilized tooth pick and kept in 1.5 ml centrifuge tube. The mycelial mass in the centrifuge tube was heated (around 100˚C - 110˚C temperature) in a microwave oven at for 4 minutes, and then suspended in 50 µl of TE buffer by vigorous overtaxing. The mycelial suspension was centrifuged for 5 minutes at 12,000 rpm. 5 µl of supernatant of the mycelial suspension was used as DNA solution for the PCR reaction. ITS region was amplified with primers ITS5and ITS4 [21]. Amplifications for the ITS regions were performed in a total PCR reaction volume 25 µl including 5 µl of template DNA. Reactions were heated to 94˚C for 5 min and then amplified for 35 cycles of 30 sec at 94˚C, 30 sec at 55˚C and 30 sec at 72˚C, with a final extension period at 72˚C for 5 min. The amplification reactions were performed in a TGradient Thermocycler (Biometra, Germany). ITS sequences of *P. oryzae* and *P. grisea* were obtained from DNA Data Bank of Japan and compared to find the sequence difference available for creating a diagnostic genetic marker (**Figure 3**). The amplicons was digested with *Dra*I (Wako Nippon Gene) according to the manufacturer's instructions and separated on a 1.5% Agarose gel containing ethidium bromide (1%) in 0.5X TBE buffer and visualized under UV light.

2.10. Hybridization

The total DNA of each isolate was digested with *Bam*HI, and fractionated on 0.8% Agarose gel in 0.5X TBE buffer. The fractionated DNA was transferred to a MSI nylon membrane (Osmonics, Westborough) and fixed by UV irradiation following the manufacturer's instruction. pSB-4 containing *Sal* I-*Bam*HI fragment (SB) from a MAGGY clone pMGY23 was used a hybridization probe. The probe was labeled with biotin by using Neblot Phototope kit (New England Biolabs). Hybridization with the biotin-labeled probe and detection of target DNA were performed [22].

3. Results

3.1. Punch Inoculation

The crabgrass blast (*P. grisea*) isolate SA13-1ME produced large (3 - 5 mm in diameter) blast lesions with typical gray center when inoculated in crabgrass following spray and punch method (**Figure 1**). Isolate TP106 and TP022 did not produced blast symptom when both of these isolates were sprayed, but at punch

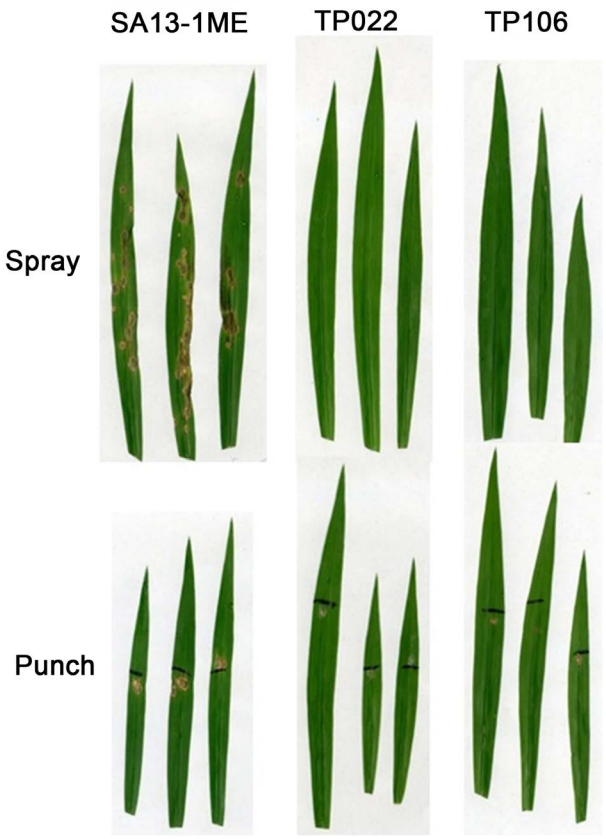

Figure 1. Pathogenicity reactions of *Pyricularia* isolates on crabgrass leaves produced by two inoculation methods.

method produced blast like symptom on crabgrass leaves (Figure 1). Following microscopic observation, sporulation was evident in 19 out of the 25 punched areas of crabgrass leaves when inoculated with SA13-1ME (Table 1). The average number of conidia of *P. grisea* found in the punched areas was about 50 (Table 1). In contrast, the rice blast (*P. oryzae*) isolates TP106 and TP022 did not develop any typical blast lesion on crabgrass leaves. However, they produced conidia on 2-3 out of the 14 punched areas, which was statistically insignificant compared to the control. The average numbers of conidia were 0.86 and 0.43 respectably for TP106 and TP022 isolates (Table 1). Therefore, it is evident from this study that the inoculation with two rice isolates induced incompatible reaction on crabgrass. These findings also suggest the capacity of rice blast fungus to cause opportunistic infection in crabgrass leaf following wound inoculation.

3.2. Double Inoculation

Double inoculation technique means produced blast disease symptom combination of two isolates (SA13-1ME/TP106 or TP022). In this method first produced disease symptom by spraying SA13-1ME isolate on SACB-1 crab grass host plant (Figure 2(a)). After developed blast symptom by spraying then an agar plug with conidia of *P. oryzae* isolate (TP106) was put on each lesion as the second

Table 1. Punch inoculation of crab grass leaves with *Pyricularia* isolates.

8	Number of spores observed on inoculated positions[a]
TP106	0 - 8 (0.86, 2, 14)
TP022	0 - 3 (0.43, 3, 14)
SA13-1ME	0 - 468 (50.16, 19, 25)

a Minimum to maximum numbers of spores. Mean numbers of spores, number of lesion with sporulation and total number of inoculated positions are in parenthesis.

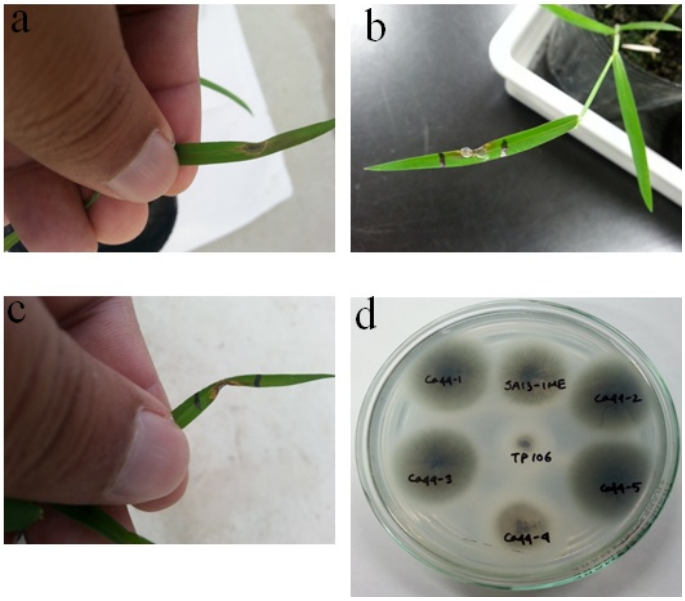

Figure 2. Procedure for double inoculation experiment. (a) Conidial suspension of *Pyricularia grisea* isolate (SA13-1ME) was sprayed on crabgrass (*Digitaria ciliaris*) as the first inoculation. Blast lesions appeared on leaves of crabgrass seven days after the first inoculation; (b) An agar plug with conidia of *P. oryzae* isolate (TP106) was put on each lesion as the second inoculation seven days after the first inoculation; (c) At most ten conidia were isolated from the lesion 14 days after the second inoculation; (d) Examples of mycelial colors of isolates from the double inoculated lesion.

inoculation, seven days after the first inoculation (**Figure 2(b)**). Then combination of spots produced comparatively bigger lesion (**Figure 2(c)**) than previously produced symptom with approach (**Figure 2(a)**) at most ten conidia were isolated from that lesion (**Figure 2(c)**). A total of 520 monoconidial isolates were collected from double inoculated lesions (**Appendix**). Of those, 319 isolates were obtained from 48 lesions double inoculated with SA13-1ME (*P. grisea*) and TP106 (*P. oryzae*) (**Table 2**). Of these, four isolates had colonies with the same white color as that of colony of TP106 (**Figure 2(d)**). On the other hands, the other 315 isolates had colonies with black in SA13-1ME and TP106 combination. Whether, 201 monoconidial isolates were derived from 28 lesions inoculated with SA13-1ME (*P. oryzae*) and TP022 (*P. oryzae*) (**Table 2**). All these 201

Table 2. Summary of *Pyricularia* isolates from double inoculated lesions on crabgrass.

Combination	Number of isolates		Number of lesions tested[c]
	P. oryzae[a]	P. grisea[b]	
SA13-1ME/TP106	4	315	48 (3)
SA13-1ME/TP 022	0	201	28 (0)

a Number of isolates having *P. oryzae* ITS type; b Number of isolates having *P. griseae* ITS type; c Number of lesions where isolates having *P. oryzae* ITS type is in parenthesises.

isolates sourced from SA13-1ME and TP022 combination colonies were black in colour providing that the colony of TP022 was whitish in colour.

3.3. PCR-RFLP Analysis

*Dra*I site in ITS2 region was found to be unique to *P. oryzae.* The ITS region was successfully amplified from all of the crude DNA samples (**Figure 3**). 319 isolates derived from the 48 lesions double inoculated with SA13-1ME (*P. grisea*) and TP106 (*P. oryzae*) and four isolates Ca32-1, Ca32-7, Ca42-4 and Ca44-4 had the PCR product that could be digested with *Dra*I (**Figure 4**). Those four isolates were sourced from three lesions (designated as Ca32, Ca42 and Ca44) double inoculated with SA13-1ME and TP106 (Ca32-1 and Ca32-7 were collected from the same lesion). Following digestion with restriction enzyme, these four isolates produced the same banding pattern with the parental isolate TP106 (*P. oryza*) (**Figure 4**). Such findings indicate that these four isolates were derived from TP106. On the contrary, of the 201 isolates sourced from the 28 lesions double inoculated with *P. grisea* isolates SA13-1ME and TP022, none produced banding pattern similar to TP022 (**Table 2**). All of this 201 isolates had PCR product that could not be digested with *Dra*I. It indicates that the isolates originated from the combination of SA13-1ME (*P. grisea*) and TP022 (*P. oryza*) were not derived from TP022 parental isolates.

3.4. Hybridization

To access the recombination genotypes, a total of 17 isolates from three lesions double inoculated with SA13-1ME and TP106 were subjected to MAGGY-DNA fingerprint analysis (**Figure 5**). The hybridization analysis using pSB-4 confirmed that MAGGY was not distributed to the genome of *Pyricularia grisea* (SA13-1ME). Except for the four isolates Ca32-1, Ca32-7, Ca42-4 and Ca44-4, the other 13 isolates could be considered to be derived from SA13-1ME based on the PCR-RFLP pattern of ITS region and their colony colors.

Therefore, MAGGY can be used for detecting recombinants in the 13 isolates since this element is transmitted only from TP106 to these isolates. However, the DNA fingerprint analysis revealed that no hybridization signals was detected from the 13 isolates, suggesting that these 13 isolates did not have recombinant genotype. An extensive study including much more single spore isolates from

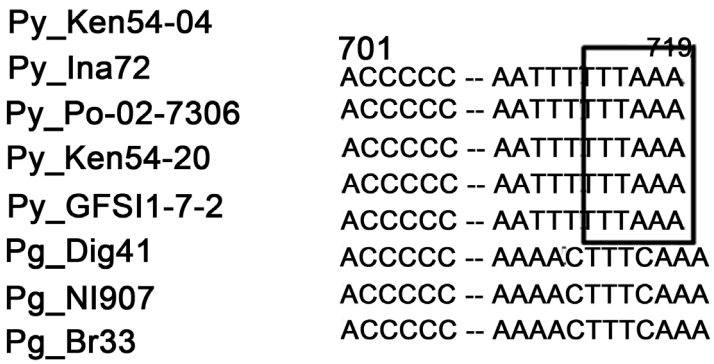

Figure 3. Alignment of ITS regions of *Pyricularia oryzae* (Py) and *P. grisea* (Pg) Restriction site of *Dra*I is shown in a square.

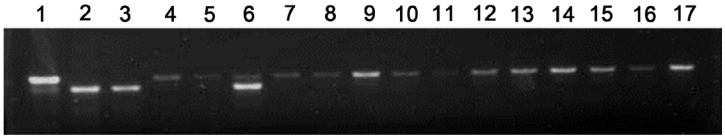

Figure 4. ITS-RFLP profiles of *Pyricularia* isolates from double inoculated leaves of crabgrass with *Pgrisea* and *P. oryzae*. ITS regions of isolates tested were amplified with PCR primers ITS5 and ITS4. Amplicons were digested with *Dra*I and fractionated in 1.5% agarose gel. Lanes: 1, SA13-1ME; 2, TP106; 3, Ca42-4; 4, Ca44-2; 5, Ca44-3; 6, Ca44-4; 7, Ca44-5; 8, Ca45-1; 9, Ca45-2; 10, Ca45-3; 11, Ca45-4; 12, Ca45-5; 13, Ca45-6; 14, Ca46-1; 15, Ca46-2; 16, Ca46-3; 17, Ca46-4.

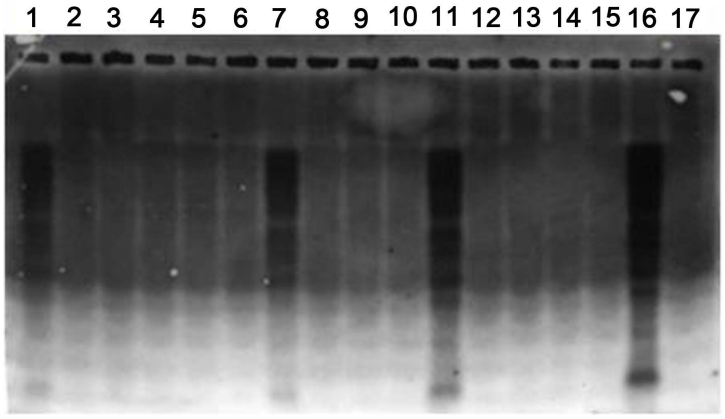

Figure 5. Southern blot analysis of MAGGY in *Pyricularia* isolates from double inoculated leaves of crabgrass with *P. grisea* and *P. oryzae*. Genomic DNA was digested with *Bam*HI, and hybridized with pSB-4. *Pyricularia* isolates were collected from lesions #32 (lanes 1 to 7), #42 (lanes 8 to 12), #44 (lanes 13 to 17), respectively. Lanes: 1, Ca32-1; 2, Ca32-2; 3,Ca32-3; 4, Ca32-4; 5, Ca32-5; 6, Ca32-6; 7, Ca32-7; 8, Ca42-1; 9, Ca42-2; 10, Ca42-3; 11, Ca42-4; 12, Ca42-5; 13, Ca44-1; 14, Ca44-2; 15, Ca44-3; 16, Ca44-4; 17, Ca44-5.

double inoculated lesions should be needed to find the parasexual recombination event between rice blast and crabgrass blast fungi.

4. Discussion

Genetic variability of plant pathogens is a critical problem for the control of plant diseases since it often results in emerging mutants which defeat resistance in plant species. Rice blast fungus has been known to be a changeable plant pathogen. The rapid emergence of new pathogenic variants enables this pathogen to overcome resistant ability of rice cultivars within 2 - 3 years after commercial cultivation. Blast resistance in rice generally follows a gene-for-gene relationship [23] [24] [25] and, as such, is dependent on the presence of a cognate *Avr* gene in rice blast fungus. Therefore, the rapid evolution of new races is attributed to rapid loss of function of *Avr* genes that correspond to the resistance genes in a gene-for-gene manner. To date, at least seven *Avr* genes have been cloned from rice blast fungus [26] [27] [28] [29] [30]. Studies of the cloned *Avr* genes have revealed that loss-of-function mutations of these genes involve point mutations, insertions of transposable elements, or deletions of entire gens. However, another aspect of changeability of this pathogen is an opposite phenomenon that rice blast populations often regain expelled *Avr* genes after cultivars containing corresponding resistance genes are removed from the field [31]. To date, reverse mutations have not been observed in the loss-of-function of *Avr* genes, and thus contribution of such rare mutations to the recovery of the expelled *Avr* genes should be very limited, if any. Sexual recombination seems to be the most appropriate to explain the mechanism for the recovery. Moreover, segregation of *Avr* genes through sexual recombination results in the loss of *Avr* genes, as well as, the gain of them in progenies. However, rice blast fungus lacks sexual lifecycle in nature, and thus sexual recombination should be excluded from the mechanisms to regain and deleted *Avr* genes. In this study, another hypothesis was that parasexual recombination play an important role in genetic variability of this pathogen. Parasexual recombination between rice blast isolates was demonstrated in several previous studies. However, it can be considered that the recovery of the expelled *Avr* genes is difficult to be accomplished by parasexual recombination only between rice blast isolates since a donor of the expelled *Avr* genes should be almost completely exterminated in the rice blast population after introducing the corresponding resistance genes. Therefore, crabgrass blast fungus was selected as a partner in parasexual recombination with rice blast isolates in this study.

One of the significances of parasexuality in asexual filamentous fungus is to produce genetic diversity [8]. Parasexuality occurred in laboratory cultures of the rice blast fungus [32]. The lineage of isolates from the Indian Himalayans and Philippines occurrence of parasexual recombination in nature indirectly, but parasexuality under field condition has never been observed [33]. The potential hyphal fusion was considered to be the reason for possible parasexual recombination. Hyphal anastomosis between isolates from the same locality was reported in the rice blast fungus [34]. Genetic exchange of DNA through parasexual recombination and segregation of pathogenicity and mating type of the pa-

rasexual recombinants might correspond to that of the progeny of the off spring of the sexual cross [6]. Rice blast fungus is non-infective to crabgrass. Therefore, it can be expected that recombination occurs in nature only when the rice blast pathogen opportunistically infect a blast lesion on crabgrass in which the crabgrass pathogen preexist. To test the ability of the rice blast pathogen for the opportunistic infection, the rice isolates were inoculated on punched areas in this study. The fact that sporulation was observed on the punched areas indicates that rice blast isolates had the ability of opportunistic infection on wounded area of crabgrass leaf. The number of conidia observed on the punched areas was low. The punching method performed in this study slightly scratched the surface of crabgrass leaf, and thus plant cells in the punched areas could keep a living condition for a long time after inoculation.

In this study, rice blast isolates TP106 and TP022 were inoculated on lesions on crabgrass produced by the crabgrass blast isolate SA13-1ME to induce parasexual recombination. Following inoculation, TP106 was recovered from 3 out of the 48 double inoculated lesions and TP022 was not recovered from the 28 double inoculated lesions (**Table 2**). In the double inoculation, the rice blast isolates was inoculated on blast lesions with collapsing center consisting of dead plant cells. It is reasonable to expect that rice blast fungus can easily access to such a dead tissue. Contrary to expectations, the rice blast isolates were not frequently recovered from the double inoculated lesions. It is unclear why the recovering rates of rice blast isolates used were not high. In double inoculation position rice blast (*P. oryzae*) isolates were allowed to stay 7 days to create conidial hyphal fusion with crab grass blast (*P. grisea*) conidia. Conidial anastomosis tube fusion between two incompatible strains of *Colletotrichum lindemuthianum* during colony initiation can survive and produce uninucleate conidia and form different phenotypic colonies comparable with parental strains [35]. At most 10 isolates were collected from each double inoculated lesion in this study. The number of sampling isolates might be too little to precisely estimate the recovering rate. Among 520 offspring of both parental combination only 4 were with white mycelial colour. The isolate TP106 could coexist with SA13-1ME in the three lesions. Pathogenic variants by the pairing inoculation of different pathogenic isolates on both agar media and rice leaves, indicating that the variants were produced by the parasexual cycle [36]. However, recombinant between the crabgrass blast isolate and the rice blast isolate was not found among the single spore isolates from the three lesions.

A total of 17 isolates was collected from the three double inoculated lesion in which opportunistic infection of TP106 was observed and was subjected to MAGGY-DNA fingerprint analysis. DNA fingerprint patterns generated by genetic recombination between TP106 and SA13-1ME in the 17 isolates could not be detected. The number of isolate collected from these lesions was possibly too little to detect any recombinant genotypes. Parasexual recombination between rice blast isolates frequently occurred on an artificial medium [8]. Furthermore,

their population genetic study suggested that parasexual recombination between rice blast isolates also could frequently occur in field populations of this pathogen. It is reasonable to expect that the parasexual recombination in field populations in their study took place in rice blast lesion with multiple infections of different rice blast isolates. It may be possible to get recombinant genotypes if higher number of single spore isolates from double inoculated lesions could be analyzed. To improve efficiency of parasexual recombination in the double inoculated lesion, it is also necessary to further modify the inoculation method. In *Colletotricum* species, hyphal fusion only occurs between germination tubes from conidia [37]. The conidial anastomosis of *Pyricularia* species would be worth testing. Frequency of parasexual recombination is a critical issue, but its exact estimate has not been reported even in laboratory experiments. Methods for efficient production of parasexual recombinants and their selection need to be developed to examine the rate of parasexual recombination in the laboratory. Characteristics concerning the stability and fitness of parasexual recombinants are important for their survival under field condition. An extensive study including much more single spore isolates from double inoculated lesions is required to find the parasexual recombination event between rice blast fungus and crabgrass blast fungus.

5. Conclusion

Rice blast pathogen shows extreme degree of polymorphic character in natural condition. In this study we are trying to find out the variant progenies of *Pyricularia* sp by punch and double inoculation method considering PCR-RFLP techniques. Finally MAGGY-DNA fingerprint analysis protocol proven that recombinant progenies were absent in studied isolates, but opportunistic infection in double inoculated lesion open a new sight of polymorphic *Pyricularia* sp pathogen.

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References

[1] Couch, B.C. and Kohn, L.M. (2002) A Multilocus Gene Geneology Concordant with Host Preference Indicates Segregation of a New Species, *Magnaporthe oryzae*, from *Magnaporthe grisea*. *Mycologia*, **94**, 683-693. https://doi.org/10.1080/15572536.2003.11833196

[2] Dean, R., Van Kan, J.A.L., Pretorius, Z.A., Kosack, K.E., Pietro, A., Spanu, P.D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J. and Foster, G.D. (2012) The Top 10

Fungal Pathogens in Molecular Plant Pathology. *Molecular Plant Pathology*, **13**, 414-430. https://doi.org/10.1111/j.1364-3703.2011.00783.x

[3] Kihoro, J., Njoroge, J.B., Murage, H., Ateka, E. and Makihara, D. (2013) Investigating the Impact of Rice Blast Disease on the Livelihood of the Local Farmers in Greater Mwea Region of Kenya. *SpringerPlus*, **2**, 308. https://doi.org/10.1186/2193-1801-2-308

[4] Eung, H. and Williams, P.H. (1985) Genetic Analysis of Electrophoresis Enzyme Variants, Mating Type and Hermaphroditism in *Pyricularia grisea* Cavara. *Canadian Journal of Genetics and Cytology*, **27**, 697-704.

[5] Notteghem, J.L. and Silve, D. (1992) Distribution of Mating Type Alleles in *Magnaporthe grisea* Populations Pathogenicon Rice. *Phytopathology*, **82**, 421-424. https://doi.org/10.1094/Phyto-82-421

[6] Noguchi, M.T., Yasuda, N. and Fujita, Y. (2006) Evidence of Genetic Exchange by Parasexual Recombination and Genetic Analysis of Pathogenecity and Mating Type of Parasexual Recombinants in Rice Blast Fungus *Magnaporthe oryzae*. *Phytopathology*, **96**, 746-750. https://doi.org/10.1094/PHYTO-96-0746

[7] Xia, J.Q. and Correll, J.C. (1995) Examination of Mitotic Stability and Hybridization Potential between Two Genetically Distinct Haplotypes of *Magnaporthe grisea*. *Experimental Mycology*, **19**, 171-177.

[8] Zeigler, R.S., Scott, R.P., Leung, H., Bordeos, A.A., kumar, J. and Nelson, R.J. (1997) Evidence of Parasexual Exchange of DNA in the Rice Blast Fungus Challenges Its Exclusive Clonality. *Phytopathology*, **87**, 284-294. https://doi.org/10.1094/PHYTO.1997.87.3.284

[9] Ou, S.H. (1987) Rice Disease Survey. The Commonwealth Mycological Institute, 109-201.

[10] Kato, H., Yamamoto, M., Yamaguchi-Ozaki, T., Kadouchi, H., Iwamoto, Y., Nakayashiki, H., Tosa, Y., Mayama, S. and Mori, N. (2000) Pathogenecity, Mating Ability and DNA Restriction Fragment Length Polimorphisms of *Pyricularia* Populations Isolated from Gramineae, Bambusideae and Zingiberaceae Plants. *Journal of General Plant Pathology*, **66**, 30-47. https://doi.org/10.1007/PL00012919

[11] Cruz, C.D., Kiyuna, J., Bockus, W.W., Todd, T.C., Stack, J.P. and Valent, B. (2015) *Magnaporthe oryzae* Conidia on Basal Wheat Leaves as a Potential Source of Wheat Blast Inoculum. *Plant Pathology*, **64**, 1491-1498. https://doi.org/10.1111/ppa.12414

[12] Hayashi, N., Li, C.Y., Li, J.L. and Naito, H. (1997) *In Vitro* Production on Rice Plants of Perithecia of *Magnaporthe grisea* from Yunan, China. *Mycological Research*, **101**, 1308-1310. https://doi.org/10.1017/S095375629700422X

[13] Mehrabi, R., Bahkali, A.H., Elsalam, K.A., Moslem, M., M'Barek, S., Gohari, A.M., Jasni, M.K., Sterqiopoulos, I., Kema, G.H. and Wit, P.J. (2011) Horizontal Gene and Chromosome Transfer in Plant Pathogenic Fungi Affecting Host Range. *FEMS Microbiology Reviews*, **35**, 542-554. https://doi.org/10.1111/j.1574-6976.2010.00263.x

[14] Yamasaki, Y. and Niizeki, H. (1965) Studies on Variation of Rice Blast Fungus *Pyriculariaoryzae* cav. I. Karyological and Genetical Studies on Variation. *Bulletin of the National Institute of Agricultural Sciences* (*Japan*), **13**, 231-273.

[15] Genovesi, A.D. and Magill, C.W. (1976) Heterokaryosis and Parasexuality in *Pyricularia oryzae* Cavara. *Canadian Journal of Microbiology*, **22**, 531-536. https://doi.org/10.1139/m76-079

[16] Fatemi, J. and Nelson, R.R. (1978) Inter-Isolate Heterokaryosis in *Pyricularia oryzae*. *Phytopathology*, **68**, 1791-1794. https://doi.org/10.1094/Phyto-68-1791

[17] Cruz, C.D., Peterson, G.L., Bockus, W.W., Kankanala, P., Dubcovsky, J., Jordan, K.W., Akhunov, E., Chumley, F., Baldelomar, F.D. and Valent, B. (2016) The 2NS Translocation from *Aegilops ventricosa* Confers Resistance to the *Triticum* Pathotype of *Magnaporthe oryzae*. *Crop Science*, **56**, 990-1000. https://doi.org/10.2135/cropsci2015.07.0410

[18] Namai, T., *et al.* (1999) Effect of True Resistance Genes Harbored by Rice Cultivars on Pathogenic Variation of Rice Blast Fungus Re-Isolated from Panicles of Resistant Rice Cultivars. *Annals of the Phytopathological Society of Japan*, **65**, 67-75. https://doi.org/10.3186/jjphytopath.65.67

[19] Zeigler, R.S., Cuoclx, Scott, R.P., Bernardo, M.A., Chen, D., Valent, B. and Nelson, R.J. (1995) The Relationship between Phylogeny and Virulence in *pyricularia grisea* in the Philippines. *Phytopathology*, **85**, 443-451. https://doi.org/10.1094/Phyto-85-443

[20] Tendulker, S.R., Gupta, A. and Chattoo, B.B. (2003) A Simple Protocol for Isolation of Fungal DNA. *Biotechnology Letters*, **25**, 1941-1944. https://doi.org/10.1023/B:BILE.0000003990.27624.04

[21] White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., Eds., *PCR Protocols*: *A Guide to Methods and Applications*, Academic Press, Inc., New York, 315-322.

[22] Luo, C.X., Yin, L.F., Koyanagi, S., Farman, M.L., Kusaba, M. and Yaegashi, H. (2005) Genetic Mapping and Chromosomal Assignment of *Magnaporthe oryzae* Avirulence Genes *AvrPik*, *AvrPiz* and *AvrPiz-t* Controlling Cultivar Specificity on Rice. *Phytopathology*, **95**, 640-647. https://doi.org/10.1094/PHYTO-95-0640

[23] Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P. and Valent, B. (2000) Direct Interaction of Resistance Gene and Avirulence Gene Products Confers Rice Blast Resistance. *The EMBO Journal*, **19**, 4004-4014. https://doi.org/10.1093/emboj/19.15.4004

[24] Kiyosa, S. (1974) Studies on Genetics and Breeding of Blast Resistance in Rice. *Miscellaneous Publication of the National Institute of Agricultural Sciences*. *Series D*. *Physiology and Genetics*, **1**, 1-58.

[25] Silue, D., Notteghem, J.L. and Tharreau, D. (1992) Evidence of a Gene-for-Gene Relationship in the *Oryza sativa-Magnaporthe grisea* Pathosystem. *Phytopathology*, **82**, 577-580. https://doi.org/10.1094/Phyto-82-577

[26] Bohnert, H.U., Fudal, I., Dioh, W., Tharreau, D., Notteghem, J.L., *et al.* (2004) A Putative Polyketide Synthase/Peptide Synthase from *Magnaporthe grisea* Signals Pathogen Attack to Resistance Rice. *Plant Cell*, **16**, 2499-2513. https://doi.org/10.1105/tpc.104.022715

[27] Leong, S.A. (2008) The Ins and Outs of Host Recognition of *Magnaporthe oryzae*. In: Gufstason, J.P., Taylor, J., Stacey, G., Eds., *The Genomics of Disease*, Springer Science + Business Media, New York, 119-216. https://doi.org/10.1007/978-0-387-76723-9_15

[28] Li, W., Wang, B., Wu, J., Lu, G., Hu, Y., Zhang, X., Zhang, Z., Zhao, Q., Feng, Q., Zhang, H., Wang, Z., Wang, G., Han, B., Wang, Z. and Zhou, B. (2009) The *Magnaporthe oryzae* Avirulence Gene *AvrPiz-t* Encodes a Predicted Secreted Protein That Triggers the Immunity in Rice Mediated by the Blast Resistance Gene *Piz-t*. *Molecular Plant-Microbe Interactions*, 22, 411-420. https://doi.org/10.1094/MPMI-22-4-0411

[29] Orbach, M.J., Farrall, L., Sweigard, J.A., Chumley, F.G. and Valent, B. (2000) A Telomeric Avirulence Gene Determines Efficacy for the Rice Blast Resistance Gene *Pi-ta*. *Plant Cell*, **12**, 2019-2032. https://doi.org/10.1105/tpc.12.11.2019

[30] Yoshida, K., Satoh, H., Fujisawa, S., Kanzaki, H., Matsumura, H., *et al.* (2009) Association Genetics Reveals Three Novel Avirulence Genes from the Rice Blast Fungal Pathogen *Magnaporthe oryzae*. *Plant Cell*, **21**, 1573-1591. https://doi.org/10.1105/tpc.109.066324

[31] Chuma, I., Isobe, C., Hotta, Y., Ibaragi, K., Futamata, N., Kusaba, M., Yoshida, K., Terauchi, R., Fujita, Y., Nakayashiki, H., Valent, B. and Tosa, Y. (2011) Multiple Translocation of *AVR-Pita* Effector Gene among Chromosomes of the Rice Blast Fungus *Magnaporthe oryzae* and Related Species. *The PLoS Pathogens*, **7**, e1002147.

[32] Crawford, M.S., Chumley, F.G., Weaver, C.G. and Valent, B. (1986) Characterization of the Heterokaryotic and Vegetative Diploid Phases of *Magnaporthe grisea*. *Genetics*, **114**, 1111-1129.

[33] Kumar, J., Nelson, R.G. and Zeigler, R.S. (1999) Population Structure and Dynamics of *Magnaporthe grisea* in the Indian Himalayas. *Genetics*, **152**, 971-984.

[34] Correll, J.C., Harp, T.L., Guerber, J.C., Zeigler, R.S., Liu, B., Cartwright, R.D. and Lee, F.N. (2000) Characterization of *Pyricularia grisea* in the United States Using Independent Genetic and Molecular Markers. *Phytopatology*, **90**, 1396-1404. https://doi.org/10.1094/PHYTO.2000.90.12.1396

[35] Ishikawa, F.H., Souza, E.A., Shoji, J., Connolly, L., Freitag, M., Read, N.D. and Roca, M.G. (2012) Heterokaryon Incompatibility Is Suppressed Following Conidial Anastomosis Tube Fusion in a Fungal Plant Pathogen. *PLoS ONE*, **7**, e31175. https://doi.org/10.1371/journal.pone.0031175

[36] Namai, T. and Yamanaka, S. (1982) Studies on Variation in Virulence of Rice Blast Fungus, *Pyricularia oryzae* Cavara I. Variant Formation by the Pairing, Cultivation and Inoculation of Two Different Pathogenic Isolates. *Annals of the Phytopathological Society of Japan*, **48**, 466-470. (In Japanese)

[37] Roca, M.G., Davide, L.C., Davide, L.M.C., Mendes-Costa, M.C., Schwan, R.F. and Wheals, A.E. (2004) Conidial Anastomosis Fusion between *Colletotrichum* Species. *Mycological Research*, **108**, 1320-1326. https://doi.org/10.1017/S0953756204000838

Appendix

Table A1. List of single conidial isolates from double inoculated lesions on crabgrass.

Isolate[a]	Code number of lesion	ITS genotype[b]	Isolate[a]	Code number of lesion	ITS genotype[b]
Ca1-1	1	A	Ca5-2	5	A
Ca1-2	1	A	Ca5-3	5	A
Ca1-3	1	A	Ca5-4	5	A
Ca1-4	1	nd	Ca5-5	5	A
Ca1-5	1	A	Ca5-6	5	A
Ca1-6	1	nd	Ca6-1	6	A
Ca1-7	1	A	Ca6-2	6	A
Ca1-8	1	A	Ca6-3	6	A
Ca1-9	1	A	Ca6-4	6	A
Ca1-10	1	nd	Ca6-5	6	A
Ca2-1	2	A	Ca6-6	6	A
Ca2-2	2	A	Ca6-7	6	A
Ca2-3	2	nd	Ca6-8	6	A
Ca2-4	2	nd	Ca6-9	6	A
Ca2-5	2	nd	Ca7-1	7	A
Ca2-6	2	A	Ca8-1	8	A
Ca2-7	2	A	Ca8-2	8	A
Ca2-8	2	A	Ca8-3	8	A
Ca3-1	3	A	Ca8-4	8	A
Ca3-2	3	A	Ca8-5	8	A
Ca3-3	3	A	Ca8-6	8	A
Ca3-4	3	A	Ca8-7	8	A
Ca3-5	3	A	Ca8-8	8	A
Ca3-6	3	A	Ca8-9	8	A
Ca3-7	3	A	Ca8-10	8	A
Ca3-8	3	A	Ca9-1	9	A
Ca4-1	4	A	Ca9-2	9	A
Ca4-2	4	A	Ca9-3	9	A
Ca4-3	4	A	Ca9-4	9	A
Ca5-1	5	A	Ca9-5	9	A
Ca16-6	16	A	Ca20-8	20	A
Ca16-7	16	A	Ca20-9	20	A
Ca16-8	16	A	Ca21-1	21	A

Continued

Ca16-9	16	A	Ca21-2	21	nd
Ca16-10	16	A	Ca21-3	21	nd
Ca17-1	17	A	Ca21-4	21	A
Ca17-2	17	A	Ca21-5	21	A
Ca17-3	17	A	Ca21-6	21	A
Ca17-4	17	A	Ca21-7	21	A
Ca17-5	17	A	Ca21-8	21	A
Ca17-6	17	A	Ca21-9	21	A
Ca18-1	18	A	Ca23-1	23	A
Ca18-2	18	A	Ca23-2	23	A
Ca18-3	18	A	Ca23-3	23	A
Ca19-1	19	A	Ca23-4	23	A
Ca19-2	19	A	Ca23-5	23	A
Ca19-3	19	A	Ca23-6	23	A
Ca19-4	19	A	Ca23-7	23	A
Ca19-5	19	A	Ca23-8	23	A
Ca19-6	19	A	Ca24-1	24	A
Ca19-7	19	A	Ca24-2	24	A
Ca19-8	19	A	Ca24-3	24	A
Ca19-9	19	A	Ca24-4	24	A
Ca20-1	20	A	Ca24-5	24	A
Ca20-2	20	A	Ca24-6	24	A
Ca20-3	20	A	Ca24-7	24	nd
Ca20-4	20	A	Ca24-8	24	A
Ca20-5	20	A	Ca24-9	24	A
Ca20-6	20	A	Ca27-1	27	nd
Ca20-7	20	A	Ca27-2	27	nd
Ca27-3	27	A	Ca31-10	31	A
Ca27-4	27	A	Ca32-1	32	B
Ca27-5	27	A	Ca32-2	32	A
Ca27-6	27	A	Ca32-3	32	A
Ca27-7	27	A	Ca32-4	32	A
Ca27-8	27	A	Ca32-5	32	A
Ca29-1	29	A	Ca32-6	32	A
Ca29-2	29	A	Ca32-7	32	B
Ca29-3	29	A	Ca33-1	33	A
Ca29-4	29	A	Ca33-2	33	A

Continued

Ca29-5	29	A	Ca33-3	33	A
Ca29-6	29	A	Ca33-4	33	A
Ca29-7	29	A	Ca33-5	33	A
Ca29-8	29	A	Ca33-6	33	A
Ca29-9	29	A	Ca33-7	33	A
Ca29-10	29	A	Ca33-8	33	A
Ca30-1	30	A	Ca34-1	34	nd
Ca30-2	30	A	Ca34-2	34	A
Ca30-3	30	A	Ca34-3	34	A
Ca30-4	30	A	Ca34-4	34	A
Ca30-5	30	A	Ca34-5	34	A
Ca31-1	31	A	Ca34-6	34	A
Ca31-2	31	A	Ca34-7	34	A
Ca31-3	31	A	Ca35-1	35	A
Ca31-4	31	A	Ca35-2	35	A
Ca31-5	31	A	Ca35-3	35	A
Ca31-6	31	A	Ca35-4	35	A
Ca31-7	31	A	Ca35-5	35	A
Ca31-8	31	A	Ca35-6	35	A
Ca31-9	31	A	Ca35-7	35	A
Ca35-8	35	A	Ca42-1	42	A
Ca36-1	36	nd	Ca42-2	42	A
Ca36-2	36	A	Ca42-3	42	A
Ca36-3	36	A	Ca42-4	42	B
Ca36-4	36	A	Ca42-5	42	A
Ca37-1	37	A	Ca44-1	44	A
Ca37-2	37	A	Ca44-2	44	A
Ca37-3	37	A	Ca44-3	44	A
Ca37-4	37	nd	Ca44-4	44	B
Ca37-5	37	A	Ca44-5	44	A
Ca38-1	38	A	Ca45-1	45	A
Ca38-2	38	A	Ca45-2	45	A
Ca38-3	38	A	Ca45-3	45	A
Ca38-4	38	A	Ca45-4	45	A
Ca38-5	38	A	Ca45-5	45	A
Ca38-6	38	A	Ca45-6	45	A
Ca40-1	40	A	Ca46-1	46	A

Continued

Ca40-2	40	A	Ca46-2	46	A
Ca40-3	40	A	Ca46-3	46	A
Ca40-4	40	A	Ca46-4	46	A
Ca40-5	40	A	Ca46-5	46	A
Ca40-6	40	A	Ca46-6	46	A
Ca40-7	40	A	Ca48-1	48	A
Ca41-1	41	A	Ca48-2	48	A
Ca41-2	41	A	Ca48-3	48	A
Ca41-3	41	A	Ca48-4	48	A
Ca41-4	41	A	Ca48-5	48	A
Ca41-5	41	A	Ca49-1	49	A
Ca41-6	41	A	Ca49-2	49	A
Ca41-7	41	A	Ca49-3	49	A
Ca49-4	49	A	Ca53-4	53	A
Ca49-5	49	A	Ca53-5	53	A
Ca49-6	49	A	Ca53-6	53	A
Ca49-7	49	A	Ca53-7	53	A
Ca50-1	50	A	Ca53-8	53	A
Ca50-2	50	A	Ca54-1	54	A
Ca50-3	50	A	Ca54-2	54	A
Ca50-4	50	A	Ca54-3	54	A
Ca50-5	50	A	Ca54-4	54	A
Ca50-6	50	A	Ca54-5	54	A
Ca50-7	50	A	Ca54-6	54	A
Ca50-8	50	A	Ca54-7	54	A
Ca51-1	51	A	Cb1-1	1	A
Ca51-2	51	A	Cb1-2	1	A
Ca51-3	51	A	Cb1-3	1	A
Ca51-4	51	A	Cb1-4	1	A
Ca51-5	51	A	Cb1-5	1	A
Ca52-1	52	A	Cb1-6	1	A
Ca52-2	52	A	Cb2-1	2	A
Ca52-3	52	A	Cb2-2	2	A
Ca52-4	52	A	Cb2-3	2	A
Ca52-5	52	A	Cb2-4	2	A
Ca52-6	52	A	Cb2-5	2	A
Ca52-7	52	A	Cb2-6	2	A

Continued

Ca52-8	52	A	Cb2-7	2	A
Ca52-9	52	A	Cb3-1	3	A
Ca52-10	52	A	Cb3-2	3	A
Ca53-1	53	A	Cb3-3	3	A
Ca53-2	53	A	Cb3-4	3	A
Ca53-3	53	A	Cb3-5	3	A
Cb4-1	4	A	Cb8-3	8	A
Cb4-2	4	A	Cb8-4	8	A
Cb4-3	4	A	Cb8-5	8	A
Cb4-4	4	A	Cb8-6	8	A
Cb4-5	4	A	Cb8-7	8	A
Cb4-6	4	A	Cb9-1	9	A
Cb5-1	5	A	Cb9-2	9	A
Cb5-2	5	A	Cb9-3	9	A
Cb5-3	5	A	Cb9-4	9	A
Cb5-4	5	A	Cb9-5	9	A
Cb5-5	5	A	Cb9-6	9	A
Cb5-6	5	A	Cb9-7	9	A
Cb5-7	5	A	Cb9-8	9	A
Cb5-8	5	A	Cb12-1	12	A
Cb5-9	5	A	Cb12-2	12	A
Cb6-1	6	A	Cb12-3	12	A
Cb6-2	6	A	Cb12-4	12	A
Cb6-3	6	A	Cb12-5	12	A
Cb6-4	6	A	Cb12-6	12	A
Cb6-5	6	A	Cb12-7	12	A
Cb6-6	6	A	Cb12-8	12	A
Cb7-1	7	A	Cb13-1	13	A
Cb7-2	7	A	Cb13-2	13	A
Cb7-3	7	A	Cb13-3	13	A
Cb7-4	7	A	Cb13-4	13	A
Cb7-5	7	A	Cb13-5	13	A
Cb7-6	7	A	Cb13-6	13	A
Cb8-1	8	A	Cb13-7	13	A
Cb8-2	8	A	Cb13-8	13	A
Cb13-9	13	A	Cb20-3	20	A
Cb13-10	13	A	Cb20-4	20	A

Continued

Cb14-1	14	A	Cb20-5	20	A
Cb14-2	14	A	Cb20-6	20	A
Cb14-3	14	A	Cb20-7	20	A
Cb14-4	14	A	Cb22-1	22	A
Cb14-5	14	A	Cb22-2	22	A
Cb15-1	15	A	Cb22-3	22	A
Cb15-2	15	A	Cb22-4	22	A
Cb15-3	15	A	Cb22-5	22	A
Cb15-4	15	A	Cb22-6	22	A
Cb15-5	15	A	Cb23-1	23	A
Cb15-6	15	A	Cb23-2	23	A
Cb15-7	15	A	Cb23-3	23	A
Cb15-8	15	A	Cb23-4	23	A
Cb16-1	16	A	Cb23-5	23	A
Cb16-2	16	A	Cb24-1	24	A
Cb16-3	16	A	Cb24-2	24	A
Cb16-4	16	A	Cb24-3	24	A
Cb16-5	16	A	Cb24-4	24	A
Cb16-6	16	A	Cb24-5	24	A
Cb17-1	17	A	Cb24-6	24	A
Cb17-2	17	A	Cb24-7	24	A
Cb17-3	17	A	Cb24-8	24	A
Cb17-4	17	A	Cb24-9	24	A
Cb17-5	17	A	Cb24-10	24	A
Cb17-6	17	A	Cb25-1	25	A
Cb17-7	17	A	Cb25-2	25	A
Cb20-1	20	A	Cb25-3	25	A
Cb20-2	20	A	Cb25-4	25	A
Cb25-5	25	A	Cb30-7	30	A
Cb25-6	25	A	Cb30-8	30	A
Cb26-1	26	A	Cb30-9	30	A
Cb26-2	26	A	Cb31-1	31	A
Cb26-3	26	A	Cb31-2	31	A
Cb26-4	26	A	Cb31-3	31	A
Cb26-5	26	A	Cb31-4	31	A
Cb27-1	27	A	Cb31-5	31	A
Cb27-2	27	A	Cb31-6	31	A

Continued

Cb27-3	27	A	Cb31-7	31	A
Cb27-4	27	A	Cb31-8	31	A
Cb27-5	27	A	Cb31-9	31	A
Cb27-6	27	A	Cb32-1	32	A
Cb27-7	27	A	Cb32-2	32	A
Cb27-8	27	A	Cb32-3	32	A
Cb28-1	28	A	Cb32-4	32	A
Cb28-2	28	A	Cb32-5	32	A
Cb28-3	28	A	Cb32-6	32	A
Cb28-4	28	A	Cb32-7	32	A
Cb28-5	28	A	Cb32-8	32	A
Cb28-6	28	A	Cb33-1	33	A
Cb28-7	28	A	Cb33-2	33	A
Cb28-8	28	A	Cb33-3	33	A
Cb28-9	28	A	Cb33-4	33	A
Cb30-1	30	A	Cb33-5	33	A
Cb30-2	30	A	Cb33-6	33	A
Cb30-3	30	A	Cb33-7	33	A
Cb30-4	30	A	Cb33-8	33	A
Cb30-5	30	A	Cb34-1	34	A
Cb30-6	30	A	Cb34-2	34	A
Cb34-3	34	A			
Cb34-4	34	A			
Cb34-5	34	A			
Cb34-6	34	A			
Cb34-7	34	A			
Cb34-8	34	A			

a Isolates with code "Ca" were obtained; From double inoculation of SA13-1ME and TP106. Isolates with code "Cb" were obtained from double inoculation of SA13-1ME and TP022. A, Pyricularia grisea type; B, P. oryzae type.

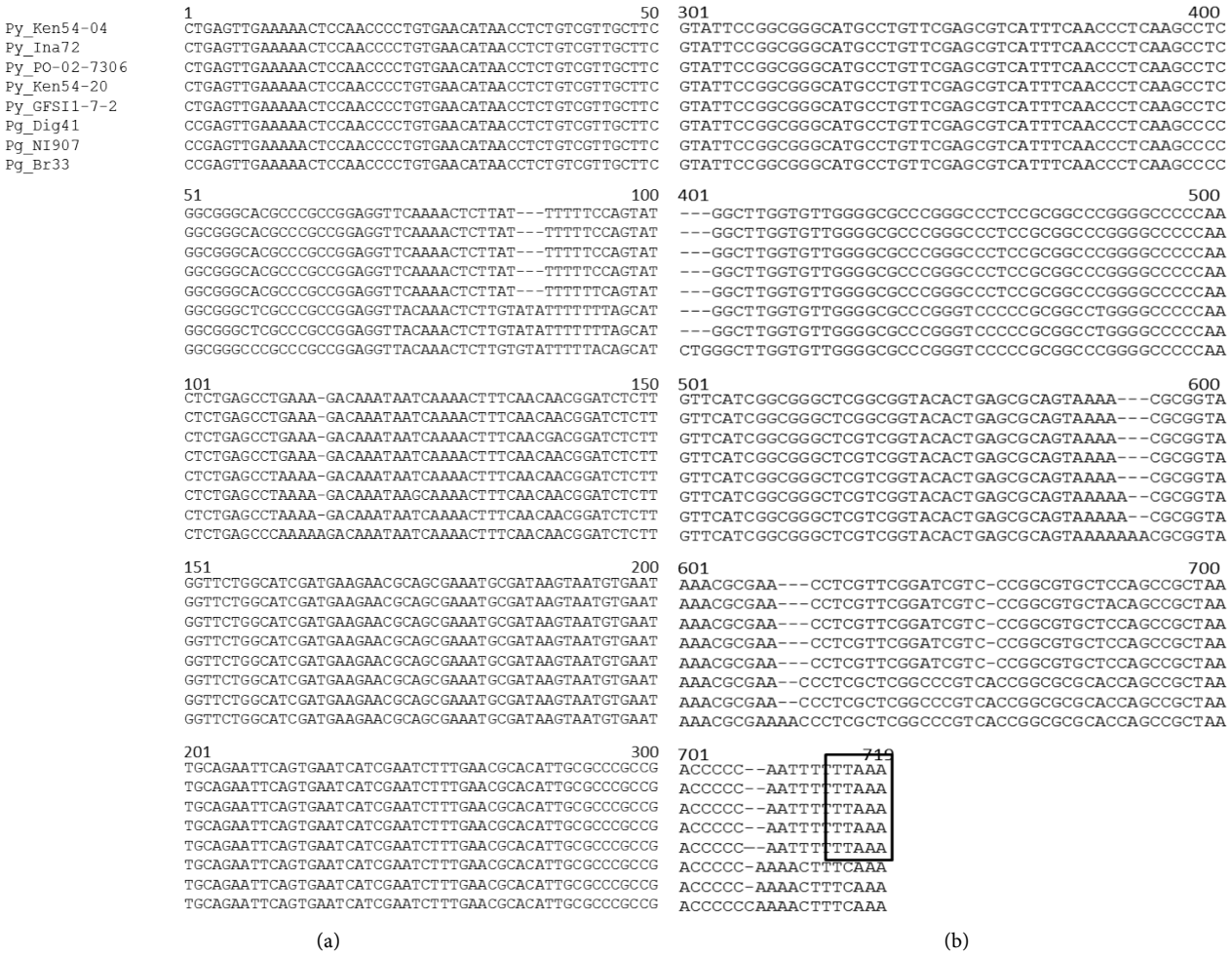

Figure A1. Alignment of ITS regions of *Pyricularia oryzae* (Py) and *P. grisea* (Pg) Restriction site of *Dra*I is shown in a square.