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Fitness Characters in Parasexual Recombinants of the Rice Blast Fungus, *Pyricularia oryzae*

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Abstract

We examined the fitness characters in parasexual recombinants derived from cocultures of two isolates, NAO-02 (race 133.1) and TH77-1 (race 047.0), of the rice blast fungus, *Pyricularia oryzae*, in liquid yeast extract medium. The cocultured isolates were transferred to oatmeal agar to produce a conidial inoculum. Then, the conidial suspension was sprayed on the japonica rice cultivar Akiyutaka, which has two resistance genes to rice blast: *Pik* and *Piz*. Three pathogenic variants were isolated from the typical leaf blast lesions that were subsequently produced. We used randomly amplified polymorphic DNA (RAPD) to examine whether the variants were derived from parasexual recombination between the parents. Eleven RAPDs indicated that the variants possessed genomic DNA from both parents. After inoculating Akiyutaka 7 times in succession, the variants maintained their original pathogenicity. The parasexual recombinants produced more conidia and larger lesions on the host than did parent TH77-1, but fewer conidia and smaller lesions than parent NAO-02. Disease development on plants inoculated with either variant or a parental isolate was also midway between the disease caused by each of the parents in a field containing a single cultivar, but the variant caused the most severe disease in a plot with a mixture of cultivars. These results suggest that parasexual recombination produces variation in fitness.

Discipline: Plant disease
Additional key words: aggressiveness, *Magnaporthe oryzae*

Introduction

Magnaporthe oryzae B. Couch (anamorph *Pyricularia oryzae* Cavara)[7], previously known as *Magnaporthe grisea* (Hebert) Barr, causes rice blast disease, which limits rice production worldwide. Resistant cultivars and fungicides can usually be used to prevent the disease. However, the extensive use of blast-resistant rice cultivars has led to the subsequent breakdown of their resistance as new pathogenic variants arise. Cultivar mixtures have been recommended as one strategy to prevent the breakdown of resistance and to reduce the use of chemicals. There are many reports on cultivar mixtures to manage cereal diseases[1,5,8,23–25,43,48], including rice blast. Cultivar mixtures also stabilize yield and cost less while reducing both disease and pest pressure. Nevertheless, cultivar mixtures have disadvantages, such as variety incompatibility and the inability to provide optimal cultivation conditions for all of the cultivars in the mixture. Multiline cultivars or near-isogenic lines (NILs), mixtures of lines that are genetically uniform except for traits such as disease resistance, have been used successfully to overcome these disadvantages[4,19,26,28,29,38,40,41]. Although multilines and NILs have been developed and cultivated for rice blast control in some prefectures in Japan[37], the usefulness of multilines may be nullified when new variants of the pathogen arise.

Many researchers have reported heterokaryosis and a parasexual cycle in this fungus[9,11,14,42,45,47]. Auxotrophic mutants have been used to study vegetative compatibility, heterokaryosis and parasexuality in *Pyricularia oryzae*[6,9,11]. While many researchers have postulated that changes in pathogenicity occur through parasexual recom-

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bination, only a few reports have dealt with the fitness of parasexual recombinants[31,32], and experimental field data on parasexual recombinants are lacking. Crow[10] defined fitness as the combined ability of an organism to survive and reproduce. Plant pathogen fitness can be estimated using such distinct components (fitness characters) as reproductive rate, infection efficiency, lesion size, efficiency of sporulation, and amount of disease (i.e., aggressiveness)[15].

We examined the fitness characters in parasexual recombinants derived from cocultures of different pathogenic races of the rice blast fungus, *P. oryzae*, to examine the potential for new isolates derived from parasexual recombination to cause severe damage in the field. The field trials that we report here compared blast development caused by a parasexual recombinant with that caused by each parental isolate in a field planted with a single cultivar or a cultivar mixture.

Materials and methods

1. Fungus

Two isolates of *P. oryzae*, NAO-02 (race 133.1, Japan) and TH77-1 (race 047.0, Japan), were used as the parents.

2. Isolation of variants

NAO-02 and TH77-1 were cocultured in liquid yeast extract medium (5 g of yeast extract and 1,000 mL of distilled water) under dark condition at 25ºC for 7 days and then transferred to oatmeal agar (50 g oatmeal, 20 g sucrose and 15 g agar in 1,000 mL of water). After subculture under dark condition at 25ºC for 11–12 days, the aerial mycelia were gently rubbed off with a water-soaked paintbrush and placed under continuous illumination with fluorescent light (18 W) at 21ºC for 3–4 days to induce sporulation. To make a conidial suspension, the mycelia were scraped and flooded with water containing 0.01% Tween 20. The suspension was then filtered through three layers of gauze mesh and adjusted to 1×10^5 conidia/mL. The conidial suspension was sprayed onto the japonica rice cultivar Akiyutaka, which has two resistance genes to rice blast: *Pik* and *Piz*, and cultivated in a greenhouse for a week. The parents are unable to cause disease on the cultivar. The races of monoconidial isolates obtained from lesions on Akiyutaka were identified in pathogenicity tests on differential cultivars.

3. Pathogenicity test

Six seedlings each of the Japanese differential cultivars Shin 2 (with the resistance gene, *Pik-s*), Aichi asahi (*Pia*), Ishikari shiroke (*Pii*), Kanto 51 (*Pik*), Tsuyuake (*Pik-m*), Fukunishiki (*Piz*), Yashiro-mochi (*Pita*), Pi No. 4 (*Pita-2*), Toride 1 (*Piz-t*), K60 (*Pik-p*), BL1 (*Pib*), and K59 (*Pit*) at the fourth-leaf stage were treated with 30 mL of the spore suspension prepared using Diaspray (Furupla, Tokyo, Japan). The inoculated plants were immediately placed in a dew chamber at 24.5ºC for 20 h, and then transferred to a greenhouse at 26–28ºC. Six or 7 days after inoculation, the pathogenic race of each isolate was determined by the reaction of the differential cultivars. We used the race number designations of Kiyosawa[18]. The largest lesion on the youngest leaves of the host was used to estimate the pathogenicity of the isolates. The experiments were conducted at least twice.

4. DNA isolation and randomly amplified polymorphic DNA (RAPD) analysis

DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method[27] with modifications[47]. A total of 400 primers (Kits A to K and AA to AI, Operon Technologies, Alameda, CA) were used in this study. Amplification reactions were performed as described by Yasuda et al.[47]. The amplification products were analyzed by electrophoresis in 1–1.5% agarose gels.

5. Successive inoculation

Successive inoculation was made to evaluate the stability of the pathogenicity of variants. Seedlings of Aichi asahi and Akiyutaka were inoculated at the fourth-leaf stage by spraying them with 15 mL of a spore suspension of each variant using an artist's airbrush PCB, 102C (Olympos, Osaka, Japan). The conidial suspension was adjusted to 5×10^5 to 8×10^5 spores/mL. Then, the inoculated plants were immediately placed in a dew chamber at 24.5ºC for 20 h, after which they were transferred to a greenhouse at 26–28ºC. Seven days after inoculation, the youngest leaves of the host at the time of inoculation were excised and placed on No. 2 filter paper (ADVANTEC, Tokyo, Japan) moistened with distilled water in a Petri dish to keep the humidity high. The Petri dishes were then placed in a growth chamber at 26ºC for conidiation. After 24 h, a lesion with conidia was placed in a small glass tube with water containing 0.01% Tween 20 and subjected to supersonic vibration to remove the conidia from the sporulating surface[44] to make the conidial suspension for the next inoculation. After repeating the inoculations 7 times as described above, single conidium isolates were obtained from single lesions to determine the race designation.

6. Assay of conidiation

The rice cultivar Aichi asahi was punch-inoculated at the 6th-leaf stage on the 6th leaves with the blast fungus

using a specially designed pressing machine to create press-injury spots (Fujihara, Osaka, Japan). A piece of filter paper (3 × 3 mm) soaked with conidia suspension adjusted to 1×10^5 to 5×10^5 conidia/mL with water containing 0.01% Tween 20 was placed on the injured spots. The inoculated plants were immediately placed in a dew chamber at 24.5ºC for 20 h, and then transferred to a greenhouse at 26–28ºC. Eight days after inoculation, a leaf with a lesion was removed and transferred to a small glass tube with 0.5 mL of distilled water to maintain high humidity. The tube was placed in a growth chamber at 26ºC for conidiation. After 24 h, 0.5 mL of water containing 0.01% Tween 20 was added to the tube and subjected to supersonic vibration to remove conidia from the sporulating surface[44]. The conidia were then counted with a hemocytometer. The number of tested rice leaves ranged from 5 to 8 per isolate for each test, and all tests were repeated three times.

7. Assay of lesion length

Lesion length was also assayed using the punch-inoculation described above. The rice cultivars Aichi asahi, Kanto 51, Fukunishiki, and Akiyutaka were inoculated. Seven, 14 and 21 days after inoculation, the lesions were measured. The number of rice leaves tested ranged from 4 to 12 per isolate for each test, and all tests were repeated twice.

8. Leaf blast development in fields

The experiments were conducted in 1997, 1998 and 1999 at the National Agricultural Research Center, Hokuriku Area, Joetsu, Japan. In 1997, trial fields were planted separately with Aichi asahi, Kanto 51 and Fukunishiki. In 1998, we used two trial fields: one planted with Aichi asahi and the other with a cultivar mixture of Aichi asahi, Kanto 51 and Fukunishiki (1 : 1 : 1). In 1999, a trial field was planted with Aichi asahi. Parental isolate NAO-02 was avirulent on Fukunishiki carrying *Piz*, but it was virulent on Aichi asahi carrying *Pia* and on Kanto 51 carrying *Pik*. The other parent, TH77-1, was virulent on Aichi asahi and Fukunishiki, but avirulent on Kanto 51. The parental isolates were avirulent on Akiyutaka carrying *Pik* and *Piz*. One of the variants isolated from Akiyutaka was named KZB. KZB was virulent on Aichi asahi, Akiyutaka, Kanto 51, and Fukunishiki. Three plots per field were infested; seedlings of cultivar Aichi asahi were infected with NAO-02, TH77-1 or KZB as a spreader and the spreader plants were placed in the center of each plot on 5 June 1997, 1 June 1998 and 4 June 1999. Each plot consisting of 8 rows was 6.0 m long with 0.3 m intervals between rows. The number of blast lesions per hill was recorded on 14 July 1997, 6 July 1998 and 13 July 1999.

Results

1. Isolation of variants

A total of 9 variants were obtained from lesions on Akiyutaka inoculated with the isolates from the coculture of the parents. One variant was race 137.1, 5 variants were race 173.1 and 3 variants were race 177.1. The pathogenicity of the parents and variants on the Japanese differen-

Table 1. Reaction of Akiyutaka and the Japanese differential cultivars to *Pyricularia oryzae* variants

Cultivars	Resistance genotype	Parental isolates		Variants		
		NAO-02	TH77-1			
Akiyutaka	*Pik*, *Piz*	R	R	R	S	S
Shin 2	*Pik-s*	S	S	S	S	S
Aichi asahi	*Pia*	S	S	S	S	S
Ishikari shiroke	*Pii*, *Pik-s*	R	S	S	R	S
Kanto 51	*Pik*	S	R	S	S	S
Tsuyuake	*Pik-m*	S	R	S	S	S
Fukunishiki	*Piz*	R	S	R	S	S
Yashiro-mochi	*Pita*	S	R	S	S	S
Pi No.4	*Pita-2*	R	R	R	R	R
Toride 1	*Piz-t*	R	R	R	R	R
K60	*Pik-p*	S	R	S	S	S
BL1	*Pib*	R	R	R	R	R
K59	*Pit*, *Pik-s*	R	R	R	R	R
Race		133.1	047.0	137.1	173.1	177.1
Number of variants				1	5	3

R: resistant, S: susceptible.

tial rice cultivars and Akiyutaka is shown in Table 1. The parents were virulent on cultivars Shin 2 and Aichi asahi, but they were avirulent on cultivars Pi No. 4, Toride 1, BL 1, K59, and Akiyutaka. The parents differed in pathogenicity on 6 cultivars, e.g., Ishikari shiroke, Kanto 51 and Tsuyuake. The variants had the same pathogenicity patterns on cultivars in which both parents incited the same reaction, with the exception of Akiyutaka. The pathogenicity of variants on cultivars reacting differently identified either parent as one of the parents. To examine the potential for a broad host range of isolates derived from parasexual recombination to cause damage in rice, three variants of race 177.1 named KZA, KZB and KZC were used in this study.

2. RAPD from parents and the variants

In the test to examine whether the race 177.1 variants were derived from the parasexual recombination between the parents, 13 of 400 primers tested produced polymorphic fragments between two parents. The primers produced banding patterns of the variants that were identical to those of either parent (Fig. 1). We detected the same RAPD patterns for the variants and NAO-02 with primers OPAA-2, OPAA-14, OPAI-18, OPF-7, OPI-16, and OPJ-8. We detected the same RAPD patterns for the variants and TH77-1 with primers OPAB-11, OPAK-12, OPE-4, OPJ-6, and OPJ-18. Primers OPK-3 and OPK-4 detected different RAPD patterns for each variant and each parent (data not shown). Primer OPK-3 produced polymorphic bands which the variants had, but the parents did not. Primer OPK-4 produced two polymorphic bands between parents, KZC had both, but KZA and KZB did not.

3. Stability of the pathogenicity of variants

After 7 successive inoculations with the variant KZB, monoconidial isolates were obtained from leaf lesions on Akiyutaka and Aichi asahi, and their pathogenicity was determined. All isolates did not change the pathogenicity of the original race 177.1 (Table 2). When variant KZC was tested on cultivars Akiyutaka and Aichi asahi, the variant also did not change its original pathogenicity (Table 2).

4. Lesion length and sporulation of the variants

Lesion length with KZB on Aichi asahi was consistently intermediate between that of the parents 7, 14 and

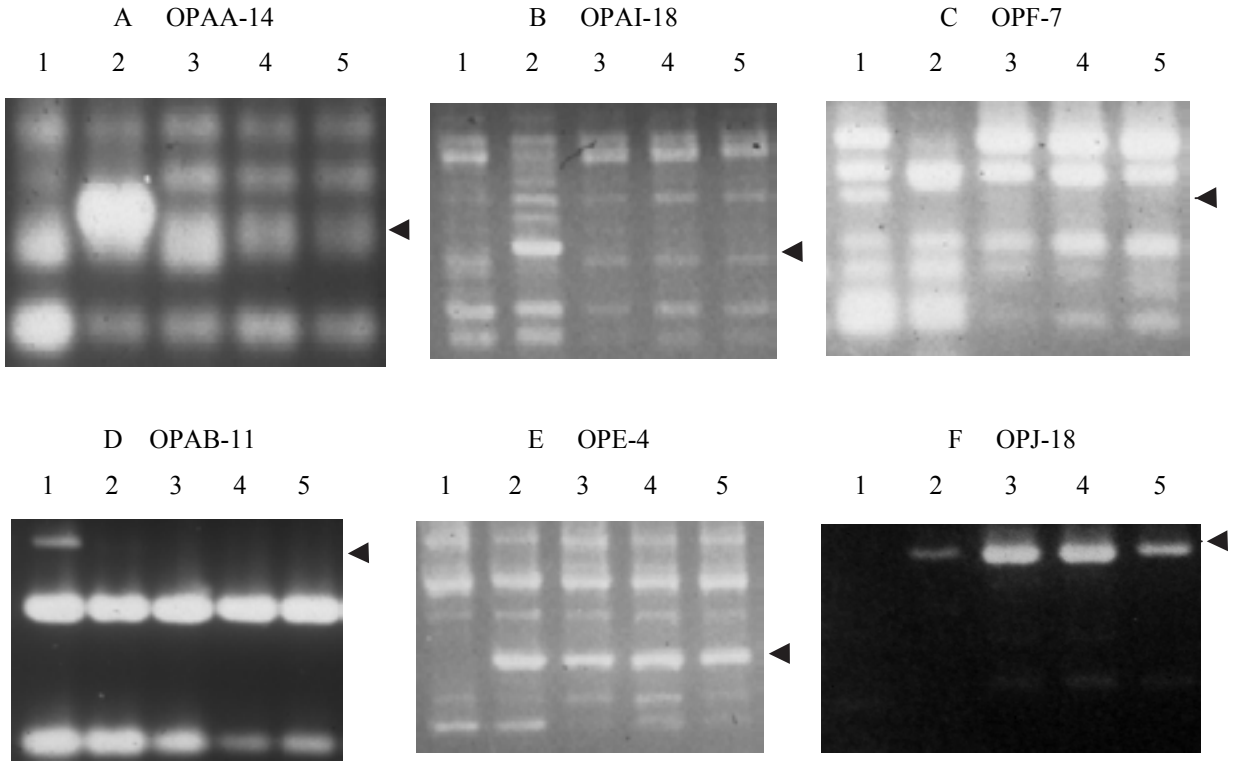

Fig. 1. Random amplification of polymorphic DNAs from parents and the variants using 10-mer primer OPAA-14 (A), OPAI-18 (B), OPF-7 (C), OPAB-11 (D), OPE-4 (E), and OPJ-18 (F)

Annealing temperature was 36ºC. Lanes 1 to 5: NAO-02, TH77-1, KZA, KZB, and KZC. Arrow heads indicate polymorphic bands between parents. (A), (B) and (C) show the same RAPD patterns for the variants and NAO-02. (D), (E) and (F) show the same RAPD patterns for the variants and TH77-1.

Table 2. Pathogenicity of the variants isolated after seven successive inoculations

Variants (race)	Cultivars (Resistance gene)	Number of isolates	%[a)]
KZB (177.1)	Akiyutaka (*Pik*, *Piz*)	10	100
	Aichi asahi (*Pia*)	9	100
KZC (177.1)	Akiyutaka (*Pik*, *Piz*)	7	100
	Aichi asahi (*Pia*)	11	100

The variants were inoculated on seedlings of Akiyutaka and Aichi asahi succesively by spraying a spore suspension of monoconidial isolates.
a): Percentages of isolates maintained same race reaction.

Table 3. Length of lesions formed on leaves of rice cultivars by the parents and the variant

	Lesion length (mm)			
Isolates	Aichi asahi	Kanto 51	Fukunishiki	Akiyutaka
Parents				
NAO-02	24.8±7.9a	22.4±5.7a	nd	nd
TH77-1	13.6±3.9b	nd	11.7±3.3a	nd
Variants				
KZB	18.8±4.8ab	12.5±5.2b	12.8±5.7a	16.8±5.5

Inoculation of the isolates was performed by press-injured method on top leaves at the 6th-leaf stages.
Lesion lengths were mesasured after 21 days inoculation.
The mean and standard deviation (mean±SD) were calculated from each isolate for 4–12 lesions.
Within columns, values followed by the same letter are not significantly different (Tukey's test; $P<0.05$).
nd: Not determined due to incompatible combination.

Table 4. Number of conidia on a single lesion on leaves of rice cultivar Aichi asahi produced by the parents and the variants

	Isolates	Conidiation ($\times 10^3$ spores)
Parents	NAO-02	50.2±39.1a
	TH77-1	5.9± 8.3b
Variants	KZA	43.3±32.1a
	KZB	29.4±29.6ab
	KZC	29.6±55.2ab

Inoculation of the isolates was performed by press-injured method on leaves of 6-leaf-stage plants.
Seven days after inoculation, a lesion was cut out and incubated for 24 h at 26ºC.
Conidia were suspended in 1 mL of sterile water and counted with a hematocytometer.
Mean and standard deviation (mean±SD) were calculated for each isolate from 5–8 lesions.
Within columns, values followed by the same letter are not significantly different (Tukey's test; $P<0.05$).

21 days after inoculation (Table 3). Statistical analysis showed no significant differences among lesion lengths with NAO-02, TH77-1 and KZB on Aichi asahi 7 days after inoculation, but showed significant differences between those of the parents 14 and 21 days after inoculation. KZB could infect Kanto 51, Fukunishiki and Akiyutaka that were resistant to either or both parents, and produced about 12–16 mm lesions on these cultivars. The variants produced more spores on single lesions on Aichi asahi than did TH77-1, but fewer than did NAO-02 (Table 4). Statistical analysis showed significant differences between the results for NAO-02 and TH77-1, but no significant differences between NAO-02 and all of the variants or between TH77-1 and the two variants KZB and KZC.

5. Leaf blast development

In the fields cultivated with a single cultivar, through

1997–1999, the most severe leaf blast disease was caused by NAO-02, followed by KZB, and then by TH77-1 (Fig. 2). The severity of leaf blast was estimated by the number of blast lesions per hill, and had severities of 129.7, 26.5 and 90.7 for NAO-02, TH77-1 and KZB, respectively in 1998 (Table 5). However, in the field containing the cultivar mixture, KZB caused more severe disease than both parents, with severities of 5.2, 0.4 and 19.1 for NAO-02,

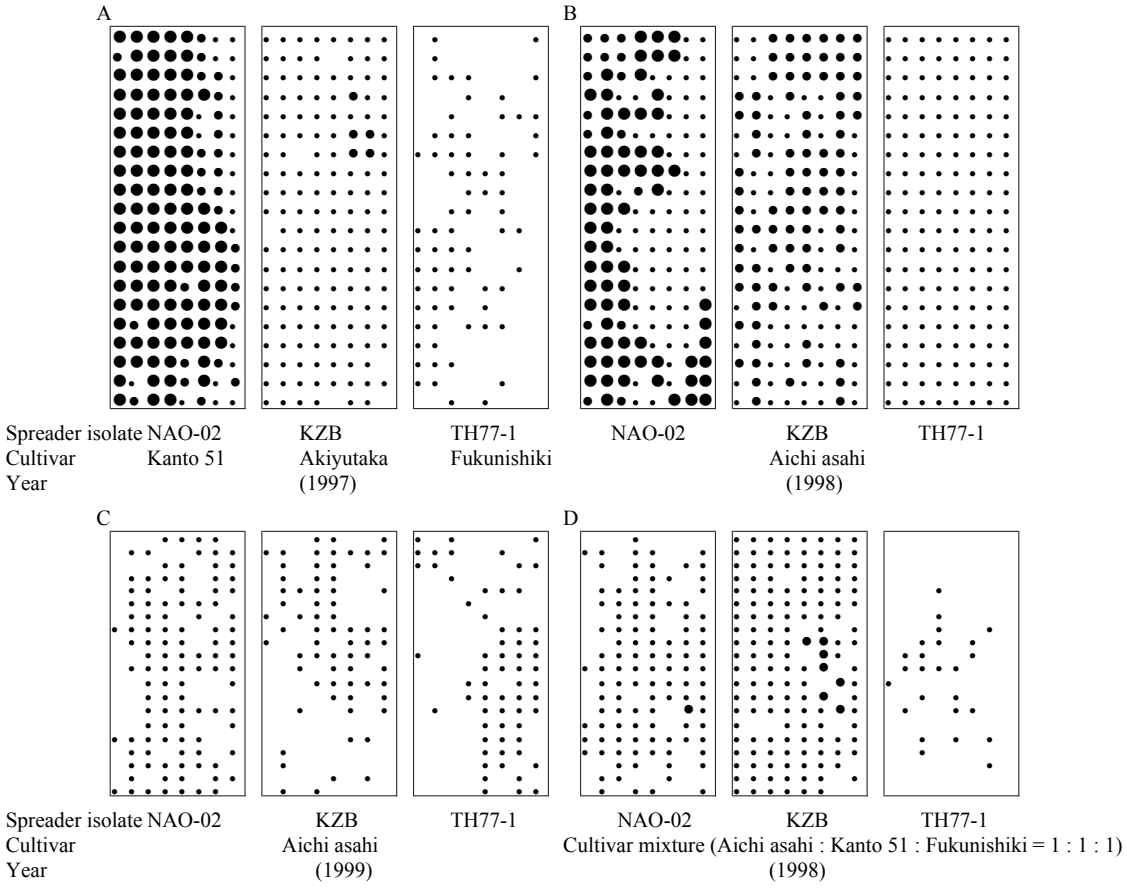

Fig. 2. Distribution of leaf blast on single cultivars and a cultivar mixture in the experimental fields

In 1997 (A), three plots per field were planted separately with Kanto 51, Fukunishiki or Akiyutaka. In 1998 (B) and 1999 (C), three plots per field planted with Aichi asahi were infested with NAO-02, TH77-1 or KZB. Three plots per field planted with a cultivar mixture (Aichi asahi : Kanto 51 : Fukunishiki = 1 : 1 : 1) were infested with NAO-02, TH77-1 or KZB in 1998 (D). Diseased seedlings of Aichi asahi infected with NAO-02, TH77-1 or KZB were planted in the center of each plot. Severity (number of blast lesions per hill) was recorded on 14 July 1997, 6 July 1998 and 13 July 1999.

Table 5. Leaf blast severities of a single cultivar and cultivar mixture in the experimental fields

The number of blast lesions per hill (Proportion of diseased plants (%))				
Spreader Isolates	Single cultivar[a)]			Cultivar mixture[b)]
	1997	1998	1999	1998
NAO-02	193.1±93.9 (100)	129.7±130.9 (100)	3.2±3.9 (67.5)	5.2± 9.6 (69.4)
TH77-1	1.2± 2.0 (41.9)	26.5± 9.0 (100)	1.1±2.1 (38.8)	0.4± 1.5 (15.6)
KZB	19.4±26.2 (96.3)	90.7± 33.0 (100)	1.2±2.3 (41.9)	19.1±23.2 (90.6)

a): In 1997, three plots per a field planted separately with Kanto 51, Fukunishiki and Akiyutaka, in 1998 and 1999, three plots per a trial field planted with Aichi asahi were infested with either NAO-02, TH77-1 or KZB.

b): Three plots per a trial field planted with cultivar mixture (Aichi asahi : Kanto 51 : Fukunishiki = 1 : 1 : 1) were infested with either NAO-02, TH77-1 or KZB. Values indicate means±standard deviation.

TH77-1 and KZB, respectively.

Discussion

Concern over the deterioration of the effectiveness of cultivar mixtures has been raised in terms of the selection for races with complex virulence. Such selection may be due to differences in the relative fitness among competing races in the mixture. The success of new pathogenic variants in a multiline field depends on fitness at either the same or a greater level than that of the prevalent races in the cultivar mixture. In this study, we showed that the pathogenic variants derived from coculture of parents differing in pathogenicity produced lesions on Akiyutaka, a cultivar that neither parent could attack, and that the pathogenic variants had the same fitness characteristics as the parents in terms of lesion length and conidium production.

Contamination from exogenous sources or mutation might explain the mechanism of the occurrence of pathogenic variants on the inoculated plants in this study. Contamination in the greenhouse was not considerable, because in the study area, the predominant race was 001.0, and the variant race 177.1, had never been observed. It was not presumed that the variants derived from mutation, because we showed that the same RAPD patterns in the variants and the other parent and RAPDs indicated that the variants were derived from parasexual recombination between the parents. From the results of RAPDs using primers OPK-3 and OPK-4, these suggested that parasexual recombination produced polymorphic bands between parents and the variants. These also support the parasexual recombination in our study.

Successive passages of two isolates of different races on rice leaves produced variants that differed in pathogenicity from their parents[31]. Ou and Ayad[35,36] reported great pathogenic variation within single-spore isolates of *P. oryzae* from a single lesion and from monoconidial cultures. By contrast, other studies revealed pathogenic stability in *P. oryzae*[3,20]. Avirulence genes in the rice blast fungus were thought to control three types of stability, i.e., genes that were highly unstable, moderately unstable or relatively stable[33]. *AVR-Pik*[21], *AVR-Pita*, and *AVR-TSUY* are known as highly unstable avirulence genes[39], while the *AVR1-CO39*, *ACE 1*[2], and *AVR1-MARA*[22] genes are relatively stable. The stability of virulence is considered related to the way virulence is gained, the chromosomal locations of the *Avr* genes, and the significance of each *Avr* gene. Our variants were selected using Akiyutaka with *Pik* and *Piz* genes. They might have maintained the virulence of *Pik* and *Piz* through successive inoculations on Akiyutaka. Although the variants had stable pathogenicity, the lack of *Pik* and *Piz* genes in Aichi asahi motivated us to examine whether the variants lost virulence to *Pik* or *Piz*. The isolate race 337, with a wide-range of pathogenicity, lost virulence to the *Pii*, *Pik*, *Pik-m*, *Pita*, and *Pita-2* genes through 9 successive passages on the rice cultivar Aichi asahi without these genes and with the *Pia* gene[32]. In our results, we found that none of the monoconidial isolates that changed virulence to *Pik* and *Piz* in both cultivars had stable pathogenicity. Our results indicate that parasexual recombination is able to produce stable pathogenic variants in the rice blast fungus.

Heterokaryosis and a subsequent parasexual cycle in the rice blast fungus and other filamentous fungi have been reported[9,11,14], and a change in pathogenicity might also arise in the rice blast fungus through parasexual recombination[30]. Mutation is also one of the major mechanisms for creating pathogenic variation, and many reports on pathogenic mutations have been published[16,17]. However, the mutants of the rice blast fungus are either less aggressive than the original isolate, or equally aggressive[12]. Our results indicate the possibility that variants derived parasexually had a level of aggressiveness that was intermediate between that of the parents; in other words, parasexual recombinants had aggressiveness that was not less than that of one of the parents. Fujita and Suzuki[13] reported that new pathogenic variants of rice blast increased in aggressiveness each year in paddy fields. New parasexual recombinants could potentially confer the relative fitness equal to the parents, and the fitness of the parasexual recombinants might increase after generations in paddy fields. The parasexual recombinants would acquire greater aggressiveness than the parents, and might become the predominant races in the field. This is the first report to determine the fitness of parasexual recombinants under field conditions, and it demonstrates that parasexual recombinants can survive in nature. In *P. oryzae*, asexual reproduction dominates under field conditions, and sexual reproduction[34] or parasexual recombination is less likely to occur in the field. Sexual recombination is most unlikely to occur in Japan, since ascospore production from crosses between Japanese field isolates has never been successful. Because a study of rice blast isolates in the Indian Himalayas suggested the occurrence of parasexual recombination in nature[47], parasexual recombination was also presumed to occur in Japan.

Complex races that can counter various resistance genes have been assumed to be unable to increase in a cultivar mixture because of the fitness costs associated with the lack of avirulence genes. However, our results suggest that a complex race can attack multiple host genotypes in a multiline system by producing variants that can

infect resistant lines through parasexual recombination. The variants had more complex virulence than the parents and exhibited fitness no less than that of one of the parents. In our field experiment with a cultivar mixture, leaf blast caused by the variants was more serious than that of both parents. These results suggest that parasexual recombination not only alters pathogenicity but also enhances fitness as determined by conidium production. We showed that a complex virulent race can arise through parasexual recombination and can cause severe losses in multiline fields.

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