Mating-type distribution of the rice blast pathogen *Pyricularia grisea* in California

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A B S T R A C T

Pyricularia grisea causes the plant disease known as rice blast and is one of most devastating diseases of rice worldwide. The fungus was discovered for the first time in California in 1996 and attempts to control the disease have been made by using resistant cultivars and fungicide applications. The long-term objective of this research project is to study the genetic structure of this fungus to determine if it has changed over time due to factors such as fungicide usage, resistant rice cultivar deployment, new introductions, and sexual reproduction of the fungus. The objective of this specific study was to determine the mating-type distribution of *P. grisea*. Each isolate of this fungus possesses a single gene for mating type with one of two alleles, either MAT 1-1 or MAT 1-2. Only isolates of opposite mating-type are able to sexually reproduce, otherwise all reproduction is from asexually derived spores. We used a PCR assay to determine the mating-type of 168 isolates collected from diseased rice in North Central California. One hundred and sixty isolates were found to be MAT1-1 and no PCR products were amplified from 8 isolates. This result suggests that *P. grisea* populations associated with rice crops in California are reproducing clonally. However, further work using additional molecular markers is being pursued to better understand the population dynamics of this important plant pathogen.

A U T H O R

R. Z. Urak

Biology and Biochemistry

Ryan Urak is studying a double major in Biology and Biochemistry with an Anthropology minor. He enjoys all aspects of research ranging from biology to humanities. His current research involves molecular work with DNA; efforts that he hopes will aid him in a profession of Forensic Anthropology.

F A C U L T Y   M E N T O R S

G. W. Douhan

Department of Plant Pathology and Microbiology

In my lab we examine the population genetics of fungal species over spatial scales ranging from centimeters to large landscapes using a number of molecular methodologies including multilocus genotyping and multi-gene genealogies. My program also focuses on developing rootstocks for avocado that are resistant to *Phytophthora cinnamomi,* the most destructive and important pathogen of avocado worldwide. Ryan started in my lab in fall of 2007 as a third year student. With his interest in anthropological forensics, he trained in molecular biology methods, including DNA isolation, polymerase chain reaction, and genotyping techniques. Ryan's research project utilized some of these tools and answered one of our first questions about the reproductive biology of *Pyricularia grisea*. This fungus is likely not going through a sexual cycle under field conditions, which is our first step at understanding the reproductive biology and population genetic structure of this important pathogen.

F. Wong

Department of Plant Pathology and Microbiology

My research and extension program focus on the management of diseases of turf and landscape in California. Of highest importance is developing control strategies for emergent and invasive diseases. *Pyricularia grisea* is an emergent fungal pathogen that was recently found to be causing significant damage to rice (1997), perennial ryegrass and kikuyugrass (2003). Because of the economic value of rice, and turf used on golf courses and sports fields, the control of *P. grisea* is important to California agriculture. As part of a project funded by the University of California Exotic and Invasive Pests and Diseases Program, Ryan helped examine the population structure of the pathogen from rice in California. Information generated from this study is important in understanding how the pathogen is reproducing, surviving and spreading in California, and how maximize the effectiveness of management programs.

Introduction

Rice is an important agricultural commodity that supplies approximately 23 % of the per capita energy for six billion people worldwide (Maclean, 1997). There are many serious plant diseases of rice, including the ascomycete fungus *Pyricularia grisea* (Telemorph: *Magnaporthe grisea*) which causes the disease known as rice blast (Correll, 2000). *Pyricularia grisea* can infect most sections of the plant, but infections of the node or the panicle are the most damaging phases of the disease (Ou, 1985). When *P. grisea* infects rice and produces neck rot or panicle blast, it will either kill the host plant or prevent seed development, respectively. *P. grisea* also causes disease in other graminacious species besides rice (Malca, 1957; Bain, 1972; Ou, 1985; Sundaram, 1972) and there are reports of this pathogen in more than 85 countries (Agarwal, 1989).

P. grisea was first identified in California in 1996 (Greer, 2001), which was unexpected due to *P. grisea's* common association with high humidity conditions (Webster, 1992) which is unlike temperate Sacramento Valley where the disease occurs. Seeds, crop residue, and secondary hosts are all possible origins for the introduction of into California and could have been the primary sources of inoculum for the disease (Agarwal, 1989; Lee, 1994; Ou, 1985; Rao, 1994; Teng, 1994). Now that *P. grisea* is present in some rice fields in California, usage of fungicides and deployment of resistant cultivars is the best course of action to control the disease.

By studying the genetic structure of this invasive pathogen, we will be able to make inferences on how the fungus spreads, reproduces, and how the population is responding or adapting to current management practices. The overall objective of this research project is to analyze the genetic structure of *P. grisea* from collections of isolates from the initial introduction of the disease in the mid 1990's to more recently sampled isolates using molecular markers. This would shed light on whether or not any changes in the genetic structure of the fungus have occurred over an approximate 10 year period which may be linked to fungicide usage, resistant rice cultivar deployment, new introductions, or due to sexual reproduction of the fungus.

P. grisea is a heterothallic fungus with a single mating type gene that produces two alleles, MAT 1-1 and MAT 1-2. The pathogen requires both mating types in order for sexual reproduction to occur (Yoder, 1986), and mating type alleles have been used as a marker to measure population diversity in this pathogen (Viji, 2002). The specific objective of this part of the project is to use mating-type to measure and assess population diversity in California populations of the pathogen from rice and determine if sexual reproduction is possible in these populations. Populations collected from both early outbreaks in 1997 and 1998, and more recent ones in 2007, were targeted. These results will provide initial information concerning potential sexual reproduction of *P. grisea* in California rice fields and give a preliminary measure of any changes in population diversity since the initial discovery of the pathogen on rice in 1996.

Results

All 33 isolates from the historical 1997 and 1998 collections were all identified through PCR as mating-type MAT 1-1 (Table 1). Of the 135 *P. grisea* isolates collected in 2007, 127 of the isolates were the mating-type MAT 1-1 (Table 1) (Figure 1). Isolates collected from fields A, D, E and F all produced the 552 bp product associated with the presence of MAT 1-1. For populations collected from fields B and C, eight isolates did not produce a PCR product specific for MAT 1-1. Subsequent PCR assays using the MAT 1-2 specific primers also failed to produce a PCR product specific for this allele.

Discussion

We detected only a single mating-type occurring in populations of *P. grisea* isolated from rice in California. This suggests that *P. grisea* is not sexually reproducing, which can be important in the population dynamics of this pathogen. Sexual reproduction can reshuffle genetic material via recombination, thereby bringing together new alleles, which may influence pathogenicity to various rice cultivars and could influence the efficacy of fungicides. MAT 1-1 has been identified in past studies as the dominant mating-type associated with rice. In a survey of 467 *P. grisea* rice isolates from 34 countries in Europe and Africa, only mating type MAT 1-1 was found (Notteghem, 1992). Research in Japan also yielded similar results, as all surveyed rice

		Mating –type		
Year	Field	MAT 1-1	MAT 1-2	No amplification
2007	A	26	0	0
“	B	26	0	1
“	C	16	0	7
“	D	16	0	0
“	E	17	0	0
«	F	26	0	0
1997	97	10	0	0
1998	98	23	0	0

Table 1. *Mating-type distribution of* **P. grisea** *based on mating-type specific PCR.*

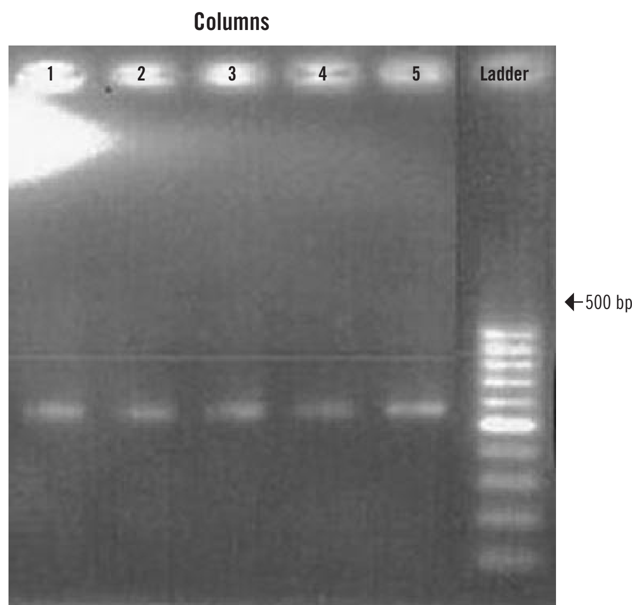

Figure 1. *Agarose gel showing the PCR product (550 bp) for the MAT1-1 allele (in columns 1-5) from some of the representative isolates of P. grisea used in this study.*

isolates belonged to MAT 1-1 (Kato, 1982). Despite these consistencies, a mating-type analysis of Californian isolates is important because sexual reproduction can affect diversity and dissemination, and both mating-types have been found in other studies. For example, Dayaker (2000) found that 39 of the 74 isolates from India were MAT 1-1 and the other 35 isolates were MAT 1-2.

California *P. grisea* isolates also showed that there was no significant change in mating-type from 1997-1998 to 2007, strongly suggesting that the pathogen survives and reproduces through asexual means. As for the few isolates that produced no results, it is believed that this is a case of failed PCR, perhaps due to user error of DNA concentration in the reactions, or that the DNA preparations contained inhibitors, which are common sources of failed reactions. Further work and more meticulous determinations of DNA concentrations in sample extracts will address these issues, but the current data suggests that the MAT 1-2 allele is not present in the California populations of *P. grisea*.

Most studies on the population structure of *P. grisea* associated with rice support the idea that this pathogen is highly clonal, as evident by the use of molecular makers and population genetic analysis to identify the fertility of the isolates (Viji, 1998). The predominance of a single mating-type in most turfgrass (Tredway, 2003) and perennial ryegrass (Viji, 2002) isolates also supports that *P. grisea* is primarily an asexual fungus, though both mating-types have been associated with other turf hosts such as kukuya grass (Wong, F. unpublished). However, *P. grisea* shows strong host preference, which suggests that gene flow does not occur between isolates associated with separate host species.

The severity of *P. grisea* has decreased since the initial 1996 introduction because, as previously postulated, *P. grisea* cannot flourish in the environmental conditions that exist in California. California rice production takes place in a climate that is permissive for rice blast but is too arid to allow the onset of significant epidemics in most years. However, control is still needed when conditions are favorable for the disease (Greer, 2001). Growers primarily use resistant rice cultivars (Ou, 1985) but fungicides are also used when disease pressure is significant. Azoxystrobin is the standard and consequently the only fungicide for rice available in California (Greer, 2001). Azoxystrobin effectively inhibits spore germination and is therefore a protectant prior to infection (Clough, 1998). The few resistant rice cultivators and single fungicide have been the only means to control rice blast in California during the past decade. Our long-term research on the population biology of *P. grisea* isolates collected approximately over a ten year period may give insight into how these cultural practices have influenced the population structure and the reproductive biology of this important pathogen.

Materials and Methods

Sampling

Isolates from 1997 and 1998 were obtained from Dr. Tom Gordon, Department of Plant Pathology, University of California, Davis; these were originally isolated and described by Greer (2001). Recovery of all of the isolates from long-term storage was not possible and recovered isolates were simply grouped by year regardless of location, and referred to as the 1997 and 1998 collections. In 2007, six rice fields (A through F) from Yuba county were sampled by randomly collecting diseased panicle tissue from along a single transect approximately five meters from the edge of the fields. The tissues were placed in paper bags, brought back to the laboratory, and air-dried in the fume hood for several days.

DNA extraction and analysis

To isolate *P. grisea*, infected tissue was surface sterilized, and the lesions were cut in half. The tissues were placed in petroleum jelly that was positioned on the lids of acid potato dextrose agar (aPDA) plates so that they would be elevated. After 48 hours, the lids were tapped to release the spores and the plates were allowed 4-7 days for growth. Five germinated single spores were randomly selected and removed from the aPDA plates. These were then transferred to clean aPDA plates. PDA was also used to regrow the 1997 and 1998 collection of *P. grisea* isolates.

Isolates were allowed to grow at room temperature for approximately one week before the hyphae were scraped and placed into cetylrimethylammonium bromide (CTAB) extraction buffer (2% CTAB, 100 mM Tris, pH 8.4, 10mM EDTA, and 0.7 M NaCl) (Gardes and Bruns, 1993). The resultant mixture was incubated at 65˚C for one hour. An equal volume of chloroform was then added to the mixture prior to it being centrifuged for 30 minutes. The extracted supernatant was transferred to a separate tube and the DNA was precipitated using 3M NaOAc and 70% isopropanol. After the mixture was centrifuged for another 30 minutes, the supernatant was poured out and the pellet cleaned with an ethanol rinse. TE buffer (10mM Tris, 1mM EDTA, 8 pH) was used to resuspend the pellet. The suspension was then incubated for an hour at 65˚C. The genomic DNA mixture was purified by the addition of RNase and one hour of heating at 35˚C. Five microliters of each DNA extraction were mixed with 0.5 µl of the nucleic acid stain SYBR Green I (Molecular Probes, Eugene, OR, USA) in TE, separated in a 0.8 % agarose gel, and visualized under UV light.

The gene encoding for the mating-type was amplified by the polymerase chain reaction (PCR) using these primers: L1 (5'- ATGAGAGCCTCATCAACGGCA) and L2 (5'- ACAGGATGTAGGCATTCGCAGGAC) for MAT 1-1 and T1 (5' ACAAGGCAACCATCTGGACCCTG) and T2 (5'-CCAAAACACCGAGTGCCATCAAGC) for MAT 1-2 (Tredway, 2003). Two microliters of Genomic DNA was used as the template in a 20 µL PCR mixture (Thermo Pol Buffer, 10mM dNTP mix, 5 µM of each primer, and TAQ polymerase) using a Mycycler (BioRad) thermalcycler. The mixture was subjected to 30 cycles of amplification. Thermocycling conditions consisted of an initial hold at 94˚C for 3 minutes, followed by 30 cycles of 94˚C (30 sec), 60˚C (30 sec), and 72˚C (1 min), and a final hold of 72˚C for 8 min. The PCR products were then stained and visualized as previously described using a 1.5% agarose gel. Any genomic DNA that was not amplified by the MAT 1-1 primer was processed again with the MAT 1-2 primer.

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