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Comparative Analysis of the Genomes of Three Field Isolates of the Rice Blast Fungus *Magnaporthe oryzae* from Southern China

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Abstract

Rice blast caused by *Magnaporthe oryzae* (*M. oryzae*) is one of the most destructive diseases, which causes significant rice yield losses and affects global food security. To better understand genetic variations among different isolates of *M. oryzae* in the nature field, we re-sequenced and analyzed the genomes of three field isolates, QJ08-2006, QJ10-10, and QJ10-3001, which showed distinct pathogenicity on Xin-Yin-Zhan, an elite variety in South China. Genome annotation indicated that these three isolates assemblies have similar genome sizes with 38.4 Mb, 38.3 Mb, and 38.4 Mb, respectively. The QJ08-2006 assembly has 2082 contigs with an N50 of 127.4 kb, the QJ10-10 assembly has 2239 contigs with an N50 of 105.13 kb, the QJ10-3001 assembly has 2025 contigs with an N50 of 133.16 kb. A total of 10,432 genes including 1408 putative secreted protein genes were identified from the annotated isolate QJ08-2006 genome, 10,418 genes including 1410 putative secreted protein genes were identified in QJ10-10, and 10,401 genes including 1420 putative secreted protein genes were identified in QJ10-3001. There are as many as 11,076 identical genes in these three isolates and contained only a few unique genes among three isolates, of which 277 unique genes in QJ08-2006 and 264 unique genes in QJ10-10, and 213 unique genes in QJ10-3001. Most of the predicted secreted protein genes had been identified, and the three re-sequenced strains contained 371, 369, and 387 small Indel, respectively. *Avr* genes were analyzed in several sequenced *Magnaporthe* strains, the results revealed that *Avr-Pi9* and *Avr-Piz-t* were present in all the sequenced isolates. The isolates QJ08-2006 contained *AvrPib*, QJ10-10, and QJ10-3001 had an insertion of a Pot3 element in the promoter of the *AvrPib* gene. Our

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results showed that, the rapid dominancy of virulence mutant isolates via clonal propagation displayed in the field after the release of the elite variety Xin-Yin-Zhan.

Keywords

Rice Blast, *Magnaporthe oryzae*, Comparative Genomics, Genetic Variation, Secreted Protein Genes

1. Introduction

Rice (*Oryza sativa*) is one of the major staple food crops for more than half of the human population worldwide [1]. However, a large portion of yield is lost due to agricultural disease and pests annually [2]. Rice blast, caused by the filamentous ascomycete fungus *Magnaporthe oryzae* (syn. *Pyricularia oryzae*), occurs in every rice-growing regions in worldwide [3] [4], and poses a major threat to global food security [5] [6]. This pathogen can infect leaves, stems, panicles, nodes, and even roots of rice at the whole stage of development and also infects wheat and other small grains [7] [8] [9]. As genomes of *M. oryzae* have been sequenced and the data of the genomes is available to the public [10], rice and *M. oryzae* have been developed as the classical model system for studying the plant-microbe interactions [11].

In many parts of the world, deployment of disease-resistance varieties is an economical and efficient strategy for the management of rice blast [2]. However, *M. oryzae* often can overcome rice resistance based on race-specific resistant genes [12]. New resistant varieties often lose their resistance within a few years after release to the fields. High genetic variations occur within the field *M. oryzae* populations, especially in the pathogenic traits *i.e.*, secreted pathogen avirulence or effectors evolve rapidly to counteract plant defense, maybe a major challenge to control rice blast disease [13].

The advent of whole-genome sequencing technology and the subsequent deployment of the next generation sequencing (NGS) technology, genome re-sequencing, comparative studies, and the deployment of PacBio sequencing technology, has been reported in *M. oryzae* in the last decade [14] [15]. More than 200 isolates of *M. oryzae* have been successfully generated genome assemblies using high throughput genome sequencing technologies [10] [14] [15]. The genome of the laboratory *M. oryzae* strain 70 - 15 was the first to be sequenced among plant-pathogenic fungi using the Sanger sequencing method [10]. Subsequently, multiple *M. oryzae* field isolates have been sequenced using NGS technology. Such as, field isolates from Japan (P131 and Ina168) and China (Y34) were sequenced using 454 sequencing technology [16] [17]. More recently field isolates FJ81278, HN19311, and 98-06 from China, isolates B157, MG01, and RML-29 from India, and isolates R88-002, Ro1-1, and V86010 from the Philippines, and

100 isolates of *M. oryzae* (*P. oryzae*) from China as well as several different regions around the world have been sequenced using Illumina high-throughput sequencing technology [15] [18] [19] [20] [21] [22]. To generate near-complete genome assembly for *M. oryzae* field isolates and evaluated the possible contribution of TEs to genomic variation events and virulence-associated secreted proteins polymorphism, field isolates FJ81278 and Guy11 have been sequenced by PacBio sequencing technology [14]. The comparison genomics analysis reported some isolate-specific genomic regions, genes under diversifying selection, hundreds of unique genes, and the specific events in the evolution of field populations of *M. oryzae* [15] [17]. These reports suggest the NGS technology is a powerful tool to elucidate the molecular basis of virulence, study pathogen genome variation, and provide a new method for cloning novel avirulence genes.

Genome-wide studies of more blast field isolates and identification of novel *Avr* effectors will help researchers to further disclose the mechanism of pathogen and plant co-evolution. QJ08-2006, QJ10-10, and QJ10-3001 were three field *M. oryzae* isolates in South China, which showed different pathogenicity on a released resistant variety Xin-Yin-Zhan. In this study, we investigated the genomic variations and DNA polymorphisms of three *M. oryzae* field isolate by NGS technology in an attempt to understand the genomic variation within field isolates after release of the resistant variety. This work will be useful for breeders and pathologists to understand the variation of *M. oryzae* virulences and develop strategies to improve the resistance of varieties against rice blast.

2. Materials and Methods

2.1. *M. oryzae* Field Isolates Used

The field *M. oryzae* isolates QJ08-2006 was collected from cv. Ma-Ba-Yin-Zhan (MBYZ), the isolates QJ10-10, and QJ10-3001 were collected from cv. Xin-Yin-Zhan (XYZ). The newly improved blast-resistant rice variety of XYZ was developed using the susceptible elite variety, MBYZ, as the recurrent line. All these three isolates were collected from the panicle neck of rice plant samples at Guangdong Province, China in the year 2008 and 2010, respectively, and have been kept at Guangdong Academy of Agricultural Sciences (GDAAS, China).

2.2. Genomic DNA Extraction and Genome Sequencing

Isolates of *M. oryzae* were cultured in liquid complete medium (CM: 6 g yeast extract, 6 g casein hydrolysate, and 10 g sucrose in 1 L of medium) to prepare the vegetative mycelia for the extraction of genomic DNA [23]. Vegetative mycelia were harvested and ground into a fine powder in liquid nitrogen. The genomic DNA of each *M. oryzae* isolate was extracted following a protocol described [24]. The whole-genome sequencing libraries of the three selected iso-

lates, QJ08-2006, QJ10-10, and QJ10-3001, were prepared using the Illumina paired-end DNA sample Prep Kit and sequenced by Illumina Hiseq2000 sequencing technology at BGI Tech (Shenzhen, China). Approximately 2 Gb clean reads from the library averaging 500 bp in size were obtained for these three sequenced isolates.

2.3. Genome Assembling

Short reads generated were mapped to the *M. oryzae* reference genome 70 - 15 (version 8, *Magnaporthe* Comparative Sequencing Project, Broad Institute of Harvard and MIT). *De-novo* genome sequence assembly was conducted with CLC Genomic Workbench 7.0 software.

2.4. Gene Prediction and Annotation

The genomic sequences of three re-sequenced isolates were filtered by using Repeatmask 3.3 (http://www.repeatmasker.org, species = "*magnaporthe_grisea*"), and repeat sequences used were from RepBase (http://www.girinst.org/). Gene predictions were conducted through the Fgenesh model of Molquest (species, *magnaporthe*; sequence length, more than 20 aa) [21]. Masked genome sequences of QJ08-2006, QJ10-10, and QJ10-3001 were compared using the MUMMER package to construct the chromosome sequence for each isolates, respectively based on the reference 70 - 15 genomic sequence [25]. Protein domain was predicted using SMART database. We used SignalP4.0, Protcomp-AN, and TMHMM2.0 package to predict secreted protein genes of *M. oryzae* [26] [27].

3. Results

3.1. Genome Sequencing and Assembly of the Genomes of QJ08-2006, QJ10-10, and QJ10-3001

Genomic DNA of isolates QJ08-2006, QJ10-10, and QJ10-3001 were prepared as paired-end libraries and sequenced by using the Illumina HiSeq2000 system at BGI Tech (Shenzhen, China). In total, 1860 Mb, 1810 Mb, and 1810 Mb of clean data for QJ08-2006, QJ10-10, and QJ10-3001 were generated, respectively, which derived from paired-end sequence libraries with an average fragment length of 500 bp in size (**Table 1**). The genomes of these three isolates were *de-novo* assembled using CLC Bio genomic workbench software and annotated using a standard procedure as described previously [28]. As showed in **Table 1**, these three assemblies resulted in similar genome size of 38.4 Mb, 38.3 Mb, and 38.4 Mb, respectively. The QJ08-2006 assembly had 2082 contigs with an N50 of 127.4 kb, the QJ10-10 assembly had 2239 contigs with an N50 of 105.13 kb, the QJ10-3001 assembly had 2025 contigs with an N50 of 133.16 kb. However, the genomic features of these three isolates described herein showed much more distinct in most features from other isolates reported previously, including 98-06, P131, Y34, FJ81278, HN19311, R88-002, and 70-15 (**Table 1**).

Table 1. Genome assembly and annotation statistics from ten *M. oryzae* isolates.

	Sequencing platform	QJ08-2006	QJ10-10	QJ10-3001	98-06	P131	Y34	FJ81278	HN19311	R88-002	70-15
		Illumina	Illumina	Illumina	Illumina	Sanger + 454	Sanger + 454	PacBio	Illumina	Illumina	Sanger
Genome	Assembly genome size (Mb)	38.41	38.28	38.40	45.3	37.95	38.87	43.85	37.1	39.08	40.95
	Coverage (fold)	50X	50X	50X	135X	20X	21X	247X	–	109X	–
	Contig Number	2082	2239	2025	1161	2601	2114	54	6249	–	219
	Average contig length (kb)	18.45	17.09	18.96	–	–	–	811.97	–	–	186.99
	N50 contig length (kb)	127.4	105.13	133.16	88.6	37.7	53		147.4	–	
	G+C content (%)	51.40	51.49	51.39	50.8	51.5	51.3	51.03	51.5	–	51.61
	Repeat sequence	2.28%	2.22%	2.28%	9.30%	2.26%	2.54%	14.43%	2.83%	2.46%	11.10%
Genes	Number of predicted genes	10,432	10,418	10,401	14,030	12,722	12,869	11,927	10,256	12,344	12,440
	Average gene length (bp)	1532	1538	1535	1410	1334	1332	–	1745	–	1356
	Number of secreted protein genes	1408	1410	1420	1732	1005	998	–	–	1008	1005

3.2. Genome Annotation and Gene Prediction

A total of 10,432 genes (≥20 aa) including 1408 putative secreted protein genes were identified from the annotated isolate QJ08-2006 genome, a total of 10,418 genes (≥20 aa) including 1410 putative secreted protein genes were identified from the annotated isolate QJ10-10 genome, and a total of 10,401 genes (≥20 aa) including 1420 putative secreted protein genes were identified from the annotated isolate QJ10-3001 genome (Table 1). The average length of predicted genes was ~1530 bp. Intriguingly, these 3 isolates showed quite similar features, e.g., genome size, number of predicted genes, and secreted protein genes. By contrast, they were distinct from other sequenced isolates in most features (Table 1).

3.3. Identification of Presence/Absence Variation in Different Isolates

To identify the unique genes in the three field isolates, the genomic content was compared to each other. To avoid false-positive gene predictions, the genes from each of the three gene sets (QJ08-2006, QJ10-10, and QJ10-3001) were aligned to genome sequences of the other two isolates using FASTA [29]. As showed in Figure 1, there were as many as 11076 identical genes in these three isolates and contained only a few unique genes among each isolates. There were 277 unique genes in QJ08-2006 and 264 unique genes in QJ10-10, and 213 unique genes in QJ10-3001 (Figure 1). To gain a functional annotation of the unique genes in isolates QJ08-2006, QJ10-10, and QJ10-3001, the gene sets were annotated by predicting secreted proteins. A small number of the secreted proteins were identified from the unique gene set: 16 (5.78%) in QJ08-2006, 31 (11.74%) in QJ10-10,

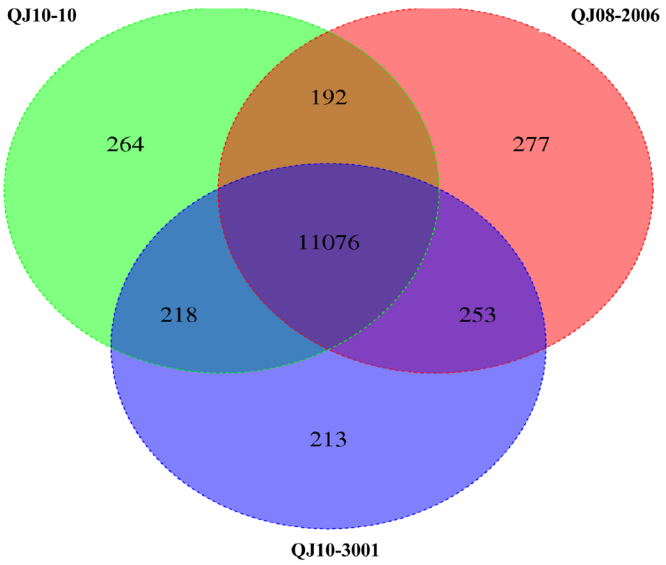

Figure 1. Venn diagram showing the presence/absence variation of the gene set from there sequenced isolates.

and 19 (8.92%) in QJ10-3001, showing that there are few of the secreted proteins in a unique gene set.

3.4. Analysis of Avirulence Genes in Some Sequenced *Magnaporthe*

We had compared *Avr* genes in some sequenced *M. oryzae* strains including 70-15, QJ08-2006, QJ10-10, and QJ10-3001 (Table 2). The avirulence gene, *Avr-CO*39 was absent in all of the analyzed strains (Table 2). *Avr-Pi*9 and *Avr-Piz-t* were present in all sequenced strains. *Avr-Pii* was absent in all of the analyzed strains except the strain P131 (Table 2). Compared with QJ10-10, QJ10-3001, the strain QJ08-2006 has a unique secreted protein gene, *AvrPib*, while the isolates QJ10-10, and QJ10-3001 had an insertion of a Pot3 element in the promoter of the *AvrPib* gene, which has led to a gain in virulence towards rice varieties carrying the *R* gene, *Pib*. We validated in silico analyzed host specificity factors and *Avr* genes by PCR amplification using gene specific primers [30]. This validation confirmed the accuracy of prediction of the presence and absence of the aforementioned *Avr* genes in QJ08-2006, QJ10-10, and QJ10-3001 (data not show).

3.5. Comparative Genome Analysis Based on Coding Genes between Three Re-Sequenced Isolates

To confirm the relationship between QJ08-2006, QJ10-10, and QJ10-3001, we compared the sequence of the predicted gene and secreted protein genes between three re-sequenced isolates with gene-genome model and Blastn alignment (-max_target_seqs 2-outfit 7, the rest are default), five kinds of variations had been identified, of which were identical (Identity), SNP, small Indel (Sindel),

Table 2. Presence/absence variation of cloned avirulence (*Avr*) genes among sequenced strains of *M. oryzae*.

Avirulence (*Avr*) genes	*M. oryzae* strains									
	QJ08-2006	QJ10-10	QJ10-3001	70-15	Guy11	FJ87278	Y34	P131	98-06	GD-05-029b
Avr-Pik	P	P	P	P	P	P	P	A	P	P
Avr1-CO39	A	A	A	A	A	A	A	A	A	A
Avr-Pi9	P	P	P	P	P	P	P	P	P	P
Avr-Pia	A	A	A	A	A	A	P	P	A	A
Avr-Pib	P	Partial	Partial	P	P	P	P	P	P	P
Avr-Pii	A	A	A	A	A	A	A	P	A	A
Avr-Pita	P	A	P	P	P	P	P	P	P	A
Avr-Piz-t	P	P	P	P	P	P	P	P	P	P

Note: P indicates presence, A indicates absence, Partial indicates partial of avirulence gene can be detected, mostly interrupted by TE insertion in promoter region.

Table 3. Comparative analysis of genes and secreted protein genes between three re-sequenced isolates.

Type of comparative[1)]	Identity	SNP	Sindel	Lindel	Unmapped
Genes					
QJ08-2006 VS QJ10-10	7541	69	4362	10	11
QJ08-2006 VS QJ10-3001	7567	63	4351	11	1
QJ10-10 VS QJ08-2006	7518	72	4357	7	2
QJ10-10 VS QJ10-3001	7574	48	4326	7	1
QJ10-3001 VS QJ08-2006	7535	60	4347	9	1
QJ10-3001 VS QJ10-10	7557	43	4334	7	11
Secreted protein genes					
QJ08-2006 VS QJ10-10	1027	4	371	1	5
QJ08-2006 VS QJ10-3001	1033	4	369	2	0
QJ10-10 VS QJ08-2006	1013	6	390	1	0
QJ10-10 VS QJ10-3001	1017	6	387	0	0
QJ10-3001 VS QJ08-2006	1029	5	384	2	0
QJ10-3001 VS QJ10-10	1030	5	378	2	5

[1)]Sindel: Small Indel, the sequence length difference is less than 60 bp, Lindel: Large Indel, the sequence length difference is more than 60 bp.

Large Indel (Lindel), and not present (Unmapped). As showed in Table 3, about 7500 predicted genes were identified between QJ08-2006, QJ10-10, and QJ10-3001, these three isolates contained 4362, 4351, and 4326 small Indel, respectively. And there were existed several SNP, large Indel, and unmapped sequences between these three isolates. Most of the predicted secreted protein genes were identified, and the re-sequenced isolates contained 371, 369, and 387 small Indel, respectively. The above results indicated that the relationship be-

tween QJ08-2006, QJ10-10, and QJ10-3001 was very closest.

4. Discussion

In several eukaryotic organisms, comparative analysis of multiple genomes of the same species has been used to improve assembly and annotation and to identify genome variations [31] [32] [33]. The rice blast fungus is well-known for its natural genetic variation in the field, resulting in the emergence of new epidemics threatening to world food production [2] [7]. Genomic comparative analyses of multiple genomics in the same pathogens not only could improve the steady of the genome assembly and annotation, and the identification of variations in the plant pathogens' genome, but also open a door to explore new virulence mechanism of the pathogen for effective plant disease control [20]. In this study, we sequenced three field isolates of *M. oryzae* from Southern China. These three field isolates were sampled from the varieties Ma-Ba-Yin-Zhan or Xin-Yin-Zhan, which have been cultivated in the rice area of northern Guangdong for a long time [30]. Genome analysis indicated that these re-sequenced field isolates were much more closely related to each other, the overall genome content and composition were similar among these three isolates, indicated that the rice blast fungus maintained a stable genomic mutation under the pressure of resistant varieties selection.

By the pathotype assays, we found QJ08-2006 was avirulent to the rice variety Xin-Yin-Zhan, while both the strain QJ10-10 and QJ10-3001 were impressively virulent to Xin-Yin-Zhan [30]. Comparative genomics found that the sequenced strains have their unique sequences, suggesting some pathogenic-related sequences may exist outside core genome regions [19]. The whole-genome re-sequenced results showed that these three strains showed quite similar features, e.g., genome size, number of predicted genes, and secreted protein genes, showed that they have a closer relationship with each other. And there were as many as 11,076 identical genes in these three isolates and contained only a few unique genes in each isolate (**Figure 1**). Among them, a small number of the secreted proteins were identified from the unique gene set: 16 (5.78%) in QJ08-2006, 31 (11.74%) in QJ10-10, and 19 (8.92%) in QJ10-3001, showing few of the secreted proteins present in the unique gene set. These data suggested that little genetic differences exist between the field isolates QJ08-2006, QJ10-10, and QJ10-3001.

Yoshida *et al.* (2009) observed that the presence or absence of effector gene polymorphisms were often associated with unstable genomic regions near the chromosome ends. It was therefore hypothesized that isolate-specific regions located at the chromosomal ends supplied new effectors and pathogenicity-related factors to drive genome evolution. The dynamic adaptation of *M. oryzae* may be primarily achieved by the deletion or recovery of *Avr* genes [34]. Such hypotheses indicate that further genome sequencing of multiple *M. oryzae* isolates is warranted for characterizing rice pathogenic strains [16]. Most of the cloned *Avr* genes of *M. oryzae* reflected the important role of transposable elements (TEs) in

variations in pathogenicity and the loss of function of *Avr* genes in *M. oryzae* [14] [21] [35]. In our study, the effector *AvrPib* also showed polymorphisms in these three re-sequenced isolates. The isolates QJ10-10, and QJ10-3001 had an insertion of a Pot3 element in the promoter of the *AvrPib* gene, which has led to a gain in virulence towards rice varieties carrying the *R* gene, *Pib* [30]. The Pot3 element inserted in the *AvrPib* promoter of QJ10-10 and QJ10-3001 suggested this gene is undergoing positive selection, which probably enables these isolates to overcome the resistant rice varieties that harbor *AvrPib* alleles recently. The loss of function of *AvrPib* was also identified in 300 isolates collected in five different rice-growing areas in China, and a Pot3 insertion in various positions of −260 bp, −236 bp, −128 bp, −52 bp, −22 bp, and +170 bp, leading to the loss of avirulence function of *AvrPib* in the five populations [36]. In the 248 virulent isolates collected from a disease hotspot in Philippine, Pot3 was present at the *AvrPib* region of −304 bp, −125 bp, and +169 bp in each of avrPib-H1, −H2, and −H3 isolates [37]. Similarly, some researchers reported that the diversification in the genome location of *Avr-Pita* was the consequence of recognition by *Pita* in rice [12] [34] [38].

To date, only 12 *Avr* genes have been cloned in *M. oryzae*. By contrast, more than 28 *R* genes in rice controlling race- and not race-specific resistance to *M. oryzae* have been cloned [39] [40]. In the traditional "gene-for-gene" model, resistance (*R*) genes in the host specifically recognize corresponding avirulence (*Avr*) genes of the pathogens. Recognition is followed by triggering a hypersensitive response (HR) [41]. Thus, there were a lot of potentials to identify and clone *Avr* genes in *M. oryzae*. Before 2009, only the *PWL* family of three members *PWL*1, *PWL*3 and *PWL*4 were cloned by homology to *PWL*2 [42], other virulence genes mainly were cloned by map-based cloning, such as *PWL*2, *Avr-Pita*, *ACE*1, *Avr1-CO*39, *Avr-Pizt*, *Avr-Pia*, *Avr-Pii*, *AvrPib* [12] [35] [36] [43] [44]. With the rapid development of biotechnology and high-throughput sequencing technology, *Avr-Piks*/*Km*/*Kp*, *Avr-Pi*9 and *Avr-Pi*54 were cloned by using the method of comparative genomics. Due to the instability of the rice blast fungus genome, and that *Avr* proteins lack conserved domains, the map-based cloning, homology cloning and other traditional cloning strategies were not be effectively utilized. Therefore, whole-genome sequencing of more field blast isolates could accelerate cloning more *Avr* genes and obtain more information about effector proteins.

Overall, this study provided a systematic genomic analysis of the rice blast field isolates QJ08-2006, QJ10-10, and QJ10-3001, collected from the same field in different years in southern China. Based on the analysis of the three isolates genome, gain, loss and changes of isolate-specific sequences will lead to variances of pathogen virulence. We found that under resistant rice selection pressure for a long time, the genome of avirulent isolate and virulent isolate was very similar, while the changes tend to take place in the genetic variation of avirulence genes corresponding to their resistance genes.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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