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Mating type and genetic diversity analysis of *Pyricularia oryzae* collected from Thai rice varieties

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Abstract Rice blast disease caused by *Pyricularia oryzae*, is one of the most damaged of rice production worldwide. The fungi are a great diversity on both genotype and pathotype. The genetic relationship of *P. oryzae* collected in Thailand during year 2016 and 2017 was analyzed. Rice plants appeared blast symptom were collected from epidemic areas and was isolated by single spore. All isolates were identified for mating type using MAT1-1 and MAT1-2 primer sets. In this result, the mating type MAT1-2 was mostly found in Thailand and only about 7.7 % of MAT1-1 was found in the population. In this observation, both MAT1-1 and MAT1-2 were found from isolates collected from the same location in Phetchabun province. Therefore, the genetic analysis of 14 *Magnaporthe grisea* using microsatellite (MGM) markers was cluster-analyzed using UPGMA method of the SHAN program. The result showed cluster analysis was separated the population into 11 groups. Group 1 to 7 showed the percentage of the population with 3.8, 5.8, 36.5, 13.5, 3.8, 25.1 and 3.8, respectively. For group 8-11, the rice blast pathogen from North and South of Thailand were separated into single isolate group. Group 6 was the highest diversity of province numbers which obtained from diverse rice varieties that showed similar genotype but difference in pathogenicity. The difference isolates of fungi could infect the same rice variety. Genetic diversity and determination of mating type of rice blast populations in Thailand are needed to study and useful for predicting an epidemic of rice blast disease and selecting the appropriate isolates for blast breeding programs.

Keywords: rice blast disease, *Pyricularia oryzae*, genetic diversity analysis, *Magnaporthe grisea* microsatellite (MGM) markers

Introduction

Pyricularia oryzae is the rice blast pathogen which caused of the mostly damaging of important rice-growing areas in Thailand. In present, the fungus is capable of rapid adaptation due to specific varietal resistant to rice blast disease in paddy field by the farmers (Gallet *et al.*, 2015). The genetic mutation are caused by changing, adding or deleting of nucleotide at gene positions

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associated with pathogenicity to produce new genes in pathogen could make to overcome resistant-rice cultivars (Chiapello *et al*., 2015) and therefore affected to genetic diversity in population of the pathogens. In addition to adaptation, the different rice cultivation systems and rice varieties including the geography of each growing region are another importance factors that caused genetic diversity of rice blast populations (Gallet *et al*., 2015). Moreover, the genetic diversity of fungi can occur by sexual reproduction itself, rice blast fungus can reproduce by both sexual and asexual reproductions. The sexual reproduction blast fungus (sexual stage refers to as *Magnaporthe oryzae*) that requires two strains with difference mating types, MAT1-1 and MAT1-2, However, few isolates with MAT1-1 type are found in the Northern of Thailand in year 1999 to 2006 (Mekwatanakarn *et al*., 1999; Saleh *et al.*, 2012; Saleh *et al.*, 2014). Therefore, the aimed of this study was to analyze genetic diversity using DNA marker and determine the mating type of rice blast populations in Thailand. The studies would useful for predicting an epidemic of rice blast disease for breeding programs.

Materials and methods

Isolation and mycelia preparation

Rice blast samples were collected from blast infected field in Thailand, during year 2016 and 2017. Rice leaves and necks were isolated for fungal conidia using single spore isolation technique and grown on Rice Flour Agar (RFA, 20 g rice flour, 2 g yeast extract, 20 g agar and 1000 ml water) with filter paper put on top of medium for storing fungal mycelium as described by Sirithunya *et al*. (2007). The filter paper containing fungal mycelium was raised on RFA for 4 days and the edge of fungal colony was transferred to Potato Dextrose Broth (PDB) using 0.5 mm diameter cork borer. PDB with fungal mycelia was shaken at 1000 rpm and 28 ℃ for 4 days.

DNA extraction and determination of mating type

The mycelium was separated from medium using sterilized filter paper and grind into powder with liquid nitrogen. DNA was extracted using Plant DNA Extraction Kit (VIVANTIS, Malaysia) and kept at 4 ℃. The DNA samples of the *P. oryzae* 70-15 (MAT1-1) and GUY11 (MAT1-2) strains were extracted and used as reference strains in this study. The mating type was determined by PCR amplification using two pairs of specific primers, MAT1-1 and MAT1-2 primer sets which each amplifying a fragment of 809 bp and 940

bp, respectively (Table 2). PCR was performed in 10 µl reaction mixtures containing sterile water, 1X *Taq* buffer with with $(NH_4)_2SO_4$, 2.5 mM of $MgCl_2$, 0.5 mM of dNTPs, 0.5 mM of primers, 0.5 U/µl of *Taq* DNA polymerase (Thermo Scientific, Lithuania) and 10 ng of genomic DNA. The thermal cycling conditions consisted of an initial denaturation at 94℃ for 1 min followed by 35 cycles of 94℃ for 30 sec, 55 ℃ for 1 min and 72℃ for 1 min, and a final extension at 72℃ for 10 min. All PCR products were verified by 1 % agarose gel electrophoresis analysis.

Magnaporthe grisea microsatellite (MGM) markers analysis

All isolates were genotyped using 14 *Magnaporthe grisea* microsatellite (MGM) markers (Table 2) by PCR. The PCR was performed in 10 µl reaction mixtures containing sterile water, 1X of Terra PCR direct buffer (with Mg^2+, dNTP), 0.3 mM of primers, 1 U/µl of Terra PCR direct polymerase mix (TaKaRa, China) and 10 ng of genomic DNA. The thermal cycling conditions consisted of an initial denaturation at 95℃ for 10 min followed by 35 cycles of 95℃ for 20 sec, 55 ℃ for 15 sec and 72℃ for 30 sec, and a final extension at 72℃ for 6 min. PCR products were verified with Polyacrylamide Gel Electrophoresis (PAGE) using 6 % polyacrylamide gel as described by Benbouza *et al*. (2006).

Clustering and Genetic diversity analysis

The alleles appeared for each MGM marker were scored based on number of bands in each isolates at specific fragment size. The similarity score was generated using Canberra similarity coefficient in the SimInt program and the cluster analysis was performed using the unweighted pair group method with arithmetic average (UPGMA) in the SHAN program of the NTSYSpc version 2.10p (Rohlf, 2004). The polymorphism level of each MGM marker was indicated by its polymorphism information content (PIC) value calculated with the formula:

$$PICi = 1 - \sum_{j=1}^{n} P^2ij$$

where *Pij* is the frequency of the *j* allele of *i* marker and *n* is the number of alleles.

Results

Isolation and mating type detection

Leaf and neck blast samples were collected from 19 provinces in the North, North East, Central, West and South of Thailand. Fifty-two isolates were isolated from rice cultivars using single spore isolation technique (Table 1). The product of PCR obtained from primer pairs revealed that 48 isolates belonged to MAT1-2 with their fragment size was similar to the reference strain, Guy11. The 4 remaining isolates included PRE59006.2, PNB59003.1, PNB59003.3 and PNB60001 showed a fragment size similar to *P. oryzae* 70-15 which therefore these isolates were MAT1-1. These 4 isolates were obtained from Phrae and Phetchabun provinces in North of Thailand (Table 1 and Figure 1).

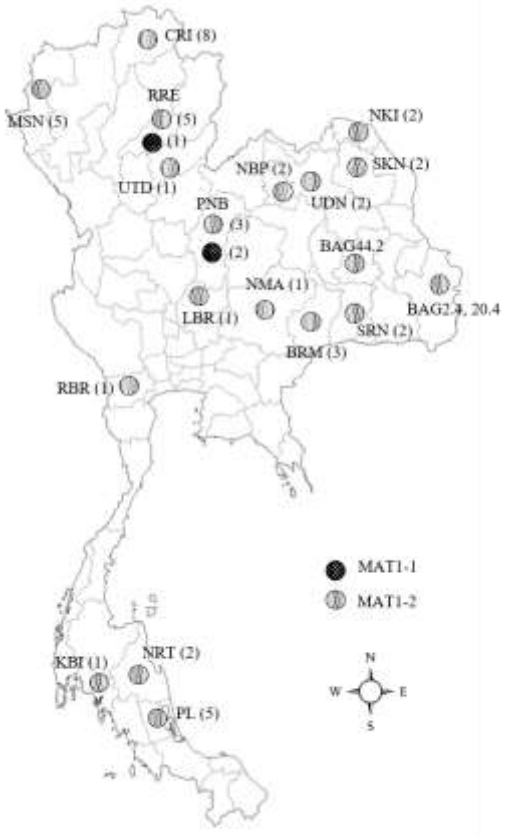

Figure 1. Mating types of 52 blast isolates obtained from 19 provinces of Thailand, MAT1-1 were indicated in black and MAT1-2 in white

Table 1. Rice blast isolates, their host variety, mating type and collecting place in 2016 (N0.1- 19) and 2017 (No.20-52)

No.	Isolates	Host	Province	Region	Mating type
1	BAG2.4	KDML105	Ubon Ratchathani	Northeast	MAT1-2
2	BAG20.4	KDML105	Ubon Ratchathani	Northeast	MAT1-2
3	BAG44.2	KDML105	Roi Et	Northeast	MAT1-2
4	CRI59001	RD6	Chiang Rai	North	MAT1-2
5	CRI59002.1	RD14	Chiang Rai	North	MAT1-2
6	CRI59004.1	Sanpatong 1	Chiang Rai	North	MAT1-2
7	CRI59005	-	Chiang Rai	North	MAT1-2
8	LBR59003.1	-	Lop Buri	Central	MAT1-2
9	PNB59001.4	-	Phetchabun	North	MAT1-2
10	PNB59003.1	-	Phetchabun	North	MAT1-1
11	PNB59003.2	-	Phetchabun	North	MAT1-2
12	PNB59003.3	-	Phetchabun	North	MAT1-1
13	PRE59001	Suphanburi 1	Phrae	North	MAT1-2
14	PRE59003	Phitsanulok 2	Phrae	North	MAT1-2
15	PRE59004.1	RD53	Phrae	North	MAT1-2
16	PRE59006.2	RD6	Phrae	North	MAT1-1
17	PRE59008.1	KDML105	Phrae	North	MAT1-2
18	RBR59001	-	Ratchaburi	West	MAT1-2
19	UTD17002	-	Uttaradit	North	MAT1-2
20	BRM60001	KDML105	Buri Ram	Northeast	MAT1-2
21	BRM60012.2	KDML105	Buri Ram	Northeast	MAT1-2
22	BRM60011.1	KDML105	Buri Ram	Northeast	MAT1-2
23	CRI60001.1	RD6	Chiang Rai	North	MAT1-2
24	CRI60003.2	RD6	Chiang Rai	North	MAT1-2
25	CRI60004.1	RD6	Chiang Rai	North	MAT1-2
26	CRI60007	KDML105	Chiang Rai	North	MAT1-2
27	KBI60001	Pathumthani 1	Krabi	South	MAT1-2
28	MSN60001	Hahngyi 71	Mae Hong Son	North	MAT1-2
29	MSN60002	Sanpatong 1	Mae Hong Son	North	MAT1-2
30	MSN60006.1	RD6	Mae Hong Son	North	MAT1-2
31	MSN60009.1	RD15	Mae Hong Son	North	MAT1-2
32	MSN60012	RD15	Mae Hong Son	North	MAT1-2
33	NBP60001	RD6	Nong Bua Lam Phu	Northeast	MAT1-2
34	NBP60002	RD6	Nong Bua Lam Phu	Northeast	MAT1-2
35	NKI60001.1	RD6	Nong Khai	Northeast	MAT1-2
36	NKI60001.2	RD6	Nong Khai	Northeast	MAT1-2
37	NMA60001	-	Nakhon Ratchasima	Northeast	MAT1-2
38	NRT60001	RD41	Nakhon Si Thammarat	South	MAT1-2
39	NRT60002	RD41	Nakhon Si Thammarat	South	MAT1-2
40	PL60001	RD41	Phatthalung	South	MAT1-2
41	PL60004	KDML105	Phatthalung	South	MAT1-2
42	PL60009	Sungyod	Phatthalung	South	MAT1-2
43	PL600010	Sungyod	Phatthalung	South	MAT1-2
44	PL600011	Riceberry	Phatthalung	South	MAT1-2
45	PNB60001	-	Phetchabun	North	MAT1-2
46	PRE60001	KDML105	Phrae	North	MAT1-1
47	SKN60001.1	-	Sakon Nakhon	Northeast	MAT1-2
48	SKN60003	-	Sakon Nakhon	Northeast	MAT1-2
49	SRN60001	RD15	Surin	Northeast	MAT1-2
50	SRN60005.1	KDML105	Surin	Northeast	MAT1-2
51	UDN60001.1	-	Udon Thani	Northeast	MAT1-2
52	UDN60005	RD6	Udon Thani	Northeast	MAT1-2

Microsatellites marker and Genetic diversity analysis

Genetic diversity analysis of 52 rice blast isolates using 14 *Magnaporthe grisea* microsatellite (MGM) markers. The result showed that polymorphism information content (PIC) value of MGM markers varied from 0.13 to 0.91 (Table 2). The best marker was MGM260 located on chromosome 6 of blast fungal genome. Cluster analysis was performed with the UPGMA of the SHAN program, the similarity coefficient at 80% separated the population into 11 groups. Group 1 to 7 showed the percentage of the population with 3.8, 5.8, 36.5, 13.5, 3.8, 25.1 and 3.8 respectively. Group 1 included blast isolates from the North, MSN60002 (Mae Hong Son) and CRI60003.3 (Chiang Rai), isolated from difference rice varieties, Sanpatong 1 and RD6, respectively. Group 2 contained isolates from Northeast and North (BRM60006.1 from Buri Ram, PNB59003.1 and PNB59003.3 from Phetchabun) isolated from difference rice varieties. Group 3 was the largest group contained 12 isolates from Northeast, 5 isolates from North and 2 isolates from South and they were isolated from difference rice varieties including RD6, RD15, KDML105 and Pathumthani 1. Group 4 contained 2 isolates from Northeast and 5 isolates from North and they were isolated from difference rice varieties including RD6, RD15, KDML105 and Hahngyi 71. Group 5 contained isolate CRI59002.1 (Chiang Rai) isolated from RD14 and NRT60001 (Nakhon Si Thammarat) form RD41, from the growing regions in the North and South respectively. Group 6 was another large group contained 1 isolate from West, 1 isolate from Central, 7 isolates from North and 3 isolates from South and they were from difference rice varieties including RD6, RD15, RD41, RD53, KDML105, Suphanburi 1, Phitsanulok 2, Sanpatong 1, Sungyod and Riceberry. Group 7 contained isolates from Northeast and North, BAG20.4 (Ubon Ratchathani) and PNR59003.2 (Phetchabun) respectively and they were from difference rice varieties. For group 8-11, the rice blast isolates from North and South of Thailand were separated into single isolate group included PNB59006.2, PNB60001, PL60001 and PL60010, respectively. In addition, 4 blast isolates that were identified as MAT1-1-mating type, PRE59006.2, PNB59003.1, PNB59003.3 and PNB60001 were in difference groups (Table 1 and Figure 2).

Discussion

Mating type and Genetic diversity analysis

In the North of Thailand, blast isolates from Phetchabun province belonged to both mating types. In this experiment, 3 isolates from the same blast lesion showed difference mating type including PNB59003.1 (MAT1-1),

PNB59003.2 (MAT1-2) and PNB59003.3 (MAT1-1). The blast fungus from Phrae also found both mating types but only one isolate was MAT1-1. It made possibility to sexual reproduction of rice blast fungus in Thailand. Rice blast isolates from the North of Thailand are reported for MAT1-1 and a female-fertility (Saleh *et al.,* 2012). Rice growing area found both mating type showed the origin of genetic diversity which caused by sexual reproduction (Tharreau *et al*., 2009; Saleh *et al.,* 2014). However, four MAT1-1 isolates were not grouped together when analyzed using MGM markers, therefore sexual reproduction may not the main cause of rice blast fungus mutation or diversity.

Genetic diversity analysis of 52 rice blast isolates collected from blast infected field in Thailand during year 2016 and 2017 resulted in 11 groups. In group 6 was the highest diversity of province numbers excepted province in Northeast. In addition, members of group 6 was obtained from diverse rice varieties. Thirteen isolates of this group were from 10 rice varieties which 4 varieties were resistant varieties (RD41, Suphanburi 1, Sanpatong 1 and Riceberry) and 6 were susceptible varieties (RD6, RD15, RD53, KDML105, Phitsanulok 2 and Sungyod). The fungi showed similar genotype but difference in pathogenicity. It explained that the fungi adapted itself to overcome the resistant gene in difference rice varieties. The low diversity of members in this group might come from nucleotide changing at gene position associated with host-pathogen interactions which important to fungal pathogenicity (Chiapello *et al.,* 2015).

Table 2. Sequence of the MGM marker and mating type primers used in this study and polymorphic information content (PIC) value

No.	Chr.	Name	Forward	Reverse	PIC
1	1	MGM447	GACTTGGACTCGGGTCTTGA	CCCTGTACACAAAATGCCTTG	0.38
2	1	MGM35	GTTGAATTACCTTTCGGACTGG	AAGGACTTTGCTCAGACCGTAG	0.70
3	2	MGM185	AATGCTTCGAGGTCCCAGT	GCTTATCGACGGCGTATTTG	0.42
4	2	MGM58	ATTACAGCGTGCACAACGAA	GAGGAGGGTGAAGGTTTCCT	0.15
5	3	MGM209	TCACCCTCAACTGCAGTCAT	GTTGCCGCTGTTGTTGAATA	0.66
6	3	MGM334	GACCCTGGTGGTAGGAGTGA	TCTTATCGTTGCAGCCAATG	0.61
7	4	MGM452	TTCTCAGTAGGCTTGGAATTGA	CTTGATTGGTGGTGGTGTTG	0.13
8	4	MGM87	GTCCACCGCTTAAACACTGC	CTCCACTCGCTATGCACGTA	0.18
9	5	MGM177	TGACTCGACCTGACATCTGC	TTCCTGGGACTGTTTCATGG	0.43
10	5	MGM255	CAAGCTTAACCCGACGGATA	TGTCACCGCAGTTGAAAGAC	0.74
11	6	MGM269	GATGGCCAGGTCAGCTTTT	ACTCTTTGCAGCCATGGAAC	0.72
12	6	MGM260	CTCCATTTCCCCCAGACTTT	ATCGTGGATTTTCGTGCAAC	0.91
13	7	MGM278	CCAACAAACAAATCGCTCAA	GCGACTTGTGCAGTTCGTAG	0.79
14	7	MGM286	CGGCTGTGGTTTAACGATTT	CCATCAGGATCCATGAACAC	0.73
15	-	MAT1-1	TCAGCTCGCCCAAATCAACAAT	ACTCAAGACCCGGCACGAACAT	-
16	-	MAT1-2	GAGTTGCCTGCCCGCTTCTG	GGCTTGGTCGTTGGGGATTGT	-

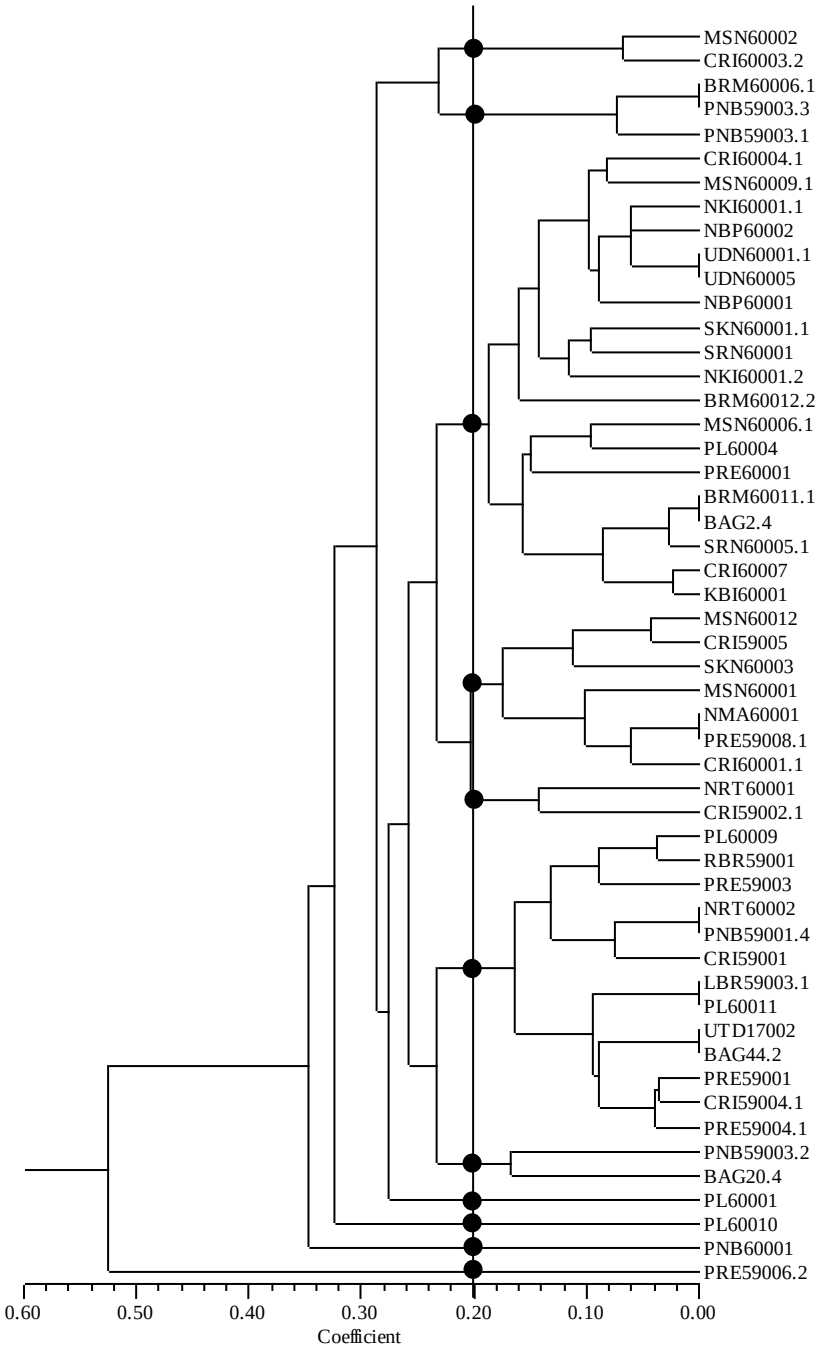

Figure 2. Dendrogram of 52 isolates of *P. oryzae* was analyzed using 14 *Magnaporthe grisea* microsatellite (MGM) markers and clustered with the UPGMA in the SHAN program of the NTSYSpc version 2.10p (Rohlf, 2004)

Rice blast fungi isolated from the same province were separated in difference groups. Isolates from Phetchabun and Phatthalung was high genetic diversity where 5 isolates from each province were separated into 4 groups. This diversity due to the adaption of fungi for different rice cultivation and rice varieties choosing by the farmers according to the geography of each province in the growing regions (Gallet *et al*., 2015). In addition, difference isolates of fungi could infect the same rice variety because these fungi are the same pathogenic gene which required for infection. PRE59008.1 and PRE59001 were collected from infected rice in 2016 and 2017 respectively, both were isolated from the same rice variety and location, but separated into difference groups.

The isolates collected from Phetchabun and Phrae province were found both MAT1-1 and MAT1-2 types. Sexual reproduction was one of the factors of genetic diversity of rice blast fungus (Tharreau et al., 2009; Konrad et al., 2012). In addition to sexual reproduction, the fungi is adapted by nucleotide sequence changes in gene positions associated with pathogenicity to overcome resistant gene in rice, different rice cultivation and rice varieties selection according to the geography of each province could become stress factors that caused genetic diversity of rice blast fungi in Thailand. Understanding of genetic diversity, population mating type and disease epidemic that obtained from this study is needed to improve the blast resistance variety.

Acknowledgement

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