Research Article

Genetic Diversity and Aggressiveness of *Bipolaris oryzae* in North-Central Thailand

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Abstract
One hundred and ten isolates of *Bipolaris oryzae*, the causal agent of rice brown spot disease were collected from paddy fields in four provinces of north-central Thailand, including Ang Thong, Chai Nat, Lop Buri, and Sing Buri. DNA polymorphism of some *Bipolaris oryzae* isolates was determined by VNTR, ISSR and RAPD markers. Only VNTR-MR primer showed different fingerprint patterns among these isolates, therefore this primer was selected to study genetic diversity of the *Bipolaris oryzae* population. In total, there were three haplotypes corresponding to the results from cluster analysis; each of the three clusters shared identical haplotype. The majority of the isolates were separated into group A (88.18%), indicating predominant asexual reproduction of clonal population. However, there was no relationship between haplotype and either collection provinces or aggressiveness on rice. Among four rice varieties tested, including Khao Dawk Mali 105 (KDML 105), RD31, Pathum Thani 1, and Jao Hom Nin (JHN), JHN was the most resistant variety, while KDML 105 was the most susceptible to brown spot disease.

Keywords: Rice, *Bipolaris oryzae*, Brown spot, Genetic diversity, Aggressiveness

1 Introduction

Rice is one of the leading staple food for more than half of the world's population and, by over the next 30 years, rice production in Asian countries is estimated to increase by 70% due to the increment of population growth [1]. Rice brown spot disease is caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker [telemorph = *Cochliobolus miyabeanus* (Ito and Kuribayashi) Drechs. ex Dastur.]. The disease is widespread throughout the rice growing areas worldwide [2] and is one of the main causes of the great Bengal famine in 1943 [3]. Furthermore, this disease causes substantial losses in grain yield both in quantitative and qualitative terms. The average of yield losses due to *B. oryzae* is about 10% whenever the disease occurs [4]; however, up to 90% of grain yield losses was also recorded [5]. Usually, the disease becomes more apparent in rice growing areas under nutrient deficiency condition [6]. The typical symptoms on rice leaves after *B. oryzae* infection show oval or cylindrical brown spots with grey center surrounded by yellow halo. Leaf spots may coalesce and later become necrotic. When *B. oryzae* infects rice glumes, it can cause discoloration of grains [5].

Despite the availability of rice brown spot disease management practices such as a proper plant nutrient supply, a use of appropriate fungicides and a use of biological control agents [2], [5], [7], planting rice brown spot resistant varieties is highly efficient and cost effective, compared to the others [8]. However, for

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development or deployment of the resistant varieties, it is necessary to have an understanding on pathogen population structure [9]. Therefore information on pathogen diversity is crucial in this regard. A high level of genetic diversity among *B. oryzae* isolates through the cluster analysis of DNA fingerprint pattern generated by variable number tandem repeat (VNTR) marker was reported in Bangladesh with no correlation between pathogenicity and genetic diversity [10]. Similar result on the high genetic variation among the isolates was also observed in north of Iran; however, low relationship between the clustering in the random amplified polymorphic DNA (RAPD) dendrogram and geographic distribution of the tested isolates was reported [11]. In Minnesota, the genetic diversity analyzed via amplified fragment length polymorphism (AFLP) pattern of rice brown spot fungal population was high. Furthermore, population differentiation by collection sites was observed [12]. With the use of VNTR markers, population subdivision by the rice varieties and the collection sites was also found among the *B. oryzae* populations in the Philippines [13]. High level of genetic variability among the *B. oryzae* isolates by the analysis of inter-simple sequence repeats (ISSR) polymorphism was reported in India with no correlation between genetic clusters and collection sites [14]. Although the information on genetic diversity of *B. oryzae* populations was reported in several countries, there is very little information on genetic diversity among *B. oryzae* isolates in Thailand. Only *B. oryzae* population obtained from the paddy fields in Phra Nakhon Si Ayutthaya province in 2007 was reported and shown to be clustered into two groups based on the analysis of DNA fingerprint patterns provided by PCR-RFLP technique of ITS1-ITS2 region [15]. In this study, the genetic diversity of *B. oryzae* isolates obtained from 4 provinces in north-central Thailand using VNTR, ISSR, and RAPD markers with the emphasis on the pathogen aggressiveness and the collection sites was investigated. In addition, the response of different Thai rice varieties against different groups of *B. oryzae* as determined by a hierarchical clustering method was also examined.

2 Materials and Methods

2.1 *Sample collection and fungal isolation*

B. oryzae isolates were obtained from rice leaves showing brown spot symptoms from different paddy fields located in 4 provinces (Ang Thong, Chai Nat, Lop Buri, and Sing Buri) of north-central Thailand (Figure 1) in August and October, 2015 (wet season) and in February, 2016 (cool season) with the average temperature of 28.9°C, 28.3°C, and 27.7°C and with the rainfall of 162.2 mm, 226.5 mm, and 0 mm, respectively as recorded at the Lop Buri weather station (The Thai Meteorological Department, Thailand). Rice leaves showing typical brown spot lesions were washed with tap water and subjected to cut into small pieces (approximately 0.5 × 0.5 cm in size). Indeed, each piece contained both infected and healthy parts of the plant tissue, and subsequently the small pieces of tissue were surface sterilized with 0.6% sodium hypochlorite for 1 min, rinsed with sterile distilled water and transferred to sterile tissue papers to get rid of the excess water. The infected tissues were then placed on Petri dishes containing potato dextrose agar, PDA (HiMedia, Bangalore, India) and incubated at 28°C for 7 days. Once fungal mycelia were produced from the infected tissues, PDA agar plugs containing the growing mycelia were cut into 1 × 1 cm in size, and transferred to new sterile Petri dishes containing sterile moist Whatman™ No.1 filter papers (Fisher Scientific, UK) with the mycelium part facing forward to the top. Petri dishes containing fungal mycelial plugs were kept inside a loose plastic bag for about 3 to 5 days until fungal sporulation. Spores of each isolate were harvested with a needle using aseptic technique and transferred into sterile 1.5 mL microcentrifuge tube containing 100 µL of sterile distilled water. Spore suspension of each isolate was then spread on a sterile Petri dish containing 1% water agar. After 12 to 24 h later, a single germinating spore of each isolate was individually harvested under compound microscope and transferred into a sterile PDA slant.

2.2 *Identification of Bipolaris oryzae*

2.2.1 Morphological identification

Colony characteristic was examined on PDA cultured at 28°C after 7 days. Conidial shape and size were investigated and examined at 400x magnification under compound microscope using the Carl Zeiss AxioVision 4.8.3.0 software. The fungal isolates were then identified based on the information described earlier [16].

Figure 1: Geographic sampling locations of *Bipolaris oryzae* isolates obtained from 4 provinces (Ang Thong, Chai Nat, Lop Buri, and Sing Buri) in north-central Thailand.

2.2.2 Molecular identification

Fungal genomic DNA was isolated based on the protocol described previously [17] with some modification. In brief, a piece of fungal mycelium of each isolate was cultured in sterile 60 × 15 mm-Petri dish containing 3 mL of potato dextrose broth, PDB (HiMedia, Bangalore, India) at 100 rpm in a shaker at room temperature for 48 h. Fungal balls were then transferred into 1.5 mL microcentrifuge tube containing 300 µL extraction buffer (100 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1 M KCl). The fungal ball suspesion was subsequently homogenized with stainless beads using the modified drilling machines for 5 min and was added up with 300 µL of chloroform and mixed by inverting. The 1.5 mL microcentrifuge tube containing lysate-chloroform suspension was then centrifuged at 12,000 rpm for 3 mins at room temperature and the upper layer solution (about 300 µL) was transferred to new 1.5 mL microcentrifuge tube. An isopropanol (180 µL) was added to the sample and immediately mixed by inverting. After centrifugation at 12,000 rpm for 3 min at room temperature, the supernatant was discarded and the pellet was washed with 300 µL of 70% chilled absolute ethanol. Centrifugation was repeated at 12,000 rpm for 3 min at room temperature, and then supernatant was discarded to obtain DNA pellet. Once DNA pellet was dried at room temperature, the DNA sample was suspended in 50 µL of sterile ultrapure water. The quantity of DNA from each fungal isolate was measured using biodrop spectrophotometer (Biodrop, Cambridge, UK).

Fungal species identity was verified based on the sequences of the rDNA internal transcribed spacer region generated through the polymerase chain reaction (PCR) technique with the ITS1 primer (5'-GCCGTAGGTGAACCTGCGC-3') and ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') [18]. PCR amplifications were performed in a 100 µL volume containing 100 ng fungal genomic DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 250 µM dNTPs, 1 µM of each primer and 1 U of FIREPol® DNA polymerase (Solis Biodyne, Tartu, Estonia) in a thermal cycler block (Thermo Fisher, Massachusetts, USA) programmed for 94°C for 2 min; followed by 25 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 2 min; plus a final extension at 72°C for 5 min. PCR products were run on a 0.8% agarose gel and amplicon sizes were

determined based on a 100 bp DNA Ladder. DNA sequencing of each PCR product was conducted by Macrogen (Seoul, South Korea). The query sequence of each isolate was then compared against the GenBank nucleotide database.

2.2.3 Pathogenicity test of B. oryze isolates

Koch's postulates were confirmed by using either the conidial suspension or mycelial plug as a source of inoculum in 45-day-old rice leaves of Khao Dawk Mali 105 (KDML 105), the Thai susceptible rice variety. To prepare *B. oryzae* spores for inoculation, the conidia were induced using the method described earlier in this study. The conidial suspension (5×10^3 spores/mL) of each isolate was sprayed on the 45-day-old rice leaves. Inoculated plants were kept overnight in sealed plastic bags containing water with approximately 100% relative humidity and transferred to a greenhouse. In the case of mycelial plug inoculation, the mycelial plug (0.5 × 0.5 cm) was prepared from the 7-day-old fungal culture on PDA. The 45-day-old rice leaves were wounded before placing the mycelial plug upside down on the wounded tissue and held in position by Scotch tape. A sterile water droplet (10 µL) was added around the mycelial plug to maintain high humidity around the infected area. Those inoculated plants with mycelial plug were then kept in a greenhouse. The symptom of brown spot was then observed and photographed at 72 h after inoculation.

2.3 *PCR amplification of genetic markers*

Genomic DNA of each *B. oryzae* isolate and *Magnaporthe oryzae*, the outgroup species was prepared according to the protocol described above in this study. PCR amplification was performed using diverse genetic markers previously used for determining the genetic diversity of *B. oryzae* population elsewhere, including one VNTR primer (MR), six nonanchored ISSR primers (GF, ISSR08, ISSR12, ISSR14, ISSR15, and ISSR22), and three RAPD primers (A01, A98, and B06). The sequences of each primers were listed in Table 1. PCR amplifications were performed in a 10 µL volume containing 10 ng fungal genomic DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 250 µM dNTPs, 1 µM of primer and 1 U of FIREPol® DNA polymerase (Solis Biodyne, Riia, Tartu, Estonia) in a thermal cycler block (Thermo Fisher, Waltham, MA, USA) programmed for 94°C for 2 min; followed by 45 cycles of 94°C for 1 min, annealing temperature specific for each primer for 1 min and 72°C for 2 min; plus a final extension at 72°C for 5 min. The 10 µL of each PCR product was electrophoresed in 1.8% agarose gel using 0.5x TBE buffer. A 100 bp DNA Ladder (100 ng) was used as a molecular weight marker. Electrophoresis was performed at 80 V for 3 h in 0.5x TBE buffer. A gel was then stained in ethidium bromide and photographed using the Bio-Rad Imaging System (Bio-Rad, Hercules, CA, USA).

Table 1: List of variable number of tandem repeat (VNTR), inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) primers used in this study

Primer	Primer Sequence (5'-3'), Annealing Temperature	Types	Ref.
MR	GAGGGTGGGTGCGGTTCT, 45°C	VNTR	[19]
GF	TCCTCCTCCTCCTCC, 35°C	ISSR	[19]
ISSR08	GACAGACAGACAGACA, 45°C	ISSR	[14]
ISSR12	GTGGTGGTG, 43°C	ISSR	[14]
ISSR14	GAGGAGGAG, 40°C	ISSR	[14]
ISSR15	CACCACCAC, 45°C	ISSR	[14]
ISSR22	CACACACACACA, 40°C	ISSR	[14]
A01	CCCAAGGTCC, 35°C	RAPD	[11]
A08	ACGCACAACC, 35°C	RAPD	[11]
B06	GTGACATGCC, 35°C	RAPD	[11]

2.4 *Cluster analysis of genetic markers*

Each PCR product was scored for the presence (1) or absence (0) of a DNA band. Different fingerprint patterns were considered as different haplotypes. A similarity matrix according to Jaccard's similarity coefficient was used to generate a dendrogram by the unweighted pair group method with arithmetic averages (UPGMA) in NTSYSpc version 2.21 u (Applied Biostatistics, Port Jefferson, NY, USA).

2.5 *Aggressiveness comparison among B. oryzae isolates*

B. oryzae aggressiveness was examined in the representative fungal isolates from genetically distinct groups based on cluster analysis that were able to produce high number of spores in the 21-day-old seedlings of susceptible rice variety, KDML 105. Each *B. oryzae* isolate was grown in PDA for 7 days and subjected to

induce the spores according to the protocols described above. Spores were harvested and prepared in sterile distilled water with 0.05% Tween 20. The conidial suspension of each isolate was then sprayed on the 21-day-old rice seedlings with the concentration of 5×10^3 spores/mL. Inoculated plants were kept overnight in sealed plastic bags containing water with approximately 100% relative humidity and transferred to a greenhouse. The percentage of damaged leaf area caused by individual isolates was examined at 5 days after inoculation. The experiment was replicated three times. Mean comparisons of the percentage of damaged leaf area caused by individual *B. oryzae* isolates were tested for significance using Tukey's test at the 0.05 probability level.

2.6 *Assessment of rice varieties response to B. oryzae isolates*

Four different rice varieties, including Khao Dawk Mali 105 (KDML 105), RD31, Pathum Thani 1 (PTT1), and Jao Hom Nin (JHN) were examined for the rice response against representative *B. oryzae* isolates from each individual genetically distinct group. The germinated seeds of different rice varieties were grown in three 20 cm pots containing a commercial soil mixture. The individual pot was divided into four parts where each part contained 5 plants of each variety. The entire pots were then kept in a greenhouse. After 21 days, the seedling plants were used for spray inoculation with *B. oryzae* conidial suspension (5×10^3 spores/mL) following the protocol described above. The percentage of damaged leaf area of each plant variety caused by the representative *B. oryzae* isolate from each individual genetically distinct group was examined at 5 days after inoculation. Mean comparisons of the percentage of damaged leaf area in each variety caused by the representative isolates were tested for significance using Tukey's test at the 0.05 probability level.

3 Results

3.1 *Collection and species confirmation of B. oryzae isolates*

A total of 110 isolates of *B. oryzae* were isolated from *B. oryzae* infected leaf samples obtained from 32 different rice paddies located in 4 provinces

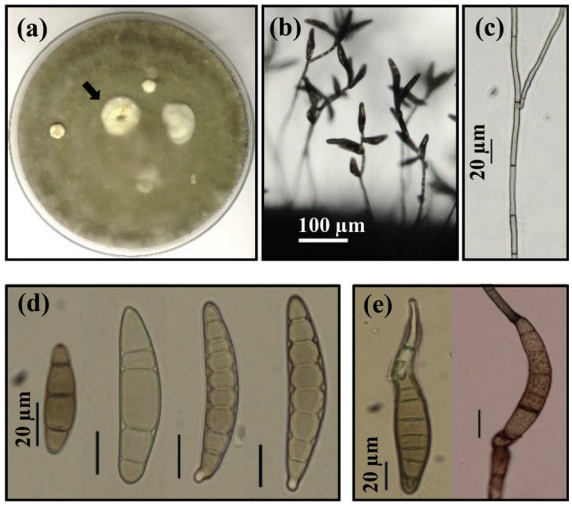

Figure 2: Morphological characteristics of *Bipolaris oryzae*. (a) Colony appearance, arrow indicates the white spot of aerial mycelium, (b) and (c) Conidiophore appearance, (d) Conidium appearance, (e) Conidium germination.

(Ang Thong, Chai Nat, Lop Buri, and Sing Buri) of Thailand during 2015–2016. The geographic origins of the isolates collected in the study are given in Table 2. The morphological characteristics of the isolates obtained in this study were identical to *B. oryzae* described by Manamgoda *et al*. [16]. In detail, the colonies of *B. oryzae* isolates on PDA were fluffy and pale grey to dark grey with small spots of white aerial mycelia [Figure 2(a)]. Conidiophores produced singly or in small groups, became dark brown in color with multiple septa [Figure 2(b) and (c)]. Conidia were 12.85 to 23.15 µM in width and 63.65 to 99.85 µM in length, hyaline during immature stage and brown in mature stage, and curved with 6 to 12 pseudosepta and had protruding hilum [Figure 2(d)]. Conidia germinated at either one end or both ends [Figure 2(e)].

Furthermore, partial ITS sequence analysis of some *B. oryzae* isolates revealed that they were genetically close to other ITS sequences (mainly located in the 5.8S and ITS2 regions) of *B. oryzae* isolates deposited in GenBank with 99 to 100% homology (Table 3). In addition, Koch's postulates confirmed that the representative isolates from each individual paddy were the causal pathogen of rice brown spot. In detail, the tested isolates either produced the typical symptom with dark brown oval spots surrounded by

Table 2: *Bipolaris oryzae* isolates with geographic sampling locations and haplotype groups identified through this study

Field No.	Isolate	Sampling Location (Subdistrict, District)	Province	Month, Year	Haplotype Group
1	AT12, AT13, AT14, AT15	Tha Chang, Wiset Chai Chan	Ang Thong	Oct, 2015	A
2	AT22, AT23, AT24, AT25	Thang Phra, Pho Thong	Ang Thong	Oct, 2015	A
3	AT32, AT33, AT34, AT35	Yi Lon, Wiset Chai Chan	Ang Thong	Oct, 2015	A
4	AT42, AT43, AT44, AT45	Thang Phra, Pho Thong	Ang Thong	Oct, 2015	A
5	AT52	Yi Lon, Wiset Chai Chan	Ang Thong	Oct, 2015	A
6	AT62, AT63	Yi Lon, Wiset Chai Chan	Ang Thong	Oct, 2015	A
7	AT82, AT83, AT84, AT85	Ekkarat, Pa Mok	Ang Thong	Oct, 2015	B
8	AT92, AT93, AT94, AT95	Bang Sadet, Pa Mok	Ang Thong	Oct, 2015	A
9	CN12, CN13, CN14, CN15	Khao Tha Phra, Mueang	Chai Nat	Aug, 2015	A
10	CN22, CN23, CN24, CN25	Khao Tha Phra, Mueang	Chai Nat	Aug, 2015	A
11	CN32, CN33	Wat Khok, Manorom	Chai Nat	Aug, 2015	C
12	CN52, CN53, CN54, CN55	Hang Nam, Manorom	Chai Nat	Aug, 2015	A
14	CN62, CN63, CN64, CN65	Ban Kluai, Manorom	Chai Nat	Oct, 2015	A
15	CN72, CN73, CN74, CN75	Ban Kluai, Manorom	Chai Nat	Oct, 2015	A
16	CN92, CN93, CN94, CN95	Nai Mueang, Mueang	Chai Nat	Oct, 2015	A
17	LB12, LB14	Phon Thong, Ban Mi	Lop Buri	Oct, 2015	B
18	LB22, LB23, LB24, LB25	Khok Salung, Phatthana Nikhom	Lop Buri	Oct, 2015	A
19	LB32	Khok Salut, Tha Wung	Lop Buri	Oct, 2015	B
20	LB62, LB63, LB64, LB65	Phon Thong, Ban Mi	Lop Buri	Feb, 2016	A
21	LB72, LB73, LB74	Phon Thong, Ban Mi	Lop Buri	Feb, 2016	A
22	LB82, LB83, LB84, LB85	Bang Kham, Ban Mi	Lop Buri	Feb, 2016	A
23	LB92, LB93, LB94, LB95	Khao Samo Khon, Tha Wung	Lop Buri	Feb, 2016	A
24	SBR12, SBR13, SBR14	Pho Prachak, Tha Chang	Sing Buri	Oct, 2015	A
25	SBR22, SBR23, SBR24, SBR25	Pho Prachak, Tha Chang	Sing Buri	Oct, 2015	A
26	SBR32, SBR33, SBR34, SBR35	Tha Kham, Khai Bang Rachan	Sing Buri	Oct, 2015	A
27	SBR42, SBR43, SBR44, SBR45	Bang Rachan, Khai Bang Rachan	Sing Buri	Oct, 2015	A
28	SBR52, SBR53, SBR54, SBR55	Thon Samo, Tha Chang,	Sing Buri	Oct, 2015	A
29	SBR72, SBR73, SBR74, SBR75	Phak Than, Bang Rachan	Sing Buri	Feb, 2016	B
30	SBR82, SBR83, SBR84, SBR85	Phak Than, Bang Rachan	Sing Buri	Feb, 2016	A
31	SBR92, SBR93, SBR94, SBR95	Phak Than, Bang Rachan	Sing Buri	Feb, 2016	A
32	SBR102, SBR103, SBR104, SBR105	Phak Than, Bang Rachan	Sing Buri	Feb, 2016	A

Table 3: The rDNA internal transcribed spacer sequence analysis of the representative isolates using blast sequence alignment

Isolate	Nearest BLAST Match	Acession Number	Identity (%)
AT12	*Cochliobolus miyabeanus* strain R82	KC315928	99
AT85	*Bipolaris oryzae* strain ACCC 36975	MK051170	99
CN32	*Bipolaris oryzae* strain ACCC 36975	MK05701	99
LB22	*Bipolaris oryzae* strain CBS 199.54	MH857291	99
LB32	*Bipolaris oryzae* isolate X88	MK304168	100
SBR32	*Bipolaris oryzae* isolate X88	MK304168	99
SBR72	*Bipolaris oryzae* isolate X88	MK304168	100

a yellow halo when observed at 5 days after spray inoculation with spore suspension or produced irregular oval shape of spots with yellow halo when observed at 5 days after mycelial plug inoculation (Figure 3). Moreover, typical characteristics of conidia described earlier were observed after re-isolation.

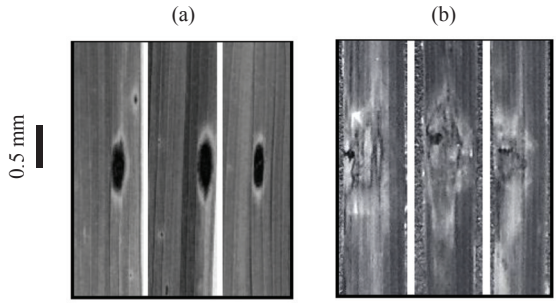

Figure 3: Koch's postulate test with *Bipolaris oryzae*. (a) An inoculated 45-day-old rice leaves with *B. oryzae* conidial suspension (5×10^3 spores/ml) by spraying method produced oval spots with yellow halo at 5 days after inoculation. (b) An inoculated 45-day-old rice leaves with *B. oryzae* mycelial plug produced irregular oval shape of spots with yellow halo at 5 days after inoculation.

3.2 *Genetic diversity of B. oryzae isolates from different geographic origins*

The genetic diversity among 110 *B. oryzae* isolates was evaluated using VNTR, ISSR, and RAPD markers. The representative isolates of *B. oryzae* from each individual paddy (N = 32) were preliminary tested with one VNTR primer (MR), six nonanchored ISSR primers (GF, ISSR08, ISSR12, ISSR14, ISSR15, and ISSR22), and three RAPD primers (A01, A98, and B06). Among all markers, only VNTR markers with MR primer indicated a variation among all tested isolates of *B. oryzae*, but not the other markers (data not shown). MR primer with the genomic DNA of the tested isolates produced 14 bands ranging from 400 to 1,500 bps. Three bands regarding polymorphism were observed among all isolates. A dendrogram constructed using the VNTR-MR data (Figure 4) shows a total of 110 *B. oryzae* isolates to be divided into three groups. There were three haplotypes in total concordant with the UPGMA dendrogram. The isolates in each group shared identical haplotype. Ninety-seven isolates (88.18%) were clustered into the main group (group A), indicating the prevalence of clonality. Eleven (10%) and two (1.82%) isolates were clustered into group B and C, respectively. There was no correlation between the haplotypes and the geographic origins because most isolates were scattered throughout group A and B regardless of collection provinces.

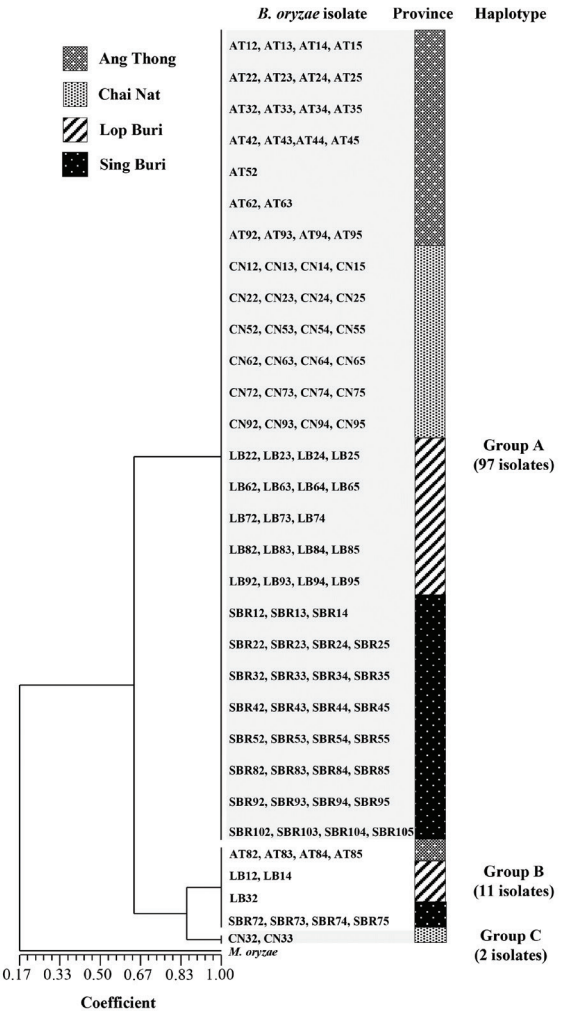

Figure 4: UPGMA dendrogram of 110 *Bipolaris oryzae* isolates and *Magnaporthe oryzae* (a representative outgroup species) derived from VNTR-MR fingerprints.

3.3 *Variation in aggressiveness of B. oryzae isolates*

Fifteen isolates of *B. oryzae*, the representative isolates from genetically distinct groups, were chosen for pathogenicity test due to the fact that these isolates were able to produce sufficient number of spores for inoculation. Spray inoculation with conidial suspension of the tested isolates on 21-day-old seedlings of rice variety KDML 105 showed some differences on aggressiveness of *B. oryzae* isolates tested (Table 4); however, there was no correlation between the haplotypes and the *B. oryzae* aggressiveness. This

interpretion was supported with the evidences that significant difference in aggressiveness of *B. oryzae* isolates belonging to haplotype group A was found. Moreover, some isolates from haplotype group A and the isolates from haplotype group B and C were able to produce the same level of aggressiveness.

Table 4: Mean percentage of damaged leaf area of 21-day-old seedlings of rice variety Khao Dawk Mali 105 (KDML 105) inoculated with isolates of *B. oryzae* from genetically distinct groups

Isolate	Haplotype Group	Damaged Leaf Area (%)[a]
AT12	A	34.8±8.2[a]
AT43	A	28.9±6.7[abc]
AT63	A	23.5±4.6[bc]
CN15	A	33.5±7.8[a]
CN23	A	32.7±8.3[ab]
CN52	A	22.7±4.7[c]
LB22	A	31.8±8.0[abc]
LB64	A	32.1±7.7[ab]
LB73	A	29.5±9.4[abc]
SBR14	A	24.0±4.4[bc]
SBR93	A	33.8±8.7[a]
AT85	B	31.4±8.4[abc]
LB14	B	34.0±6.5[a]
LB32	B	32.4±8.7[ab]
CN32	C	32.8±8.8[a]

[a] Means ± SD percentage damaged leaf area (N = 15) followed by same letter are not significantly different as indicated by Tukey's test at P < 0.05.

3.4 *Response of Thai rice varieties to B. oryzae isolates*

Four different rice varieties were selected for investigation of the rice response against five representative *B. oryzae* isolates from each individual genetically distinct group. After spore inoculation of each isolate on the 21-day-old seedling of different rice varieties, KDML 105 was the most susceptible to all isolates tested (33.8±8.5 to 36.0±7.0% of damaged leaf area), whereas JHN showed the most resistant against all *B. oryzae* isolates (6.7±4.4 to 8.0±4.0% of damaged leaf area). In addition, RD31 and PPT1 showed 22.7±5.2 to 25.4±7.2% and 14.3±3.7 to 17.6± 4.5% of damaged leaf areas, respectively, which were significantly higher than JHN despite being the suggested varieties against brown spot disease for rice cultivation in central Thailand elsewhere (Figure 5). Furthermore, the results also indicated that each rice variety similarly responded to *B. oryzae* from genetically distinct groups.

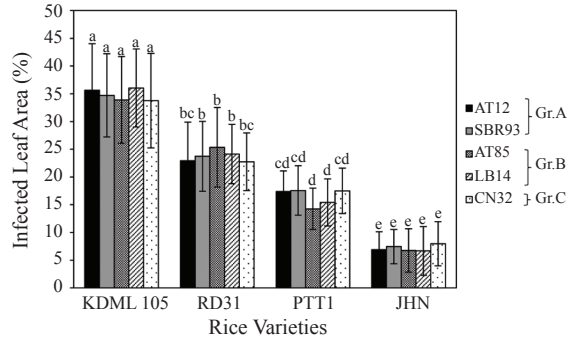

Figure 5: Mean ± standard derivative values (N = 24) of the infected area percentages in 4 Thai rice varieties: Khao Dawk Mali 105 (KDML 105), RD31, Pathum Thani 1 (PTT1), and Jao Hom Nin (JHN) after inoculating with representative isolates of each genetically distinct group. Same letter are not significantly different as indicated by Tukey's test at P < 0.05.

4 Discussion

Despite the fact that *B. oryzae* is found to be present in all the rice growing countries including Thailand and causes significant quantitative and qualitative grain-yield losses [5], the information on genetic diversity of *B. oryzae* population in Thailand is still very limited. Among various types of genetic markers tested in this study, including VNTR, ISSR, and RAPD, only VNTR markers with MR primer showed the DNA polymorphism among the *B. oryzae* isolates. Therefore, VNTR markers were selected to analyze the genetic diversity of the *B. oryzae* isolates. Previously VNTR markers have been successfully used to differentiate closely related isolates of fungi [19]. Particularly, VNTR analysis was adopted to uncover the genetic diversity among the isolates of *B. oryzae*, the populations of Bangladesh [10] and the Philippines [13]. In the present study, with the analysis of VNTR fingerprints provided by MR primer, there was low genetic diversity among 110 isolates of *B. oryzae* collected from the paddies located in 4 provinces in the north-central part of Thailand. Indeed, the *B. oryzae* population was clustered into three groups where the majority of the isolates (88.18%) sharing identical haplotype was clustered into group A, indicating the prevalence of clonality in the population. Low genetic diversity in the *B. oryzae* population found in this study can be attributed to the fact that *B. oryzae*

may primarily reproduce through asexual mode in the cultivated areas. As indicated in the Philippines, intensive analysis of *B. oryzae* population in an individual field showed near clonal population due to asexual reproduction [13]. Although different mating types could be found in the *B. oryzae* population in some countries [12], [20], the evidence supporting the existence of sexual reproduction in the field was only obtained from the report of Dickson (as cited in Castell-Miller and Samac [12]) in the USA. In addition, all *B. oryzae* isolates in this study were obtained from the same growing environment where rice plants were cultivated in the rice-based irrigation system. These factors may also interfere the heterogeneity of the pathogen population. Unlike this study, moderate to high genetic diversity were reported in Bangladesh and the Philippines [10], [13], [20]. This might be due to the fact that *B. oryzae* isolates were obtained from diverse locations throughout the countries where different parts of a country may have different climates, cropping systems and rice varieties. Such biophysical characteristics of the environments, rice varieties, soil nutrients and cropping patterns could play role in the level of pathogen genotypic diversity [13].

Most likely there was no correlation between VNTR markers and collection sites (based on the provinces) particularly when considering the *B. oryzae* isolates belonging to group A and B. Both groups contained the isolates from different provinces. However, the relationship between them remained unclear in those isolates belonging to group C due to the fact that this group comprised only two isolates obtained from the same paddy fields. No or little relationship of *B. oryzae* genetic markers and geographic region was also reported in the previous studies [11], [14]. The occurrence of more than one haplotype in a single province may be the consequence of infected seed transportation from one to other cultivated areas due to seed distribution among farmers, as *B. oryzae* is usually seed-transmitted pathogen [21]–[22]. Similar to geographic location, this study showed no relationship between genetic markers and *B. oryzae* aggressiveness which was in accordance with the previous reports elsewhere [10], [13], indicating that polymorphic DNA bands given by the VNTR-MR primer may not be linked to the pathogen aggressiveness.

Although all the representative isolates of each genetically distinct group did not show the differences on their aggressiveness in all rice varieties tested, different responses among the rice varieties against the *B. oryzae* isolates could be observed. In this study, Jao Hom Nin displayed the highest incomplete resistance compared to the recommended varieties for management of rice brown spot disease, including Pathum Thani 1 and RD31. Hence, Jao Hom Nin may provide potential source of disease resistance to *B. oryzae*.

5 Conclusions

In conclusion, the present study reports the low level of genetic diversity in the *B. oryzae* population in north-central Thailand. Since the collection sites were restricted within four provinces close to each other in this area, future research should further investigate the genetic diversity of *B. oryzae* isolates in other different regions throughout Thailand. In addition, mating type identification should be carried on. This knowledge on the population genetic diversity of the pathogen will be crucial for developing crops that are resistant to the rice brown spot disease.

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