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Molecular and pathotype analysis of the rice blast fungus in North Vietnam

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Abstract

Rice blast caused by *Pyricularia oryzae* is a devastating disease worldwide. In Vietnam, rice blast is especially severe in the Red River Delta in the North. The genetic diversity of 114 *P. oryzae* isolates collected from rice in 2001 in the Red River Delta and nine additional Vietnamese *P. oryzae* isolates was analysed using Amplified Fragment Length Polymorphism (AFLP). DNA similarity and cluster analysis based on 160 polymorphic AFLP markers showed twelve different AFLP genetic groups among the 123 field isolates. Isolates collected from *japonica* hosts clustered separately from *indica* host isolates with at least 60% dissimilarity with little evidence for gene flow between the two populations. In the 2001 population originating from *indica* hosts, three genetic groups were predominant and represented 99% of the isolates sampled. One predominant clonal lineage represented 59% of the 2001 *indica* host population and was found in eleven provinces in the Red River Delta of North Vietnam. Significant genotype flow could be demonstrated between the *indica* population south of Red river and the *indica* population north of Red river. There was significant linkage disequilibrium between the AFLP loci within the *indica* population, indicating that this is not a random mating population. Pathogenicity tests of 25 isolates selected from the 12 AFLP groups on a set of 29 differential rice lines revealed two avirulent isolates and 23 pathotypes. Different combinations of known resistance genes were found to have potential for blast resistance breeding for North Vietnam.

Introduction

Rice production is very important in Vietnam with a total area of 7.45 million ha and a yearly production of 34 million tonnes. Vietnam is the world's second largest rice exporter after Thailand and exported in 2004 around 4 million tonnes of rice. In Vietnam, irrigated rice is mainly produced in the Mekong River Delta in the South with 3.79 million ha and in the Red River Delta in the North with 1.18 million ha (General Statistical Office, Vietnam).

Rice blast, caused by *Pyricularia oryzae* (teleomorph: *Magnaporthe oryzae*; Couch and Kohn, 2002) is a widespread and damaging disease in most rice-growing areas of the world (Ou, 1985). In Vietnam, rice blast is especially severe in the Red River Delta with its humid, tropical climate that is highly conducive to fungal pathogens. *Pyricularia oryzae* can be controlled by fungicides, the use of resistant cultivars and agricultural practices. The most effective and economical

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strategy to control rice blast is the use of resistant cultivars (Bhatt and Singh, 1992). To date more than 40 major blast resistance genes have been mapped, although some of these genes could be identical or allelic (Sallaud et al., 2003). Introduced resistance often breaks down after a few years due to the high diversity within *P. oryzae* populations and the appearance of new pathogenic races (Correa-Victoria and Zeigler, 1995). To efficiently control rice blast disease, efforts have been made to characterize the structure of *P. oryzae* populations using pathogenicity tests or molecular techniques.

For more than 50 years, rice differentials have been used to identify pathotype groups in *P. oryzae* populations. Different sets of standard differential lines with characterized resistance genes have been developed, which have been used to characterize *P. oryzae* populations in various countries (Correa-Victoria and Zeigler, 1993; Thinlay et al., 2000; Mekwatanakarn et al., 2000; Xia et al., 2000; Chen et al., 2001).

Pyricularia oryzae populations have also been studied using Restriction Fragment Length Polymorphism (RFLP) and PCR-based markers. These studies have revealed that *P. oryzae* populations are composed of different genetic groups. American, European, Japanese and Iranian *P. oryzae* populations appear to have a relatively simple structure with only a few distinct genetic groups (Levy et al., 1991; Roumen et al., 1997; Don et al., 1999b; George et al., 1998; Javan-Nikkhah et al., 2004). *Pyricularia oryzae* populations in tropical Asia, however, are much more complex, probably because Asia, centre of origin of rice, could also be the centre of origin of *P. oryzae*. Distinct genetic groups cannot easily be defined (Zeigler, 1998; Kumar et al., 1999).

Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995) is used increasingly to study genetic variation below the species level in a variety of taxa, including bacteria, fungi, animals and plants. The quantity of information generated, replicability, resolution, ease of use and cost efficiency are at least as good, if not superior, to those of other standard molecular markers (Mueller and Wolfenbarger, 1999).

Breeding for resistance in Asia can only be successful if it is based on knowledge about the pathogen population in the target area. Up to now, management of the rice blast disease in Vietnam using bred resistant cultivars has not been very efficient, in part because little is known about its *P. oryzae* population. We are only aware of the work of Noda et al. (1999) and Don et al. (1999a) who studied the *P. oryzae* population from the Mekong River Delta and included a few isolates from the Red River Delta in their analysis.

The first objective of this work was to use AFLPs to determine the genetic structure of a *P. oryzae* population sampled in 2001 from the Red River Delta of North Vietnam. We wanted to identify an optimal set of AFLP primer combinations for use in the characterization of Vietnamese blast populations and to initiate pathotype analysis using local Vietnamese cultivars and a large set of internationally used rice blast differentials. In the long run, a combined genetic and pathotype assessment of Vietnamese *P. oryzae* isolates might help to identify resistance genes for use in Vietnamese rice breeding programmes. The second objective was to determine whether the genetic structure of the *P. oryzae* population from North Vietnam was more consistent with random mating as observed in the Indian Himalayas (Kumar et al., 1999) or asexual reproduction as observed in rice-growing areas outside the centre of origin of rice.

Materials and methods

Pyricularia oryzae *isolates*

Isolates of *P. oryzae* were collected from rice panicles with blast symptoms in 2001 from 66 fields in 11 provinces in the Red River Delta of North Vietnam (Red River Delta population 2001, see Table 1 and Figure 1). For comparison, nine additional isolates were obtained from CIRAD, France or from the Agricultural Genetics Institute in Hanoi, Vietnam (Table 1). Monoconidial cultures of the fungus were recovered from blast lesions by placing surface-sterilized and air-dried samples on moist filter paper in Petri dishes. Sporulation was obtained by incubating the Petri dishes at 28 °C for 2–3 days. Conidia were picked up by gently touching the spores on top of the conidiophores under a binocular using a thin glass needle. Conidia were spread on 2% water agar medium. Three hours after incubation at 28 °C, single germinated conidia from each sample were

transferred to half-strength oatmeal agar medium (DIFCO, Sparks, USA) (30 g of oatmeal and 12.5 g of agar for 1 l) and incubated at 28 °C for 3–4 days. Isolates were routinely kept on dry filter paper at −20 °C as described by Valent et al. (1986).

Fungal DNA isolation

Pyricularia oryzae isolates were grown in YEG medium (Valent et al., 1986) for mycelium production. Lyophilized mycelium was used for DNA isolation following a modified version of the CTAB method (Murray and Thompson, 1980) as described below. One ml of extraction buffer (NaCl: 0.7 M; Tris–HCl: 50 mM pH 8; EDTA: 10 mM pH 8; CTAB: 1%) was added to the ground tissue and incubated at 65 °C for 1 h. After centrifugation, the supernatant was transferred to a new tube, purified with 1 ml of chloroform/isoamyl alcohol (24:1) and centrifuged. The supernatant was further purified by adding 85 μl (1/10 v/v) of CTAB 10% and 1 ml of chloroform/isoamyl alcohol (24:1) until the interphase became transparent. Nucleic acids were precipitated by using 1 ml CTAB precipitation buffer (Tris–HCl: 50 mM, pH 8; EDTA: 10 mM, pH 8; CTAB: 1%) followed by incubation at room temperature for 30 min. After centrifugation, the pellet was dissolved in 500 μl of high-salt Tris–EDTA buffer (NaCl: 1 M; Tris–HCl: 10 mM, pH 8; EDTA: 1 mM, pH 8). The nucleic acid was precipitated again with 1500 μl of cold absolute ethanol and the pellet was dissolved in 600 μl of TE buffer (Tris–HCl: 10 mM, pH 8; EDTA: 0.1 mM, pH 8). RNA was removed by adding 4 μl of RNAse (10 mg ml^{-1}), followed by incubation at 37 °C for 2 h. DNA was precipitated with 30 μl of NaCl (5 M) and 1500 μl of cold absolute ethanol and rinsed with 500 μl of 70% ethanol. DNA was collected by centrifugation; the pellet was air-dried and gently dissolved in 200 μl of TE.

Amplified fragment length polymorphism

AFLP analysis on all *P. oryzae* isolates was performed following the protocol of Vos et al. (1995) with minor modifications. Genomic DNA was digested with restriction enzymes *Eco*RI and *Mse*I (BIOLAB). *Eco*RI adapters (adp1: 5′-CTCGTAGACTGCGTACC-3′, adp2: 5′-AATTGGTACGCAGTCTAC-3′) and *Mse*I adapters (adp1: 5′-GACGATGAGTCCTGAG-3′, adp2: 5′-TACTCAGGACTCAT-3′) were ligated to the DNA fragments in one step. The primers and adaptors used in this research were obtained from GENSET (Paris, France). For each sample, 500 ng of genomic DNA was used. The digestion–ligation mixtures were four times diluted before being used as templates for the pre-amplification. Primers *Eco*RI and *Mse*I without selective nucleotides (*Eco*RI + 0 and *Mse*I + 0) were employed for pre-amplification as described by Vos et al. (1995). The pre-amplified product was quantified in a 1% agarose gel along with a 1 kb ladder (GIBCO). For selective amplification, 10-fold dilutions of pre-amplified products were used as a template. Different primer combinations of *Eco*RI + 2/*Mse*I + 2 were used in this study (Table 2). Primer *Eco*RI with selective nucleotides was labelled with γ-[^{33}P]-ATP (Amersham Biosciences Europe, Roosendaal, the Netherlands). All PCR reactions were carried out using a Perkin Elmer 9600 Thermal Cycler. After selective amplification, an equal volume of formamide loading dye (98% v/v Formamide, 10 mM EDTA, 0.005% Xylene Cyanol FF and 0.005% Bromophenol blue) was added to the PCR product. The samples were denatured at 95 °C for 5 min, and 3 μl of each sample were loaded onto a pre-heated 5% polyacrylamide gel. Electrophoresis was carried out at 100 W for 2.5 h. The SequaMark DNA size marker (Research Genetics) was used to determine the size of the AFLP fragments. The polyacrylamide gels were vacuum-dried on 3 MM Whatman paper at 80 °C for 30 min and exposed to the Phosphoimager screen. After 12 h, the image of DNA patterns was obtained by scanning the screen. For each primer combination, distinct, major, polymorphic AFLP fragments were visually scored as the presence/absence of bands. From these data a binary presence/absence matrix was constructed.

Rice cultivars and experimental layout

Pathogenicity tests were carried out on 29 differential rice lines (Table 3). 'Tetep', 'Chiembac' and 'Thamthom' are traditional Vietnamese indica cultivars. 'CR203' is an improved IRRI cultivar. Rice seeds were germinated in humid plates (≥92% RH), at 28 °C for 4 days. Six to seven germinated seeds of each differential host were sown in

Table 1. Origin of the different Vietnamese *Pyricularia oryzae* isolates used in this study

Field	Isolates	Cultivars	Type	Province	Date
Red River Delta population (2001) – indica isolates					
North of Red River					
V38	V38-A	Q6	*indica*	Bac Ninh	28/05/2001
V40	V40-A	Q5	*indica*	Bac Ninh	28/05/2001
V41	V41-A, V41-B	Q5	*indica*	Bac Ninh	28/05/2001
V42	V42-A	C70	*indica*	Bac Ninh	28/05/2001
V43	V43-A,V43-B	C70	*indica*	Bac Ninh	28/05/2001
V59	V59-A	Q5	*indica*	Bac Ninh	28/05/2001
V64	V64-A, V64-B	Q5	*indica*	Bac Ninh	28/05/2001
V107	V107-A, V107-B	Unknown	*indica*	Hai duong	2/06/2001
V109	V109-B	Unknown	*indica*	Hanoi	29/05/2001
V11	V11-B	Unknown	*indica*	Hanoi	29/05/2001
V24	V24-A	Unknown	*indica*	Hanoi	29/05/2001
V26	V26-A	DT10	*indica*	Hanoi	29/05/2001
V31	V31-B	Unknown	*indica*	Hanoi	29/05/2001
V33	V33-A, V33-B	Khang dan	*indica*	Hanoi	29/05/2001
V83	V83-A, V83-B	Khang dan	*indica*	Hanoi	29/05/2001
V87	V87-A	Unknown	*indica*	Hanoi	29/05/2001
V92	V92-B	Unknown	*indica*	Hanoi	31/05/2001
V115	V115-A, V115-B, V115-C	Unknown	*indica*	Hung yen	30/05/2001
V116	V116-A, V116-B	Unknown	*indica*	Hung yen	30/05/2001
V14	V14-A, V14-B	Unknown	*indica*	Hung yen	30/05/2001
V78	V78-A	Unknown	*indica*	Hung yen	30/05/2001
V12	V12-A	Unknown	*indica*	Quang ninh	2/06/2001
V111	V111-B	Unknown	*indica*	Thai binh	30/05/2001
V75	V75-A	Unknown	*indica*	Thai binh	30/05/2001
V76	V76-A	Unknown	*indica*	Thai binh	30/05/2001
V23	V23-A	Unknown	*indica*	Thai nguyen	29/05/2001
V27	V27-A	DT13	*indica*	Thai nguyen	29/05/2001
V29	V29-B	Unknown	*indica*	Thai nguyen	29/05/2001
V81	V81-A	DT10	*indica*	Thai nguyen	29/05/2001
V84	V84-A, V84-B	DT10	*indica*	Thai nguyen	29/05/2001
V85	V85-A, V85-B	Q5	*indica*	Thai nguyen	29/05/2001
V86	V86-A	Khang dan	*indica*	Thai nguyen	29/05/2001
South of Red River					
V100	V100-A, V100-B	DV108	*indica*	Ha nam	30/05/2001
V101	V101-A, V101-B	CR203	*indica*	Ha nam	30/05/2001
V103	V103-B	Unknown	*indica*	Ha nam	30/05/2001
V117	V117-A, V117-B	Unknown	*indica*	Ha nam	30/05/2001
V13	V13-B	AIT 77	*indica*	Ha nam	30/05/2001
V17	V17-B	Unknown	*indica*	Ha nam	30/05/2001
V18	V18-A, V18-B	Unknown	*indica*	Ha nam	30/05/2001
V35	V35-A, V35-B	unknown	*indica*	Ha nam	30/05/2001
V36	V36-B	Unknown	*indica*	Ha nam	30/05/2001
V5	V5-A, V5-B, V5-C, V5-E	Unknown	*indica*	Ha nam	30/05/2001
V80	V80-A	Unknown	*indica*	Ha nam	30/05/2001
V99	V99-B	CR203	*indica*	Ha nam	30/05/2001
V44	V44-A, V44B	Unknown	*indica*	Ha tay	31/05/2001
V46	V46-A	Unknown	*indica*	Ha tay	31/05/2001
V47	V47-B	Unknown	*indica*	Ha tay	31/05/2001
V57	V57-A	Unknown	*indica*	Ha tay	31/05/2001
V58	V58-A	Unknown	*indica*	Ha tay	31/05/2001
V60	V60-B	Unknown	*indica*	Ha tay	31/05/2001
V89	V89-A	Unknown	*indica*	Ha tay	29/05/2001
V98	V98-A	Unknown	*indica*	Ha tay	31/05/2001
V19	V19-A	Unknown	*indica*	Nam dinh	30/05/2001

Table 1. Continued

Field	Isolates	Cultivars	Type	Province	Date
V34	V34-A, V34-B	Unknown	*indica*	Nam dinh	30/05/2001
V4	V4-A, V4-F, V4-G, V4-H	Unknown	*indica*	Nam dinh	30/05/2001
V6	V6-A, V6-B, V6-D, V6-E, V6-G	Unknown	*indica*	Nam dinh	30/05/2001
V118	V118-A	Unknown	*indica*	Ninh binh	30/05/2001
V1	V1-A, V1-B, V1-C, V1-D, V1-E, V1-F, V1-G, V1-J	Unknown	*indica*	Ninh binh	30/05/2001
V2	V2-B, V2-D	Unknown	*indica*	Ninh binh	30/05/2001
V3	V3-A,V3-B, V3-C, V3-D, V3-E, V3-H, V3-I, V3-J	Unknown	*indica*	Ninh Binh	30/05/2001
Red River Delta population (2001) – japonica isolates					
V110	V110-A, V110-B	Unknown	*japonica*	Hanoi	29/05/2001
V21	V21-A, V21-B	Nep	*japonica*	Hanoi	29/05/2001
V22	V22-A-G, V22-A-W	Nep Hai phong	*japonica*	Hanoi	29/05/2001
V16	V16-B	Nep May	*japonica*	Nam dinh	30/05/2001
V20	V20-A, V20-B	Nep Rau	*japonica*	Nam dinh	30/05/2001
V15	V15-A	Unknown	*japonica*	Thai binh	30/05/2001
Other Vietnamese Pyricularia oryzae isolates					
I1	I1	CR203	*indica*	Hanoi	1997
I2	I2	VN10	*indica*	Thai Binh	1997
I6	I6	CR203	*indica*	Nghe An[1]	1997
I11	I11	IR17494	*indica*	Phu Yen[1]	1997
VT1	VT1	Unknown	*indica*	Hochi Minh, city[2]	9/10/1989
VT3	VT3	Nep Lun	*japonica*	Thuathine Hue[1]	16/02/1994
VT5	VT5	IR38	*indica*	Thuathine Hue[1]	16/02/1994
VT6	VT6	IR38	*indica*	Thuathine Hue[1]	16/02/1994
VT7	VT7	IR38	*indica*	Thuathine Hue[1]	16/02/1994

V-isolates: Vietnamese isolates collected for this study; I-isolates: Vietnamese isolates provided by Agricultural Genetics Institute, Vietnam; VT-isolates: Vietnamese isolates provided by CIRAD, France.
All provinces are in North Vietnam, except [1]from Central Vietnam and [2]from South Vietnam.

trays in a potting compost (Klassmann–Deilman, Geeste, Germany) and grown under greenhouse conditions at 30 ± 4 °C with a 16-h photoperiod. Plants were fertilized after 8, 15 and 22 days with 5 g of $(NH_4)_2SO_4$ m^{-2}. One day after the last fertilization, plants with 5–6 leaves were used for inoculation tests.

Pyricularia oryzae *inoculum production*

Pyricularia oryzae isolates were grown for sporulation at 28 °C on half-strength oatmeal agar (DIFCO, Sparks, USA) or on rice-starch agar (20 g of rice starch, 20 g of agar and 2 g of yeast extract for 1 l). Five day-old mycelium was flattened onto the medium using a sterile spoon and exposed to blue light (combination of Philips TLD 18W/08 and Philips TLD 18W/33) for 7 days to induce sporulation. Upon sporulation, plates were flooded with 10 ml of demineralized water and spores were removed with a painting brush. The spore suspension was filtered through four layers of 0.25×0.25 mm nylon mesh and the number of spores estimated with a haemacytometer. The inoculum concentration was adjusted to 5×10^4 spores ml^{-1} in 0.5% gelatin solution for spray inoculation.

Inoculation method and disease evaluation

Rice plants were inoculated by spraying the *P. oryzae* spore suspension with a painting air brush (Model 150-M) connected to an air compressor. Each isolate was inoculated on all 29 rice cultivars. Six to seven plants per rice cultivar were used. Each isolate-host cultivar combination was assessed in at least two trials.

Inoculated plants were kept for 18 h in humid chambers (≥92% RH) at 30 ± 4 °C. Six days after inoculation, blast symptoms were evaluated using a 0–6 scale based on the type and size of lesions (Roumen et al., 1997). Rice lines that only showed scores from 0 to 2 (no sporulating lesions) were considered resistant; rice lines were considered susceptible if the majority of the lesions that developed were type 3–6.

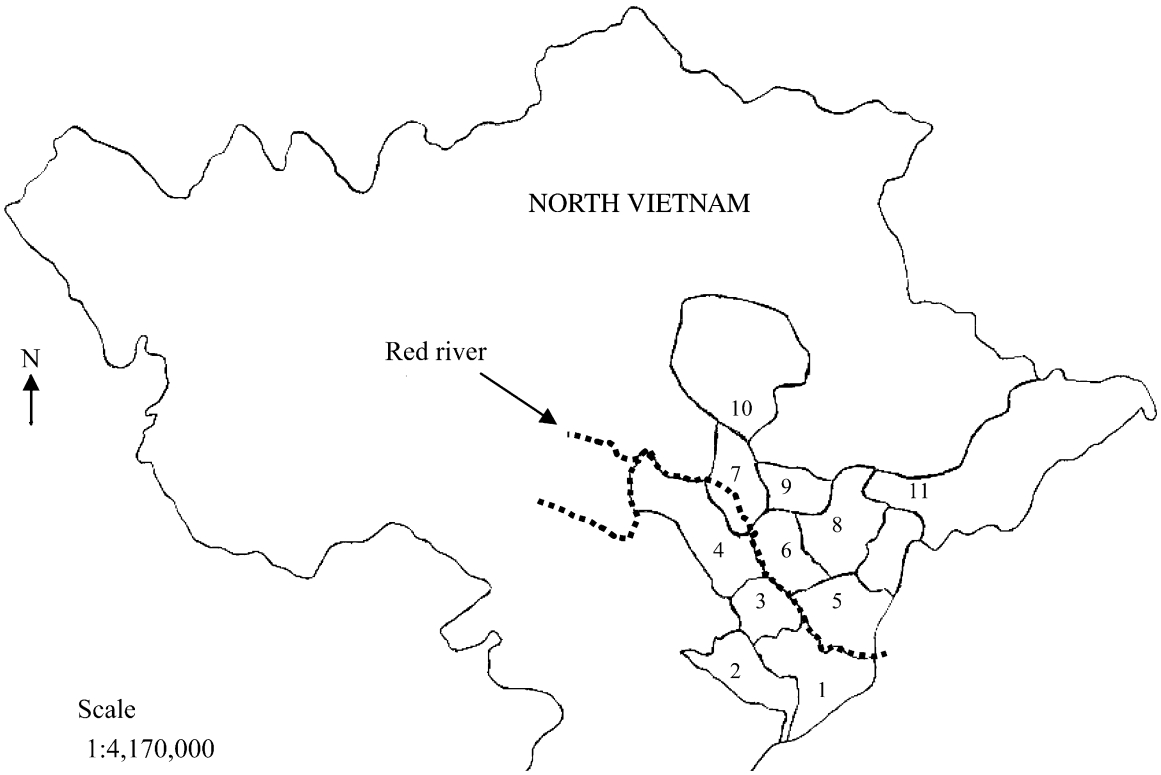

Figure 1. Origin of characterized *Pyricularia oryzae* isolates collected in 2001. Isolates were from 11 Provinces in the Red River Delta of North Vietnam. South of Red River: 1 = Nam Dinh; 2 = Ninh Binh; 3 = Ha Nam; 4 = Ha Tay; North of Red River: 5 = Thai Binh; 6 = Hung Yen; 7 = Ha Noi; 8 = Hai Duong; 9 = Bac Ninh; 10 = Thai Nguyen; 11 = Quang Ninh.

Data analysis

Data matrices were analyzed using the Treecon programme (Version 1.3b; Van de Peer and De Wachter, 1994). Genetic similarities based on Jaccard's coefficient were calculated. Cluster analysis was performed on the generated similarity matrices using UPGMA algorithm. All dendrograms were created with the DRAW option of Treecon. Based on visual assessment of the dendrogram, *P. oryzae* isolates with at least 75% similarity were considered as belonging to the same genetic group. Bootstrap node frequency values were calculated with 1000 replications using Treecon. Similarity matrices constructed from data generated by different primer-enzyme combinations (PECs) were compared using the MCOMP module in NTSYS pc. A test for allele association among loci (Brown et al., 1980) was conducted using POPGENE programme version 1.32 (available at http://www.ualberta.ca/~fyeh). POPGENE was also used to estimate gene flow based on the average G_{ST} (population differentation) and Nm (effective migration rate) as described by Nei (1987). The data set was clone-corrected, using only one representative isolate from each clone, for all analyses based on POPGENE.

Results

Genetic structure

To obtain an optimum number of scorable polymorphic bands, 64 *EcoR*I + 2/*Mse*I + 2 primer combinations were tested on a set of six *P. oryzae* isolates. Eight primer combinations were selected (Table 2) based on the number of fragments amplified and the polymorphism rate observed. These primer pairs were applied to the 123 Vietnamese *P. oryzae* isolates listed in Table 1. In total, 733 fragments were scored of which 160 bands were polymorphic (22%) (Table 2).

DNA similarity analysis using Jaccard's coefficient and UPGMA cluster analysis showed that 12

Table 2. Selected primer combinations and polymorphism rates for Amplified Fragment Length Polymorphism (AFLP) analysis of 123 Vietnamese *Pyricularia oryzae* isolates

Primer pair code	*Eco*RI Primers[1]	*Mse*I primers[2]	Number of amplified bands	Number of polymorphic bands	% Polymorphism
PEC1	+CA	+AC	59	23	39
PEC2	+CA	+AG	101	16	16
PEC3	+CA	+GG	123	32	26
PEC4	+CA	+AT	107	23	21
PEC5	+CA	+CG	98	14	14
PEC6	+GC	+GA	69	6	9
PEC7	+CA	+GC	101	23	23
PEC8	+AC	+GC	75	23	31
Total			733	160	22

[1]*Eco*RI primer + 0 = 5′ GACTGCGTACCAATTC 3′.
[2]*MSE*I primer + 0 = 5′ GATGAGTCCTGAGTAA 3′.

Table 3. Rice lines used in the pathogenic characterization of Vietnamese *Pyricularia oryzae* isolates

Cultivar[1]	Type	Resistance genes identified
Near isogenic lines based on CO39		
C039	*indica*	*Pi-C039*
C101LAC	*indica*	*Pi-1, Pi-33*
C104LAC	*indica*	*Pi-1*
C101A51	*indica*	*Pi-2*
C104PKT	*indica*	*Pi-3*
C101PKT	*indica*	*Pi-4*^{a}
Japanese differential cultivars		
K2	*japonica*	*Pi-a*
K3	*japonica*	*Pi-k*^{h}
K59	*japonica*	*Pi-t*
Shin2	*japonica*	*Pi-sh, Pi-k*^{s}
Fujisaka5	*japonica*	*Pi-i, Pi-k*^{s}
Pi no. 4	*japonica*	*Pi-ta*^{2}, *Pi-sh*
Fukunishiki	*japonica*	*Pi-z, Pi-sh*
Toride1	*japonica*	*Pi-z*^{t}
Kanto51	*japonica*	*Pi-k*
ST1	*japonica*	*Pi-f*
Zenith	*japonica*	*Pi-z, Pi-a, Pi-i*
Vietnamese cultivars		
Tetep	*indica*	*Pi-3(t), Pi-4*^{b} *(= Pi-ta), Pi-ta*^{2}
Thamthom	*indica*	
Chiembac	*indica*	*Pi-ta*^{2}
CR203	*indica*	
Other cultivars		
Azucena	*japonica*	Pi-24(t), Pi-26(t), Pi-28(t)
IR64	*indica*	*Pi-25(t), Pi-27(t), Pi-29(t), Pi-30(t), Pi-31(t), Pi-32(t), Pi-ta*
IR1529–860–3	*indica*	
NATO	*japonica*	
NILTH-F145–2	*japonica*	*Pi-b*
Maratelli	*japonica*	Susceptible check
Bala	*indica*	
Moroberekan	*japonica*	*Pi-7(t), Pi-10(t), Pi-12(t), Pi-44(t), Pi-157*

[1]All seeds were obtained from CIRAD, France, except CR203, 'Chiembac' , 'Tamthom', 'Tetep' from Agricultural Genetics Institute, Vietnam.


Similarity (%)
40
50
60
70
80
90
V41-B
V59-A
V36-B
V41-A
V14-B
V33-A
V34-A
V35-B
V4-G
V5-C
V3-E
V3-J
V5-E
V6-E
V6-G
V12-A
V85-B
V89-A
V83-B
V85-A
V100-A
V115-C
V116-B
V1-C
V1-E
V1-F
V3-D
V2-D
V3-I
V3-B
V35-A
V101-A
V101-B
V4-A
V64-A
V5-B
V1-A
V43-B
V92-B
V3-A
V3-C
V19-A
V43-A
V118-A
V4-H
V115-A
V42-A
V1-D
V99-B
V100-B
V111-B
V117-B
V83-A
V24-A
V78-A
V4-F
V86-A
V103-B
V107-B
V1-J
V14-A
VNG1
V3-H
I11
I2
VT1
I6
I1
VT6
VNG2
V75-A
V76-A
V84-A
V27-A
V46-A
V57-A
V26-A
V44-B
V11-B
V47-B
V81-A
V31-B
V87-A
V29-B
V44-A
V21-A
V107-A
V84-B
V98-A
VNG3
V60-B
V5-A
V109-B
V1-B
V80-A
V18-A
V33-B
V1-G
VT7
V13-B
V23-A
V18-B
V58-A
V64-B
V116-A
V6-A
V6-B
V6-D
V38-A
V117-A
V34-B
V115-B
V17-B
V40-A
VNG4
V2-B
VT5
V15-A
V16-B
V20-B
V20-A
VNG5
VT3
V110-A
V110-B
VNG6
V22-A-W
V21-B
V22-A-G
VNG7
indica host isolates
japonica host isolates

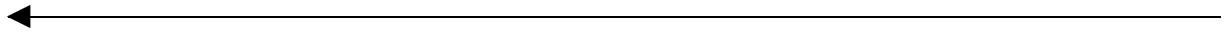

Figure 2. Cluster analysis of 123 Vietnamese *Pyricularia oryzae* isolates using Amplified Fragment Length Polymorphism (AFLP) markers obtained by eight different primer combinations, based on Treecon analysis with Jaccard's similarity coefficients and the UPGMA clustering method. All bootstrap values are indicated. Isolates indicated with a black dot do not belong to the 2001 Red River Delta population and were included for comparison.

genetic groups could be recognized when all isolates were taken into consideration. The 2001 Red River Delta population was comprised of eight genetic groups (Figure 2). Within groups, isolates were at least 75% similar at DNA level. Isolates from japonica hosts (VNG5, VNG6 and VNG7) clearly clustered separately from indica host isolates (VNG1, VNG2, VNG3 and VNG4) (Figure 2). Within the 2001 indica population (see also Table 1) three predominant groups (VNG1, VNG3 and VNG4) contained 54, 17 and 20% of the isolates, respectively. Within these groups, several clones with identical AFLP profiles could be distinguished. Of the nine additional, older isolates that were included in the analyses, four clustered in the VNG2 group (I2, I6, I11, VT1) together with one isolate from the 2001 indica population (V3-H), while one isolate clustered with the VNG4 group (VT7).

AFLP optimization

We tried to optimize the choice of AFLP primer pairs to minimize labour and costs for others who want to apply AFLP for genetic diversity studies. Similarity matrices generated by markers resulting from each individual PEC were compared with each other and were also compared with the similarity matrix generated by the 160 markers obtained by the eight PECS using the NTSYS-MCOMP module (Table 4). Similarity matrices generated from PEC1, PEC3 and PEC8 showed the best correlation with the similarity matrix generated from the 160 markers by all eight PECs. The population structure based on UPGMA cluster analysis using 78 markers generated by the three PECS 1, 3, and 8 is shown in Figure 3. The correlation analysis between the two similarity matrices (eight PEC matrix versus three PEC matrix) yielded a correlation value $r = 0.99$. The dendrogram obtained with only three PECs showed the same grouping as the dendrogram obtained with all eight PECs. Interestingly, these three PECs also produced the highest percentage of polymorphism when compared to the other PECs (Table 2). However, most bootstrap values for the tree obtained with three PECs (Figure 3) were clearly lower and the order of the *japonica* groups and single isolate *indica* groups was slightly changed when compared with the tree obtained with all eight PECs.

Linkage disequilibrium

The number of polymorphic AFLP loci in the indica host populations defined in Table 5 ranged from 80 to 90. In all the populations, the observed variance V_O was higher than the estimated 95% cut-off value for significance L, indicating that there is significant linkage disequilibrium between the AFLP loci within these populations.

Table 4. Matrix comparison between the similarity matrices given by each of the eight AFLP primer combinations and the similarity matrix given by all primer combinations

	PEC1	PEC2	PEC3	PEC4	PEC5	PEC6	PEC7	PEC8
PEC1	1							
PEC2	0.78	1						
PEC3	0.83	0.77	1					
PEC4	0.06	0.07	0.06	1				
PEC5	0.73	0.71	0.84	0.07	1			
PEC6	0.76	0.64	0.74	0.04	0.55	1		
PEC7	0.8	0.68	0.78	0.04	0.73	0.58	1	
PEC8	0.85	0.73	0.85	0.06	0.78	0.69	0.8	1
All markers	0.93	0.83	0.94	0.07	0.83	0.79	0.88	0.94

Correlation values (r) are indicated.


Similarity (%)
40
50
60
70
80
90
V1-C
V1-E
V117-B
V115-C
V116-B
V89-A
V100-A
V101-B
V107-B
V41-A
V41-B
V35-B
V36-B
V59-A
V83-B
V85-A
V85-B
V4-H
V5-C
V3-J
V4-G
V1-F
V2-D
V3-D
V3-E
V6-G
V12-A
V5-E
V6-E
V14-B
V33-A
V34-A
V35-A
V3-I
V99-B
V100-B
V111-B
V1-D
V42-A
V101-A
V4-A
V64-A
V1-A
V5-B
V19-A
V3-C
V4-F
V43-A
V43-B
V92-B
V1-J
V3-A
V86-A
V3-B
V103-B
V115-A
V118-A
V83-A
V14-A
V78-A
V24-A
V2-B
VNG1
VT1
I6
I2
V3-H
I11
I1
VNG2
V75-A
V76-A
V84-A
V27-A
V46-A
V57-A
V47-B
V29-B
V44-B
V11-B
V26-A
V107-A
V21-A
V84-B
V98-A
V44-A
V87-A
V31-B
V81-A
VT6
VNG3
V60-B
V18-B
V58-A
V38-A
V64-B
V116-A
V17-B
V34-B
V115-B
V40-A
V6-D
V6-A
V6-B
V117-A
V1-G
V5-A
V109-B
V1-B
V80-A
V18-A
V33-B
VT7
V13-B
V23-A
VNG4
VT5
V21-B
V22-A-G
V22-A-W
VNG7
V110-A
V110-B
VNG6
VT3
V20-A
V20-B
V15-A
V16-B
VNG5

Figure 3. Cluster analysis of 123 Vietnamese *Pyricularia oryzae* isolates using Amplified Fragment Length Polymorphism (AFLP) markers obtained by primer combinations PEC1, PEC3 and PEC8, based on Treecon analysis with Jaccard's similarity coefficients and the UPGMA clustering method. All bootstrap values are indicated.

Table 5. Multilocus associations among AFLP loci in a population of *Pyricularia oryzae* isolated from *indica* hosts in 2001 from the Red River Delta of North Vietnam

Population[a]	*n*	*m*	*M* (%)	*h*	V_E	V_O	*L*	I_A
Clone-corrected 100%	66	90	56.25	0.125	12.830	111.862	17.191	7.719
Clone-corrected 95%	31	86	53.75	0.133	13.930	93.463	20.840	5.710
Clone-corrected 90%	18	80	50.00	0.135	14.356	94.960	23.702	5.615

Based on multilocus analysis (Brown et al., 1980; Kumar et al., 1999).
n: clone-corrected sample-size; *m*: number of AFLP polymorphic loci; *h*: mean single-locus diversity; V_E: expected variance; V_O: observed variance; *L*: upper 95% confidence limit for the observed variance; I_A: index of association ($I_A = V_O/V_E$-1) and has an expected value that does not differ from zero if there is no association between loci.
[a]Only one isolate of clones or clonal lineages defined by 100%, 95% or 90% similarity was used in the analysis.

Table 6. Distribution of Vietnamese blast AFLP groups[1] across various provinces in Vietnam (only the 2001 population from North Vietnam has been taken into consideration)

Provinces	# isolates	Vietnamese AFLP groups						
		Indica group				Japonica group		
		VNG1	VNG2	VNG3	VNG4	VNG5	VNG6	VNG7
North Vietnam								
North of red river								
Bac Ninh	10	7			3			
Ha Noi	17	5		5	2		2	3
Hai Duong	2	1		1				
Hung Yen	8	6			2			
Quang Ninh	1	1						
Thai Binh	5	1	1	2		1		
Thai Nguyen	9	3		5	1			
South of red river								
Ha Nam	20	13			7			
Ha Tay	9	1		6	2			
Nam Dinh	15	8			4	3		
Ninh Binh	17	15			2			
Number of isolates	113	61	1	19	23	4	2	3
N provinces		11	1	5	8	2	1	1

[1]*Pyricularia oryzae* V2-B, not assigned to VNG1–7 was isolated from Ninh Binh.

Population differentiation

When the *indica* population north of the Red river was compared with the population south of the Red river, the estimated G_{ST} was very low ($G_{ST} = 0.021$), indicating genetic exchange between populations north and south of the Red river (Nm = 24 which means that 24 individuals would need to be exchanged each generation to account for the actual degree of similarity between the two populations).

Distribution of the 2001 Red River Delta Pyricularia oryzae *population*

The 61 isolates belonging to VNG1 were widely distributed in 11 provinces in the North of

Table 7. Host response[1] to 23 Vietnamese *Pyricularia oryzae* isolates from different AFLP groups on 29 rice lines

			Rice differentials												
Indical/Japonica			I	I	I	I	I	I	I	I	I	I	I	I	I
AFLP	Isolate	Province	CO39	C101LAC	C1041AC	C104PKT	C101PKT	C101A51	IR64	Tetep	Chiembac	Thamthom	CR203	IR1529–860–3	Bala
VNG1	V1-C	Ninh Binh	S	R	R	S	R	R	S	R	R	S	S	S	R
VNG1	V1-E	Ninh Binh	S	R	R	S	R	R	R	R	R	S	S	S	R
VNG1	V12-A	Quang Binh	S	R	R	S	R	R	R	R	R	R	S	S	R
VNG1	V14-B	Hung Yen	S	R	R	S	R	R	R	R	R	R	S	S	S
VNG1	V14-A	Hung Yen	S	R	R	S	R	R	S	R	R	S	S	S	S
VNG2	V3-H	Ninh Ninh	S	R	R	R	R	R	S	R	R	R	S	S	S
VNG2	I2	Thai Binh	S	R	R	R	S	R	R	R	R	R	S	R	S
VNG2	VT1	Ho Chi Minh	S	R	R	R	S	S	R	R	R	R	R	S	S
VNG2	I6	Nghe An	S	R	R	S	R	R	S	R	R	R	S	S	S
	I1	Hanoi	S	R	R	R	S	R	S	R	S	R	S	S	S
	VT6	Hue	R	R	R	R	R	R	R	S	S	S	R	S	R
VNG3	V11-B	Hanoi	S	R	S	S	S	R	R	R	R	R	S	R	R
VNG3	V27-A	Thai Nguyen	S	R	R	R	S	R	R	R	R	R	S	R	R
VNG3	V98-A	Ha Tay	S	R	R	S	S	R	R	R	R	R	S	R	S
VNG4	V1-G	Ninh Binh	S	R	R	S	S	R	S	R	R	R	S	S	S
VNG4	VT7	Hue	S	R	R	S	S	R	R	R	R	R	S	R	R
VNG4	V40-A	Bac Ninh	S	R	R	S	R	R	S	R	R	S	S	S	S
VNG4	V60-B	Ha Tay	S	R	R	S	R	R	S	R	R	S	S	S	R
	V2-B	Ninh Binh	S	R	R	S	R	S	S	R	R	S	S	S	R
	VT5	Hue	R	R	R	R	R	R	R	S	S	S	R	S	R
VNG5	V20-B	Nam Dinh	R	R	R	R	R	R	R	R	S	R	R	R	R
	VT3	Hue	S	R	S	R	S	R	R	R	R	R	S	R	R
VNG6	V110-B	Hanoi	R	R	R	R	R	R	R	R	R	R	R	R	R
Compatible interaction(%)			83	0	9	57	39	9	39	9	17	35	78	65	43

[1]R: resistance interaction (score 0–2); S: susceptible interaction (score 3–6) based on the 0–6 lesion type scale, –: data missing.

Vietnam (Table 6). Groups VNG3 and VNG4 were found in five and eight provinces in the North of Vietnam, respectively. Within these three groups, isolates that belonged to the same AFLP haplotype (putative clones) were also found in several provinces. Within VNG1, for instance, 41% of the isolates belonged to the same clone that could be found in nine different provinces, indicating the importance of genotype flow in the North of Vietnam. Among the provinces studied, Hanoi had the most diverse *P. oryzae* population with the presence of five different AFLP groups (Table 6).

Pathogenicity analysis

Pathogenicity tests were carried out with five isolates from VNG1, four isolates from VNG2, three isolates from VNG3, four isolates from VNG4 and one isolate from each of the other genetic groups (Table 7). Isolates V22-A-G and V21-B, both belonging to genetic group VNG7 were avirulent on all 29 differentials and were omitted from the table. Twenty-three pathotypes were detected among the 23 Vietnamese isolates tested. Isolates V2-B, V60-B, V14-A and V40-A belonging to different AFLP groups had a wide virulence spectrum and could infect at least 55% of the rice blast differentials tested. Isolates V20-B, V110-B and VT5 were found to have a narrow virulence spectrum since they could infect a maximum of 17% of the differentials.

Inoculation results showed that 'Maratelli', 'Azucena' 'CO39' and 'NILTH-F145-2' were broadly susceptible lines. These lines showed 83% of compatible interaction to the *P. oryzae* isolates tested. 'Moroberekan' and 'C101LAC' were resistant to all Vietnamese blast isolates tested. 'Pi no. 4' and the Vietnamese traditional cultivars 'Tetep' and 'Chiembac' were resistant to the three predominant genetic groups VNG1, VNG3 and VNG4 and the very virulent V2-B isolate from Ninh Binh. Also 'C101A51' (*Pi-2*) and 'Kanto51' (*Pi-k*) showed resistance to all isolates from the three predominant genetic groups, but both cultivars were sensitive to V2-B.

I	J	J	J	J	J	J	J	J	J	J	J	J	J	J	J	J
Zenith	Fukunishiki	Pin 4	Shin 2	Fujisaka5	Kanto51	NIL TH-F145–2	Toridel	K59	Azucena C	Moroberekan	NATO	Maratelli	K3	K2	ST1	Compatible interaction(%)
R	R	R	S	S	R	S	S	S	S	R	S	S	R	R	R	48
R	R	R	R	S	R	S	S	S	S	R	S	S	R	R	R	41
R	R	R	R	S	R	S	S	S	S	R	S	S	R	R	R	38
R	R	R	R	S	R	S	R	S	S	R	S	S	R	R	R	38
R	R	R	S	S	R	S	S	S	S	R	S	S	R	R	S	55
R	R	R	S	R	R	S	S	S	S	R	S	S	R	R	S	45
R	R	R	R	R	R	S	–	S	S	R	S	S	R	R	–	31
R	R	R	R	R	R	S	R	R	S	R	R	R	R	R	S	28
R	R	R	S	S	R	S	S	S	S	R	S	S	R	R	R	48
R	R	S	R	R	R	R	R	S	S	R	S	S	R	R	R	41
R	R	S	R	R	R	S	–	R	R	R	R	R	R	R	S	24
S	R	R	S	S	R	S	R	S	S	R	S	S	R	S	R	48
S	R	R	S	R	R	S	R	S	S	R	S	S	R	S	S	41
S	R	R	S	R	R	S	R	S	S	R	S	S	R	R	S	45
R	S	R	S	S	R	S	R	S	S	R	S	S	R	R	R	52
R	R	R	R	S	R	S	–	R	S	R	S	S	R	R	–	31
R	R	R	S	S	R	S	R	S	S	R	S	S	S	S	R	55
S	S	R	S	S	R	S	R	S	S	R	S	S	R	S	S	59
S	R	R	S	S	S	S	R	S	S	R	S	S	R	S	S	62
R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	17
R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	3
R	R	R	S	R	S	R	R	R	S	R	S	S	S	S	S	41
R	R	S	R	R	R	S	R	R	R	R	R	S	R	R	R	10
22	9	17	52	52	9	83	30	70	83	0	78	83	9	26	43	

Discussion

The genetic structure of the *P. oryzae* population of the Red River Delta in North Vietnam appears to be highly diverse. AFLP analysis clearly separated *japonica* host isolates from *indica* host isolates with a similarity of less than 40%. This result is consistent with limited gene flow between the two groups. All *japonica* host isolates belonged to minor AFLP groups, probably because *japonica* rice is not widely grown in Vietnam. In the year 2000 for instance, the area for *japonica* rice was only 5.38% of the total rice growing area in North Vietnam (Pham et al., 2004). In Vietnam, *japonica* rice is only used in special dishes, while *indica* rice is widely grown for daily consumption. Isolates from *japonica* hosts in 2001 have a narrow virulence pattern compared to isolates from *indica* hosts: V20-B was virulent only on 3% and V110-B on 10% of tested cultivars, while V22-A and V21-B were not virulent to any of the used rice lines. These results indicate a specialization of *P. oryzae* according to the subspecies of the rice lines from which the fungus is isolated, but more isolates from *japonica* hosts should be tested to study this new property in the rice-*P. oryzae* relationship.

Within the *indica* host isolates, VNG1 is the most predominant group (54%), supported by a bootstrap value of 99%. Isolates within this clonal lineage are closely related with 90% or more DNA similarity. Within the VNG1 lineage, even clones with identical AFLP profiles were widely distributed in various provinces in the North of Vietnam, indicating the importance of genotype flow in this region, which is most likely caused by movement of infested seed. None of the older isolates that we included in our study belonged to the VNG1 group. The minor group VNG2, supported by a bootstrap value of 87%, comprises genetically diverse isolates obtained in 1989, 1994, 1997 and 2001 from the South, Central and North of Vietnam (Table 1). This suggests that the genetic group VNG2 already existed in 1989, and at least before 2001 it appeared to be widespread in Vietnam. The other two predominant genetic groups VNG3 and VNG4 contain isolates which are genetically more diverse with low bootstrap values at the nodes. Despite this observed diversity, a test

for randomness of association among loci indicated that the 2001 Red River Delta population is not a random mating population and that asexual reproduction is predominant. In addition, only mating type MAT1-2 was observed in this Vietnamese population (N.T. Ninh Thuan, Ghent University, Belgium, unpubl.). Our data are in agreement with observations in most rice-growing areas in the world, where asexual reproduction of *P. oryzae* is predominant (Zeigler, 1998).

Since the sampling in Vietnam was done in the field, we were not able to identify the host cultivar for many isolates. However, for those isolates for which the cultivar is known, we found no obvious link between isolate grouping and the cultivar from which they were obtained. VNG1, for instance, contains isolates obtained from at least five different cultivars (see Table 1 and Figure 2). This is in contrast with the genetic structure of *P. oryzae* populations in Iran, where certain lineages were specifically adapted to certain host cultivars (Javan-Nikkhah et al., 2004).

Hanoi appeared to be the province with the highest blast diversity. This could be due to the fact that in this province more than 38 cultivars are grown on a relatively small area (53,000 ha). In other provinces, the rice growing area is larger, with fewer different cultivars per hectare (Pham et al., 2004).

Previous work done by Don et al. (1999a) using RFLP-MAGGY showed that the genetic structure of the *P. oryzae* population from the Mekong River Delta in South Vietnam is less diverse than the population from North Vietnam. Sixty-one *P. oryzae* isolates from the South clustered in one lineage while 17 isolates from the provinces Ha Tay and Thai Binh in North Vietnam clustered into four lineages. Although we used different markers, our data appear to be in agreement with these findings. The 14 isolates collected from Ha Tay and Thai Binh in this study clustered in three predominant and two minor AFLP groups.

The North-Vietnamese *P. oryzae* population also appears to have a more variable pathogenicity than the population in the South. In the 2001 population of the Red River Delta of North Vietnam, we identified 13 pathotypes from 13 tested *P. oryzae* isolates. Noda et al. (1999) studied the pathogenicity of 129 *P. oryzae* isolates from the Mekong River Delta in South Vietnam. Isolates were classified into 12 pathogenic groups based on their virulence to 12 Japanese differential rice varieties. Don et al. (1999a) identified five different races when 17 *P. oryzae* isolates from the Red River Delta of North Vietnam were tested on nine Japanese differential lines. When in our 2001 population we exclude the *indica* differentials from our pathogenicity test, we still obtain 12 pathotypes from the 13 isolates tested.

Genetic groups VNG1, VNG3 and VNG4 are most widespread in North Vietnam and should be considered when breeding for resistance. Moroberekan and 'C101LAC' (*Pi-1*, *Pi-33*), based on the 'CO39' genetic background, showed broad-spectrum resistance against all Vietnamese isolates. Moroberekan is a well-known resistant cultivar, which has been tested in many countries. This land race cultivar has a number of major resistance genes and QTLs, which makes it hard to know which genes are effective against the Vietnamese population. The resistance gene *Pi-ta*^{2} could be of interest for rice breeding in North Vietnam since it is most likely responsible for the resistance to the three predominant genetic groups VNG1, VNG3 and VNG4 observed in 'Pi no. 4' (*Pi-ta*^{2}) and the Vietnamese traditional cultivars, 'Tetep' and 'Chiembac'. 'Tetep' and 'Chiembac' were reported to have a broad-spectrum resistance to blast disease in Vietnamese rice fields (Ninh Thuan et al., 2000). 'Tetep' is known to carry several resistance genes including *Pi-ta* and *Pi-ta*^{2} (Bryan et al., 2000). A broad-spectrum resistance gene in 'Chiembac' was characterized and is probably identical to *Pi-ta*^{2} (N.T. Ninh Thuan, Ghent University, Belgium, unpubl.). Interestingly, isolates VT5 and VT6 that are virulent on *Pi-ta*^{2} had a narrow host range and showed a resistant reaction on broadly susceptible cultivars such as 'CO39', 'Maratelli' (reported to be used as susceptible check) (Roumen et al., 1997), 'CR203' and 'NATO'. This could indicate that compatibility with *Pi-ta*^{2} resistance limits compatibility with other resistance genes.

Within the Japanese differentials, Kanto 51 (*Pi-k*) appeared to be resistant to the predominant genetic groups in Vietnam, indicating that *Pi-k* may be of interest for rice breeding in North Vietnam. Kanto 51 was also resistant to the 17 isolates from the Red River Delta tested by Don et al. (1999a).

The information obtained from Don et al. (1999a), Noda et al. (1999) and from this study

may ultimately be useful to rice blast disease management in Vietnam. Blast resistance breeding for North Vietnam probably needs more study and effort than for South Vietnam because the *P. oryzae* population in the North is genetically more diverse and more variable in pathogenicity compared to the population in South Vietnam.

In conclusion, we identified an optimal set of AFLP primer combinations that can be used for characterization of *P. oryzae* populations. The 2001 population of *P. oryzae* in North Vietnam appears to be diverse, but we did not find gametic equilibrium consistent with sexual recombination. Ideally, the sampling of *P. oryzae* isolates from North Vietnam should be repeated so that the biological evolution of this population can be determined.

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