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RESEARCH ARTICLE

Avirulence gene based RFLP and rep-PCR distinguish the genetic variation of *Xanthomonas oryzae* pv. *oryzae* pathotypes in Bangladesh

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Abstract

Bacterial blight (BB) caused by *X. oryzae* pv. *oryzae* is one of the devastating diseases of rice mostly in Asia. Genomes of *X. oryzae* pv. *oryzae* is highly variable due to rearrangement of the large contents of transposable elements and dynamic changes of *X. oryzae* pv. *oryzae* population regulated efficiency of the control measures used for BB management of rice worldwide. In this study, genetic variation of *X. oryzae* pv. *oryzae* pathotypes of Bangladesh was studied using aviruelnce gene based RFLP and rep-PCR techniques aimed to formulate pathogen targeted effective control measures against BB of rice. Eight pathotypes of *X. oryzae* pv. *oryzae* field isolates were identified based on their reactions against 10 Near Isogenic Lines (NILs). Among eight pathotypes, pathotypes IV and V contained higher number of isolates which were 30.13% and 23.01% respectively while pathotype VIII revealed as minimum containing only 2.51% of total isolates. These eight pathotypes were studied for their genetic variation by RFLP using *avrBs3* repeat domain as probe. The results conceded that Bangladeshi *X. oryzae* pv. *oryzae* strains seem carrying a minimum of two and maximum of nine *avrBs3* family genes homologs. The resistance phenotype on IRBB7 and IRBB10 NILs also indicated presence of two major *avrBs3* family genes viz. *avrxa7* and *avrXa10* in some pathotypes. Relationship of phylogenicity exhibited that *X. oryzae* pv. *oryzae* pathotypes assorted into two RFLP haplotypes as well as these haplotypes are largely distributed in Bangladesh. Phylogenetic analyses carried out by (REP, ERIC), rep-PCR and BOX depicted the presence of two main molecular haplotypes of *X. oryzae* pv. *oryzae* pathotypes. The relationship between pathotypes and molecular haplotypes of *X. oryzae* pv. *oryzae* in Bangladesh indicated that the same lineage possesses different pathotypes and different lineage possesses different pathotypes. The results indicated that eight different pathotypes might have originated from common inherited haplotypes with a wide genetic variation.

Keywords

RFLP, Rep-PCR, Pathotypes

Introduction

Co-evolution of reciprocal, ''arms race'' like relationship between bacterial pathogens and their hosts for adaptation has been observed during plant-microbe interactions (1, 2). Bacterial pathogens overcome host defence or to establish successful infection in novel hosts through the development of new virulence factors such as effectors by maintaining a high level of ge-

nomic polymorphism (3). A number of genetic factors such as gene insertion, deletion, point mutations, duplication, fission, fusion and wide range of genomic reorganization were accounted for extreme levels of genomic plasticity within several bacterial populations (4). A genetic process named horizontal gene transfer is fundamental for genomes to get novel DNA segments from remotely more or less similar organism through a number of process like conjugation, transduction and transformation as well as which booms genetic variability of bacteria (5). To coherent evaluation of mechanism and potentiality of pathogenic bacteria, the level of genetic variation must be analysed deeply (6).

A vascular pathogen named *Xanthomonas oryzae* pv. *oryzae* is the causal agent of Bacterial blight (BB) disease of rice (7, 8) is one of the utmost devastating disease of rice throughout the globe (9). *X*. oryzae pv. *oryzae* (*Xoo*) is a round-ended, rod-shaped, gram-negative bacterium whose cells vary in length and are motile by means of a single polar flagellum. Colonies on nutrient broth yeast extract media containing glucose are round, convex, mucoid and yellow in colour due to the development of the pigment Xanthomonadin, the characteristic of the genus (10). Extreme rain, humidity and temperature are having been designated to be significant factors creating larger amount of disease incidence and severity of this disease (11). Normally, in different growth stages of rice plant BLB appears, disease symptoms predominantly exhibit on leafs showing leaf blight or revealing wilting symptoms of young plants which has been known as Kresek, to infect such way, wound or water pores are served as a prime invading option for the pathogen *X*. *oryzae* pv. *oryzae* lesions with wavy edges emerge from the leaf tip and margins and then coalesce and enlarge in size, turn yellow, and then ultimately dying of the plant (12). 20 to 40% reduction of yield was accounted when plants infected at tillering stage with the highest rate (13). Up to 50% yield reduction was also manifested in the early detection of bacterial blight disease in rice (14). Throughout the last few years, a serious BB epidemic happened in many areas of Bangladesh with substantial yield losses of around 50-70% especially in irrigated hybrid varieties possibly due to the emergence of new races of its pathogen (15). It has been reported the pathogenic variability of *X*. *oryzae* pv. *oryzae* in Bangladesh (16, 17). Twelve races of the *X*. *oryzae* pv. *oryzae* have been identified until 1995 in Bangladesh and the study indicated that some aggressive strains of *Xoo* occur in Bangladesh (16). Severe outbreak was observed in Bangladesh during the season of Boro 2007-2008 in both hybrid and inbred varieties (18). Bacterial blight is prevalent in both tropical and temperate areas and is endemic to much of Asia and parts of West Africa. In Asia specifically in Bangladesh, this disease occurs destructively during the monsoon (14) and it is a serious threat to agriculture and global food security (19). The diseases become one of the most devastating of rice because of the extensive cultivation of nitrogen-responsive modern rice cultivars (13). Damage due to BB increased significantly following the widespread cultivation of high yielding and nitrogen-responsive dwarf hybrid varieties of rice in the 1960s (20).

However, several practices are included in controlling BB include biological control, chemical control, cultural methods, host genetic resistance and disease forecasting all around the globe (21). In Bangladesh, pathotypic and genotypic analyses were carried in some selected rice-growing areas during the last few years and around 24 distinct pathotypes were detected around four years ago (15, 22). The results also revealed an admixture of the *X*. *oryzae* pv. *oryzae* races in the North to Mid-eastern districts of the country and indicated the existence of widespread genetic variation such as physiological, morphological and biochemical differences among *Xoo* strains (23). Another significant factor described was host–pathogen interactions ultimately modified in the pathogenic structures through mutation process accounted for high degree of DNA polymorphism and also observed that molecular variation was utmost between strains of several virulence phenotype (15). Genetic variation mainly influenced by widespread cultivation of rice varieties as pathogen is exceedingly dynamic in nature thus a shift in pathogen population structure and varieties possessing several resistance genes have transformed into susceptible cultivars against BB in prevalent rice producing areas of the world (6). Regional variation and diversity of *X*. *oryzae* pv. *oryzae* (*Xoo*) was remarkable in Asia because of polymorphic virulent strains (24). A single crop cycle leads to the modification in pathogen population structure through either recombination or mutation to adapt itself in several unfavourable ambience (25).

Therefore, distribution of resistant cultivars containing foremost resistance (*R*) genes by the breeding programme could be the effective method to control BB. Still now, against bacterial blight 42 resistant genes has been studied coming from japonica varieties, indica cultivars as well as a number of resembling wild species such as *O*. *officinalis, O. rufipogon*, *O. minuta* and *O. longistaminata* (26-28). Due to overused of few resistance genes which might triggered the selection of new pathogenic races of the isolates followed by the selection of resistant host plants when a crop cycle is fully complete. Thus, this activity initiates either mutation or recombination changing the population arrangement of pathogen to accustom itself to the novel resistant host and changes of associated environment. Through several members of the *avrBs*3/*pthA* family in numerous strains or races, virulance disintegration is seem to be mediated. In Korean strain (KACC10311), 15 members are included in the family of *avrBs3/pthA* indicated by genome sequence (29), 19 in USA (PXO99A) (30) and 17 in Japan (MAFF311018) (31). Moreover, *Xoo* African strains hold up to eight members of *avrBs3/pthA* and on the other hand, in a number of Asian strains, 16 copies were identified excluding Bangladesh (32, 33).

To evaluate the *X*. *oryzae* pv. *oryzae* strains globally and analyze the molecular haplotyping excluding Bangladeshi strains, two different approaches have been manifested those are polymerase chain reaction (rep-PCR) with repetitive sequence-based and RFLP (Restriction fragment length polymorphism) (34). Identification and functional

characterization of *avrBs*3 family members genes in many *Xanthomonas* spp. more importantly in *X. oryzae* pv. *oryzae* have been reported to clarify the relation between races and genotypes (4, 35). *AvrBs*3 family proteins contains a central repeat domain, a nuclear localization signals (NILs) and an acidic transcriptional activation domain (36). Generally, the repeat number at specific position determines host specificity or adaptation to virulence. It is possible that the evolution of the 102-bp repeat unit is related to the mechanisms of virulence evolution in *X. oryzae* pv. *oryzae* under selection pressure by host *R* genes. To unravel the relationship between virulence phenotypes and the genotypes of Bangladeshi *X. oryzae* pv. *oryzae* races, the present study was designed to identify copy numbers and distribution patterns of *avrBs*3 family genes in *X. oryzae* pv. *oryzae* isolates in Bangladesh using RFLP analyses with *avrBs*3 family gene as probe. Repetitive sequence-based rep-PCR (polymerase chain reaction) analyses have been demonstrated to assess molecular haplotyping and evolution of *X. oryzae* pv. *oryzae* strains worldwide (34) except strains from Bangladesh. Epidemiological experiments of numerous mammalian pathogens were exhibited to detect the efficacy level of rep-PCR technique (37, 38). These techniques were also used to differentiate pathovars and strains of plant-pathogenic Xanthomonads and Pseudomonads (39). Oligonucleotide primers for the amplification of DNA from three families of irrelevant repetitive DNA sequences corresponding to REP (repetitive extragenic palindromic) (40) are required for build this technique, ERIC (enterobacterial repetitive intergenic consensus) (41) and elements of BOX (42). The reasons to use the sequences of REP, ERIC and BOX are to know the evolution of lineages of *X. oryzae* pv. *oryzae*. In this study, eight pathotypes of *X. oryzae* pv. *oryzae* were used to analyze the haplotypic variation using RFLP and rep-PCR (REP, ERIC) and BOX.

Materials and Methods

Isolation and identification of X. oryzae pv. oryzae isolates

Rice leaf samples infected with BB (Bacterial Blight) were coalesced from diverse rice fields, plots and farmers' plantings representing 30 Agro-ecological Zones (AEZs) in Bangladesh, during 2015-2016. From those infected samples, 239 isolates of *X. oryzae* pv. *oryzae* were isolated by following method described earlier by (23) and each isolates comprised of three replicates. In net house, a pathogenicity test by using a check cultivar IR24 which is susceptible was used to confirm the isolates of *X. oryzae* pv. *oryzae*. Wizard® genomic DNA purification kit (Promega, Madison, WI, USA) was used for the extraction of the genomic DNA of *X. oryzae* pv. *oryzae*. By using this kit genomic DNA was extracted from 1 ml of liquid culture of each isolate. Extraction of genomic DNA from each strain of *X. oryzae* pv. *oryzae* were quantified using a UV spectrophotometer absorbance at 260 nm with a model T-80 UV/VIS and stored at -20 °C (23). Genomic DNA was stored at -20 ^{0}C. DNA concentration were adjusted to 100ng/µl and verified for PCR on 1.5% agarose gel. Very specific primers XOR-F (GCATGACGTCA TCGTCCTGT) and XOR-R2 (CTCGGAGCTATATGCCGTGC) and XOR-F (GCATGACGTCATCGTCCTGT) were also utilised for the confirmation of *X. oryzae* pv. *oryzae* has documented earlier *(43)* with the help of GoTaq® G2 Green Master Mix (Promega, Madison, WI, USA). PCR conditions were maintained by sequentially as follows: 2 min initial denaturation at 95 °C, 30 sec initial denaturation with 30 cycles at 95 °C, 30 sec annealing at 63 °C, 1 min extension at 72 °C and finally 7 min extension at 72 °C. The molecular size marker was a 100-bp DNA ladder (Invitrogen).

Pathotype analysis

A method described (23) that ten (10) near-isogenic lines (NILs) of rice those were IRBB2, IRBB4, IRBB1, IRBB5, IRBB8, IRBB13, IRBB14, IRBB21, IRBB11 and IRBB10 carrying a BB *R* -gene in the background of IR24 (a susceptible check cultivar) gone through a pathotypic analyses of the pathogen of BB.

RFLP analysis using avrBs3 repeat domain as probe

The genetic diversity of the pathogen of BB pathotypes were assessed using RFLP analyses with *avrBs*3 repeat region as probe as described previously by (44) with some modifications using Alkphos direct labelling kit (GE Healthcare/Amersham) where two replicates were used for each pathotypes. Genomic DNA (5 µg) of each pathotype of *X. oryzae* pv. *oryzae* was digested with *2 µ*l of *Eco*RI (Invitrogen) in a reaction volume 20 µl containing 1 µl of RNAse A (10 mg/ml) at 37 °C for at least 8 hrs and the digested DNA was then separated on 0.7% agarose gel for overnight (18 hrs) at 40V. The DNA was then transferred to Hybond N+-membrane by capillary action for overnight and was fixed by exposing the membrane under UV light for 5 min. Probe was prepared by PCR amplification of the *avrBs*3 repeat domain containing the 2.0 kb fragment using primers *AvrBs3*-FP: 5´-AGACGGTGCAGCGGCTGTT-3´, *AvrBs*3-RP: 5´-TGGGATGGCCACGACCTGGT-3´ with PCR conditions: 2 min initial denaturation at 95 °C, 30 sec initial denaturation with 30 cycles at 95 °C, 30 sec annealing at 63 °C, 1 min extension at 72 °C and finally 7 min extension at 72 °C. The PCR products were confirmed by sequencing according to the standard protocols for the ABI 3730xl DNA genetic analyzer (Applied Biosystems, Foster City, CA, USA) with BigDye® Terminator v1.1 and 3.1 Cycle Sequencing Kits. The probe was then labelled with Alkphos direct labelling kit (GE Healthcare/Amersham) according to manufacturer's instruction, Pre-hybridization, hybridization and post-hybridization washes were performed according the manufacturer's instruction.

Identification of avirulence genes from X. oryzae pv. oryzae

In order to confirm the presence of the known *avrBs*3 family members, phenotypic characterization was performed by assessing the avirulence and aggressiveness of *X. oryzae* pv. *oryzae* pathotypes or races on rice NILs containing R genes (*Xa7* and *Xa10)* and IR-24 was used as control (45). The virulence assays were performed by leaf infiltration method according to (46, 47). Briefly, bacterial suspensions were inoculated to rice leaves of 16 days old rice seedlings by infiltration of bacterial suspension (5×10^{7} CFU/ml) with hypodermal syringe after pricking the leaves with insect pin

(47). By the method of infiltration the development of symptoms were analysed after inoculation at least 5 days later. 2, 4 and 5 days after infiltration lesion lengths were scored after leaf-clip inoculation because incompatible responses involving *Xa*7 and *Xa*10 take longer to develop than those involving *Xa*7 (3-4days) and *Xa*10 (4-5 days).

Rep-PCR analysis of X. oryzae pv. oryzae

Molecular haplotyping of *X. oryzae* pv. *oryzae* pathotypes was carried out by rep-PCR. Rep-PCR analyses of *X. oryzae* pv. *oryzae* pathotypes were performed as described previously (34). For the process of rep-PCR primers were as follows: for ERIC, ERIC (5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC2 (5'-AAGAAGTGACTGG GGTGAGCG-3'), for REP, REP (5'-CGICGICATCIGGC-3') and REP2-1 (5'-ICGICITATCIGGCCTAC-3') and for BOX, BOX (5'-CTACGGCAA GGCGA CGCTGACG-3'). The conditions for PCR amplification were: for the REP primers, the cycles were as follows: 6 min for 1 cycle at 95 °C , 1 min for 30 cycles at 94 °C, 1 min at 40 °C, and 8 min at 65 °C; 16 min for 1 cycle at 65 °C; For the ERIC primers, 7 min for the first cycle at 95 °C followed by at 94 °C 30 cycles for 1 min, 1 min at 52 °C, and 8 min at 65 °C; 16 min for 1 cycle at 65°C (39) and for the BOX primers, 7 min for the first cycle at 95 °C followed by 1 min for 30 cycles at 94 °C, 1 min at 53 °C, and 8 min at 65 °C; 16 min for 1 cycle at 65 °C; and at 4 °C for the final soak (48). Temperature for each PCR conditions were varied due to having different melting temperatures of forward and reverse primers of REP, ERIC and BOX. Template DNA (50 ng) was used per reaction. 1.5% agarose in 1XTBE buffer (Tris base, boric acid and 0.5 M EDTA [pH 8.0]) was used for the analysis of PCR products using 0.5 µg/ml ethidium bromide. After electrophoresis, the gel was placed under UV transilluminator (GelView Master, Dynamica, UK) for visualization of DNA bands.

Data analysis

An unweighted pair group method of averages (UPGMA) was used for making dendograms belong to SAHN programme of NTSYSpc 2.02i (Exeter Software, Setauket, NY) and a dice coefficient option was exploited from making similarity matrix as well as by using Winboot (a computer program) was directed to assess the dendrogram robustness. To detect the confidence limits of each dendrogram 1000 replications of bootstrap analysis were employed by the exact computer program.

Results

Confirmation of X. oryzae pv. oryzae by PCR

X. oryzae pv. *oryzae* isolates were confirmed by PCR amplification of genomic DNA of the 8 representative pathotypes (I-VIII) and negative control was maintained with nuclease-free water. Results showed that the primers XOR-F and XOR-R amplified a specific DNA fragment in the size of 470-bp with DNA of all *X. oryzae* pv. *oryzae* pathotypes *(*Fig. 1).

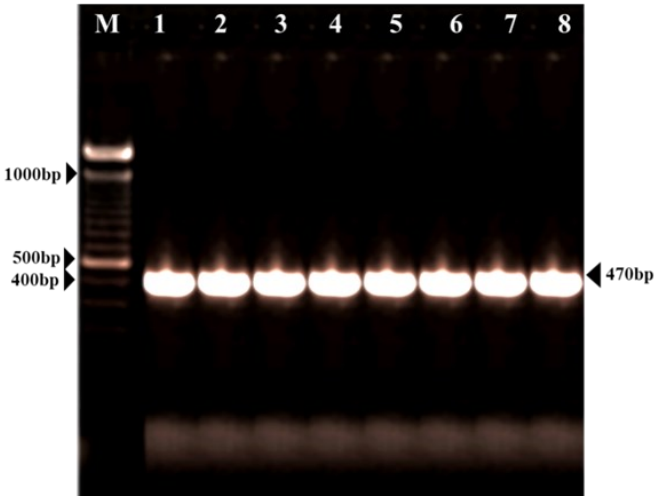

Fig. 1. PCR confirmation of *X. oryzae* pv. *oryzae* isolates representing eight pathotypes using primer XOR-F and XOR-R. M: the molecular size marker is a 100-bp DNA ladder.

Determination of pathotypes of X. oryzae pv. oryzae field isolates

239 *X. oryzae* pv. *oryzae* pathotypes of field isolates were determined depend on their reactions against ten Near Isogenic Lines (NILs) and a total eight pathotypes (I-VIII) were determined. Pathotype IV consisted of maximum 72 isolates followed by Pathotype V- 55, Pathotype III- 36, Pathotype II- 28, Pathotype I- 17, Pathotype VI- 13, Pathotype VII- 12 and Pathotype VIII- 6. Pathotype I showed the uppermost virulence congenial with all NILs, conversely, pathotype VIII manifested minimum virulence inharmonious with NILs. Except *Xa1*, *Xa4* and *Xa5,* pathotype VIII manifested avirulence reactions against all *R*-genes. Pathotype II performed better virulence by showing maximum compatible reaction to NILs. Pathotype II showed avirulence reaction only with *Xa1*, *Xa8* and *Xa21.* Pathotype III performed avirulence interactions to four *R*-genes and showed virulence responses to *Xa4*, *Xa5, Xa8, Xa13, Xa14* and *Xa21.* The major pathotype IV exposed virulence response to *Xa2*, *Xa4, Xa8, Xa10, Xa14* and *Xa21* whereas pathotype V exhibited virulence reactions to *Xa2*, *Xa5, Xa8, Xa13* and *Xa14.* Pathotype VI exhibited virulence response to *Xa2*, *Xa4, Xa5, Xa11* and *xa13* whereas pathotype VII exposed virulence reactions with *Xa4, Xa10, Xa13* and *Xa14.* Likewise, virulence and avirulence reactions was found in all other pathotype to each other with NILs tested (Table 1).

RFLP analysis

RFLP based genotyping of eight pathotypes of *X. oryzae* pv. *oryzae* was performed by southern hybridization using *avrBs3* repeat region as DNA fingerprinting probe to determine their genetic relationship. The results conceded that two to nine fragments were detected after hybridization of *EcoR1* genomic DNA fragments of these eight pathotypes of *X. oryzae* pv. *oryzae* with molecular weight approximately 500bp to 13kb (Fig. 2). This result indicated that the isolates representing each pathotype encoded multiple *avrBs3* homologs. Among the isolates representing eight pathotypes, *X. oryzae* pv. *oryzae* isolates representing pathotype I generated only two bands and the isolates representing pathotypes I and VII generated highest number (nine) of

Table 1. Response of NILs to eight pathotypes of *X. oryzae* pv. *oryzae* isolates in irrigated and rainfed season during *2016*

Pathotype	Total Isolates	% of Isolates	Near-Isogenic Lines (NILs) and single known resistance genes[a]										
			IRBB1 *(Xa1)*	IRBB2 *(Xa2)*	IRBB4 *(Xa4)*	IRBB5 *(xa5)*	IRBB8 *(Xa8)*	IRBB10 *(Xa10)*	IRBB11 *(Xa11)*	IRBB13 *(xa13)*	IRBB14 *(Xa14)*	IRBB21 *(Xa21)*	IR24 (susceptible)
I	17	7.11	S[b]	S	S	S	S	S	S	S	S	S	S
II	28	11.72	R	S	S	S	R	S	S	S	S	R	S
III	36	15.06	R	R	S	S	S	R	R	S	S	S	S
IV	72	30.13	R	S	S	R	S	S	R	R	S	S	S
V	55	23.01	R	S	R	S	S	R	R	S	S	R	S
VI	13	5.44	R	S	S	S	R	R	S	S	R	R	S
VII	12	5.02	R	R	S	R	R	S	R	S	S	R	S
VIII	6	2.51	S	R	S	S	R	R	R	R	R	R	S

[a]IRBB7 and IRBB10 contain the *Xa-7* and *Xa-10* genes, respectively and susceptible check IR 24.
[b] R indicates the infiltration site turns brown, characteristics of an incompatible interaction.
[c] S indicates the infiltration site is water soaked, characteristics of an compatible interaction.

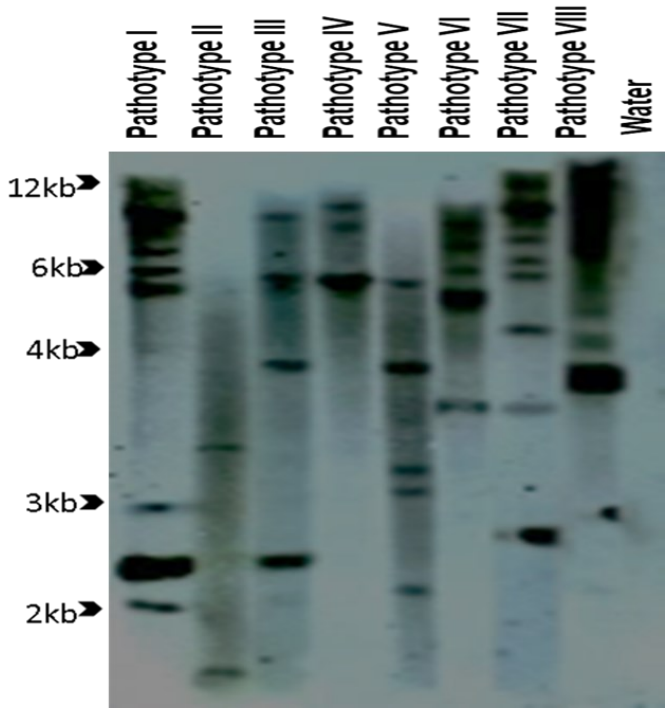

Fig. 2. RFLP (Restriction Fragment Length Polymorphism) analysis of *X. oryzae* pv. *oryzae* pathotypes using *avrBs3* repeat region as probe. Genomic DNA was isolated from eight *X. oryzae* pv. *oryzae* pathotypes and southern blot hybridization was performed using the *avrBs3* probe as described in Methodology. Four different RFLP clades of *X. oryzae* pv. *oryzae* isolates representing eight pathotypes were identified. M: a 1 kb DNA ladder (Invitrogen) was added as a size marker. Lanes 1-8 represent the DNAs obtained from *X. oryzae* pv. *oryzae* isolates of representing pathotypes, lane 9: W-water.

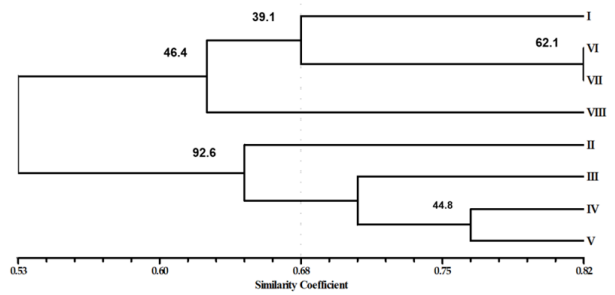

Fig. 3. Dendrogram illustrating the genetic relationship among eight pathotypes of *X. oryzae*pv. *oryzae* based on RFLP data by UPGMA clustering. A data matrix was generated for the presence of band as 1 and absence of band as 0 for the construction of dendrogram. A similarity matrix was derived with the SIMQUAL program (NTSYSpc, version 2.02i, Exeter Biological Software, Setauket, NY) using Jaccard's coefficient of similarity

bands. The UPGMA cluster analysis was shown in Fig. 3 obtaining from Jaccard similarity coefficients. Phylogenetic relationship revealed that *X. oryzae* pv. *oryzae* isolates representing eight pathotypes distributed into four RFLP clades and these clades are widely distributed all over Bangladesh as depicted in the dendrogram at 68% homology. Clade I contained 42 strains belonging to pathotypes I, VI, VII and these isolates were obtained from 3 different districts viz. Bogura (AEZ 4), Sylhet (AEZ 20) and Lakshmipur (AEZ 17), Clade II contained 6 strains belonging to pathotype VIII which were isolated from 2 districts viz. Khulna (AEZ 13) and Barishal (AEZ13), Clade III contained 28 strains belonging to pathotype II isolated from the districts Rangpur (AEZ 27) and Dinajpur (AEZ 1) and Clade IV contained 163 strains belonging to pathotype III, IV, V obtained from the districts Rajshahi (AEZ 26), Panchagarh (AEZ 1) and Mymensingh (AEZ 8). This depicts that pathotypes I, VI, VII comprised of genetically nearly related isolates from diverse locations and pathotypes III, IV and V composed of isolates from several widely distributed locations in Bangladesh.

Identification of avirulence gene from X. oryzae pv. oryzae

To confirm the presence of any known *avrBs*3 homolog in representative *X. oryzae* pv. *oryzae* isolates, isolates of each pathotype were undergone a pathogenicity test on rice lines IRBBXa7 and IRBBXa10 containing *Xa*7 and *Xa*10 *R*-genes respectively. The reactions of the phenotypes fluctuated with involving resistance genes (*Xa1*, *Xa2, Xa4 , Xa5 , Xa8, Xa10 , Xa11 , Xa13 , Xa14 , Xa21)* in the interaction between *X. oryzae* pv. *oryzae* pathotypes and the rice lines IRBB. Within 24-48 hrs a dark brown colour is formed all the area of infiltrated site when an interaction happened between rice line *Xa*10 and *X. oryzae* pv. *oryzae* pathotypes, on the other hand, at 48 hrs a dark margin colour formed around the perimeter of the water-soaked area containing rice line with *Xa*7 as well as tan to brown colour formed in tissue locating within the site at 72 hrs (Table 2). In the infiltration site congenial interactions in too combinations stayed water-soaked through at 5 days, after that time the leaf had gone wilting and the water-soaked lesion had resulted into spreading. The resistance phenotype character-

Table 2. Interactions of *X. oryzae* pv. *oryzae* strains with near-isogenic rice cultivar

Pathotypes	Rice cultivar[a]		
	IRBB7	IRBB10	IR24
I (BDXO91)	R[b]	R	S
II (BDXO68)	S[c]	S	S
III (BDXO98)	R	S	S
IV (BDXO34)	R	S	S
V (BDXOS6)	R	R	S
VI (BDXO210)	S	S	S
VII (BDXO251)	S	S	S
VIII (BDXO319)	S	R	S

[a]IRBB7 and IRBB10 contain the *Xa-7* and *Xa-10* genes respectively and susceptible check IR24.
[b] R indicates the infiltration site turns brown, characteristics of an incompatible interaction.
[c] S indicates the infiltration site is watersoaked, characteristics of an compatible interaction.

ized by turning brown of the infiltration site that was observed after inoculation of 16 days old rice seedlings in case of pathotype I (BDXO91), pathotype III, Pathotype IV and Pathotype V (BDXOS6) on IRBB7 and suggesting the pathotypes containing *avrXa*7 gene. On the other hand, resistance phenotype that was noticed in case of pathotypes I (BDXO91), V (BDXOS6) and pathotype VIII (BDXO319) on IRBB10 line conceded that these pathotypes containing *avrXa10 (*Fig. 4*)*.

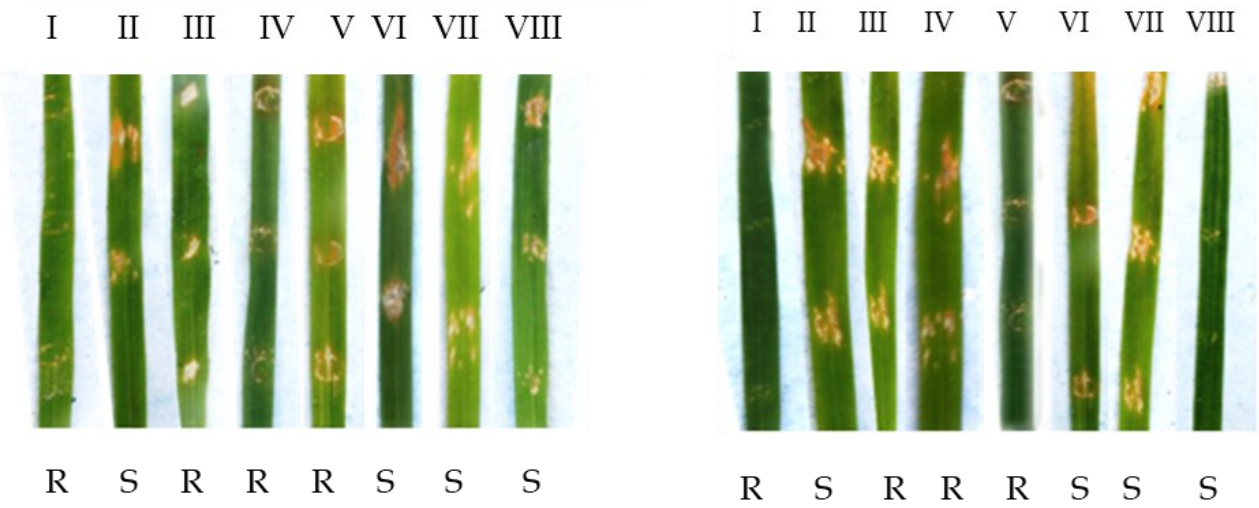

Fig. 4. Virulence (S) and avirulence (R) activity of different pathotypes on IRBB7 (A) and IRBB10 (B) containing *Xa7* and *Xa10 R* genes, respectively. *R* indicates the infiltration.

Rep-PCR analysis

The genetic relationships among *X. oryzae* pv. *oryzae* isolates collected from 30 rice growing AEZs in Bangladesh representing eight pathotypes were determined by analysing banding patterns of rep-PCR (Fig. 5). Rep-PCR (REP, ERIC) and BOX primers produced a total of 13, 18 and 10 bands respectively of which all bands (100%) were considered as polymorphic respectively. Among the pairing site of the DNA strand and the primer of low homology perhaps showed delicate bands. Amplification products size ranged from approximately 400-3000 bp (base pair) in case of REP, 150-3000 bp in case of ERIC and 400-2500 bp in case of BOX primers. Dendrogram constructed based on the REP primer from the pooled data had three main clades. Clade I consisted of isolates of pathotypes I (BDXO91), II (BDXO68) and VIII (BDXO319) obtained from the districts Bogura (AEZ 4), Rangpur (AEZ 27) and Barishal (AEZ 13). Clade II formed by pathotypes III (BDXO98), pathotypes IV (BDXO34) and pathotypes V (BDXOS6) belong to the districts Tangail (AEZ9), Thakurgaon (AEZ1), Panchagarh (AEZ1) respectively. The Clade III contained pathotypes VI (BDXO210) and pathotypes VII (BDXO251), all these isolates belonged to Natore (AEZ5), Habiganj (AEZ22), Feni (AEZ19), Lakshmipur (AEZ17) and Bhola (AEZ13). The similarity among strains among these Clades varied at around 60% (Fig. 6A). The dendrogram constructed based on ERIC from the pooled data had three main clades. Clade I consisted of isolates of pathotypes I obtained from the district Bogura (AEZ 4). Clade II formed by pathotypes II, pathotypes VII and pathotypes VIII belongs to Rangpur (AEZ 27), Habiganj (AEZ 22) and Barishal (AEZ 13). The Clade III contained pathotypes III, IV, V and VI under the districts Tangail (AEZ 9), Thakurgaon (AEZ 1), Panchagarh (AEZ 1) and Natore (AEZ 5). The similarity among strains among these clades varied at around 60% (Fig. 6B). Dendrogram constructed based on BOX primer from the pooled data had two clades. Clade I consisted isolates of pathotypes I, VIII and V. Clade II was represented by *X. oryzae* pv. *oryzae* isolates of pathotypes II, III, IV, VI and VII collected from Rangpur (AEZ 27), Natore (AEZ 5), Panchagarh (AEZ 1), Netrokona (AEZ 9), Habiganj (AEZ 22), and Lakshmipur (AEZ 18) districts. The similarity among strains between these sub-clusters varied at around 68% (Fig. 6C). However, dendrogram constructed based on rep-PCR (REP, ERIC, and BOX) from the pooled data had two clades. Clade I consisted isolates of pathotypes I and Clade II was represented by *X. oryzae* pv. *oryzae* isolates of pathotypes II, III, IV, V, VI, VII and VIII. The similarity among strains between these sub-clusters varied at around 70% (Fig. 6D).

Comparison of RFLP and rep-PCR analyses on field isolates

RFLP with the *avrBs3* probe and rep-PCR with the ERIC and REP Primers were used to detect the level of diversity of *X.*

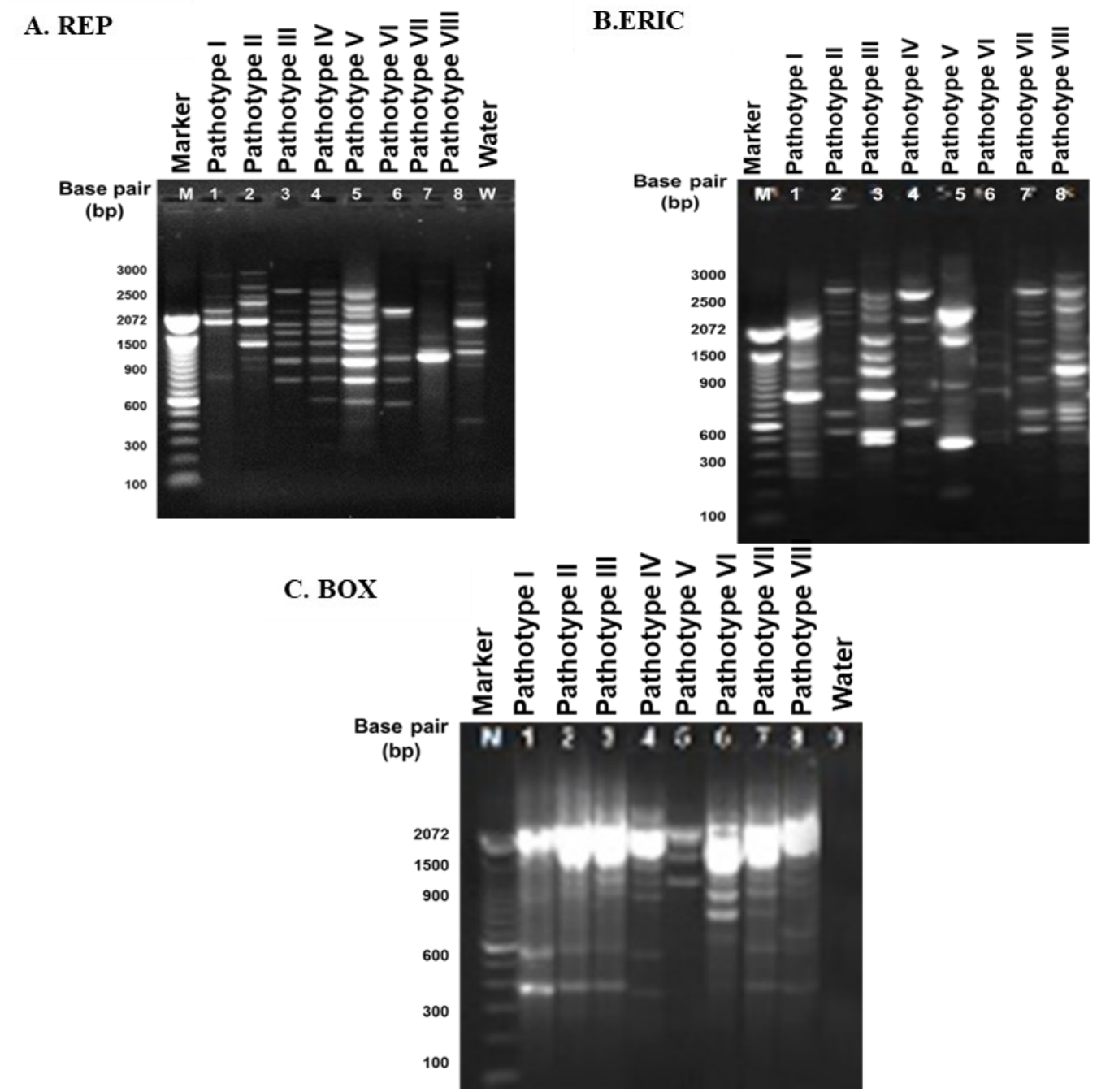

Fig. 5. Agarose gel showing representative banding patterns of each pathotypes of *X. oryzae* pv. *oryzae* generated by repetitive sequence-based polymerase chain reaction (rep-PCR) with repetitive extragenic palindromic (REP, ERIC and BOX) Primers. After amplification by PCR, the DNA fragments were separated in agarose gels and visualized by staining with ethidium bromide. Molecular weight marker M: 100 bp DNA Ladder (Invitrogen). Numbers to the left indicate molecular weight in base pairs. lane 1-8: *X. oryzae* pv. *oryzae* pathotypes and lane *9:* Water (control).

oryzae pv. *oryzae* isolates from field. The 239 isolates of BB pathogen distributed in eight pathotypes and were distinctly separated into two lineages, with only 62% similarity in RFLP analysis (Fig. 7A) and with 76% similarity in rep-PCR (Fig. 7B). The two lineages discerned by rep-PCR contained the different isolates as the lineages defined by RFLP analysis. Lineage A was separated into three haplotypes (designated pathotypes I, VI, VII and VIII) whereas B as detected by RFLP with *avrBs3* separated into three haplotypes composed of pathotypes II, III, IV & V. In the combined rep-PCR and RFLP data analyses of field populations of *X. oryzae* pv. *oryzae* characterise two lineages, A contains isolates of pathotype I and B contains isolates of pathotypes II, III, IV, VII, VIII, V and VI (Fig. 7C). The results indicated the same pathotypes distributed in different lineages and different lineages possess same pathotype. Bootstrap values (100% for each lineage) indicated highly reliable groups.

Correlation between pathotypes and molecular haplotypes of Xoo in Bangladesh

A phenogram was structure depending on the virulence of isolates (*X. oryzae* pv. *oryzae*) to the ten (NILs) Near Isogenic Lines of rice carrying single *R*-gene to elucidate the relationship between haplotypes and the pathotypes (Fig. 8). Constructed based on the outcomes of inoculation in the phenogram where four haplotypes were exploited at 63% level of similarity. From all of these haplotypes, haplotypes II considered as most important which comprises number of pathotypes III, V containing 91 isolates out of 239. Haplotype I contained number of isolates (89) belongs to patho-

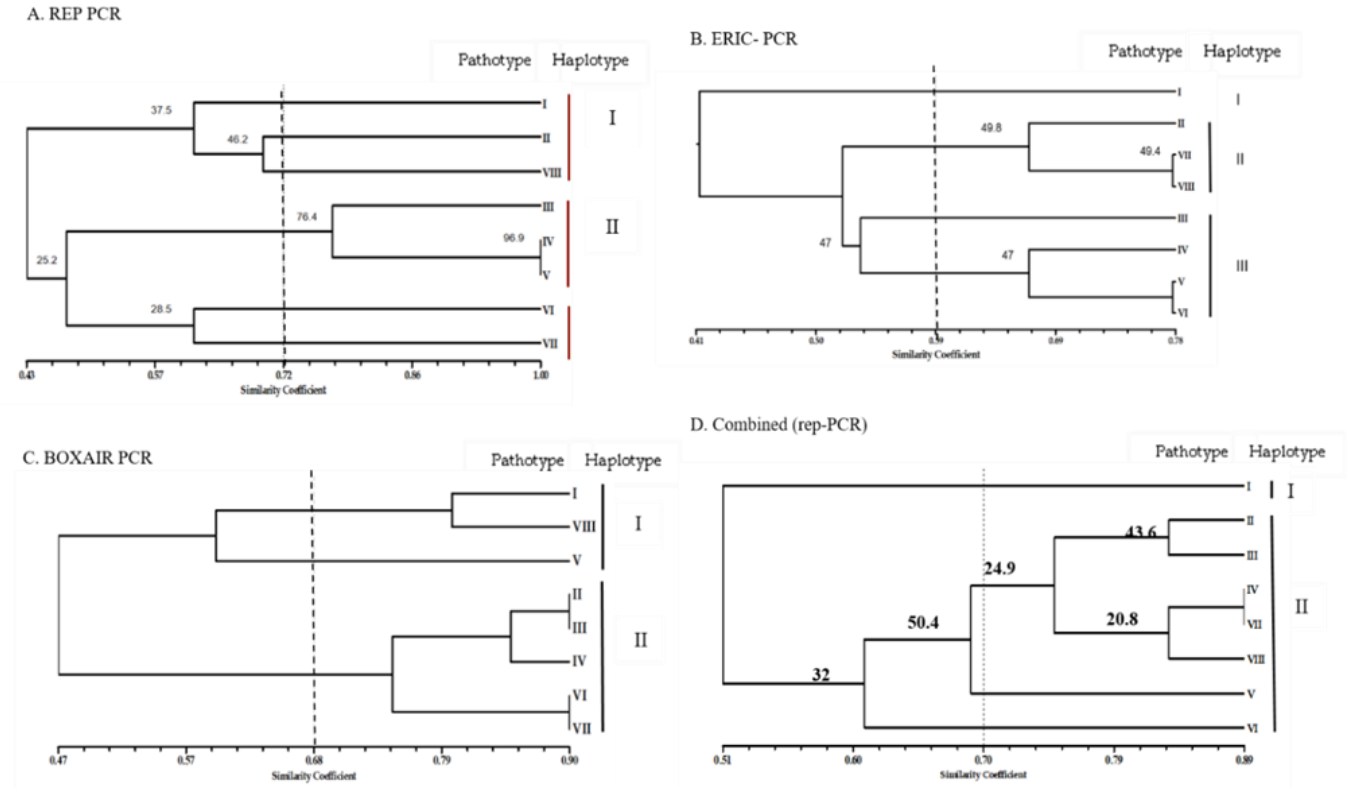

Fig. 6. Dendrograms illustrating the genetic relationship among eight pathotypes of *X. oryzae* pv. *oryzae* based on REP (A), ERIC (B) and BOX (C)RFLP data by UPGMA clustering. A data matrix was generated for the presence of band as 1 and absence of band as 0 for the construction of dendrogram. A similarity matrix was derived with the SIMQUAL program (NTSYSpc, version 2.02i, Exeter Biological Software, Setauket, NY) using Jaccard's coefficient of similarity. A. REP; B. ERIC; C. BOX elements; D. Combined Repetitive sequence-based polymerase chain reaction (rep-PCR) analyses of *X. oryzae* pv. *oryzae* pathotypes distinguished two haplotypes, Similarity between pairs of strains was determined by Jaccard's method, and clusters were generated by the UPGMA by NTSYS-pc. The numbers at the base of each node are bootstrap values analyzed by the program Winboot that represent statistical reliability of the nodes.

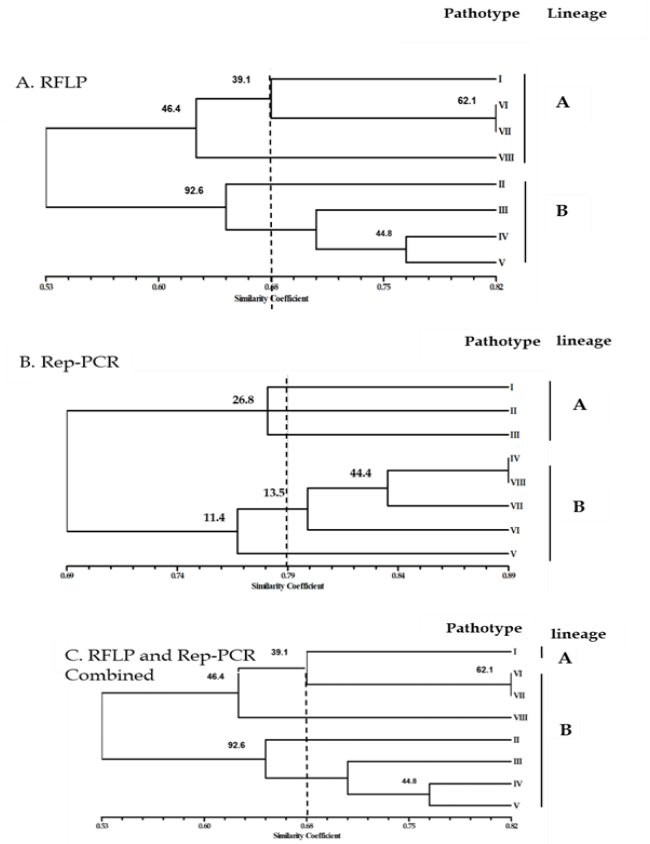

Fig. 7. Restriction fragment length polymorphism (RFLP) (A), repetitive sequence–based polymerase chain reaction (rep-PCR) (B); and Combined RFLP -rep-PCR analyses (C) of *X. oryzae* pv. *oryzae* field populations distinguished two lineages, A and B. Similarity between pairs of strains was determined by Jaccard's method, and clusters were generated by the UPGMA by NTSYS-pc. The numbers at the base of each node are bootstrap values analyzed by the program Winboot that represent statistical reliability of the nodes. Nomenclature of RFLP derived haplotype is based on [64].

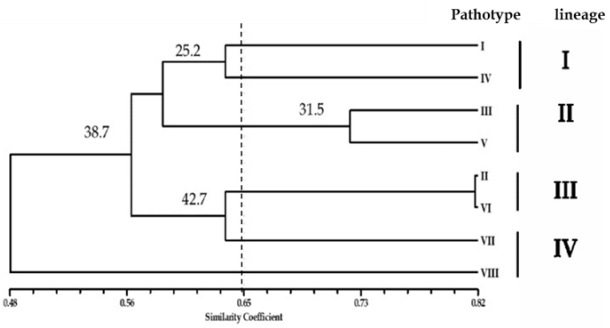

Fig. 8. Phenogram of *X. oryzae* pv. *oryzae* strains based on virulence to 10 near -isogenic lines containing a single *R*-gene. A data matrix was generated for the virulence data by scoring avirulence as 0 and virulence as 1. From these data, a similarity matrix was derived with the SIMQUAL program (NTSYSpc, version 2.02i, Exeter Biological Software, Setauket, NY) using Jaccard's coefficient of similarity. A phenogram was reproduced by the unweighted pair group method for arithmetic average in the SAHN Program

types I and IV. Haplotype III comprises 2 pathotypes (II and VI) containing 41 isolates and haplotype IV represented by pathotypes VII, VIII, diverse than other haplotypes. From the phenogram, i) Plentiful haplotypes were exposed from different population with exact pathotypes of the group of isolates of *X. oryzae* pv. *oryzae,* ii*)* those diverse populations had same type of haplotypes in a lot of pathotypes and iii) the group of isolates of *X. oryzae* pv. *oryzae* from multifarious population had the identical haplotypes and pathotypes.

Discussion

The evolutionary process of an organism includes genetic polymorphisms which are occurred due to numerous phases such as genetic recombination, horizontal gene transfer

and mutations (4). These genetic processes called polymorphism including genetic drift, gene transfer and selection are added or removed from different populations eventually (49). Thus, pathogens capability of adaption into diverse ambience more or less depend on its genetic polymorphism. For building completely new virulent ability to infect hosts more accurately bacterial genomes need to be a greater level of polymorphism as it is fundamental (50). In the present research, we analysed the variation of genetic processes of *X. oryzae* pv. *oryzae* pathotypes in Bangladesh using two DNA markers viz. RFLP using *avrBs3* repeat domains and rep-PCR. Results obtained from these DNA markers supported that *X. oryzae* pv. *oryzae* pathotypes showed a substantial genetic variation in their genomes. RFLP results showed that Bangladeshi *X. oryzae* pv. *oryzae* isolates representing eight pathotypes carrying a minimum of two and maximum of nine *avrBs3* family gene homologs. Phylogenetic relationship exposed that *X. oryzae* pv. *oryzae* strains dispersed into two haplophytes (RFLP) and these are largely distributed all round Bangladesh. The virulence differentiation of Bangladeshi *X. oryzae* pv. *oryzae* pathotypes might be due to the copy number of *avrBs3* family genes present in the genome. These results agreed with the notion that *X. oryzae* pv. *oryzae* possess several homologs and possibly a *avrBs3* gene family (35). Variable copy number and genomic location of multiple *avrXa27*, a member of *avrBs3* family gene were also reported in the genome of *X. oryzae* pv. *oryzae (4).* Avirulence genes of *X. oryzae* pv. *oryzae* are one of the main virulence determinants in eliciting BB (bacterial blight) on rice. The presence or absecnce of an avirulence gene in the host gives rise to a compitable (resistant) or incompatible (susceptible) reaction as stated in the gene for gene hypothesis (51). The *X. oryzae* pv. *oryzae* avirulence genes, *avrXa7* and *avrXa10* are very similar to *avrBs3* and account for the differences in the sizes of the *Bam*HI fragments con-tained within the genes (*avrBs3*, 3.3 kb; *avrXa7,* 4.1 kb; *avrXa10*, 3.1 kb) by the differences in estimated copy numbers of the 102-bp repeat structure (17.5, 25 and 15.5 respectively) (52). To validate our RFLP fingerprinting using *avrBs3* repeat domain, the presence of two known *avr* genes viz. *avrXa7* and *avrXa10* were determined by pathogenicity test on rice lines containing *Xa7* and *Xa10* respectively. The resistance phenotype that was observed in the present study after inoculation of 16-days-old rice seedlings in case of pathotypes I, pathotypes III, Pathotype IV and Pathotype V on *IRBBXa7* and suggesting these pathotypes containing *avrXa7* gene on the other hand, resistance phenotype that was noticed in case of pathotypes I, V and VIII on IRRBB10 line conceded that these pathotypes may contain *avrxa10*. *X. oryzae* pv. *oryzae* race 2 strain was also found avirulent on rice cultivars carrying resistance genes *Xa5, Xa7* and *Xa10* (35).

The rep-PCR technique (repetitive sequence PCR) consists of analysing DNA polymorphism of repetitive sequences occurring in the bacterial genome. The distribution of these sequences is unique for each strain which allows the strains to be differentiated. The rep-PCR is based on the fact that outwardly facing oligonucleotide primers complementary to interspersed repetitive sequences will enable the amplification of differently sized DNA fragments consisting of sequences lying between these elements, however, rep-PCR was introduced to differentiate microbes by combining the advantages of DNA amplification with the application of repetitive sequence-based oligonucleotide primers (53). These repetitive bacterial DNA sequences are described as repetitive extragenic palindromic sequence (REP), enterobacterial repetitive intergenic consensus (ERIC) and the BOX element. REP, ERIC and BOX elements are not only restricted to the DNA sequence and hybridization of eubacterial species but also it is also used for phylogenetic analysis and differentiation of diverse bacterial genera (54) including *Rhizobium* (55), *Frankia* (56), *Staphylococcus* (57), *Legionella (*46*,* 58). In this study, the genetic variation of eight pathotypes of *X. oryzae* pv. *oryzae* was estimated by using REP, ERIC and BOX primers. The similarity among strains between these haplotypes varied at around 60%, 60% and 68% respectively. Two molecular haplotypes were identified of *X. oryzae* pv. *oryzae* isolates representing eight pathotypes at similarity level 70% based on rep-PCR data (Fig. 6D). It was observed that remarkable genome variability in both clonal and field isolates of *X. oryzae* pv. *oryzae* by the amplification of REP, ERIC and BOX-AIR primers despite having cultural and morphological similarities of *X. oryzae* pv. *oryzae* isolates of rice (59). Haplotypic variation of *X. oryzae* pv. *oryzae* population was also observed by rep-PCR (34, 60). Rep-PCR results revealed that among the African and Asian strains of *X. oryzae* pv. *oryzae* have dissimilarities in their genomic characteristics (61).

The 239 *X. oryzae* pv. *oryzae* strains collected from the field were distinctly separated into two lineages based on combined RFLP and rep-PCR data, lineage A was separated into 3 haplotypes and lineages B was separated into 4 haplotypes. Bootstrap values (100% for each lineage) indicated highly reliable groups. In RFLP, cluster I was comprised of four pathotypes where pathotypes VI and VII were closely related to each other at 62% similarity level as well as pathotypes VIII was equally closely related to pathotypes I, VI, VII at 46% similarity level. Conversely, in case of Rep-PCR, cluster I consisted of three pathotypes named pathotype I, II, III exhibiting 26% similarity to each other, thus no significant correlation was found in cluster I between RFLP and Rep-PCR. In RFLP, cluster II was comprised of four pathotypes (II, III, IV, V) in which IV and V were closely related to each other at 44.8% similarity as well as pathotype II was equally closely related to III, IV, V at 92% similarity level, on the other hand, in Rep-PCR, cluster II was consisted of five pathotypes (IV, VIII, VII, VI, V) where 44% similarity was observed between VII and closely related pathotypes IV and VIII, pathotypes V was equally related to all four pathotypes. Above correlation suggested that eight pathotypes were same but they may vary in the same cluster when compared to RFLP and Rep-PCR.

Present study revealed that due to the exchanging of contaminated germplasm and national movement, there might be dispersion of the pathogen *X. oryzae* pv. *oryzae* which were detected from several regions of Bangladesh. This diversity might explore a number of complications encountered historically to modify several rice cultivars

which showed resistance to *X. oryzae* pv. *oryzae* throughout Bangladesh. RFLP and PCR markers were used to evaluate Indonesian and Philippinan strains of *X. oryzae* pv. *oryzae* (62). Based on their research they found that strains those were predominant from the collection of pathogens of both of those countries having regional movement because of exchanging germplasm and those both strains were found highly matching (63) declared that a number of resistance genes have the ability to trigger the selection of pathogenic races which are new at a rate exact to 1.64 times the surge in individual virulence of the isolates after one crop cycle following selection pressure of resistance host-plant. Depending on the DNA polymorphism with a large degree, a phenogram and a dendrogram was constructed among *Xoo* strains (239) isolated from variegated geographic regions of Bangladesh.

All strains exhibiting a multifarious relationship between molecular haplotypes and pathotypes which are genetically, geographically and pathogenically variegated. Identical experimental outcomes was depicted by (15, 64-66). They indicated that the relationship between *X. oryzae* pv. *oryzae* pathotypes and molecular haplotypes were composite. Similar observations were also recorded (67). They reported that some haplotypes were geographically dispersed and most frequent which occurred in diverse geographic populations in Nepal. Taken together, genetic variability of *X. oryzae* pv. *oryzae* pathotypes might be due to their variation of places or origins where cultivars with diverse *R*-genes cultivated. These *R*-genes might be responsible for evolving new haplotypes and pathotypes of *X. oryzae* pv. *oryzae.* RFLP analyses using *avrBs3* repeat region conceded that Bangladeshi *X. oryzae* pv. *oryzae* strains carrying *avrBs3* family gene homolog which is crucial and one of the important determinants for the development resistant rice varieties through gene pyramiding. However, analyses of whole genome sequences of some selected Bangladeshi *X. oryzae* pv. *oryzae* strains would clarify the complex genetic relationship between pathotypes and molecular haplotypes.

Conclusion

Genetic polymorphisms within the genomes of *X. oryzae* pv. *oryzae* signifies their evolutionary potential during long-term interaction with its hosts. Information related to the presence of *avrBs3* family gene homologs *X. oryzae* pv. *oryzae* Bangladeshi strains will be useful for breeding to develop BB resistant rice varieties through corresponding *R* gene pyramiding to each avirulence gene of *X. oryzae* pv. *oryzae* pathotype (s) exist in Bangladesh. Identification of the homolog *avr* genes in the dominant pathotypes of *X. oryzae* pv. *oryzae* exist in Bangladesh and their corresponding *R* genes would be the next steps of the present study.

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Authors contributions

MRI: conceptualization, methodology, supervision, and editing; MMH: investigation, formal analysis, performed the statistical analysis and writing original draft, MMM: edited the manuscript, SB: edited the manuscript, MZA: edited the manuscript, MIH: edited the manuscript. All authors carefully read and authorized the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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