Short Communication

REP-PCR DISTINGUISHES RICE BACTERIAL BLIGHT PATHOGEN (*XANTHOMONAS ORYZAE* pv. *ORYZAE*) STRAINS OF INDIAN MAINLAND AND BAY ISLANDS

K. Manigundan[1], P. Puneeth Kumar[1], R. Singh[1], K. Sakthivel[1], R.K. Gautam[1], P.K. Singh[1] and G.S. Laha[2]

[1]*Division of Field Crop Improvement and Protection, ICAR- Central Islands Agricultural Research Institute, Port Blair-744101, India*
[2]*Division of Plant Pathology, ICAR- Indian Institute of Rice Research, Rajendranagar, Hyderabad-500 030, India*

SUMMARY

Thirty-two strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) representing 12 pathotypes from major rice growing Indian mainland states and Andaman Islands were analyzed using Rep-PCR and IS*1112*-based PCR approaches. All four PCR primers (BOX, REP, ERIC and JEL primers) confirmed the presence of high genetic variability among the *Xoo* strains studied. The REP primers generated more PCR fragments than other set of primers and the amplification pattern of BOX, REP, ERIC and JEL primers were 277, 171, 147 and 149 respectively with the band size varying from 200 to 3000 base pairs. Cluster analysis of 32 *Xoo* strains revealed that the combined dendrogram constructed using Rep-PCR analysis was effective in distinguishing Islands *Xoo* strains from Indian mainland strains with the similarity index of Islands clusters of about 53%. In case of *IS1112* repeat element based PCR approach, the two of the mainland strains (DX_027 and DX_143) were grouped with one mainland isolate ANBB_4 in one sub cluster whereas remaining three mainland isolates grouped separately in a main cluster. This indicated that Islands strains were genetically distinct from mainland strains. Also, the Rep-PCR approach was found to be more precise and thus suitable for discriminating the variability among *Xoo* strains emanating from geographically diverse locations.

Keywords: Rice, bacterial Blight, Rep-PCR, *IS1112* element, Andaman Islands.

Xanthomonas oryzae pv. *oryzae* is the predominant bacterial pathogen of rice causing bacterial blight (BB) disease worldwide (Mew *et al*., 1993). In India, the bacterial blight disease is endemic in all rice growing areas including Andaman and Nicobar Islands (Sakthivel *et al*., 2015, 2017). The Andaman and Nicobar (A&N) group of Islands, situated in the Bay of Bengal at about a distance of 1200 km from Indian mainland ports comprises of 30 rice growing Islands with approximately 8000 ha under rice (Gautam *et al*., 2015). The yield loss due to BB disease incidence in the Islands is quite prominent and this problem is highly accentuated by prevailing warm and humid climate and frequent introduction of paddy germplasm from different parts of mainland states in India. Gene for gene hypothesis historically propounded by Flor (1956) is quite relevant even today for deploying specific resistance gene(s) in light of evolving genetic nature of pathogen. Though about 38 *Xa/xa* genes of rice conferring resistance to bacterial blight disease caused by *Xanthomonas oryzae* have been identified till date, there is comparatively less understandings about precise molecular genetic structure of pathogen due to its complex and dynamic nature. The management of rice bacterial disease mainly depends on the use of resistant varieties introgressed with one or more resistant gene(s), but the breakdown of resistance is the common phenomena due to emergence of new races/pathotypes. Though native island paddy varieties are grown by farmers, there is also introduction of seed improved varieties of paddy from main land to the islands. The evolution and adaptation of local *Xoo* strains influenced by interaction with mainland strains emanating from different ports (Chennai and Vizag in South India and Kolkata in the East India) might present an interesting pathogen population. Hence, knowledge on the genetic variability of the local pathogen population is very important for developing a matching resistant variety. With the advent of molecular biology, the use of molecular markers in population genetic studies has unraveled epidemiological information to levels of precision which was not previously possible in plant pathogens (Sakthivel *et al*., 2016). In *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), multiple molecular techniques have been used to characterize the pathogen but most of them are costly and time consuming. But few PCR based approaches like IS*1112* based genomic fingerprinting and repetitive sequence based polymerase chain reaction (Rep-PCR) were known as the most sensitive, cost effective and reliable technique to understand the genetic nature of the *Xoo* pathogen. IS*1112* based JEL primers differentiates the *Xoo* strains based on the amplification of IS*1112* repeat element, which is present in multiple copies (more than

Corresponding author: K. Sakthivel
E-mail: veluars@gmail.com

Table 1. Details of *Xanthomonas oryzae* pv. *oryzae* strains used in the study.

S. No.	Origin	Isolate	Pathotypic group*	Place of collection	District/ Islands	Variety	Year of collection	Reference/ Source
1	Strains of Indian Islands (Andaman)	ANBB1	IV	Uttara, Kadamtala	Middle Andaman	Jaya	2014	Sakthivel *et al*., 2015
2		ANBB2	VI	Baratang	Middle Andaman	Swarna Jaya	2014	
3		ANBB3	V	Bakultala	Middle Andaman	Swarna Jaya	2014	
4		ANBB4	II	Baratang	Middle Andaman	C14-8	2014	
5		ANBB5	IV	Uttara	Middle Andaman	Jaya	2014	
6		ANBB6	V	Kadamtala	Middle Andaman	C14-8	2014	
7		ANBB7	VI	Mangultan	South Andaman	C14-8	2014	
8		ANBB8	VII	Kodiyagadu	South Andaman	C14-8	2014	
9		ANBB9	VII	Swaraj gram	North Andaman	Jagannath	2014	
10		ANBB10	VI	Kalipur	North Andaman	Jaganath	2014	
11		ANBB11	V	Nimbudera	North Andaman	Jaya	2014	
12		ANBB12	V	Billiground	North Andaman	Jaya	2014	
13		ANBB13	III	Harinagar	North Andaman	Jaganath	2014	
14		ANBB14	III	Rangat 3	Middle Andaman	C14-8	2014	
15		ANBB15	VI	Kadamtala	Middle Andaman	C14-8	2014	
16		ANBB16	VII	Nimbudera	North Andaman	C14-8	2014	
17		ANBB17	III	Tushnabad	South Andaman	C14-8	2014	
18		ANBB18	I	Ferrarganj	South Andaman	C14-8	2014	
19		ANBB19	II	Meethagadi	South Andaman	C14-8	2014	
20		ANBB20	II	Hut Bay	Little Andaman	Swarna Jaya	2014	
21		ANBB21	I	Ram Nagar	North Andaman	C14-8	2014	
22		ANBB22	V	Hut Bay	Little Andaman	Jaya	2014	
23		ANBB23	III	Burmadera	North Andaman	Khushbayya	2014	
24		ANBB24	VI	North Andaman	North Andaman	C14-8	2013	
25		ANBB25	VI	Middle Andaman	Middle Andaman	C14-8	2013	
26		ANBB26	IV	South Andaman	South Andaman	C14-8	2013	
27		ANBB27	V	Havelock Islands	South Andaman	C14-8	2013	
28	Strains of Indian mainland states	DX-002	M-I	Faizabad	Uttar Pradesh	-	2013	This study
29		DX-020	M-II	Hyderabad	Telangana	-	2013	This study
30		DX-027	M-III	Chinsurah	West Bengal	-	2013	This study
31		DX-148	M-IV	Cuttack	Odisha	-	2013	This study
32		DX-321	M-V	Ludhiana	Punjab	-	2013	This study

80 copies in some strains) in the *Xoo* pathogen genome, whereas the Rep-PCR targets the naturally occurring, highly conserved, repetitive DNA sequences like REP (repetitive extragenic palindromic), ERIC (enterobacterial repetitive intragenic consensus) and BOX elements of any bacterial genome (Kumar *et al*., 2004).

Although reports are available on molecular diversity of *Xoo* pathogen all over India and Asia, the precise information on molecular diversity of *Xoo* population in Andaman Islands *versus* mainland isolates is still lacking and the comparative molecular variation with other Indian mainland strains has not been carried out previously. Thus, we attempted to assess the genetic diversity and relatedness of the local (Andaman) *Xoo* population compared to highly predominant and evolved Indian *Xoo* population, using Rep-PCR and IS*1112*-based PCR approaches.

A total of 27 isolates were collected from different locations of Andaman Islands (Table 1). The pathogenic nature of each isolate was confirmed by pathogenicity tests on susceptible rice genotypes *viz*., TN1 and C14-8. In addition, to understand the genetic relationship between Islands and mainland strains, five *Xoo* isolates belonging to different states of mainland India were obtained from the Division of Plant Pathology, Indian Institute of Rice Research (IIRR), Hyderabad and used in the study.

All the isolates were identified up to subspecies level (*Xanthomonas oryzae* pv. *oryzae*), through multiplex PCR approach with a set of primers (Table 2) as suggested by Lang *et al*. (2010). A 25 µl PCR reaction mix was performed in C1000™ Thermal Cycler (Bio-Rad, USA) using the following cycle: initial denaturation of 94°C for 3 min; 35 cycles of 94°C for 30 s, 62.5°C for 1 min 30 s, 72°C for 30 s and final extension of 7 min at 68°C. A 5 µl aliquot of amplified PCR products was resolved in 1.5% agarose in 1X Tris acetate EDTA buffer for 90 min at 60 V. The gel was stained with ethidium bromide and photographed on Gel documentation unit (Bio-Rad, USA).

The isolates were further confirmed for the species identity through the PCR amplification of a 964 bp fragment using species-specific TXT primers (data not shown). Further, the multiplex PCR analysis revealed identity of all the isolates at subspecies level by amplifying two specific bands *viz*., one at 331 bp which is species specific (*X. oryzae*) and the other at 162 bp which is specific to sub species (*Xanthomonas oryzae* pv. *oryzae*) (data not shown). In addition, all the *Xoo* isolates in this study were earlier classified into 12 races/pathotypes (P I-IV and M 1-V) based upon its individual pathogenic potential in a set of rice differentials carrying one or more resistance genes (Table 1).

Table 2. List of primers used in the present study.

	Primer	Primer sequences (5'-3')
Multiplex PCR analysis	*Xoo 80*	Xoo 80F: GCC GCT AGG AAT GAG CAAT Xoo 80R: GCG TCC TCG TCT AAG CGA TA
	Xoo 3756	Xoo 3756 F: CAT CGT TAG GAC TGC CAG AAG Xoo 3756 R: GTG AGA ACC ACC GCC ATC T
	Xoc 3864	Xoc 3864 F: GTG CGT GAA AAT GTC GGT TA Xoc 3864 R: GGGATG GAT GAA TAC GGA TG
	Xoc 3866	Xoc 3866 F: ATC TCC CAG CAT GTT GAT CG Xoc 3866 R: GCG TTC AAT CTC CTC CAT GT
Rep-PCR analysis	BOX	BOX: 5'-CTACGGCAAGGCGACGCTGACG-3'
	ERIC	ERIC I R: 5'-ATGTAAGCTCCTGGGGATTCAC-3' ERIC 2: 5'-AAGTAAGTGACTGGGGTGAGCG-3'
	REP	REPIR-1: 5'-IIIICGICGICATCIGGC-3' REP2-I: 5'-ICGICTTATCIGGCCTAC-3'
Jel-PCR analysis	*IS1112*	JEL 1: CTCAGGTCAGGTCGCC JEL 2: GCTCTACAATCGTCCGC

*Pathotypes of all the *Xoo* strains were earlier classified based on the reaction on IRBB differentials.

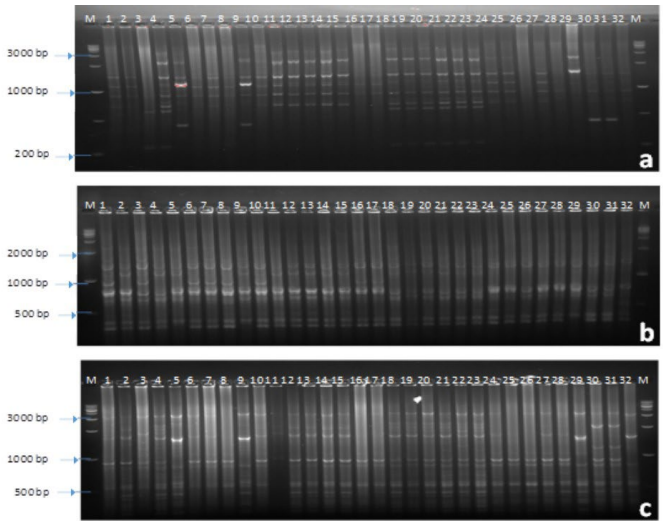

Fig. 1. Rep-PCR patterns of *Xanthomonas oryzae* pv. *oryzae*. (a) REP- Primer, (b) BOX- Primer and (c) ERIC- Primer. M, 10 kb ladder; lanes 1 to 27, ANBB1-27; 28-32, mainland *Xoo* strains.

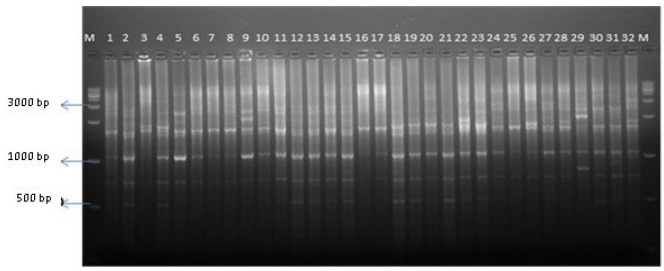

Fig. 2. JEL-PCR profile of *Xanthomonas oryzae* pv. *oryzae* strains. M, 10 kb ladder; lanes 1 to 27, ANBB1-27; 28-32, mainland *Xoo* strains.

Genetic variability analysis was performed with the 32 *Xoo* isolates (which comprise 27 Island and mainland isolates) using Rep-PCR and *IS1112* based PCR approaches (Table 2). The number of amplified products of genomic DNA in 32 *Xoo* isolates varied with the different PCR approach. In repetitive sequence based PCR fingerprint method, BER primers (BOX, ERIC and REP) were used according to Versalovic *et al.* (1994). For DNA amplification, 25 µl PCR reaction mix was performed in a thermocycler (Bio-Rad) with an initial denaturation step of 95°C for 7 min, followed by 30 cycles of 94°C for 1 min, and annealing at 53°C, 40°C and 52°C for 1 min for BOX, REP and ERIC primers, respectively, and 65°C for 8 min with a final extension step of 65°C for 15 min before cooling at 4°C. A 10 µl aliquot of amplified PCR products was resolved in 2.0% agarose in 1× Tris acetate EDTA buffer for 4 h at 80 V. The gel was stained with ethidium bromide and photographed on Gel documentation unit (Bio-Rad).

For IS*1112* based genomic fingerprinting analysis, two outwardly directed JEL1 and JEL2 primers specific to the ends of repetitive element IS*1112* present in *Xoo* were used to amplify the variable length sequences between copies of the element IS*1112*. PCR reaction was carried out with the initial denaturation of 94°C for 1 min; 35 cycles of denaturation at 94°C for 10 s, annealing at 62°C for 1 min, extension at 65°C for 8 min and final extension of 65°C for 8 min. A 10 µl aliquot of amplified PCR products were resolved in 2.0% agarose in 1× Tris acetate EDTA buffer for 4 h at 80 V. The gel was stained with ethidium bromide and photographed on Gel documentation unit (Bio-Rad). The molecular sizes of fragments generated by electrophoresis were compared with a concurrently running 1 kb DNA ladder (1-3,000 kb). The presence and absence of a band for each strain was scored as 1 (plus) or 0 (minus), respectively. Cluster analysis was attempted using the Unweighted Pair Group Method with Arithmetic averages (UPGMA) using NTSYS-PC software, version 2.1. Similarities were calculated using Jaccard's coefficient.

Among PCR methods studied with four set of primers (REP, ERIC, BOX, JEL), REP primers generated more PCR fragments than other set of primers. There were 277 total numbers of amplified bands with REP primers and the number of bands varied from 3 to 11 per isolate (Fig. 1). The ERIC primers could amplify 147 bands with the banding pattern varying from 2 to 8 bands per isolate. In case of Box-PCR, a total of 152 PCR fragments were obtained with the maximum of 7 and minimum of 4 fragments with the size ranging from 200 to 2000 bp, respectively (Fig. 1). In case of ERIC primers, the banding pattern ranged from 300 to 3,000 bp in length with the PCR products varying from 1 to 11 per isolate (Fig. 1). Cluster analysis of the combined Rep-PCRs performed across BOX, REP and ERIC primer–BER primers showed that all the *Xoo* clustered into two major clusters I and II (Fig. 3). The main cluster I was further divided into six sub clusters which comprised of all the 27 Islands *Xoo* isolates segregating into eight different sub clusters with 55-84% similarity index. The cluster II consisted of all the five *Xoo*

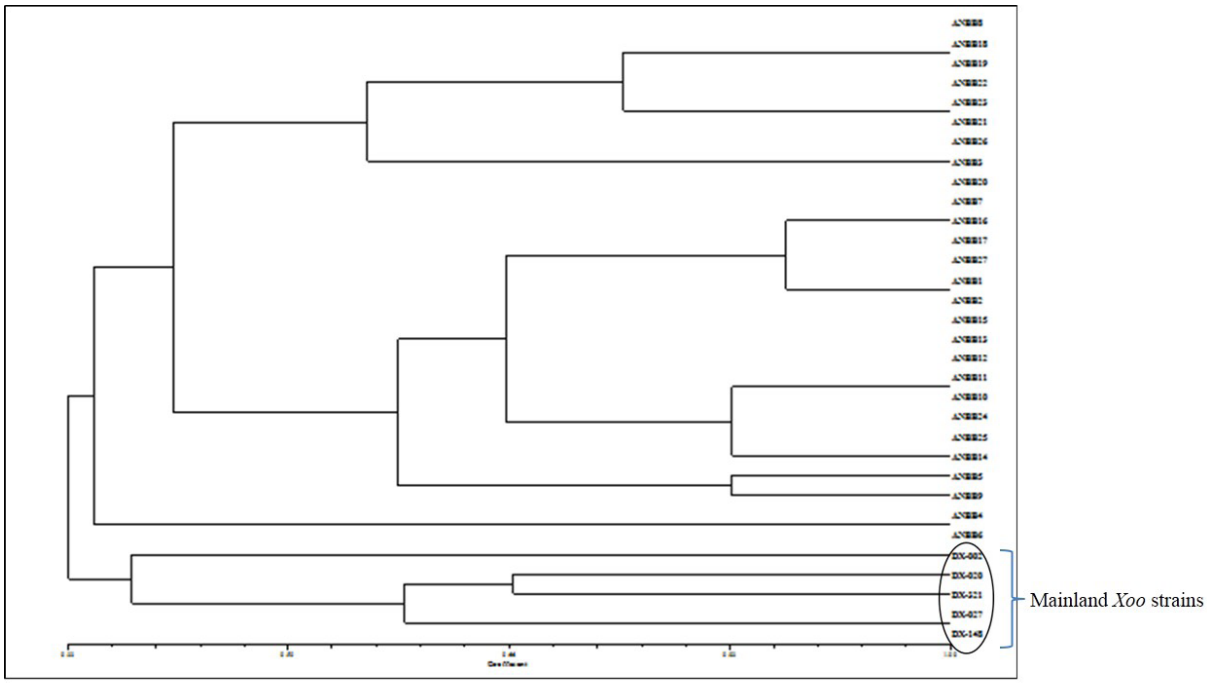

Fig. 3. Dendrogram constructed with combined REP-PCR analysis (Box, Rep and ERIC primers – BER primers).

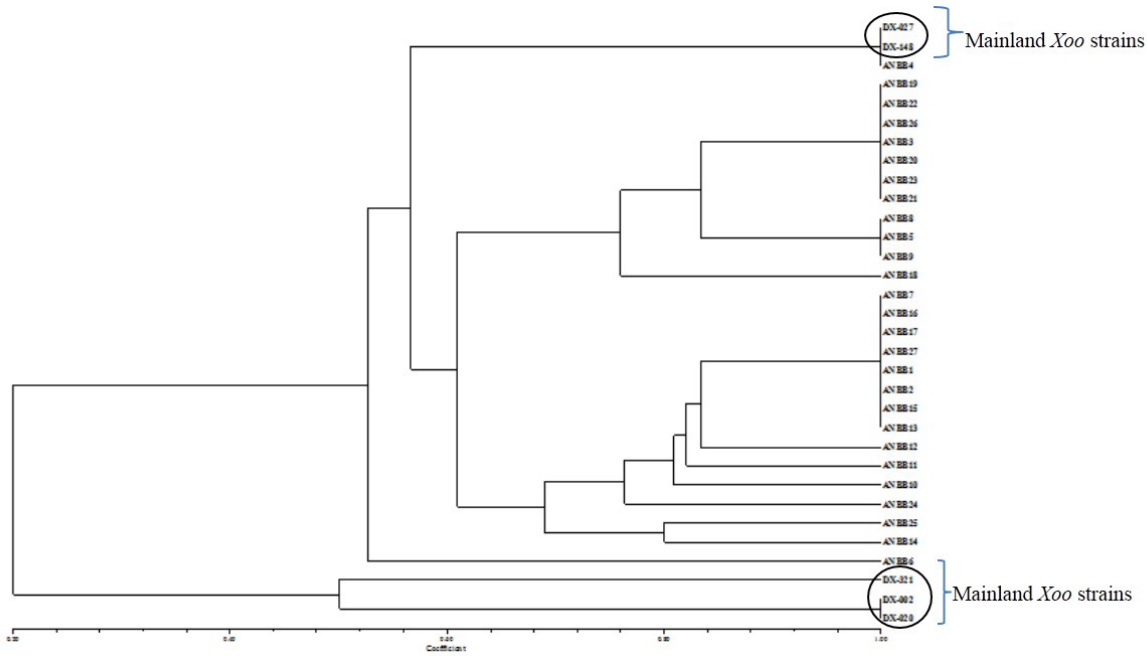

Fig. 4. Dendrogram constructed with JEL-PCR analysis.

isolates from Indian mainland states in three sub clusters with the similarity index ranging from 38 to 100%.

DNA fingerprinting using JEL primers which targets IS*1112* repeat element resulted in a total of 149 bands with the banding pattern ranging from 300 bp to 3000 bp. (Fig. 2). Cluster analysis using JEL primers divided the *Xoo* isolates again into two main clusters with the first main cluster comprising of 30 isolates in five sub clusters which includes all Islands strains and two mainland *Xoo* strains with the similarity index of 53 to 82% (Fig. 4). The second main cluster was obtained from remaining three isolates from mainland (Dx_321, Dx_002 and Dx_020).

From the results of both studies, it is inferred that all the 32 isolates of *Xoo* representing 12 important pathotypes exhibited high level of genetic differences at molecular level. However genetic diversity was not correlated with the pathogenic/race potential. This genetic variation among same pathogenic group of *Xoo* isolates might be

due to selection pressure imposed on the pathogen population by varying level of host resistance and recent diversification of rice varieties throughout the country including Andaman Islands (Hajri *et al*., 2012; Gautam *et al*., 2015; Sakthivel *et al*., 2015, 2017).

In the context of geographical origin, among the two approaches used, it is found that Rep-PCR could well separate mainland *Xoo* strains from Islands strains. The potential of Rep-PCR in differentiating the *Xoo* strains is also evident from several reports. Rep-PCR using primers BOX, ERIC and REP and the combined BER fingerprint data were previously used to generate genomic fingerprints of 339 *Xanthomonas* strains comprising of 80 pathovars (Rademaker *et al*., 2005). Rep-PCR results of *Xoo* showed that there are differences between genomic characteristics of the Asian and African strains (Gonzalez *et al*., 2007). Based on the result of Rep-PCR, the differentiation of the *Xoo* may be ascribed to the geographical areas in Malaysia, irrespective of the influence of the host cultivars (Keshavarz *et al*., 2011).

In case of IS*1112* based JEL primers approach, the grouping of *Xoo* strains slightly differed with the Rep-PCR. Here the two mainland isolates (DX_027 and DX_148) were grouped together with one Islands strain (ANBB_4). This might be due to the well-known fact that the pathogen might have entered into Islands from Indian mainland by different means which includes import of seed materials for both cultivation and research purposes. Also, the presence of two different pathotypes/isolates in single lineage as identified during molecular study might be due to co-evolution of one isolate from the other during the course of time or due to the presence of some transposable elements (Lore *et al*., 2011; Gautam *et al*., 2015).

The genetic grouping of *Xoo* strains by both the PCR approaches was also not related to the host varietal origin. This might be due to the practice of adopting multiple rice varieties by the different farmers of same locality in the Islands and the adaptation of pathogen to all those rice varieties, since none of the hoist varieties studied posed any BB resistance selection pressure.

The overall results of the study showed that Rep-PCR approach is effective and precisely responsible than IS*1112*-based PCR approach in discriminating geographically isolated mainland and Islands *Xoo* isolates. However, the results obtained through IS*1112*-based PCR primers should also be considered in the context of day to day pathogen migration mediated by movement of infected paddy seeds from mainland to Islands through various shipping and aerial routes.

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