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Genetic diversity and pathotype profiling of *Xanthomonas oryzae* pv. *oryzae* isolates from diverse rice growing ecosystems of Karnataka state of India

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Abstract: Bacterial leaf blight (BLB) disease of rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is one of the significant constraints for rice production. A study was carried out to analyze the virulence, genetic diversity, and population structure of Xoo isolates collected from different rice ecosystems in Karnataka, India. The taxonomic identity of all 54 isolates was confirmed using a Xoo-specific PCR assay. The virulence assay on a set of twenty-eight near-isogenic differentials identified nine pathotype groups (KPthX-1 to KPthX-9). Among them, KPthX-6 was highly virulent, whereas KPthx-2 was less virulent. Differentials with four and five resistance (R) genes (*Xa*4 + *xa*5 + *xa*13 + *Xa*21, *Xa*4 + *Xa*7 + *xa*13 + *Xa*21, and *Xa*4 + *xa*5 + *Xa*7 + *xa*13 + *Xa*21) were effective against all the pathotypes. Further, genetic diversity was deduced using a universal marker set, JEL-1 and JEL-2, complementary to a conserved repetitive sequence IS1112. The amplicon size and numbers varied from 100 bp to 3 Kbp and 1 to 25. Based on the amplicon counts, Jaccard's co-efficient and phylogenetic analyses were carried out and categorized the 54 isolates into three clusters (I to III). Cluster II contains the maximum number of isolates (27), followed by cluster I (23 isolates) and cluster III (one isolate). The structure analysis categorized the isolates into five subgroups, viz SG1, SG2, SG3, SG4, and SG5 indicate the existence of five subpopulations. The present study has identified the genetic and virulence diversity of the Xoo population and reported the effective R gene/s for different rice ecosystems, which can be used in the breeding program for the eco-specific management of BLB of rice.

Keywords: rice; disease; bacteria; leaf blight; pathotypes; virulence; diversity

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Rice (*Oryza sativa* L.) crop is affected by many diseases, including bacterial diseases such as bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), bacterial leaf streak (*X. oryzae* pv. *oryzicola*) (Fang et al. 1957), panicle blight/bacterial grain rot (*Burkholderia glumae*) (Mondal et al. 2015) have been reported to cause severe damage in India (Saha et al. 2015). The BLB disease affects the crop at the seedlings (kresek/wilt), vegetative (leaf blight), and reproductive stages. Disease severity can vary with location, weather, season, growth stage, crop nutrition, and rice cultivar (Rao & Kauffman 1977). However, infection at the tillering stage causes severe blighting of leaves resulting in yield losses of up to 74 percent (Reddy et al. 1978). Chemical control of BLB in the tropical climate of East Asia is unsuitable, and there is a lack of effective commercial bactericides for its control (Ou 1985). The only effective, economical and eco-friendly way to manage BLB disease is breeding for disease resistance. Globally, BLB-resistant rice cultivars were identified/developed, and as many as 46 *Xa* genes conferring resistance against Xoo have been identified so far (Fiyaz et al. 2022). However, the resistance durability under climate change scenario depends upon the frequency of the emergence of pathogen races (Adhikari et al. 1999; Noda et al. 2001).

This is because the Xoo pathogen is highly diverse, and more than 30 races/pathotypes have reported the presence of different pathotypic groups in the Xoo population (Mondal et al. 2014; Yugander et al. 2017; Chen et al. 2019); it mainly occurs due to its dynamic nature and existence of a high degree of variability within the pathogen population (Noer et al. 2018; Chen et al. 2019). This condition also holds good for the Indian Xoo population, where earlier works revealed the existence of six races and 22 pathotype groups, suggesting the existence of a high degree of variability among the Xoo isolates (Mondal et al. 2014; Yugander et al. 2017). Karnataka occupies a significant area of rice in India, grown in different ecosystems (Muniraju et al. 2017; Amoghavarsha et al. 2021; Sharanabasav et al. 2021). In Karnataka, the BLB causes significant yield damage in all ecosystems; however, the pathogen population structure, such as virulence and genetic diversity, is limited to only a few isolates (five only) collected from a limited (only four) fields (Yugander et al. 2017).

The population structure analysis provides essential information about the genetic diversity existing in the pathogen population. Previously, several studies attempted to deduce the genetic diversity of the Xoo population using different genetic markers such as amplified fragment length polymorphism (AFLP); restriction fragment length polymorphism (RFLP); entero-bacterial repetitive intergenic consensus sequences (ERIC); BOX-conserved repetitive elements; repetitive extragenic palindromic sequence (REP)-polymerase chain reaction (PCR); and endogenous insertion sequence element-based (IS)-PCR (Leach et al. 1992; Adhikari et al. 1995; Louws et al. 1995; Rademaker et al. 2000; Gonzalez et al. 2007; Hu et al. 2007; Que et al. 2011). However, molecular markers targeting an insertion sequence (IS) of Xoo, i.e., *IS1112*, have been reported as more effective in deducing the genetic diversity among bacterial isolates (George et al. 1997; Zhang et al. 1997). The IS elements are prominent and conserved features of bacterial genomes; they are generally considered to play essential roles in adapting phytopathogenic bacteria in the prevailing environment. These elements act as useful markers for identifying bacterial lineages and characterizing the structure or composition of bacterial populations. The presence of IS elements in the Xoo population was first reported by Leach and co-workers in 1990 (Leach et al. 1990). Genetic diversity analyses by using primers complementary to *IS1112* repetitive elements depicted variation within the pathogen population of Xoo (Yugander et al. 2017; Sandhu et al. 2018). In Karnataka, the BLB of rice is one of the major limiting factors in all rice ecosystems, and understanding the virulence diversity and genetic makeup, which has been limited to date, is essential to plan the disease resistance breeding programs. In this present investigation, we have identified the virulence diversity using a set of 28 international Xoo differentials (near-isogenic lines harboring different resistance genes) and deduced the genetic diversity among the Xoo isolates collected from the different ecosystems of Karnataka, a southern state of India.

MATERIAL AND METHODS

Collection of diseased leaf samples and isolation of the pathogen. A roving survey was carried out

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in Kharif, 2019. About 70 diseased samples showing the typical BLB symptoms were collected from five rice ecosystems of Karnataka, India (Figure 1) (TBP, UKP, Bhadra, Hilly, and Kaveri ecosystems). During the survey, the disease grades/ratings were recorded (in the field where the pathogen isolate was collected) as per the 0–9 scale of IRRI (2013). In each randomly selected plot of 1 m^2 area, about ten hills were selected randomly. About 3–5 leaves were given a 0–9 score based on per cent leaf damage on each hill. Using the grades, percent disease index (PDI) was calculated using a previously reported formula (McKinney 1923).

$$PDI = \frac{\text{Sum of all disease ratings}}{\text{Total number of leaves examined} \times \text{maximum disease rating}} \times 100 \quad (1)$$

Most of the samples were collected from farmers' rice fields. After collection, samples were placed in auto-seal plastic packets with silica gel

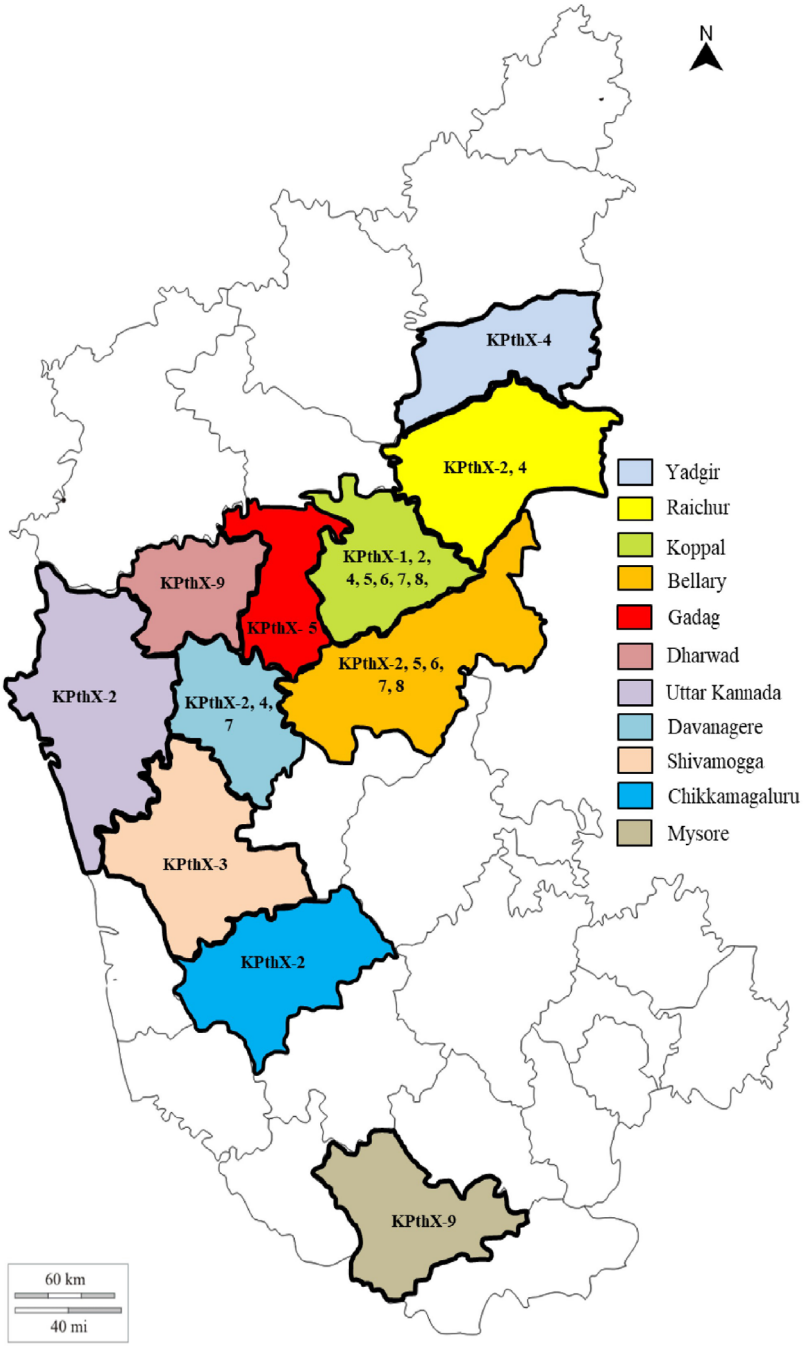

Figure 1. Karnataka state map showing the distribution of different pathotypes of Xoo in distinct districts of Karnataka

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and stored at 4 °C until the pathogen was isolated. The pathogen was isolated from the symptomatic leaves as described previously (Shankara et al. 2017). Three single colonies were isolated from each infected leaf sample, and one strain per field was selected as a representative strain for this study. The inoculated petriplates were kept under incubation at 28 ± 2 °C for 4–5 days. After 4–5 days of incubation, petriplates were observed for the growth of the yellow bacterial colonies. After four days of incubation, the bacterial growth obtained in the plates was recorded, and single-round-yellowish-mucoid colonies characteristics of Xoo were selected and further sub-cultured on the fresh sterile petriplates to get pure cultures. Yellow colonies which appeared for up to four days were discarded. Finally, pure culture for 54 Xoo isolates was recovered and multiplied in nutrient broth (NB) and stored as glycerol stocks (30% v/v) in a refrigerator at 4 °C for further use.

Isolation of genomic DNA. Pure cultures of 54 isolates were grown overnight in NB media and transferred to 2 mL Eppendorf tubes. The suspension was centrifuged at 10 000 *g* for 2 min to harvest the bacterial cells as pellets. The genomic DNA was extracted from the pellets using a HiPura® Bacterial Genomic DNA Purification Kit (Himedia Laboratories Pvt. Ltd, India) following the manufacturer's protocol. The DNA quality was assessed on a 1% agarose gel electrophoresis; later, it was visualized under a gel documentation unit (UVITEC, Essential V6, UK). The DNA was quantified using a Qubit 4.0 fluorometer (Qubit™ Invitrogen, USA).

Primer synthesis. To confirm the taxonomic identity of isolates, a set of Xoo-specific primers X002976 (F-GCC-GTT-TTT-CTT-CCT-CAG-C; R-AGG-AAA-GGG-TTT-GTG-GAA-GC) reported previously by Lang and co-workers, were synthesized (Lang et al. 2009). Similarly, for deducing the genetic diversity, *IS1112* specific primer JEL-1 (5'-CTC-AGG-TCA-GGT-CGC-C-3') and JEL-2 (5'-GCTCTACA-ATC-GTC-CGC-3') reported previously by George and co-workers, were synthesized (George et al. 1997). All primers were synthesized at a commercial facility (Eurofins, Luxembourg).

PCR-assay for taxonomic confirmation. The PCR assay was carried out in an ABS-Veriti™ 96-well thermocycler (Applied Biosystems, USA). The PCR reaction mixture (20 µL) consisted of 2 µL template DNA, 2 µL each of forward and reverse primers (5 pM/µL), 2 µL dNTPs mix (2.5 mM), 2 µL of 10X assay buffer with 15 mM $MgCl_2$, 1 µL of *Taq* DNA polymerase (6 U/µL) and 9 µL of sterile distilled water. The PCR reaction was configured to the following set of conditions; initial denaturation at 94 °C for 1 min followed by 35 cycles of denaturation at 96 °C (1 min), annealing at 58 °C (1.5 min), extension at 72 °C (1.5 min) and final extension at 72 °C for 10 min (Lang et al. 2009). The amplified PCR amplicons were separated through 1% agarose gel electrophoresis and visualized under a gel documentation system (UVITEC, Essential V6, UK).

Source of rice differentials. The seeds of the 28 rice differentials developed as the international differentials for BLB strains harboring different resistance (R) genes were obtained from the All India Coordinated Rice Improvement Programme, Gangavathi, Karnataka, India. The resistance genes of these varieties are given in Table 1 (IRBB lines and gene combination). The seeds were multiplied following the standard seed production procedures, and harvested seeds were packed in air-tight aluminum pouches and stored at 4 °C for further use. The seeds of all differentials were treated with streptocycline sulphate (100 ppm) and sowed in plastic pots. The plants were maintained in an environmentally controlled glasshouse following standard agronomic practices.

Virulence profiling and pathotype grouping. The virulence of Xoo isolates was determined by inoculating all 54 Xoo isolates on test differentials [28 near-isogenic lines (NILs) harboring different R gene/s, one rice cv. DV-85 and a resistant check cv. RP-BIO 226] at 45 days after sowing. Seeds of monogenic rice differentials were sown in plastic boxes, and seedlings were further transplanted to 25 cm diameter plastic pots after three weeks. Rice seedlings were grown under controlled greenhouse conditions for 90 days. The bacterial suspensions for inoculations were prepared in 10 mL of sterile distilled water at 10^8 CFU/mL. To test the virulence of the strains, ten plants with fully expanded leaves in each rice line in three replications were inoculated at the maximum tillering stage by the leaf clip-inoculation method described previously (Kauffman et al. 1973). The lesion lengths on inoculated leaves were recorded 12 days post-inoculation using a standard evaluation scale for BLB of rice developed by IRRI (2013). The pathogenicity tests were replicated three times. The isolates were classified into different pathotype groups based on

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Table 1. Grouping of Xoo isolates* from varied rice ecosystems of Karnataka into different pathotypes based on their reaction pattern to rice differentials

Sl. No.	NILs	Genes/gene combination	KPthX-1 (3)	KPthX-2 (18)	KPthX-3 (3)	KPthX-4 (9)	KPthX-5 (6)	KPthX-6 (3)	KPthX-7 (8)	KPthX-8 (2)	KPthX-9 (2)
1	IRBB-1	*Xa*1	S	MR	S	S	S	S	S	MR	S
2	IRBB-3	*Xa*3	MR	R	S	MR	MR	S	MR	R	S
3	IRBB-4	*Xa*4	S	MR	MR	S	S	S	S	MR	S
4	IRBB-5	*xa*5	S	MR	MR	S	R	S	S	S	S
5	IRBB-7	*Xa*7	S	MR	S	MR	MR	S	S	S	S
6	IRBB-8	*xa*8	MR	R	R	S	MR	S	R	R	S
7	IRBB-10	*Xa*10	R	R	R	S	R	MR	R	MR	MR
8	IRBB-11	*Xa*11	R	R	S	R	R	S	R	R	S
9	IRBB-13	*xa*13	MR	MR	MR	S	MR	S	R	R	S
10	IRBB-14	*Xa*14	R	MR	S	S	S	S	MR	MR	MR
11	IRBB-21	*Xa*21	MR	R	MR	R	MR	S	R	R	R
12	IRBB-50	*Xa*4 + *xa*5	R	MR	MR	S	S	MR	MR	MR	MR
13	IRBB-51	*Xa*4 + *Xa*13	MR	R	R	MR	MR	S	MR	MR	S
14	IRBB-52	*Xa*4 + *Xa*21	MR	R	MR	R	R	S	R	MR	MR
15	IRBB-53	*xa*5 + *xa*13	S	R	MR	R	R	R	MR	MR	R
16	IRBB-54	*xa*5 + *Xa*21	MR	R	R	MR	MR	S	MR	R	MR
17	IRBB-55	*Xa*21 + *Xa*13	R	R	R	S	MR	S	R	MR	S
18	IRBB-56	*Xa*4 + *xa*5 + *xa*13	R	R	MR	S	R	S	R	MR	S
19	IRBB-57	*Xa*4 + *xa*5 + *Xa*21	R	R	R	S	R	S	R	MR	MR
20	IRBB-58	*Xa*4 + *xa*13 + *Xa*21	MR	R	R	S	R	S	R	MR	S
21	IRBB-59	*Xa*5 + *xa*13 + *Xa*21	R	R	R	MR	R	MR	R	MR	MR
22	IRBB-60	*Xa*4 + *xa*5 + *xa*13 + *Xa*21	R	R	MR	R	R	R	MR	MR	S
23	IRBB-61	*Xa*4 + *xa*5 + *Xa*7	R	R	R	MR	R	S	R	S	S
24	IRBB-62	*Xa*4 + *Xa*7 + *Xa*21	MR	R	R	S	R	MR	R	MR	R
25	IRBB-63	*xa*5 + *Xa*7 + *xa*13	MR	R	R	S	R	S	MR	MR	MR
26	IRBB-64	*Xa*4 + *xa*5 + *Xa*7 + *Xa*21	R	R	R	S	R	S	R	R	R
27	IRBB-65	*Xa*4 + *Xa*7 + *xa*13 + *Xa*21	R	R	R	S	R	S	R	R	S
28	IRBB-66	*Xa*4 + *xa*5 + *Xa*7 + *xa*13 + *Xa*21	R	R	MR	MR	R	MR	R	MR	R
29	DV-85	*xa*5 + *Xa*7	S	MR	MR	S	R	S	R	R	S
30	RP-BIO 226	*Xa*5 + *xa*13 + *Xa*21	R	R	R	MR	R	MR	R	MR	MR
31	TN-1	Susceptible check	S	S	S	S	S	S	S	S	S

MR = moderately resistant; NIL = near-isogenic line; R = resistant; S = susceptible; Sl. No. = serial number
*Number of isolates belonging to the given pathotype is given in brackets

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the severity of the symptoms (disease grade) produced in different isolate-NIL combinations. Further, the pathotypes were classified into different virulent types such as less, moderate, and highly virulent based on the number of R gene/s against which the virulence developed, viz, < 4 R genes (less virulent), 4–5 R genes (moderately virulent), and > 6 R genes (highly virulent). To know the dissimilarity between the isolates based on virulence nature, isolates were categorized into two classes (1 = virulent, 0 = avirulent) by considering virulence as a variable factor. The dissimilarity was depicted by dendrogram using Darwin software (Jaccob's dissimilarity index)

PCR amplification using JEL primers and diversity analysis. For JEL-primers based genotyping, the PCR reaction mixture (total of 20 µL) was composed of template DNA (2 µL), 2 µL (5 pM/µL) of each forward and reverse primers, 2 µL of the dNTPs mix (2.5 mM), 2 µL of 10X assay buffer with 15 mM $MgCl_2$, 1 µL of *Taq* DNA polymerase (6 IU/µL) and 9 µL of sterile distilled water. The PCR reactions were carried out in an ABS-Veriti^{TM} 96-well thermocycler (Applied Biosystems, USA). The PCR reaction was configured with an initial denaturation at 94 °C for 1 min followed by 35 cycles of denaturation at 94 °C for 10 s, annealing at 62 °C (1 min), and extension at 65 °C (10 min) followed by one cycle of final extension at 65 °C for 10 minutes. Later, 4 µL of PCR products and 100 bp DNA ladder (Invitrogen, USA) were subjected to agarose electrophoresis using 1% agarose gel for 5 h at 60 V/cm. Finally, the results were visualized under the gel documentation unit (UVITEC, Essential V6, UK) and photographed using the default settings of the instruments.

The gel photograph was used to profile the amplicons (size and numbers) produced by each isolate. The differential amplicon profile was prepared using binary codes (1 = presence of amplicon, 0 = absence of amplicon) and analyzed using DARwin software (version 6.0.021). Jaccob's co-efficient analysis analyzed the diversity among the 54 isolates, and clustering was carried out using UPGMA and neighbor-joining (NJ) method.

Structure analysis. The amplicons size based on JEL-primers among the 54 isolates was converted into binary codes and used to determine the presence of any genetic structure through the program STRUCTURE version 2.3.4 (Pritchard et al. 2000), and the program was run with the number of clusters from K = 1 to K = 10, with five independent replications per K using the admixture model and correlated allele frequencies, a 200 000 burn-in period and 200 000 MCMC to determine approximations of posterior distributions. Then, the optimum K value was determined by estimating the peak value ΔK using Evanno and co-workers (Evanno et al. 2005) method through the STRUCTURE HARVESTER version 0.6.193 (Earl & VonHoldt 2012).

RESULTS

Disease distribution in different rice ecosystems. During the roving survey conducted in five rice ecosystems of Karnataka, the PDI was calculated for each field and each ecosystem where the BLB isolates were collected. The disease was recorded in all the ecosystems, and the PDI varied from 11.10 to 88.80. The PDI in the TBP ecosystem varied from 13.32 to 88.80%. Similarly, the PDI in the UKP ecosystem ranged from 11.10 to 26.64%. The PDI in the Bhadra ecosystem varied between 33.30 to 73.26%. Likewise, PDI in the hilly ecosystem ranged from 22.20 to 46.62, and the Kaveri ecosystem recorded a PDI of 31.08%. The PDI recorded in all the fields has been listed in Table 2.

Pathogen isolation and taxonomic confirmation. During the roving survey, approximately 70 BLB-infected diseased samples showing the typical BLB symptoms were collected from different rice ecosystems of Karnataka, India. The associated bacterial pathogen was isolated following the standard protocol. After 4–5 days of incubation, smooth, yellow, round individual colonies were selected and sub-cultured. A set of 54 Xoo isolates were isolated and maintained through the single colony methods. The taxonomic identity of all 54 isolates was confirmed using a set of Xoo-specific primers, i.e., Xoo-2976 (Lang et al. 2009). In a PCR assay, an expected amplicon of 337 bp was detected in the DNA of all 54 isolates confirming their identity as Xoo (Table 2 and Figure 1).

Pathotype grouping and distribution. All 54 Xoo isolates produced the characteristics disease symptoms on susceptible check DV-85. Pathotypic analyses of all Xoo isolates were conducted based on their reactions against 28 NILs. All 54 Xoo isolates were grouped into nine pathotypes based on their virulence reaction, namely, KPthX-1 to KPthX-9 (Table 1). KPthX-2 was the

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Table 2. Different isolated collected and studied from diverse rice ecosystems of Karnataka, India

Ecosystem	Districts	Villages	Variety	PDI	GPS coordinates		Altitude (m)	Isolate number	Isolate code	PCR confirmation*
					N^0	E^0				
TBP ecosystem	Ballari	Sugur	RNR 15048	55.50	15.486 0	76.744 3	381	21	Blr-Sgr-XOO-21	+
		Nittur	Sona mahsuri	46.62	15.564 4	76.838 9	371	10	Blr-Ntr-XOO-10	+
		Mannur	BPT 5204	48.84	15.477 1	76.711 2	387	11	Blr-Mnr-XOO-11	+
		Kamlapura	Nellur sona	44.40	15.305 3	76.476 6	460	41	Blr-Kmp-XOO-41	+
		Venkatapura	BPT 5204	71.04	15.596 9	76.371 9	515	19	Blr-Vkp-XOO-19	+
		Ittagi	BPT 5204	68.82	15.102 1	75.894 7	507	49	Blr-Itg-XOO-49	+
		Basaweshwara camp	Kaveri sona	35.52	15.431 9	76.649 1	412	17	Blr-Bwc-XOO-17	+
		Rajwala	RNR 15048	73.26	15.027 9	75.856 3	519	14	Blr-Rjl-XOO-14	+
		Kaganoor	Kaveri sona	39.96	15.062 3	75.923 2	478	20	Blr-Kgn-XOO-20	+
		Shivapura	RNR 15048	66.60	14.903 8	76.283 8	581	15	Blr-Spr-XOO-15	+
		Devani	Kaveri sona	44.40	15.613 1	76.863 5	371	18	Blr-Dvn-XOO-18	+
	Koppal	Chikkajanthakal	RNR 15048	53.28	15.415 6	76.583 6	397	2	Kpl-Ckj-XOO-2	+
		Basavapattana	Kaveri sona	35.52	15.463 2	76.496 1	430	33	Kpl-Bvp-XOO-33	+
		Vaddaratti	Gangavathi sona	35.52	15.457 3	76.502 4	430	3	Kpl-Vdt-XOO-3	+
		Ulenoor	RNR 15048	33.30	15.506 6	76.722 1	398	1	Kpl-Unr-XOO-1	+
		Arhal	Kaveri sona	42.18	15.473 5	76.486 7	438	43	Kpl-Ahl-XOO-43	+
		Siddhikeri	BPT 5204	64.38	15.429 5	76.492 1	433	9	Kpl-Sdk-XOO-9	+
		Hanwal	BPT 5204	59.94	15.539 2	76.560 6	433	13	Kpl-Hnl-XOO-13	+
		Kadebaagilu	Kaveri sona	37.74	15.367 8	76.501 2	418	16	Kpl-Kdb-XOO-16	+
		Sangapura	Kaveri sona	59.94	15.389 0	76.516 9	418	48	Kpl-Sng-XOO-48	+
		Ayodhya	BPT 5204	88.80	15.423 3	76.572 7	394	29	Kpl-Ayd-XOO-29	+
		Budagumpa	BPT 5204	42.18	15.393 3	76.313 8	506	30	Kpl-Bdg-XOO-30	+
		Hulgi	BPT 5204	73.26	15.311 8	76.338 3	475	47	Kpl-Hlg-XOO-47	+
		Munirabad	BPT 5204	37.74	15.302 5	76.337 7	468	27	Kpl-Mnb-XOO-27	+
		Agalakera	RNR 15048	59.94	15.339 5	76.334 8	467	24	Kpl-Agl-XOO-24	+
	Raichur	Deosugur	Kaveri sona	19.98	16.369 0	77.361 2	354	54	Rcr-Dsg-XOO-54	+
		Shaktinagar	Nandhyal sona	24.42	16.367 6	77.338 2	350	7	Rcr-Skt-XOO-7	+
		Karekal	Nellur sona	39.96	16.389 3	77.315 2	349	53	Rcr-Krk-XOO-53	+
		Neermanvi	BPT 5204	17.76	16.042 2	77.100 3	380	5	Rcr-Nmv-XOO-5	+
		Amarapur	RNR 15048	15.54	15.705 5	74.670 2	662	8	Rcr-Amp-XOO-8	+
		Budhihal camp	RNR 15048	13.32	15.780 0	76.770 0	385	36	Rcr-Bdc-XOO-36	+
		Dhadesugur	BPT 5204	24.42	15.693 3	76.897 4	360	37	Rcr-Dhs-XOO-37	+

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Table 2 to be continued

Ecosystem	Districts	Villages	Variety	PDI	GPS coordinates		Altitude (m)	Isolate number	Isolate code	PCR confirmation*
					N^0	E^0				
TBP ecosystem	Gadag	Singataluru	RNR 15048	44.40	15.060 2	75.882 6	505	38	Gdg-Sgt-XOO-38	+
		Nagarahalli	RNR 15048	59.94	15.158 8	75.887 6	508	12	Gdg-Ngh-XOO-12	+
UKP ecosystem	Yadgiri	Benakanahalli	Sona II	26.64	16.682 8	76.869 3	402	46	Ydg-Bnk-XOO-46	+
		Hunkal	Sona	11.10	16.740 7	76.795 0	445	50	Ydg-Hnk-XOO-50	+
		Hanumanahala	Sona II	66.60	16.381 5	76.536 6	433	44	Ydg-Hnl-XOO-44	+
Bhadra ecosystem	Shivamogga	Talaguppa	Jyothi	66.60	14.214 5	74.908 7	594	31	Smg-Tgp-XOO-31	+
		Matkalaga	Intan	73.26	14.225 1	74.905 3	571	6	Smg-Mtk-XOO-6	+
		Barandooru	Nallur sona	57.72	13.796 0	75.728 0	618	52	Smg-Bnd-XOO-52	+
	Chik-kamagaluru	Lakkavalli	Mangala	33.30	13.701 3	75.653 1	634	40	Ckm-Lkv-XOO-40	+
		Bhavikere	Jyothi	35.52	13.082 0	77.365 3	889	39	Ckm-Bvk-XOO-39	+
	Davanagere	Vaddinahalli	Sreeream sona	42.18	14.430 5	75.961 2	606	45	Dvg-Vdh-XOO-45	+
		Kumbalur	RNR 15048	44.40	14.375 5	75.753 1	586	25	Dvg-Kmb-XOO-25	+
		Nallur	Sona Mahsuri	53.28	14.100 0	75.883 0	816	26	Dvg-Nlr-XOO-26	+
		Thyavanige	Sreeream sona	34.41	14.249 3	75.884 6	591	22	Dvg-Tvg-XOO-22	+
		Dheetur 1	Kempu Jyothi	39.96	14.405 9	75.949 2	612	32	Dvg-Dtr 1-XOO-32	+
		Harihara	RNR 15048	73.26	14.515 2	75.807 2	548	34	Dvg-Hrh-XOO-34	+
		Dheetur 2	Nallur sona	55.50	14.576 7	75.825 1	547	28	Dvg-Dtr 2-XOO-28	+
Hilly ecosystem	Uttara Kannada	Bhagwati	Jyothi	37.74	15.195 0	74.455 3	546	35	Uk-Bgw-XOO-35	+
		Kegdal	Mysuru Mallige	22.20	15.162 2	74.711 4	443	42	Uk-Kgd-XOO-42	+
		Isloor	Intan	26.64	14.679 9	74.881 8	672	51	Uk-Ilr-XOO-51	+
	Dharwad	Mandihala	Bili Kagga	46.62	15.451 7	74.904 9	709	4	Dwd-Mdl-XOO-4	+
Kaveri ecosystem	Mysore	Hansoge	Jaya	31.08	12.542 3	76.184 1	801	23	Mys-Hng-XOO-23	+

PDI = percent disease index; TBP = Tunga Bhadra Project; UKP = Upper Krishna Project

*Taxonomic identity of all isolates was confirmed in PCR assay using a Xoo-specific primer pair (Lang et al. 2009) where, PCR positive result is represented as '+' sign

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most predominantly distributed pathotype in Karnataka, containing 18 isolates. In the case of monogenic NILs, IRBB-1 (*xa*1), IRBB-4 (*xa*4), IRBB-5 (*xa*5), and IRBB-7 (*xa*7) were the most in-effective R gene, whereas, *Xa*21 was the most effective, as it offered a broad spectrum of resistance against 45 isolates. Based on the number of R genes affected, KPthX-6 was found to be the most virulent, followed by KPthX-4; however, both these pathotypes were avirulent on IRBB-53 (*xa*5 + *xa*13) and IRBB-60 (*Xa*4 + *xa*5 + *xa*13 + *Xa*21) pyramids. The KPthX-2 was the least virulent strain. Among the five rice ecosystems, the TBP ecosystem (Ballari, Koppal, Raichur, Gadag) comprised maximum isolates (34) with the occurrence of seven different pathotypes, followed by the Bhadra ecosystem (Shivamogga, Chikkamagaluru, Davanagere) with ten isolates and four pathotypes, Hilly ecosystem (Uttara Kannada, Dharwad) with two pathotypes whereas, UKP (Yadgiri, KPthX-4) and Kaveri ecosystem (Mysore, KPthX-9) consist of one isolate each (Figure 1).

The isolates were grouped into three clusters (Figure 2). Cluster I consists of three pathotypes, KPthX-1, KPthX-7, and KPthX-8, whereas cluster II consists of only one pathotype, KPthX-2. The highest number of pathotypes were grouped in cluster III with five pathotypes such as KPthX-3, KPthX-4, KPthX-5, KPthX-6, and KPthX-9.

Genetic diversity and phylogenetic analysis. Understanding genetic diversity within the pathogen population is essential for the effective management of plant disease. The genetic diversity of 54 Xoo isolates of the Karnataka region was estimated using the PCR-based JEL primers that produced multiple DNA amplification products in 0.1 to about 3 Kbp. DNA fingerprinting using PCR revealed significant genetic diversity among fifty-

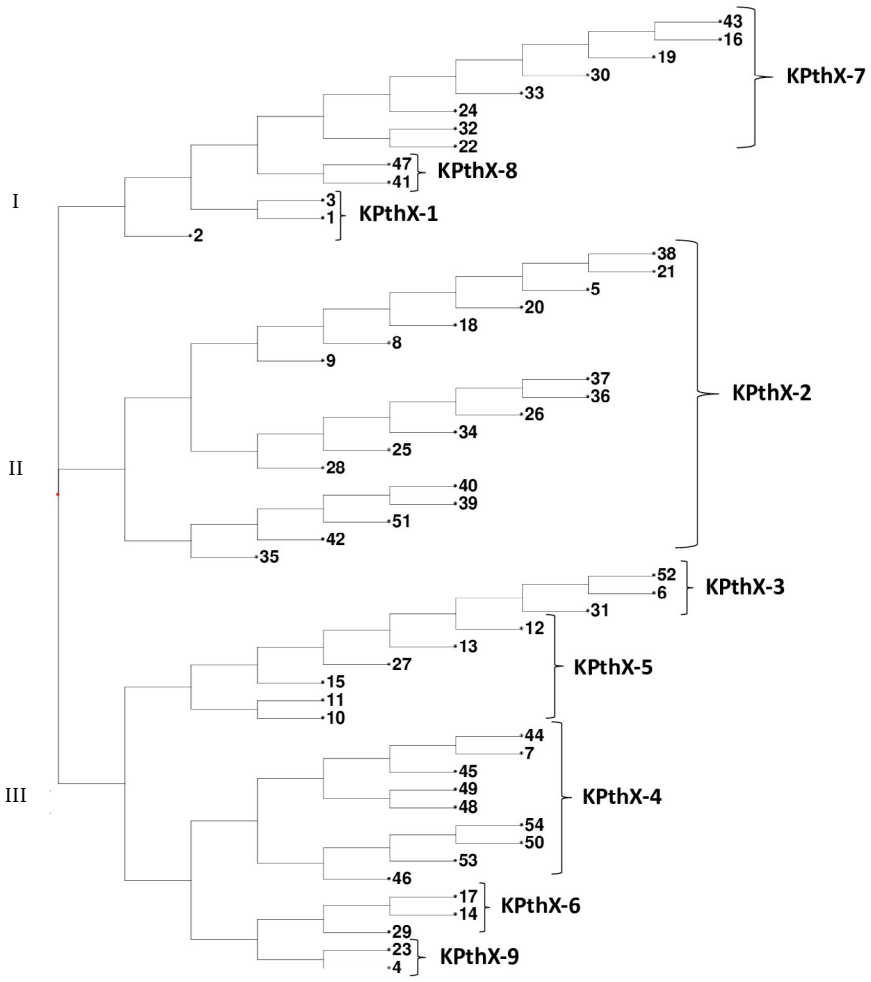

Figure 2. Cluster analysis of 54 isolates (or pathotypes) of Xoo based on their reaction against monogenic differentials using the neighbour joining method

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four isolates. Gel-electrophoresis of PCR amplified product showed a distinct amplicon profile in each isolate. In this investigation, the amplicons numbers varied from 1 to 25 among the isolates. A representative electrophoretic gel of JEL-PCR-amplified products of different Xoo isolates is shown in Figure 3.

A phylogenetic study using Darwin software grouped the isolates into three distinct clusters (Figure 4). Cluster I comprises 23 isolates, cluster II comprises 27, and cluster III with only one isolate. Further, cluster I was sub-clustered into I-A and I-B, comprised of 17 and six isolates. Interestingly, only one isolate (Mys-Hng-Xoo-23) completely diverged into a separate major cluster (cluster III). Further, the data was co-related with the pathotype group of the isolates. Cluster I includes seven pathotypes viz, KPthX-1, KPthX-2, KPthX-3, KPthX-4, KPthX-5, KPthX-6, and KPthX-8. Cluster II includes nine pathotypes viz., KPthX-1, KPthX-2, KPthX-3, KPthX-4, KPthX-5, KPthX-6, KPthX-7, KPthX-8, and KPthX-9, and cluster III contains only one pathotype KPthX-9.

Structure analysis results. Based on JEL-primers genotyping, the amplicons (size and numbers) produced by each isolate were converted into binary codes (1 = presence of amplicon, 0 = absence of am-

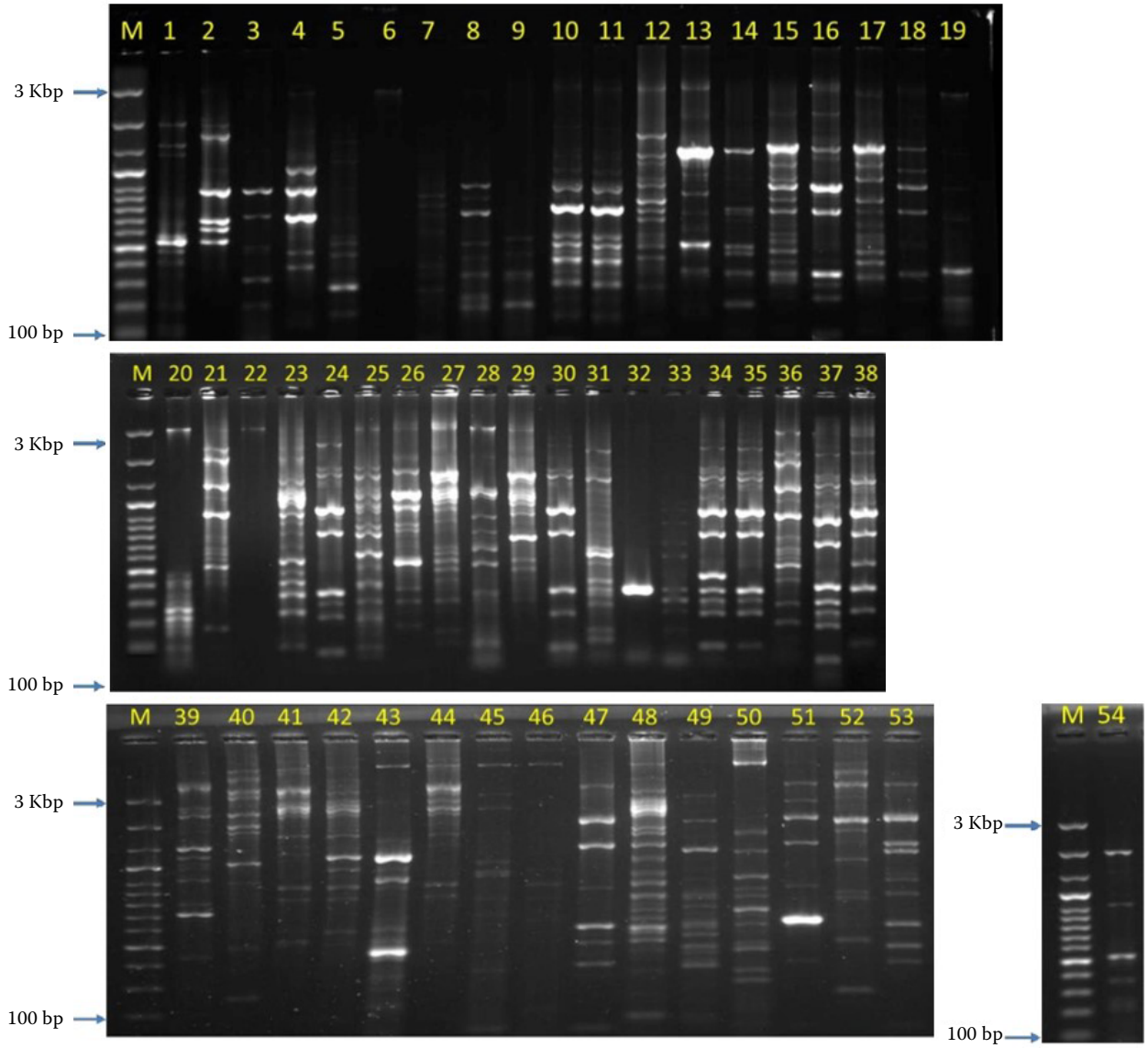

Figure 3. Agarose gel electrophoresis showing the amplified fragments of *IS1112* insertion element in the *X. oryzae* pv. *oryzae* isolates using *IS1112*-specific JEL primers

M = 100 bp DNA marker; lane 1–54 = Xoo isolates collected across different rice ecosystems of Karnataka

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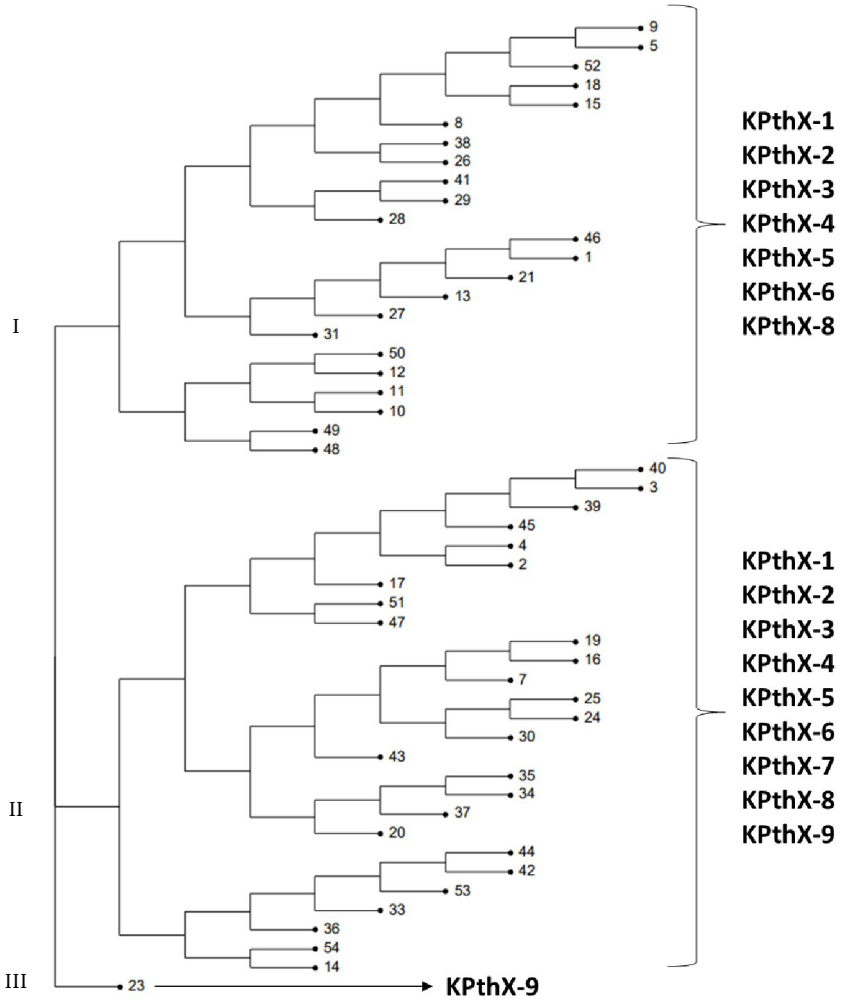

Figure 4. Neighbor-joining tree shows 54 Xoo isolates clustering based on DNA fingerprinting data using *IS1112*-based PCR

The tree was generated using DARWin software (version 6) through un-weighted neighbor joining based on dissimilarity matrices using single data based on the presence or absence of amplicons and Jaccard's coefficient

plicon) and analysed to determine the presence of population structure among the 54 isolates using STRUCTURE software. Based on the STRUCTURE HARVESTER program (http://taylor0.biology.ucla.edu/structureHarvester/), the peak plateau of the ad hoc measure ΔK was detected at K = 5 (Figure 5). The 54 genotypes were distributed into five subgroups based on this data, viz SG1, SG2, SG3, SG4, and SG5 indicate five subpopulations (Table 3). Classification of isolates to the subgroups based on the ancestry threshold value of 70% was conducted to identify the isolates belonging to specific subgroups, whereas the rest below the threshold value were classified as Admixture (AD) (Table 3). Out of the 54 isolates, the largest number of isolates (31.48%) were classified as AD. The SG-1 consisted of nine isolates, whereas the rest of the subgroups, SG2, SG3, SG4, and SG5, were distributed seven isolates each (Table 3).

DISCUSSION

The variability in virulence of Xoo is well documented in almost all rice-growing regions of the world. Similarly, there is significant information on virulence diversity in India (Mishra et al. 2013; Mondal et al. 2014; Yugander et al. 2017). Karnataka occupies a significant area under rice, and BLB is major biotic stress due to the limited availability of cultural, biological, and chemical methods. Host plant resistance-based strategies are ef-

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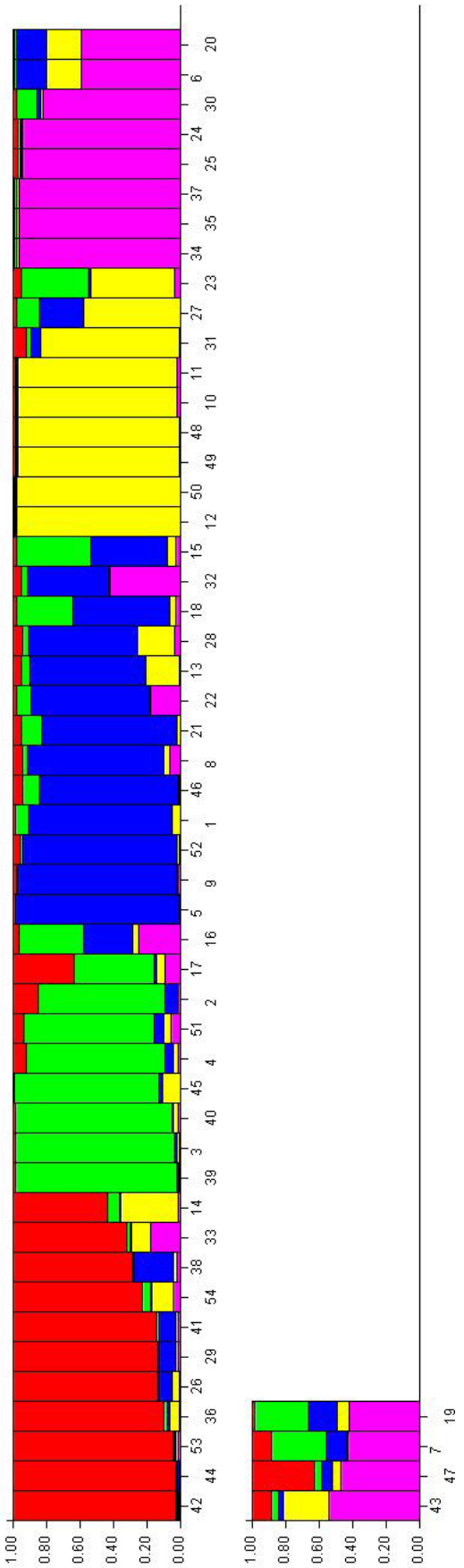

Figure 5. The estimated population structure of the 54 isolates
Colored segments are representing the estimated membership for K = 5, indicating that the entire population can be grouped into five subgroups. The numbers in the *x*-axis represent the isolate number as per Table 2

Table 3. Population structure group of 54 isolates based on inferred ancestry values

Isolates	Inferred clusters					Subgroup
	Q1	Q2	Q3	Q4	Q5	
1	0.012	0.077	0.861	0.044	0.007	SG3
2	0.148	0.759	0.072	0.004	0.017	SG2
3	0.01	0.95	0.015	0.015	0.01	SG2
4	0.078	0.823	0.051	0.03	0.017	SG2
5	0.009	0.005	0.972	0.007	0.007	SG3
6	0.006	0.012	0.18	0.206	0.596	AD
7	0.109	0.327	0.123	0.007	0.434	AD
8	0.054	0.029	0.811	0.037	0.07	AD
9	0.02	0.006	0.95	0.005	0.019	SG3
10	0.008	0.008	0.011	0.95	0.023	SG4
11	0.008	0.008	0.012	0.95	0.022	SG4
12	0.007	0.007	0.005	0.975	0.006	SG4
13	0.046	0.047	0.696	0.203	0.009	AD
14	0.564	0.067	0.009	0.345	0.015	AD
15	0.018	0.442	0.455	0.052	0.033	AD
16	0.035	0.385	0.294	0.031	0.255	AD
17	0.36	0.481	0.012	0.051	0.097	SG5
18	0.018	0.332	0.585	0.031	0.034	AD
19	0.013	0.316	0.177	0.066	0.428	AD
20	0.006	0.012	0.179	0.209	0.594	AD
21	0.047	0.124	0.804	0.02	0.005	SG3
22	0.016	0.091	0.706	0.003	0.184	SG3
23	0.043	0.403	0.013	0.505	0.036	AD
24	0.027	0.01	0.008	0.01	0.945	SG5
25	0.027	0.01	0.008	0.01	0.946	SG5
26	0.861	0.01	0.078	0.043	0.008	SG1
27	0.018	0.134	0.267	0.574	0.007	AD
28	0.053	0.036	0.652	0.218	0.04	AD
29	0.858	0.012	0.097	0.017	0.016	SG1
30	0.017	0.125	0.022	0.008	0.827	SG5
31	0.078	0.027	0.055	0.832	0.007	SG4
32	0.044	0.038	0.486	0.004	0.428	AD
33	0.676	0.018	0.009	0.117	0.18	AD
34	0.007	0.008	0.005	0.01	0.97	SG5
35	0.007	0.008	0.005	0.01	0.97	SG5
36	0.899	0.019	0.012	0.059	0.011	SG1
37	0.007	0.008	0.005	0.01	0.97	SG5
38	0.711	0.01	0.23	0.026	0.023	SG1
39	0.011	0.961	0.008	0.01	0.009	SG2
40	0.008	0.942	0.007	0.026	0.018	SG2
41	0.854	0.013	0.099	0.017	0.017	SG1
42	0.97	0.007	0.008	0.007	0.007	SG1

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Table 3 to be continued

Isolates	Inferred clusters					Subgroup
	Q1	Q2	Q3	Q4	Q5	
43	0.108	0.047	0.026	0.274	0.544	AD
44	0.97	0.007	0.009	0.007	0.008	SG1
45	0.007	0.858	0.024	0.104	0.007	SG2
46	0.053	0.103	0.826	0.006	0.012	SG3
47	0.365	0.047	0.064	0.046	0.477	AD
48	0.011	0.008	0.007	0.965	0.008	SG4
49	0.011	0.008	0.007	0.966	0.008	SG4
50	0.007	0.007	0.005	0.975	0.006	SG4
51	0.059	0.777	0.061	0.039	0.064	SG2
52	0.04	0.016	0.917	0.018	0.008	SG3
53	0.951	0.01	0.007	0.014	0.018	SG1
54	0.765	0.054	0.006	0.13	0.044	SG1

fective and eco-friendly (Mizukami & Wakimoto 1969). However, the development of resistant varieties requires a clear knowledge of the population structure and virulence distribution of the target pathogen. Although many research groups studied the virulence diversity of Xoo in India, the majority of work was limited to Northern India (Shanti et al. 2001; Lore et al. 2011; Mishra et al. 2013; Mondal et al. 2014). Although representative isolates of Karnataka were included in the previous studies, the number of isolates studied was very few and collected from a limited geographical area (Yugander et al. 2017).

The recognition of the pathogenic race of Xoo depends on its ability to induce a combination of a susceptible and resistant reaction on a set of standard differential host (Adhikari et al. 1999). The Near-isogenic rice lines (NILs) possessing different major genes for resistance to Xoo were developed by IRRI and were used for the identification of Xoo races. In the present investigation, a field survey was conducted in five major rice ecosystems of Karnataka and collected 54 isolates. Our study revealed the presence of significant virulence diversity among the isolates.

All 54 Xoo isolates were pathotyped using NILs harboring different Xoo resistance genes and one rice cv. DV-85 and a resistant check (cv. RP-BIO 226). The present study revealed the presence of nine distinct pathotypes. Similarly, 11 pathotypes were detected using nine NILs lines overall in India (Mishra et al. 2013); 24 pathotypes were identified based on their virulence patterns on the NILs tested in Bangladesh (Islam et al. 2016), and 14 pathotypes were identified among the Sri Lankan strains (Ochiai et al. 2000). The pathotype KPthX-2 was the most frequently distributed pathotype in Karnataka, including 18 isolates. Likewise, pathotype III was the most frequent pathotype distributed over all of India and accounted for 40.7% of the isolates (Mishra et al. 2013), three pathotypes namely; VII, XII, and XIV, were considered major pathotypes in Bangladesh (Islam et al. 2016), and only pathotype I was virulent to all major resistance genes in Sri Lanka (Ochiai et al. 2000).

In the present study, the *Xa*21 gene was the most effective R gene, as it offered a broad spectrum of resistance against 45 isolates. Interestingly, Mishra and co-workers (Mishra et al. 2013) also reported that, *Xa*21 was the most effective and broad resistance gene, conferring resistance against 88% of the Indian isolates, and *Xa*21 was found effective against all Sri Lankan pathotypes except pathotype 1 (Ochiai et al. 2000). The *Xa*21, BLB resistant gene from *Oryza longistaminata*, is effective due to its broad spectrum of resistance against multiple strains of BLB pathogen. Among the five major rice ecosystems of Karnataka, the TBP ecosystem included maximum isolates (Mishra et al. 2013) and seven pathotypes, while UKP and Kaveri ecosystems included one isolate each.

In our present study, the most virulent, KPthX-6 and KPthX-4, were found avirulent on IRBB-53 (*xa*5 + *xa*13) and IRBB-60 (*Xa*4 + *xa*5 + *xa*13 + *Xa*21) pyramids lines. Our results are in line with the previous study, where the gene pyramid line carrying *Xa*21, *xa*13, and *xa*5 was resistant to the Tripura strain. Our present study revealed that differentials with four and five R genes (*Xa*4 + *xa*5 + *xa*13 + *Xa*21, *Xa*4 + *Xa*7 + *xa*13 + *Xa*21, and *Xa*4 + *xa*5 + *Xa*7 + *xa*13 + *Xa*21) were effective in managing disease against all the pathotypes of Karnataka. Previous workers also reported a high BLB resistance against BLB pathotypes by using three-gene and two-gene pyramided lines in several parts of India (Singh et al. 2003; Sundaram et al. 2008). All these genes combination appeared to be good candidates to be deployed in different rice cultivars. This study helps in the strategic planning and deployment of different *Xa* gene combinations in the genetic background of important Indian rice cultivars.

We identified the distribution of nine pathotypes in Karnataka. Previously, seven pathotype

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groups have been reported in Karnataka (Shankara 2015). However, we used internationally accepted monogenic and oligogenic differentials. The grouping was carried out separately based on isolate reactions on the monogenic differential, oligogenic differentials, and a monogenic and oligogenic differential combination. Among three types of grouping, reaction on monogenic differential was considered due to the appearance of contrasting phenotypes such as R and S. Whereas, in the case of oligogenic differentials, phenotype on most of the isolate-differential combination was MR in addition to S, and R. MR-phenotype is highly confusing to decide whether the pathotype is either virulent or avirulent on a particular R gene and its combination. Therefore, we have considered the pathotype grouping in this study based on the monogenic differential. Previous studies have also reported the superiority of monogenic differential in resolving the virulence diversity of Xoo isolates (Lore et al. 2011; Mondal et al. 2014; Kumar et al. 2018). Nine pathotypes identified in this study were distributed across all ecosystems, and no correlation was found between the pathotypes and ecosystems. This could be due to the free exchange of seed material between the ecosystems and the cultivation of similar cultivars in all irrigated ecosystems.

Information on the genetic diversity among the different pathogen isolates is a prerequisite for understanding phylogeny and evolution better. Among the different techniques, the characterization of IS elements, such as *IS1112* and *IS1113,* is widely accepted globally. The *IS1112* is a transposable element found only in the Xoo pathogen and absent in non-pathogenic xanthomonads. This marker can be used for both detection and the characterization of Xoo simultaneously. Several attempts were made in India to characterize the Xoo population using IS-sequences (Shanti et al. 2001; Singh et al. 2003; Reddy et al. 2009; Ladha & Rabindran 2012; Mondal et al. 2014; Yugander et al. 2017). However, most of the studies have not included the isolates from Karnataka or included very few isolates.

To prove our hypothesis, we have collected 54 isolates representing all the rice ecosystems of the state, except the coastal (due to the absence of disease during the survey). Cruz et al. (1996) reported a thumb rule to decide the sample size to determine the haplotype diversity of the Xoo population within a location. In our study, the sample size from the different ecosystems was uneven; however, each ecosystem was represented by at least three isolates except the Kavery ecosystem, where we could isolate pure culture for only one isolate from Mysore.We used the *IS1112* marker for deducing diversity, as this marker is known for its higher resolution to distinguish isolates of Xoo. All 54 isolates showed diversity (in terms of number and size of insertion sequence). The phylogenetic analysis of the amplicon profile revealed the three distinct clustering patterns, where most of the isolates clustered in cluster I and cluster II. Surprisingly, one isolate (Mys-Hng-Xoo-23) collected from the Kaveri ecosystem, i.e., the Mysore district, was divergent and clustered separately from all remaining isolates. This could be because the disease was recently reported in that region, and the pathogen of that region may be having distinct evolutionary history. Moreover, the rice genotypes were grown, and the agro-climatic conditions of the Kaveri ecosystem are different from other ecosystems in the state.

We did not observe any significant correlation between the pathotype group and isotype cluster based on diversity in genomic insertions. However, a partial relationship was found between the determined phylogenetic groups and pathotypes for Sri Lankan and Nepal isolates (Adhikari et al. 1999; Ochiai et al. 2000); this might be because virulence is not related to insertion sequences.

Based on structure analysis, 54 Xoo isolates were categorized into five subgroups, viz, SG1, SG2, SG3, SG4, and SG5, indicating the existence of five subpopulations. Among them, the maximum isolates (31.48%) were classified as AD. The sub-group included a maximum of nine isolates. We did not observe any correlation between cluster and structure analysis. The present study of evaluating the pathotypes and genetic diversity in the Xoo population in Karnataka is an initial step to understanding the pathogen population structure and would help in a more comprehensive understanding of this pathogen population.

CONCLUSION

This genetic diversity might explain solving some of the problems encountered in develop-

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ing rice cultivars with specific and broad-spectrum resistance against the Xoo population of Karnataka. The major obstacle in deciphering the biology of the Xoo population and its management has been one of the biggest challenges. Understanding the distribution of different pathotypes would help better monitor the spread of Xoo pathogens in other major rice-growing producing areas in Karnataka and India. Further current study might help to provide a platform for the characterization of new uncharacterized isolates. Further, a combination of four or five genes in different rice cultivars appeared to be the best strategy for managing the menace caused by this disease. This present analysis would aid in deploying better strategies and planning the incorporation of different *Xa* gene combinations in important Indian rice cultivars for effective management against this pathogen.

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