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PCR-MEDIATED IDENTIFICATION AND CHARACTERIZATION OF RICE BACTERIAL BLIGHT PATHOGEN (*XANTHOMONAS ORYZAE* PV. *ORYZAE*) ISOLATES COLLECTED FROM THE SINDH PROVINCE OF PAKISTAN

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Abstract

Bacterial leaf blight (BLB) is one of the most devastating diseases of rice crop. Its severe incidence has been reported in the Sindh province of Pakistan in recent years. It can cause up to 50% crop losses, whereas in favorable conditions, the disease can result in premature plant death. This study was conducted to identify and characterize the BLB causing pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in Sindh province through molecular approaches. Pure cultures of 120 bacterial isolates, obtained from various locations of the province, were developed using the streaking method. The purified cultures were grown overnight in LB medium until saturation. Cultures of the bacterial isolates were then subjected to DNA isolation employing the CTAB method. The extracted bacterial DNA was subjected to PCR reaction. XOR primer pair [XOR-F (5'-GCATGACGTCATCGTCCTGT-3') and XOR-R2 (5'-CTCGGAGCTATATGCCGTGC-3')] was employed for amplification of 16S-23S rDNA spacer region-specific to *Xoo* bacterium. The PCR product was run in 1.2% gel electrophoresis in TBE buffer. The targeted band of 470 bp was observed in a total of 34 isolates analyzed through PCR. The identified *Xoo* isolates were further subjected to RAPD-PCR, which yielded various polymorphic bands. The binary data collected from RAPD analysis was used for investigating genetic relatedness and diversity of the *Xoo* isolated using the un-weighted pair-group (UPGMA) method. The UPGMA analysis showed extensive genetic variability among the studied isolates as the isolates were observed to cluster into seven clusters. This is the first report of PCR-based confirmation of *Xoo* in Sindh province, as per our knowledge. Rapid, reliable, and accurate identification of *Xoo* is very important to adopt disease management strategies to minimize crop losses. Moreover, the RAPD based genetic analysis of these isolates may serve as a platform study for refined characterization of new isolates for which racial classification has not been determined in Sindh province.

Key words: Bacterial leaf blight, Molecular identification, PCR, Rice, *Xanthomonas oryzae* pv. Oryzae.

Introduction

Xanthomonas oryzae pv. *oryzae* (*Xoo*), the causative agent of bacterial leaf blight, is one of the most devastating rice crop pathogens. *Xoo* enters the plant through lesions or water pores, subsequently producing a systemic infection (Mew, 1987). Once invaded by *Xoo*, tannish grey to white lesions are formed along the veins of the plant. Symptoms start appearing from tillering stage of the crop, and the severity progresses as the plant matures, reaching an apex at the flowering stage. In case of acute invasion, the plant leaves turn yellow and wilt, ultimately leading to partial or total crop failure (Mizukami & Wakimoto, 1969; Sharma *et al*., 2017).

Bacterial leaf blight (BLB) affects both conventional and hybrid rice (Mew, 1987; Noroozi *et al*., 2015). It can cause up to 50% crop losses, whereas in severe conditions, the disease can result in premature plant death and lodging of the infected plant population (Niño-Liu *et al*., 2006). BLB is prevalent among rice varieties cultivated all around the world (Singh *et al*., 2015). The disease was initially reported in Japan from where it has spread to many areas globally, including Pakistan (Mew & Majid, 1977). With time, alarming incidents of BLB have been observed in numerous regions of the country (Shah *et al*., 2009; Yasmin *et al*., 2017).

Rice is one of the top agricultural commodities of Pakistan (Yasmin et al., 2017). The country ranks at 10th position in rice production globally, with a total production of 6.85 million tonnes (MT). In 2017, Pakistan farmed rice on 2.72 million hectares (M ha) (Ministry of Finance, 2017). It is cultivated in all four provinces of the country. Sindh province, the second-largest rice producer in Pakistan, harvested 2.66 MT of rice in 2017 (Agriculture Marketing Information Service Pakistan, 2017). Rice cultivation is being jeopardized in Sindh because of serious attacks of BLB, as the region has favorable conditions for the growth of *Xoo* (Rafi *et al*., 2013).

Severe yield losses have been observed as a result of BLB. Unlike other diseases of rice, resistant cultivars for this disease are not available. Moreover, *Xoo* has a high rate of variability. Therefore, the introduction of resistant genotypes is not expected to be a sustainable solution attributed to the fast rate of mutations in the pathogen (Mohan Babu *et al*., 2003). Rapid and accurate identification of *Xoo* is very important to adopt disease management strategies to minimize crop losses. Containment and elimination of the pathogen can only be ensured through an apposite response to disease invasion within urgent timeframe. The identification of *Xoo* on colony morphology, biochemical tests, or disease symptoms is intricate and has various limitations. Symptoms of bacterial leaf blight overlap with the bacterial leaf streak of rice, and it isn't easy to differentiate the two (Kong *et al*., 2002; Lang *et al*., 2010). The biochemical and culture-based methods, on the other hand, are not accurate and specific. Moreover, such approaches are also tedious and time-consuming (Ash *et al*., 2014; Lang *et al*., 2014; Cui *et al*., 2016). These limitations complicate the urgent application of appropriate and specific disease control measures.

Molecular approaches have recently been used for specific, accurate, and rapid identification of *Xoo* (Pramod *et al*., 2015). These methods amplify unique sequences in the highly variable 16S-23S rDNA intergenic spacer region of the pathogen. In this study, an attempt was made to target the 16S-23S rDNA intergenic spacer region using XOR primers specific to *Xoo*. These primers amplify the specific sequences existing in *Xoo* only. Hence, this strategy can be used as a rapid and accurate diagnostic tool to ascertain the infestation of *Xoo* (Adachi & Takashi, 2000).

Isolates from ten different locations of Sindh province were subjected to PCR reaction in this study. Scant literature is available regarding evaluation and identification of *Xanthomonas oryzae* pv. *oryzae* from Sindh province. Additionally, in any previous reports, specific molecular approaches have not been used to confirm *Xoo* in this region. Hence, this is one of the first reports of molecular-based confirmation of *Xanthomonas oryzae* pv. *oryzae* in Sindh province, as per our knowledge.

Materials and Methods

Collection of Diseased Samples: Strategies were made to collect BLB infected samples from ten districts across the Sindh province. The isolates were collected from five districts of lower and five districts of upper Sindh by the Plant Protection Division, Nuclear Institute of Agriculture (NIA) Tando Jam.

Isolation and purification of BLB isolates: Samples collected from survey sites were processed for isolation and purification of the pathogen. Infected leaf pieces of rice were excised with a sterile scalpel. The leaf surface was sterilized using 70% ethanol followed by washing. The infected leaf pieces were then transferred to nutrient agar medium and incubated at 28-30 ± 2°C for 72 hours. Bacterial colonies of different shapes, sizes, and colors appeared on nutrient agar medium. The bacterial colonies exhibiting *Xoo* characteristics were then purified using the streaking method.

DNA extraction: Pure cultures of the bacterial isolates were subjected to DNA extraction employing the CTAB method. Cultures were inoculated in the liquid medium until the growth was saturated. Subsequently, the microbial cultures were centrifuged for collecting compact bacterial pallets. The pallet was suspended in TE buffer containing SDS and Proteinase K. After incubation at 37°C, a higher concentration of NaCl was added, followed by the addition of CTAB/NaCl solution. The samples were then incubated at 65°C, followed by three purifications using chloroform/ isoamyl alcohol, phenol/ chloroform/ isoamyl alcohol, and again chloroform/ isoamyl alcohol to ensure high purity. DNA precipitation was done using isopropanol, followed by ethanol washing. The extracted DNA was finally suspended in TE buffer.

DNA quantitation: Quantification of the DNA was carried-out using Thermo Spectronic Biomate 3 spectrophotometer.

Amplification of 16s r DNA, 16S-23S r DNA intergenic spacer region: Isolates of *Xanthomonas oryzae* pv. *oryzae* were identified through Polymerase Chain Reaction (PCR). XOR primer pair [XOR-F (5'-GCATGACGTCATCGTCCTGT-3') and XOR-R2 (5'-CTCGGAGCTATATGCCGTGC-3')] was used for PCR amplification of 16S-23S rDNA spacer region-specific to *Xoo* bacterium.

PCR optimization: The final reaction mixture contained 10 µl of Wizpure PCR master mix, 6 µl deionized water, 1 µl forward primer, 1 µl reverse primer, and 2 µl of bacterial DNA. Successful PCR reactions were observed using the PCR profile provided in (Table 1). Thirty-five cycles of PCR reaction were used for amplification purpose.

Table 1. PCR conditions for optimal amplification of targeted DNA sequence using XOR primers.

Parameter	Temperature	Duration
Initial denaturation	95°C	5 min.
Denaturation	95°C	30 sec.
Annealing	56°C	45 sec.
Extension	72 °C	1 min.
Final extension	72 °C	10 min.

Gel electrophoresis: The PCR product was run in 1.2% agarose gel electrophoresis in TBE buffer at 90 V. Ethidium bromide staining was employed to visualize the PCR product. Bands were observed under UV light, and the photographs were recorded using Uvitec Gel Documentation System. 100 bp DNA ladder was used as a molecular size reference to compare the bands' size yielded from PCR.

RAPD-based characterization of identified *Xoo* isolates: The identified *Xoo* isolates were subjected to molecular characterization. DNA isolation and purification were done using the CTAB method. Final purification was done by phenol/chloroform/isoamyl alcohol extraction. Isopropanol was used for precipitating the DNA, followed by ethanol washing. Purified bacterial DNA of *Xoo* isolates was subjected to PCR reaction using RAPD primers. Twelve different decamer RAPD primers belonging to OPA and OPK series were used in this analysis. Wizpure master mix (2X) was used for amplification of the bacterial DNA. PCR was performed in a 25 µl reaction mixture. Generally, initial denaturation was done at 94°C for 4 minutes, denaturation at 94°C for 1 min, annealing at 36°C for 1 min; extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. Forty-five cycles of the PCR reaction were performed for each analysis. The PCR product was run in 1.2% agarose gel electrophoresis in TBE buffer at 90 V. PCR bands were observed and recorded using the Uvitec Gel documentation system.

The PCR bands observed in RAPD analysis were scored for calculating the similarity index values. The presence or absence of particular PCR bands at different positions was converted into binary data, i.e., the presence or absence of a band at identical molecular weight was coded as 1 or 0, respectively. This scoring matrix was then used for investigating genetic relatedness and diversity of the *Xoo* isolates using the Un-weighted Pair Group method (UPGMA).

Results and Discussion

A total of 120 isolates were collected from suspected plant samples. CTAB extraction yielded high, quality DNA used as a template for amplifying the targeted product using XOR–F/XOR-R2 primer pair. Accurate, rapid, and sensitive detection of *Xoo* was accomplished by this primer set as it specifically targets the 16S-23S rDNA intergenic spacer region of *Xanthomonas oryzae* pv. *Oryzae*. A single PCR product of 470 bp was produced by *Xoo* strains only. The method was first developed by Adachi & Takashi (2000) in Japan.

At an annealing temperature of 56°C for 45 sec., robust PCR amplification was observed. Out of 120 isolates subjected to PCR reactions, 34 isolates produced the mentioned PCR product of 470 bp, while no PCR band was observed for the remaining isolates (Fig. 1). Also, compared with the samples collected from non-infected samples, the PCR reaction did not produce any band.

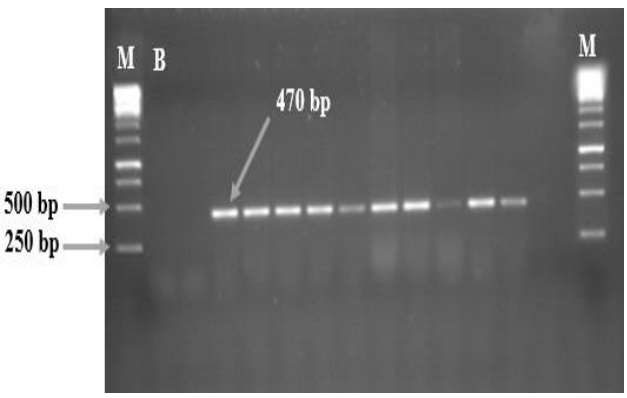

Fig. 1. Agarose gel electrophoresis of products from PCR performed on DNA of suspected isolates using *Xoo* specific primers i.e. XOR-F and XOR-R2. Lane M represents 1kb DNA size ladder, Lane B represents Blank while other lanes represent PCR from different bacterial isolates subjected to the analysis. Lane 4-13 referring to *Xoo* bacterial isolates showed the specific PCR product of 470 bp.

The *Xoo* PCR product was observed in isolates belonging to various survey locations of the Sindh province (Table 2). Overall, 17 isolates originated from upper Sindh, while the remaining 17 belonged to the lower Sindh area. The confirmation of *Xoo* invasion in numerous lower and upper Sindh samples indicated the widespread prevalence of BLB in rice-growing areas of the province.

Table 2: The bacterial isolates presenting characteristics of *Xoo* were subject to PCR identification. The PCR analysis showed that the samples both from upper as well as lower Sindh exhibited *Xoo*.

Rafi *et al*., (2013) collected *Xoo* isolates from different infected areas of Khyber Pakhtunkhwa province and characterized them on biochemical tests. Nevertheless, it was seen that the biochemical variations existed among the isolates indicating that such a system would not be accurate. Therefore, the authors suggested the use of molecular approaches to identify the plague specifically. Earlier, a phage-based approach has also been used to identify *Xoo* (Yoshimura, 1963). Yet, its protocols are too complicated, time-consuming, and not feasible for analyzing larger batches of samples.

On the contrary, the reported PCR-based approach is highly specific, is not labor-intensive, and can be used for a large number of samples simultaneously. Moreover, multiplexing this procedure for other diseases can provide complete profiling of important diseases of rice in a single run (Lang *et al*., 2010; Cui *et al*., 2016). In a traditional single PCR reaction, hundreds of samples can be loaded, indicating that the technique is feasible for many samples. This procedure is also sufficiently sensitive to detect the pathogen even at extremely low CFU ml^{-1}. Additionally, the 16s-23s spacer region has a large copy number in *Xoo* which further facilitates the identification process (Adachi & Takashi, 2000).

Table 2. Isolates confirmed as *Xanthomonas oryzae* pv. *oryzae*.

Serial number	Isolate	Location
1.	16 e3	Lower Sindh
2.	11B	Upper Sindh
3.	453 g3	Upper Sindh
4.	53g3	Upper Sindh
5.	Talhar	Lower Sindh
6.	11 3f	Upper Sindh
7.	22 3	Lower Sindh
8.	17 3	Lower Sindh
9.	243G	Lower Sindh
10.	P11	Lower Sindh
11.	Talhar g3	Lower Sindh
12.	1b	Upper Sindh
13.	19 3	Lower Sindh
14.	32 D	Lower Sindh
15.	39 3A	Upper Sindh
16.	363	Upper Sindh
17.	16B	Lower Sindh
18.	45A	Upper Sindh
19.	39 3	Upper Sindh
20.	24 3	Lower Sindh
21.	163	Lower Sindh
22.	343G	Lower Sindh
23.	10B	Upper Sindh
24.	12B	Upper Sindh
25.	2B	Upper Sindh
26.	39A3	Upper Sindh
27.	783	Lower Sindh
28.	31 3	Lower Sindh
29.	3B	Upper Sindh
30.	16G4	Lower Sindh
31.	351	Upper Sindh
32.	39 3A	Upper Sindh
33.	363	Upper Sindh
34	16G3	Lower Sindh

Comparing the amplification of sequences from 16S rDNA, IS1113, and 16S-23S r DNA intergenic regions of *Xoo*, Pramod *et al*., (2015) proposed that 16S rDNA-based primers are not specific enough, whereas, for IS1113 region-based primers, there is a possibility of false negatives. On the other hand, XOR primers for 16S-23S r DNA intergenic region are reliable, specific, accurate, and sensitive. Also, recently, Cho *et al*., (2011) developed a method of *Xoo* identification using RT-Bio PCR which amplifies rhs family gene, while Song *et al*., (2014) used AFLP markers for distinguishing the K3a race of *Xoo*.

However, these approaches are highly expensive, involve costly resources, and are laborious than simple and rapid procedure employing XOR primer pair as proposed in this study. Our results agreed with previous reports of Adachi & Takashi (2000) and Lang *et al*., (2010), who have used these primers to successfully identifying the *Xoo* pathogen in rice samples from other countries.

Being the second-largest rice-growing province in the country, Sindh cultivates this crop on an area of 0.54 M ha (Sindh Board of Investment, 2018). BLB has already developed a history in the province, and as high as 90% of prevalence has been observed when suitable agro-climatic conditions existed (Rafi *et al*., 2013). Akhtar & Zakria (2003) reported the presence of bacterial leaf blight in Sindh province in 2002. Mannan *et al*., (2009) and Jabeen *et al*., (2012) also conducted a survey of BLB in Pakistan and reported the presence of the disease in some regions of Sindh as well. Moreover, Bhutto *et al*., (2018) have also recently described the presence of BLB in this area. However, the identification of *Xoo* in all such studies was based on disease symptoms and culture-based or biochemical approaches. To reliably advance disease identification for designing control strategies and investigate the pathogenic diversity existing in this region, this study carries substantial importance ascertaining the pathogen through molecular approaches.

The RAPD based genetic analysis of the isolates may serve as a platform study for refined characterization of new isolates for which racial classification has not been determined in Sindh province. It is practically laborious to handle many pathogenic isolates. Therefore, grouping into representative clades – based on molecular differences – may find applications in easing such studies. Moreover, these investigations may also help determine the population structures of *Xoo* and ultimately relating the pathogenicity of the isolates to genetic factors involved in the long run.

The identified BLB isolates in this study were subjected to molecular characterization. It was observed that the RAPD primers yielded multiple DNA amplification products for these isolates (Fig. 2). The sizes of PCR products were seen to vary in the range of 0.1 to 3.5 kb. Overall, 168 polymorphic bands were observed. The PCR bands were scored for calculating the similarity index values. This scoring matrix was then used to investigate the genetic relatedness and diversity of the *Xoo* isolates using the Un-weighted Pair Group method (UPGMA). The dendrogram was constructed as shown in Fig. 3.

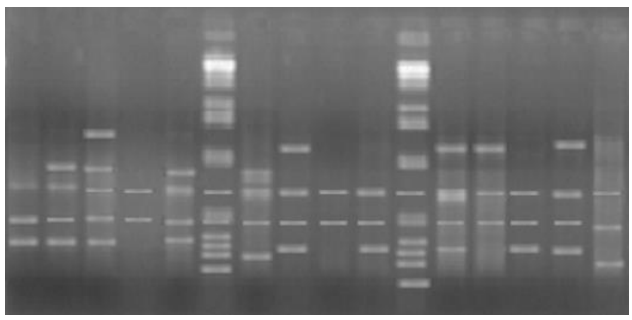

Fig. 2. DNA fingerprinting showing polymorphic bands. DNA fingerprinting of the isolates was done using RAPD primers. RAPD primers yielded multiple DNA amplification products, the sizes of which were observed to vary. Various polymorphic bands were observed as a result of RAPD-PCR.

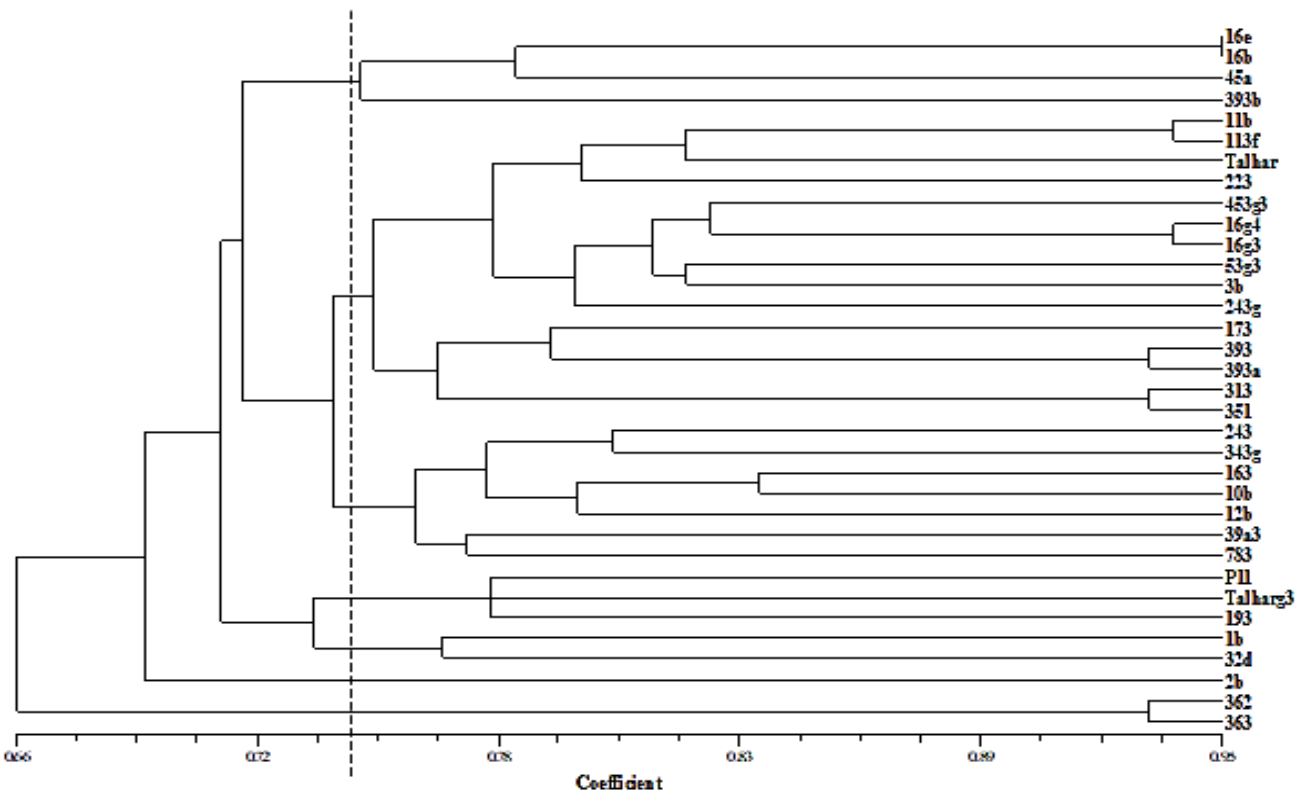

Fig. 3. Dendrogram showing grouping of isolates into clusters. At a cut off value of 0.75 the isolates grouped into six main clusters, while one isolate loosely grouped with them. The isolate 2b was observed to appear distantly from all other isolates in the study. Cluster II comprised of highest number of isolates i.e., 15. While I, III, IV, V, and VI comprised of 4, 7, 5, and 2 isolates respectively.

The isolates were grouped into a total of seven clusters (Table 3). The relationship between clusters and isolates' locations appeared to be complex, as observed in earlier studies (Hu *et al.,* 2007; Lore *et al.,* 2011). The results observed in this investigation agreed with the reports proposing that a high degree of genetic variability exists among the *Xoo* isolates. Hence, isolates from different regions can cluster together or vice versa (Gautam *et al*., 2015). Several other reports have also suggested a similar weak association of pathogens' location and genetic diversity (Adhikari *et al.,* 1995; Shanti *et al.,* 2007 Li *et al*., 2009; Pandey *et al*., 2014).

Conventional approaches of BLB identification are neither simple nor accurate. Moreover, such methods are also not sufficiently reliable when the pathogen populations occur in low numbers (Kong *et al.*, 2002). Therefore, using molecular-based approaches can provide higher certainty in identifying the disease at initial stages of invasion, isolate the suspected *Xoo* communities for further characterization, and apply correct disease control measures within the proper timeframe. There is a strong need to carry-out pathogen characterization, investigate its diversity, and ultimately develop resistant genotypes. Simple molecular approaches, as reported in this study, can predominantly facilitate such investigations.

Table 3. Distribution of isolates into different clusters.

Clusters	Strains in cluster
I	16e, 16b, 45a, 393b
II	11b, 113f, Talhar, 223, 453g3, 16g4, 16g3, 543, 3b, 243g, 173, 393, 393a, 313, 351
III	243, 343g, 163, 10b, 12b, 39a3,783
IV	P11, Talharg, 193
V	1b, 32d
VI	2b
VII	362, 363

Conclusion

It was seen that XOR primers could specifically and sensitively detect *Xoo*. We have identified 34 bacterial isolates from the cultures which were isolated from infected plants collected from several areas of Sindh province. The molecular characterization based on RAPD primers classified the isolates into seven clusters. The identified cultures were later used to conduct screening of the rice genotypes against bacterial leaf blight using artificial inoculation and were seen to have pathogenic attributes. Such studies can help in finding moderately resistant genotypes against this dilemma of the rice crop.

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