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Development of a Combined Molecular Diagnostic and DNA Fingerprinting Technique for Rice Bacteria Pathogens in Africa

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Abstract: A combined molecular diagnostic and DNA fingerprinting PCR technique for *Xanthomonas oryzae* pv. *oryzae* (Xoo), *Xanthomonas oryzae* pv. *oryzicola* (Xoc), *Pseudomonas fuscovaginae* (Pf) and *Pseudomonas syringae* pv. *syringae* (Pss) pathogens from rice has been developed in Africa by this study, using four primer pairs designed from Xoo (NC_007705.1), Xoc (NZ_AAQN01000001.1), Pf (AB021381.1) and Pss (NC_007005.1) complete genome sequence. Molecular PCR diagnostic showed that the presence of at least a band indicates positive detection of a bacterial pathogen and absence of a band indicates negative for no bacterial pathogen detected, while in the same PCR assay the presence of one or more band at different position revealed the DNA fingerprint of a bacterial pathogen. Out of 95 bacterial cultured isolates analyzed, 84 contained Xoo, 50 contained Xoc, 19 contained Pf and 16 contained Pss pathogens. DNA fingerprinting of the 84 Xoo pathogens revealed seven Xoo genotypes, four Xoc genotypes were identified from 50 Xoc pathogens and 19 Pf pathogens produced three Pf genotypes while 16 Pss pathogens formed three Pss genotypes. Development of a reliable molecular technique for Xoo, Xoc, Pf and Pss identification and differentiation is a prerequisite into understanding the genetics of Xoo, Xoc, Pf and Pss population structure in Africa and deployment of durable resistance cultivars.

Key words: *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas oryzae* pv. *oryzicola*, *Pseudomonas fuscovaginae*, *Pseudomonas syringae* pv. *syringae*, molecular diagnostic, DNA fingerprinting, PCR technique, genotype, Africa

INTRODUCTION

Rice (*Oryza sativa* L.) is the primary food grain consumed by almost half of the world's population, making it the most important food crop currently produced (Cottyn *et al*., 2001). It provides 27% of energy and 20% of proteins in developing countries (Kennedy *et al*., 2002). More than 70 diseases caused by fungi, bacteria, viruses and nematodes have been recorded on rice. *Xanthomonas oryzae* pv. *oryzae* (Xoo), *Xanthomonas oryzae* pv. *oryzicola* (Xoc), *Pseudomonas fuscovaginae* (Pf) and *Pseudomonas syringae* pv. *syringae* (Pss) are bacterial pathogens capable of causing disease on different rice cultivars. Bacterial Leaf Blight

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(BLB) caused by Xoo is a very destructive disease in Asia and Africa (Adhikari *et al*., 1995; Sere *et al*., 2005). In a more recent survey in West Africa, bacterial blight incidence ranged from 70-85%, indicating a wide spread of the disease in 14 farmers' fields (Sere *et al*., 2005). Research studies have also revealed that BLB is an important rice disease in irrigated rice ecosystems in West Africa, mainly in Sahelian and soudano-sahelian countries. Bacterial Leaf Streak (BLS) caused by Xoc is restricted largely to tropical and subtropical Asia, including southern China, Thailand, Malaysia, India, Viet Nam, the Philippines and Indonesia, but it also affects rice-growing regions of northern Australia and Africa (Ou, 1985; Awoderu *et al*., 1991; Sigee, 1993). Although, documentation does not exist for many areas in which BLS is present, available reports suggested that yield losses due to this disease typically up to 20% depending on the rice variety and climatic conditions (Ou, 1985). Pf is a fluorescent pseudomonad that causes sheath brown rot of rice in temperate regions (Miyajima *et al*., 1983; Tanii *et al*., 1976). This pathogen has been described in countries (Japan, Nepal, Madacascar, Burundi and Colombia) where cold temperatures represent a limiting factor for rice cultivation (Duveiller *et al*., 1988; Rott *et al*., 1989; Zeigler and Alvarez, 1990). The most important damage associated with the bacterium is grain sterility and yield loss up to 58% has been reported by Jauhet *et al*. (1996). In Asia, America, Europe and Africa (mainly Burundi, Zaire, Rwanda and Madagascar), Pss has been reported as the cause of rice bacterial sheath brown rot and 75% of the panicle can be affected (Zeigler and Alvarez, 1990).

Early field detection and identification of Xoo, Xoc, Pf and Pss pathogens will prevent further spread of the pathogens and allow early disease control. Several indirect and direct methods aid in identification of the pathogen. The phage technique involves the incubation of seed samples with a species phage. The increase in phage number by plaque count would detect the presence of bacterium. Serological methods also serve as sensitive tools for detection of the pathogen because the outer membrane of gram negative bacteria has a variety of potentially antigenic molecules which may be detected by monoclonal antibodies. Gnanamanickam *et al*. (1999) demonstrated the detection of Xoo in rice seeds inoculated with the pathogen using Enzyme-Linked Immuno Sorbent Assay (ELISA), whereby the bacterial colonies that reacted positively to monoclonal antibodies specific to the bacterium were examined by direct immunofluorescence (IF). Though ELISA and IF provide conclusive evidence for the presence of the pathogen, neither technique is sensitive enough to detect low numbers of the pathogen, which necessitates enrichment (Sakthivel *et al*., 2001). Molecular probes, on the other hand, facilitate detection of even low numbers of the pathogen through Polymerase Chain Reaction (PCR) analyses (Sakthivel *et al*., 2001). Random amplified polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphism (RFLP) markers have been used for DNA fingerprinting analyses of bacterial pathogens (Sakthivel *et al*., 2001; Onasanya *et al*., 2003).

Therefore, the present study aimed at developing a combined molecular diagnostic and DNA fingerprinting PCR technique for rice bacteria pathogens in Africa in order to reveal the distribution, movement and epidemiology of Xoo, Xoc, Pf and Pss rice pathogens in Africa.

MATERIALS AND METHODS

Research location: Bacterial isolate propagation and primer design were carried out at Plant Pathology Unit, Africa Rice Center (AfricaRice), Cotonou, Benin Republic, while the molecular PCR analysis was carried out at Central Biotechnology Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. This study was conducted between February and May 2009.

Bacterial isolates and propagation: Ninety five bacterial isolates (Table 1, 2) used in this study were obtained from Plant Pathology Unit, Africa Rice Center (AfricaRice), Cotonou, Benin Republic, where they were isolated from different diseased plants in West and East Africa. Two bacterial isolates from Asia were obtained from International Rice Research Institute (IRRI) in the Philippines and used as control isolates during the study. The bacterial isolates were first propagated using a modified procedure developed by Kado and Keskett (1970). Nutrient broth (75 mL; pH 7.5) was prepared inside a 250 mL conical flask. Each Xoo isolate (200 μL) from storage was transferred into 75 mL of nutrient broth and kept under constant shaking at 30°C for 24 h for bacterial growth. The bacterial cells were removed by centrifugation, washed with 0.1 mM Tris-EDTA (pH 8.0) and kept at -20°C for DNA extraction.

Genomic DNA extraction: DNA extraction was according to Roeder and Broda (1987) and Thottappilly *et al*. (1999) with some modification: 0.3 g of washed bacterial cell was suspended in 200 μL of Cetyl trimethylammonium bromide (CTAB) buffer (50 mM Tris, pH 8.0; 0.7 mM NaCl; 10 mM EDTA; 2% hexadecyltrimethylammonium bromide; 0.1% 2-mercaptoethanol), followed by 100 μL of 20% sodium

Table 1: List of rice bacterial isolates used for the study

S/No.	Bacterial isolate	Host plant	Locality	Country
1	NX229	*Leersia hexandra*	Bonféba	Niger
2	NX80	Cultivated rice	Daibéri	Niger
3	NX246	*Leersia hexandra*	Daïkaina	Niger
4	NX242	*O. longistaminata*	Diambala	Niger
5	NX243	*Leersia hexandra*	Diambala	Niger
6	NX289	Cultivated rice	Diffa	Niger
7	NX292	Cultivated rice	Diffa	Niger
8	NX226	*O. longistaminata*	Diomana	Niger
9	NX225	*Eulesine indica*	Diomana	Niger
10	NX280	*O. longistaminata*	Gaya	Niger
11	NX284	*Dactyloctenium aegyptium*	Gaya	Niger
12	NX275	Cultivated rice	Karégorou	Niger
13	NX273	*Leersia hexandra*	Karégorou	Niger
14	NX276	*Panicum repens*	Karégorou	Niger
15	NX257	*Echinocloa* sp.	Karma	Niger
16	NX258	*Leersia hexandra*	Karma	Niger
17	NX220	Cultivated rice	Kirkissoye	Niger
18	NX221	*Leersia hexandra*	Kirkissoye	Niger
19	NX247	*Kyllinga squamulata*	Kokkotroni	Niger
20	NX128	*Eulesine indica*	Kouloukalé	Niger
21	NX101	Cultivated rice	Liboré	Niger
22	NX223	*Leersia hexandra*	Liboré	Niger
23	NX255	*Cyperus esculentus*	Lossa	Niger
24	NX102	Cultivated rice	Namardé	Niger
25	NX268	*Eulesine indica*	Namardé	Niger
26	NX272	*Panicum repens*	Namardé	Niger
27	NX237	*Cyperus rotondus*	Namarigoungou	Niger
28	NX241	Cultivated rice	Namarigoungou	Niger
29	NX162	Cultivated rice	N'Douriga	Niger
30	NX111	Cultivated rice	Saadia	Niger
31	NX3	V06R1 Xa7	Saga	Niger
32	NX2	V06R1 Xa7	Saga	Niger
33	NX13	V06R2 Xa7	Saga	Niger
34	NX30	V20R3	Saga	Niger
35	NX263	*Penisetum pedicelatum*	Say I	Niger
36	NX266	*Panicum laetum*	Say I	Niger
37	NX267	*O. barthii*	Say I	Niger
38	NX118	Cultivated rice	Say II	Niger
39	NX259	*Echinocloa* sp.	Say II	Niger
40	NX261	*Panicum repens*	Say II	Niger
41	NX262	*Brachiaria* sp.	Say II	Niger
42	NX122	Cultivated rice	Sébéri	Niger
43	NX253	*Panicum repens*	Sona	Niger
44	NX250	Cultivated rice	Sona	Niger
45	NX252	*Cyperus* sp.	Sona	Niger
46	NX245	*Leersia hexandra*	Toula	Niger
47	NX138	Cultivated rice	Toula	Niger
48	NX214	Cultivated rice	Yelwani	Niger
49	*Xoo-1*	Cultivated rice	-	Philippines
50	*Xoo-2*	Cultivated rice	-	Philippines

dodecyl sulfate and incubated at 65°C for 20 min. The DNA was purified by two extractions with phenol: chloroform: isoamyl alcohol (24:25:1) and precipitated with -20°C absolute ethanol. After washing with 70% ethanol, the DNA was dried and resuspended in 200 μL of sterile distilled water; its concentration was measured using DU-65UV spectrophotometer (Beckman Instruments Inc., Fullerto CA, USA) at 260 nm. DNA quality was also checked by electrophoresis on a 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer (45 mM Tris-acetate, 1 mM EDTA, pH 8.0). Finally it was kept at -20°C freezer before Polymerase Chain Reaction (PCR) analysis was carried out.

PCR primers design: Using *Xanthomonas oryzae* pv. *oryzae* complete genome sequence (NCBI reference sequence: NC_007705.1), *Xanthomonas oryzae* pv. *oryzicola* whole genome shotgun sequence (NCBI reference sequence: NZ_AAQN01000001.1), *Pseudomonas fuscovaginae* genome sequence (NCBI reference sequence: AB021381.1) and *Pseudomonas syringae* pv. *syringae* complete genome sequence (NCBI reference sequence: NC_007005.1) obtained in previous studies (Anzai *et al*., 2000; Feil *et al*., 2005; Ochiai *et al*., 2005; Salzberg *et al*., 2008), four diagnostic and fingerprinting primers (Table 3) were designed using Primer Express™ software version 2·0 (Applied

Table 2: List of rice bacterial isolates used for the study

S/N	Bacterial isolate	Host plant	Locality	Country
51	MOZ292	Cultivated rice	IIAM res station at Chokwo	Mozambique
52	UGA621	*Pusa Basmati*	Tilda Scheme	Uganda
53	RWA111	Cultivated rice	Nyazatare Experimental Station	Rwanda
54	UGA211	WITA9	Tilda Scheme	Uganda
55	RWA2101	IR64	Nyazatare Experimental Station	Rwanda
56	UGA213	WITA9	Tilda Scheme	Uganda
57	RWA2103	IR64	Nyazatare Experimental Station	Rwanda
58	RWA551	Cultivated rice	Nyazatare Experimental Station	Rwanda
59	RWA112	Cultivated rice	Nyazatare Experimental Station	Rwanda
60	RWA4114	Cultivated rice	Nyazatare Experimental Station	Rwanda
61	UGA731	WITA9	Tilda Scheme	Uganda
62	RWA113	Cultivated rice	Nyazatare Experimental Station	Rwanda
63	RWA2102	IR64	Nyazatare Experimental Station	Rwanda
64	UGA214	WITA9	Tilda Scheme	Uganda
65	UGA212	WITA9	Tilda Scheme	Uganda
66	RWA554	Cultivated rice	Nyazatare Experimental Station	Rwanda
67	RWA2104	IR64	Nyazatare Experimental Station	Rwanda
68	RWA552	Cultivated rice	Nyazatare Experimental Station	Rwanda
69	RWA555	Cultivated rice	Nyazatare Experimental Station	Rwanda
70	MOZ291	Cultivated rice	IIAM station at Chokwo	Mozambique
71	UGA622	Pusa Basmati	Tilda Scheme	Uganda
72	MOZ293	Cultivated rice	IIAM station at Chokwo	Mozambique
73	RWA553	Cultivated rice	Nyazatare Experimental Station	Rwanda
74	UGA623	Pusa Basmati	Tilda Scheme	Uganda
75	UGA215	WITA9	Tilda Scheme	Uganda
76	RWA4112	Cultivated rice	Nyazatare Experimental Station	Rwanda
77	MOZ294	Cultivated rice	IIAM station at Chokwo	Mozambique
78	RWA4113	Cultivated rice	Nyazatare Experimental Station	Rwanda
79	RWA4111	Cultivated rice	Nyazatare Experimental Station	Rwanda
80	UGA216	WITA9	Tilda Scheme	Uganda
81	NXC40	Cultivated rice	Daikaina	Niger
82	NXC11	V17R2	Saga	Niger
83	NXC41	Cultivated rice	Karégorou	Niger
84	NXA26	V07R1	Bonféba	Niger
85	NXC1	V23R1	Saga	Niger
86	NXC43	Cultivated rice	Liboré	Niger
87	NXA3	V06R1	Saga	Niger
88	NXA35	Cultivated rice	Saga	Niger
89	NXC38	V11R3	Bonféba	Niger
90	NXC32	V24R3	Bonféba	Niger
91	NXA5	V07R1	Saga	Niger
92	NXC42	Cultivated rice	Liboré	Niger
93	NXA10	V06R2	Saga	Niger
94	NXC39	V11R3	Bonféba	Niger
95	NXC15	V19R2	Saga	Niger

Table 3: Identity of rice bacterial diagnostic and fingerprinting primers used for the study

S/N	Bacteria	Primer	Sequence (5'- 3')
1	*Xanthomonas oryzae* pv. *oryzae*	XooF1	TGGTAGTCCACGCCCTAAAC
		XooR1	CCTGAGCTACAGACCCGAAG
2	*Xanthomonas oryzae* pv. *oryzicola*	XocF2	CTGAAGAGGGTCGCAGAAAC
		XocR2	AACACCAGAGGTTCGTCCAC
3	*Pseudomonas fuscovaginae*	PfF3	AACGGGTGTACTTGGTCAGG
		PfR3	CTCCGAGATTACCCACAAGC
4	*Pseudomonas syringae* pv. *syringae*	PssF4	GGGCCACAAAAGTATCCTGA
		PssR4	AGCGAGCTTCCATACTCTGC

F: Forward direction; R: Reverse direction

Biosystems). The specificities of the primers were computer-tested, as was the theoretical PCR products. For direct use in PCR analysis, the designed primers sequences (Table 3) were synthesized at Eurogentec, B-4102 Seraing, Belgium.

Molecular diagnostic and DNA fingerprinting PCR analysis: The PCR analysis was according to Onasanya *et al.* (2003) with some modifications. Amplifications were performed in 25 μL reaction mixture consisting of bacterial genomic DNA, reaction buffer

(Promega), 100 mM each of dATP, dCTP, dGTP and dTTP, 0.2 mM each for forward and reverse primer, 2.5 mM $MgCl_2$ and 1U of Taq polymerase (Boehringer, Germany). The reaction mixture was overlaid with 50 μL of mineral oil to prevent evaporation. All the DNA of the 95 bacterial isolates (Table 1, 2) were analyzed. Amplification was performed in a thermowell microtitre plate (Costa Corporation) using Perkin Elmer programmable Thermal Controller model 9600. The cycling program was (1) 1 cycle of 94°C for 3 min; (2) 35 cycles of 94°C for 1 min for denaturation, 55°C for 1 min for annealing of primer and 72°C for 2 min for extension and (3) a final extension at 72°C for 7 min. Amplification products were maintained at 4°C until electrophoresis. The amplification products were resolved by electrophoresis in a 1.4% agarose gel using Tris-Acetate-EDTA (TAE) buffer (45 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 100 V for 2 h. A 1 kb ladder (Life Technologies, Gaithersburg, MD, USA) was included as molecular size marker. Gels were visualized by staining with ethidium bromide solution (0.5 μg mL^{-1}) and banding patterns were photographed over UV light using UVP-computerized gel photo documentation system.

Diagnostic band scoring and cluster analysis: Positions of unequivocally scorable amplified DNA bands were transformed into a binary character matrix (1 for the presence and 0 for the absence of a band at a particular position). Pairwise distance matrices were compiled by the Numerical Taxonomy System (NTSYS) 2.0 software (Rohlf, 2000) using the Jaccard coefficient of similarity (Ivchenko and Honov, 1998). Phylogenetic tree was created by Unweighted Pair-Group Method Arithmetic (UPGMA) cluster analysis (Sneath and Sokal, 1973; Jakó *et al*., 2009).

RESULTS

A combined molecular diagnostic and DNA fingerprinting PCR technique for *Xanthomonas oryzae* pv. oryzae (Xoo), *Xanthomonas oryzae* pv. *oryzicola* (Xoc), *Pseudomonas fuscovaginae* (Pf) and *Pseudomonas syringae* pv. *syringae* (Pss) rice pathogens has been developed in Africa by this study.

Out of 95 bacterial isolates analyzed for Xoo diagnosis using the Xoo specific diagnostic primer pair (XooF1 and XooR1), 84 of the bacterial isolates contained Xoo pathogen from which 24 were confirmed as pure Xoo pathogen and no mixture with Xoc, Pf or Pss pathogens (Table 4, 5 and Fig. 1, 2). Besides, the DNA fingerprinting of the diagnosed 84 Xoo pathogens have been revealed by the same primer pair (XooF1 and XooR1) in the same PCR assay used for the diagnosis (Fig. 1, 2). DNA fingerprinting of 84 Xoo pathogens using primer pair (XooF1 and XooR1) produced 216 fragments from which 162 (75%) were polymorphic with genotype index of 2.6 leading to the identification of seven Xoo genotypes, which were *Xoo-1*, *Xoo-2*, *Xoo-3*, *Xoo-4*, *Xoo-5*, *Xoo-6* and *Xoo-7* respectively (Table 6, Fig. 3). *Xoo-1*

Table 4: Molecular diagnosis for *Xanthomonas oryzae* pv. *oryzae* (Xoo), *Xanthomonas oryzae* pv. *oryzicola* (Xoc), *Pseudomonas fuscovaginae* (Pf) and *Pseudomonas syringae* pv. *syringae* (Pss) rice pathogen by Polymerase Chain Reaction (PCR) assay

			Molecular PCR diagnostic assay			
S/N	Bacterial isolate	Country	Xoo	Xoc	Pf	Pss
1	NX229	Niger	+	-	+	+
2	NX80	Niger	+	-	+	+
3	NX246	Niger	+P	-	-	-
4	NX242	Niger	+P	-	-	-
5	NX243	Niger	+P	-	-	-
6	NX289	Niger	+P	-	-	-
7	NX292	Niger	+	+	+	-
8	NX226	Niger	+P	-	-	-
9	NX225	Niger	+P	-	-	-
10	NX280	Niger	+P	-	-	-
11	NX284	Niger	+	+	+	-
12	NX275	Niger	+P	-	-	-
13	NX273	Niger	+P	-	-	-
14	NX276	Niger	+P	-	-	-
15	NX257	Niger	+	-	+	-
16	NX258	Niger	+	+	-	-
17	NX220	Niger	+P	-	-	-
18	NX221	Niger	+P	-	-	-
19	NX247	Niger	+	+	-	-
20	NX128	Niger	+P	-	-	-
21	NX101	Niger	+P	-	-	-
22	NX223	Niger	+	+	-	+
23	NX255	Niger	+	-	+	-
24	NX102	Niger	+	+	-	-
25	NX268	Niger	+	+	+	-
26	NX272	Niger	+P	-	-	-
27	NX237	Niger	+P	-	-	-
28	NX241	Niger	+	-	+	+
29	NX162	Niger	+	+	-	+
30	NX111	Niger	+	+	-	-
31	NX3	Niger	+	+	+	-
32	NX2	Niger	+	+	-	-
33	NX13	Niger	+	+	-	-
34	NX30	Niger	+	+	-	-
35	NX263	Niger	+P	-	-	-
36	NX266	Niger	+P	-	-	-
37	NX267	Niger	+P	-	-	-
38	NX118	Niger	+	-	+	-
39	NX259	Niger	+P	-	-	-
40	NX261	Niger	+P	-	-	-
41	NX262	Niger	+	+	-	-
42	NX122	Niger	+	+	-	-
43	NX253	Niger	+	-	-	+
44	NX250	Niger	+	+	-	-
45	NX252	Niger	+	+	+	-
46	NX245	Niger	+P	-	-	-
47	NX138	Niger	+	+	-	-
48	NX214	Niger	+	-	-	+
49	*Xoo-1*	Philippines	+	-	+	+
50	*Xoo-2*	Philippines	+	-	+	+

-: Absence, +: Presence, P: Pure isolate

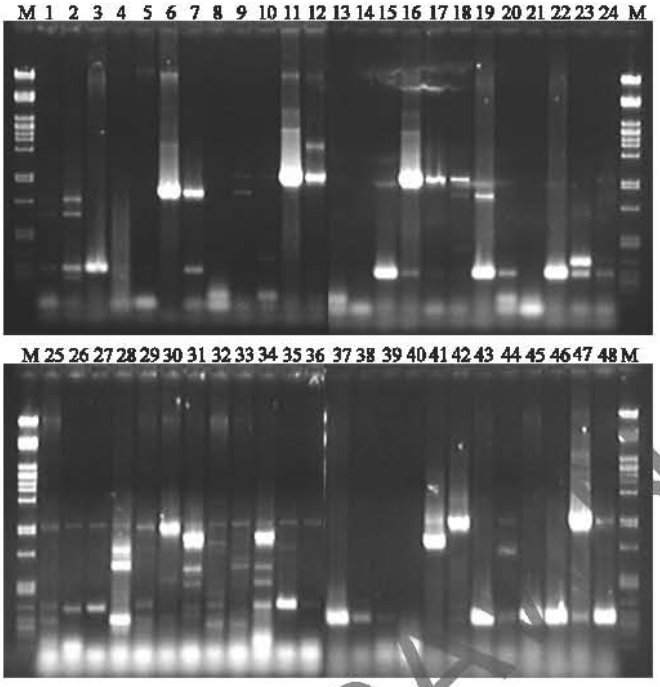

Fig. 1: *Xanthomonas oryzae* pv. *oryzae* (Xoo) diagnosis and DNA fingerprint as revealed PCR analysis using XooF1 and XooR1 Xoo specific primers. M= Molecular size marker. Molecular PCR diagnostic showed that the presence of at least a band indicates positive (+) detection of Xoo pathogen and absence of a band indicates no Xoo pathogen detected. In the same PCR assay the presence of one or more band at different position revealed the DNA fingerprint of Xoo pathogen

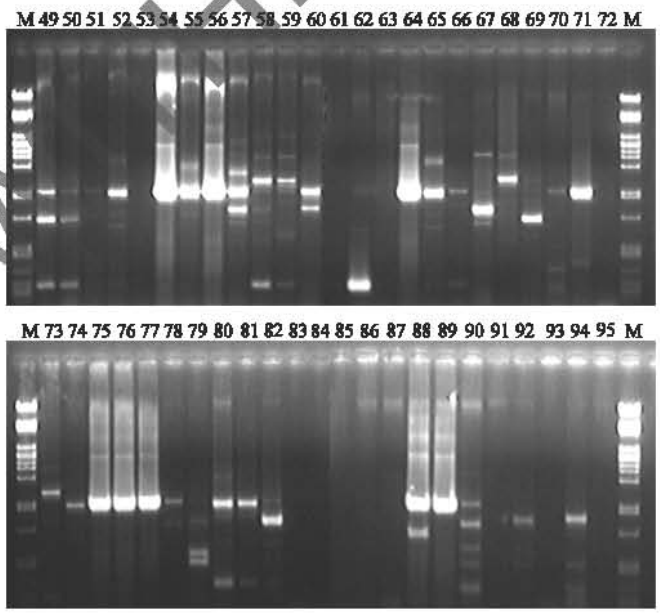

Fig. 2: *Xanthomonas oryzae* pv. *oryzae* (Xoo) diagnosis and DNA fingerprint as revealed PCR analysis using XooF1 and XooR1 Xoo specific primers. M = Molecular size marker. Molecular PCR diagnostic showed that the presence of at least a band indicates positive (+) detection of Xoo pathogen and absence of a band indicates no Xoo pathogen detected. In the same PCR assay the presence of one or more band at different position revealed the DNA fingerprint of Xoo pathogen

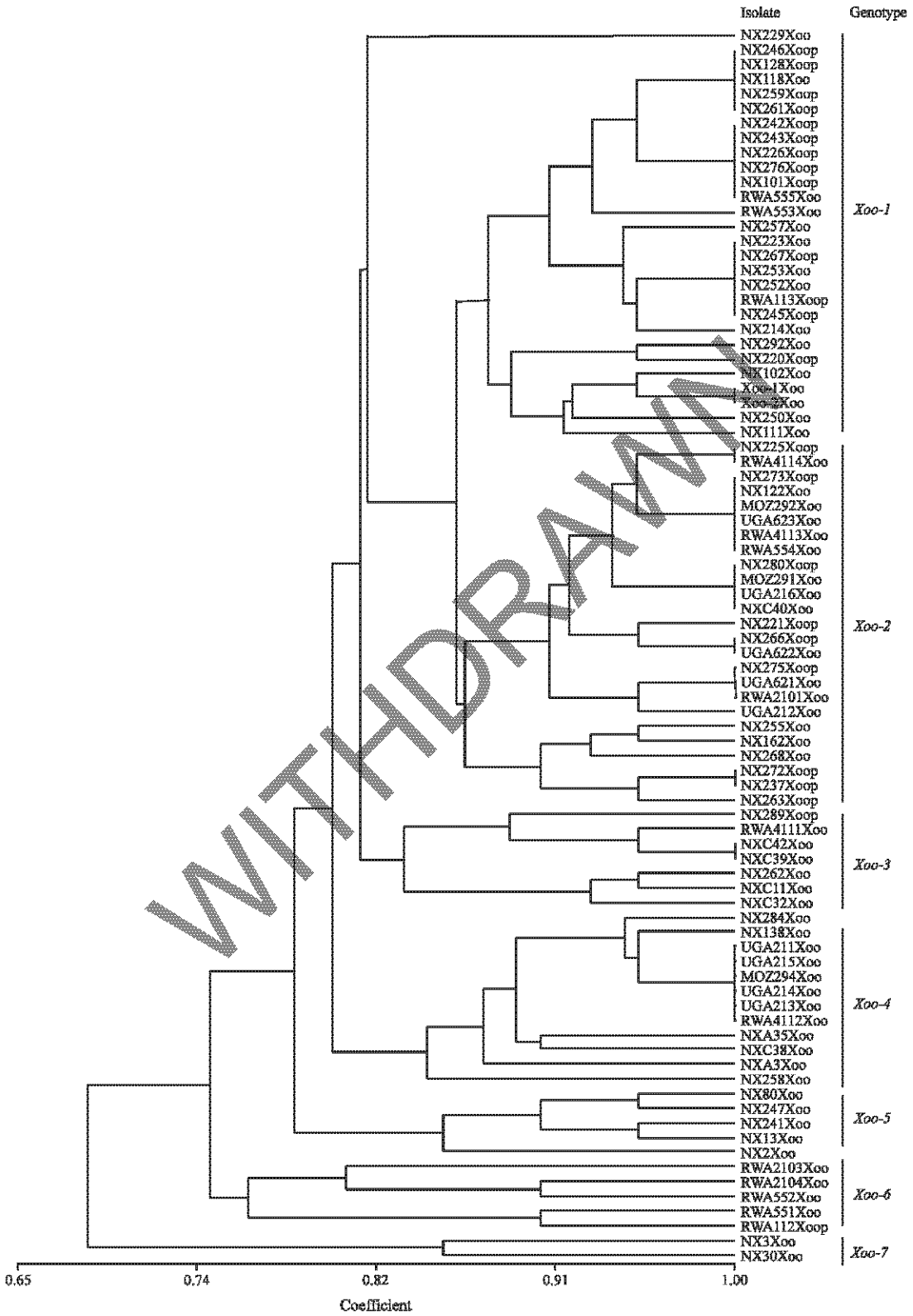

Fig. 3: Genetic diversity among 84 *Xanthomonas oryzae* pv. *oryzae* (Xoo) isolates as revealed PCR analysis using XooF1 and XooR1 Xoo specific primers

Table 5: Molecular diagnosis for *Xanthomonas oryzae* pv. *oryzae* (Xoo), *Xanthomonas oryzae* pv. *oryzicola* (Xoc), *Pseudomonas fuscovaginae* (Pf) and *Pseudomonas syringae* pv. *syringae* (Pss) rice pathogen by Polymerase Chain Reaction (PCR) assay

			Molecular PCR diagnostic assay			
S/N	Bacterial isolate	Country	Xoo	Xoc	Pf	Pss
51	MOZ292	Mozambique	+	+	-	-
52	UGA621	Uganda	+	+	-	-
53	RWA111	Rwanda	-	-	-	-
54	UGA211	Uganda	+	+	-	-
55	RWA2101	Rwanda	+	+	-	-
56	UGA213	Uganda	+	+	-	-
57	RWA2103	Rwanda	+	+	-	+
58	RWA551	Rwanda	+	+	-	+
59	RWA112	Rwanda	+P	-	-	-
60	RWA4114	Rwanda	+	+	-	-
61	UGA731	Uganda	-	-	+	+
62	RWA113	Rwanda	+P	-	-	-
63	RWA2102	Rwanda	-	+	-	+
64	UGA214	Uganda	+	+	-	-
65	UGA212	Uganda	+	+	-	-
66	RWA554	Rwanda	+	-	+	-
67	RWA2104	Rwanda	+	+	-	+
68	RWA552	Rwanda	+	-	+	-
69	RWA555	Rwanda	+	+	-	+
70	MOZ291	Mozambique	+	+	-	-
71	UGA622	Uganda	+	+	-	-
72	MOZ293	Mozambique	-	-	-	-
73	RWA553	Rwanda	+	+	-	+
74	UGA623	Uganda	+	+	-	-
75	UGA215	Uganda	+	+	-	-
76	RWA4112	Rwanda	+	+	-	-
77	MOZ294	Mozambique	+	+	-	-
78	RWA4113	Rwanda	+	+	+	-
79	RWA4111	Rwanda	+	+	-	-
80	UGA216	Uganda	+	+	-	-
81	NXC40	Niger	+	+	-	-
82	NXC11	Niger	+	+	-	-
83	NXC41	Niger	-	-	-	-
84	NXA26	Niger	-	-	-	-
85	NXC1	Niger	-	+	-	-
86	NXC43	Niger	+	-	-	-
87	NXA3	Niger	+	+	-	-
88	NXA35	Niger	+	+	+	-
89	NXC38	Niger	+	+	-	-
90	NXC32	Niger	+	+	+	-
91	NXA5	Niger	-	-	-	-
92	NXC42	Niger	+	+	-	-
93	NXA10	Niger	-	-	-	-
94	NXC39	Niger	+	+	-	-
95	NXC15	Niger	-	+P	-	-

-: Absence, +: Presence, P: Pure isolate

genotype consists of 28 Xoo isolates from Niger, Rwanda and Philippines while *Xoo-2* genotype consists of 25 Xoo isolates from Niger, Rwanda, Mozambique and Uganda, *Xoo-3* genotype consists of 7 Xoo isolates from Niger and Rwanda while *Xoo-4* genotype consists of 12 Xoo isolates from Niger, Rwanda, Mozambique and Uganda, *Xoo-5* and *Xoo-7* genotypes consist of 7 Xoo isolates from Niger and *Xoo-6* genotype consists of 5 Xoo isolates from Rwanda (Table 7, Fig. 3). However, Xoo genotype occurrence and distribution among countries was between 2.4 to 33.3% with *Xoo-1* genotype having the highest of 33.3% and *Xoo-7* genotype having the lowest of 2.4% (Table 7).

However, the same 95 bacterial isolates were analyzed for Xoc diagnosis using the Xoc specific diagnostic primer pair (XocF2 and XocR2) and 50 of the bacterial isolates contained Xoc pathogen from which only one was confirmed pure as Xoc pathogen and no mixture with Xoo, Pf or Pss pathogens (Table 4, 5 and Fig. 4, 5). Besides, the DNA fingerprinting of the diagnosed 50 *Xoc* pathogens have been revealed by the same primer pair (XocF2 and XocR2) in the same PCR assay used for the diagnosis (Fig. 4, 5). DNA fingerprinting of 50 Xoc pathogens using primer pair (XocF2 and XocR2) produced 69 fragments from which 33 (47.9%) were polymorphic with genotype index of 1.4 leading to the identification of four Xoc genotypes, which were *Xoc-1*, *Xoc-2*, *Xoc-3* and *Xoc-4* respectively (Table 6, Fig. 6). *Xoc-1*, *Xoc-2* and *Xoc-4* genotypes consist of 9, 4 and 6 Xoc isolates respectively from Niger and Rwanda, while *Xoc-3* genotype consists of 31 Xoc isolates from Niger, Rwanda, Mozambique and Uganda (Table 7, Fig. 6). Besides, Xoc genotype occurrence and distribution among countries was between 8 to 60% with *Xoc-3* genotype having the highest of 62% and *Xoc-2* genotype having the lowest of 8% (Table 7).

The same 95 bacterial isolates were also analyzed for Pf diagnosis using the Pf specific diagnostic primer pair (PfF3 and PfR3) and 19 of the bacterial isolates contained Pf pathogen (Table 4, 5 and Fig. 7, 8). However, the DNA fingerprinting of the diagnosed 19 Pf pathogens

Table 6: Genetic polymorphism and genotype index characteristics of the four primers

S/N	Primer	Bacterial pathogen	No of isolate	Total No of fragments amplified	Total No of polymorphic bands	Polymorphism (%)	Genotype index
1	XooF1 XooR1	Xoo	84	216	162	75	2.6
2	XocF2 XocR2	Xoc	50	69	33	47.9	1.4
3	PfF3 PfR3	Pf	19	23	23	100	1.2
4	PssF4 PssR4	Pss	16	27	27	100	1.7

Xoo: *Xanthomonas oryzae* pv. *oryzae*, Xoc: *Xanthomonas oryzae* pv.*oryzicola*, Pf: *Pseudomonas fuscovaginae*, Pss: *Pseudomonas syringae* pv. *syringae*

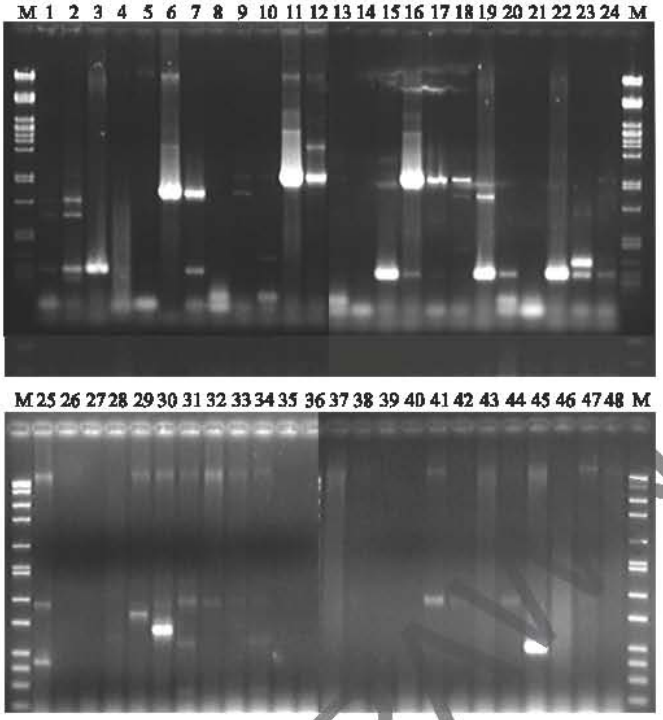

Fig. 4: *Xanthomonas oryzae* pv. *oryzicola* (Xoo) diagnosis and DNA fingerprint as revealed PCR analysis using XocF2 and XocR2 Xoc specific primers. M = Molecular size marker. Molecular PCR diagnostic showed that the presence of at least a band indicates positive (+) detection of Xoc pathogen and absence of a band indicates no Xoc pathogen detected. In the same PCR assay the presence of one or more band at different position revealed the DNA fingerprint of Xoc pathogen

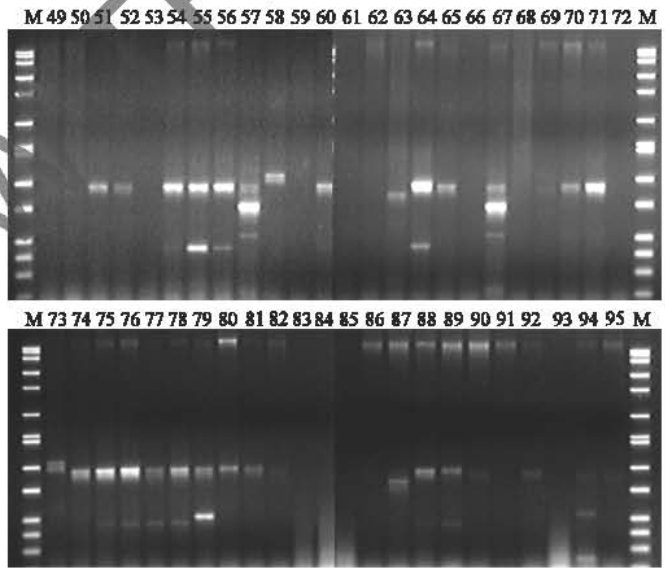

Fig. 5: *Xanthomonas oryzae* pv. *oryzicola* (Xoc) diagnosis and DNA fingerprint as revealed PCR analysis using XocF2 and XocR2 Xoc specific primers. M = Molecular size marker. Molecular PCR diagnostic showed that the presence of at least a band indicates positive (+) detection of Xoc pathogen and absence of a band indicates no Xoc pathogen detected. In the same PCR assay the presence of one or more band at different position revealed the DNA fingerprint of Xoc pathogen

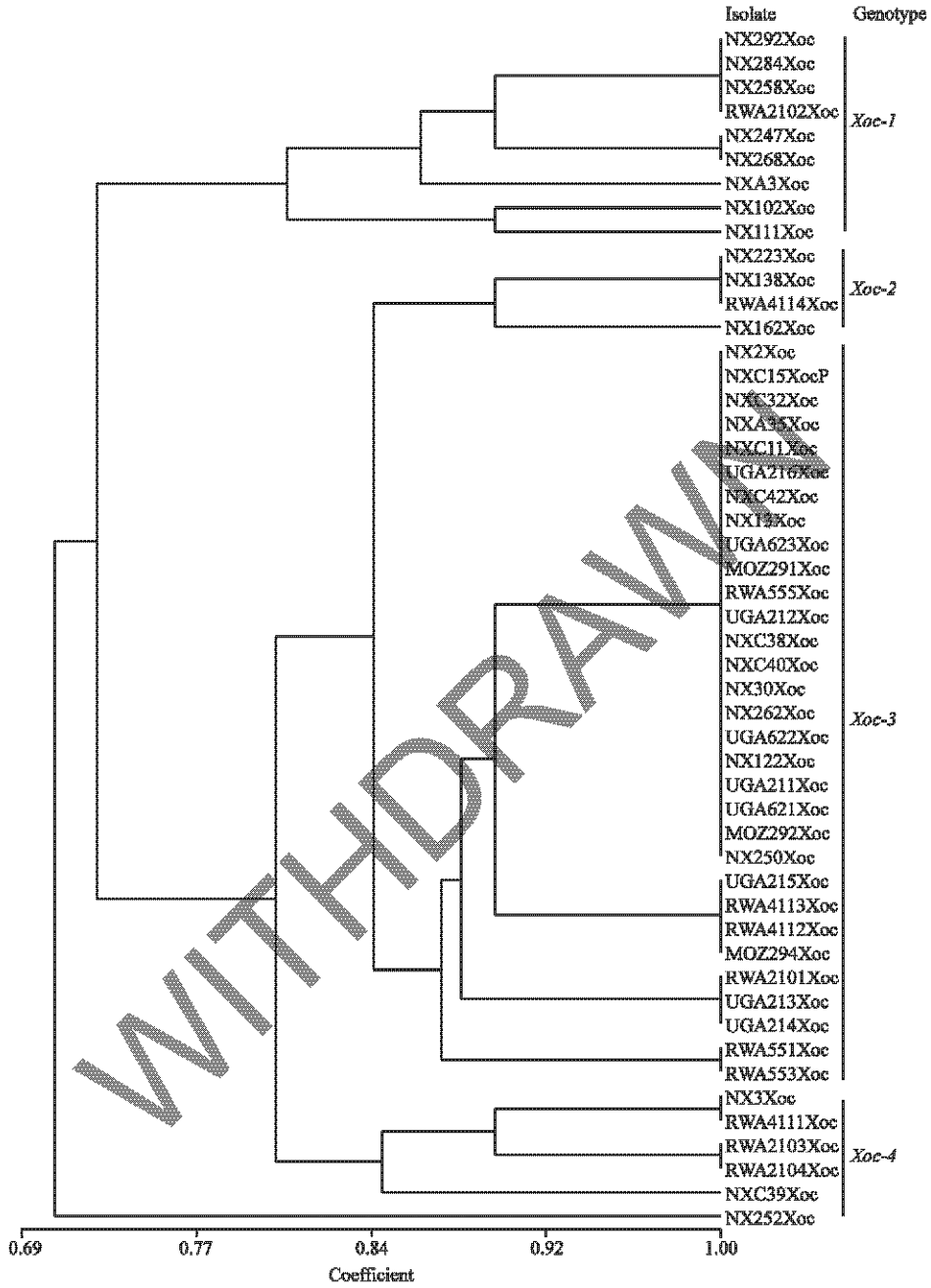

Fig. 6: Genetic diversity among 50 *Xanthomonas oryzae* pv. *oryzicola* (Xoc) isolates as revealed PCR analysis using XocF2 and XocR2 Xoc specific primers

have been revealed by the same primer pair (PfF3 and PfR3) in the same PCR assay used for the diagnosis (Fig. 7, 8). DNA fingerprinting of 19 Pf pathogens using primer pair (PfF3 and PfR3) produced 23 fragments and all were polymorphic with genotype index of 1.2 leading to the identification of three Pf genotypes, which were *Pf-1*, *Pf-2* and *Pf-3* respectively (Table 6, Fig. 9). *Pf-1* genotype consists of 15 Pf isolates from Niger, Rwanda, Uganda and Philippines while *Pf-2* genotype consists of 2 Pf isolates from Niger and *Pf-3* genotypes consists of 2 Pf isolates from Rwanda (Table 7, Fig. 9). Besides, Pf genotype occurrence and distribution among countries was between 10.5 to 78.9% with *Pf-1* genotype having the highest of 78.9% (Table 7).

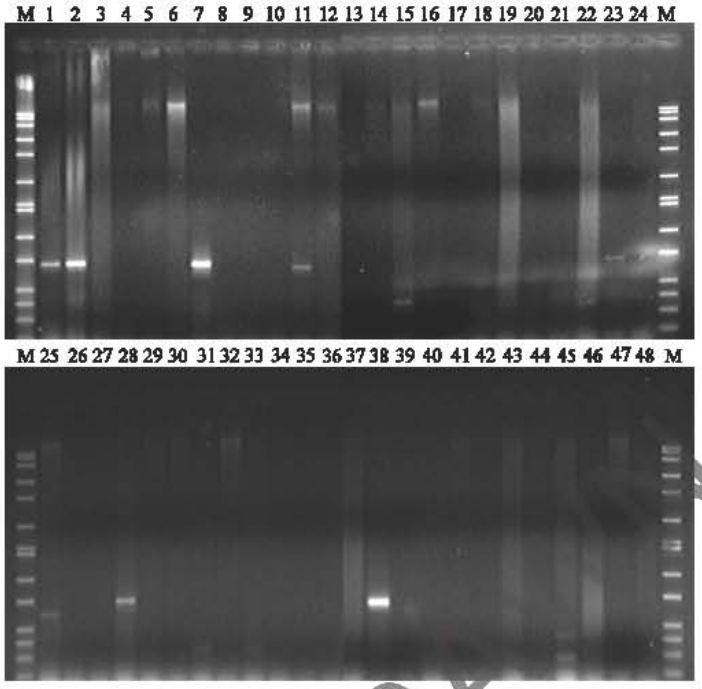

Fig. 7: *Pseudomonas fuscovaginae* (Pf) diagnosis and DNA fingerprint as revealed PCR analysis using PfF3 and PfR3 Pf specific primers. M = Molecular size marker. Molecular PCR diagnostic showed that the presence of at least a band indicates positive (+) detection of Pf pathogen and absence of a band indicates no Pf pathogen detected. In the same PCR assay the presence of one or more band at different position revealed the DNA fingerprint of Pf pathogen

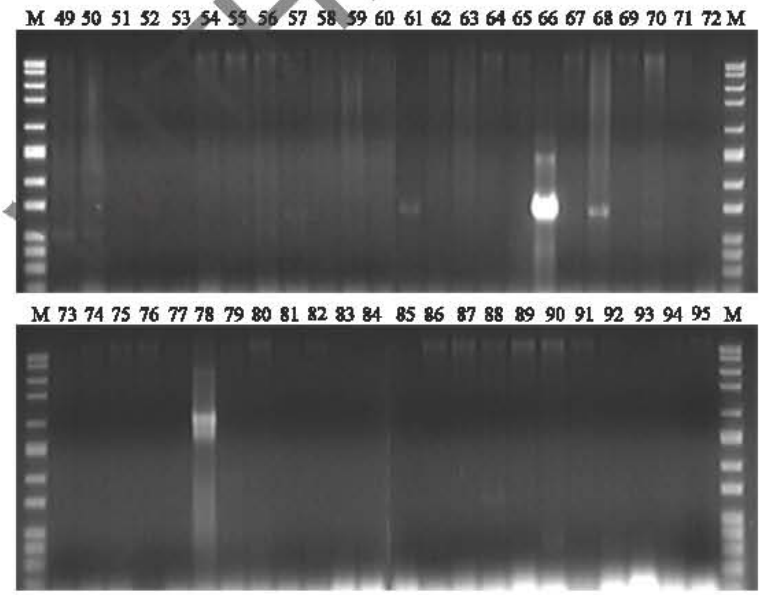

Fig. 8: *Pseudomonas fuscovaginae* (Pf) diagnosis and DNA fingerprint as revealed PCR analysis using PfF3 and PfR3 Pf specific primers. M = Molecular size marker. Molecular PCR diagnostic showed that the presence of at least a band indicates positive (+) detection of Pf pathogen and absence of a band indicates no Pf pathogen detected. In the same PCR assay the presence of one or more band at different position revealed the DNA fingerprint of Pf pathogen

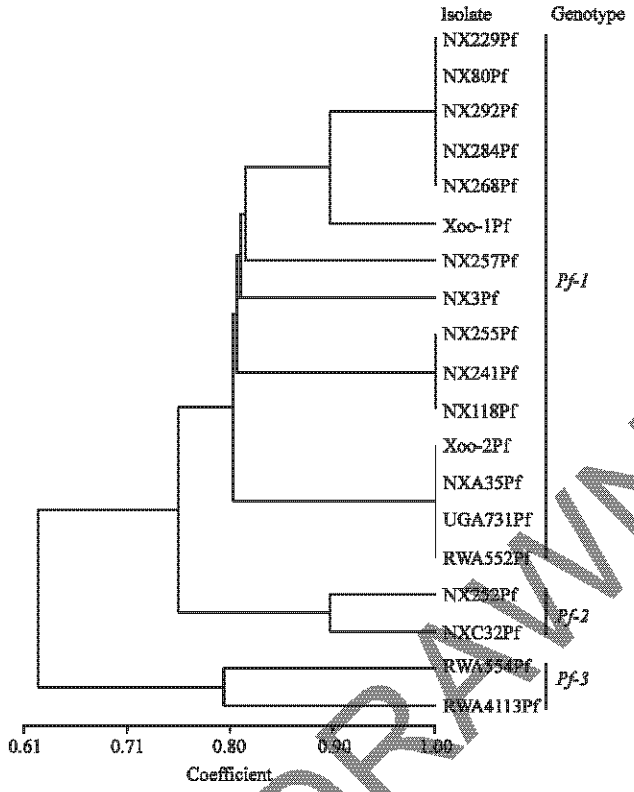

Fig. 9: Genetic diversity among 19 *Pseudomonas fuscovaginae* (Pf) isolates as revealed PCR analysis using PfF3 and PfR3 Pf specific primers

Table 7: Bacterial genotype, occurrence and distribution relative to country of origin

			Isolate origin and distribution					
Bacterial pathogen	No. of isolate	Genotype	Niger	Rwanda	Mozambique	Uganda	Philippines	Occurrence (%)
Xoo	84	*Xoo-1*	23	3	-	-	2	33.3
		Xoo-2	14	4	2	5	-	29.8
		Xoo-3	6	1	-	-	-	8.3
		Xoo-4	6	1	1	4	-	14.3
		Xoo-5	5	-	-	-	-	6.0
		Xoo-6	-	5	-	-	-	6.0
		Xoo-7	2	-	-	-	-	2.4
Xoc	50	*Xoc-1*	8	1	-	-	-	18.0
		Xoc-2	3	1	-	-	-	8.0
		Xoc-3	13	6	3	9	-	62.0
		Xoc-4	3	3	-	-	-	12.0
Pf	19	*Pf-1*	11	1	-	1	2	78.9
		Pf-2	2	-	-	-	-	10.5
		Pf-3	-	2	-	-	-	10.5
Pss	16	*Pss-1*	3	1	-	1	-	31.3
		Pss-2	3	5	-	-	1	56.3
		Pss-3	1	-	-	-	1	12.5

Xoo: *Xanthomonas oryzae* pv. *oryzae*, Xoc: *Xanthomonas oryzae* pv. *oryzicola*, Pf: *Pseudomonas fuscovaginae*, Pss: *Pseudomonas syringae* pv. *syringae*

Out of the same 95 bacterial isolates analyzed for Pss diagnosis using the Pss specific diagnostic primer pair (PssF4 and PssR4), 16 of the bacterial isolates contained Pss pathogen (Table 4, 5 and Fig. 10, 11). Moreover, the DNA fingerprinting of the diagnosed 16 Pss pathogens have been revealed by the same primer pair (PssF4 and PssR4) in the same PCR assay used for the diagnosis (Fig. 7, 8). DNA fingerprinting of 16 Pss pathogens using primer pair (PssF4 and PssR4) produced 27 fragments and all were polymorphic with genotype index of 1.7 leading to the identification of three Pss genotypes, which were *Pss-1*, *Pss-2* and *Pss-3* respectively (Table 6, Fig. 12). *Pss-1* genotype consists of 5 Pss isolates from Niger, Rwanda and Uganda while

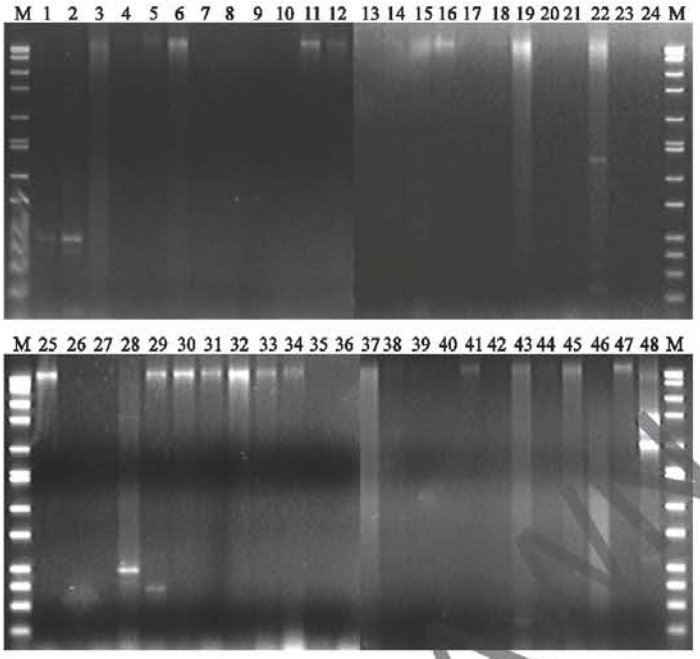

Fig. 10: *Pseudomonas syringae* pv. *syringae* (Pss) diagnosis and DNA fingerprint as revealed PCR analysis using PssF4 and PssR4 Pss specific primers. M = Molecular size marker. Molecular PCR diagnostic showed that the presence of at least a band indicates positive (+) detection of Pss pathogen and absence of a band indicates no Pss pathogen detected. In the same PCR assay the presence of one or more band at different position revealed the DNA fingerprint of Pss pathogen

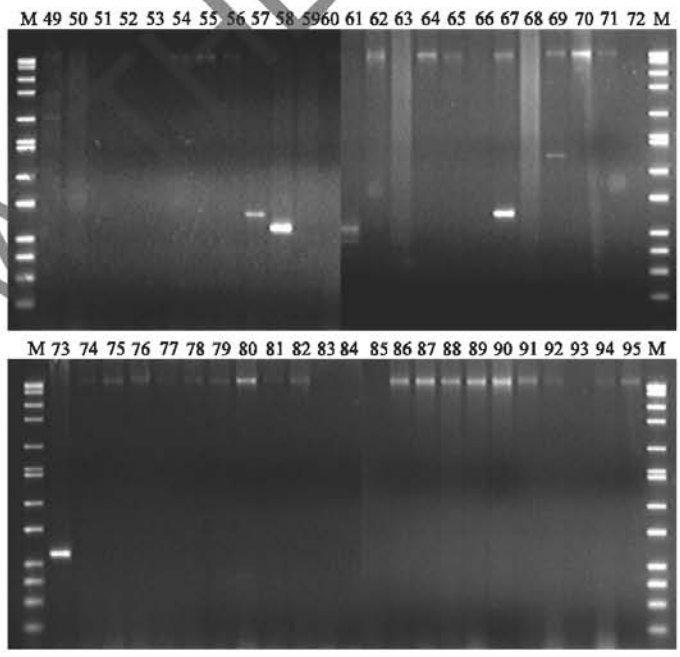

Fig. 11: *Pseudomonas syringae* pv. *syringae* (Pss) diagnosis and DNA fingerprint as revealed PCR analysis using PssF4 and PssR4 Pss specific primers. M = Molecular size marker. Molecular PCR diagnostic showed that the presence of at least a band indicates positive (+) detection of Pss pathogen and absence of a band indicates no Pss pathogen detected. In the same PCR assay the presence of one or more band at different position revealed the DNA fingerprint of Pss pathogen

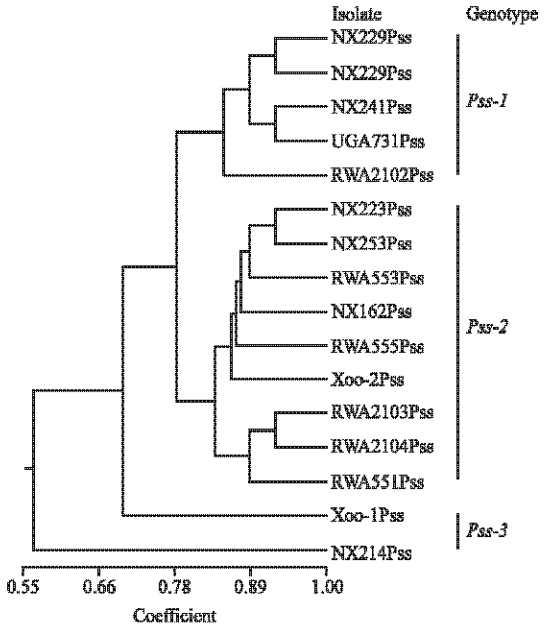

Fig. 12: Genetic diversity among 16 *Pseudomonas syringae* pv. *syringae* (Pss) isolates as revealed PCR analysis using PssF4 and PssR4 Pss specific primers

Pss-2 genotype consists of 9 Pss isolates from Niger and Philippines (Table 7, Fig. 12). However, Pss genotype occurrence and distribution among countries was between 12.5 to 56.3% with *Pss-2* genotype having the highest of 56.3% and *Pss-3* genotype having the lowest of 12.5% (Table 7).

DISCUSSION

Molecular PCR diagnostic showed that the presence of at least a band indicates positive (+) detection of a bacterial pathogen and absence of a band indicates negative (-) as no bacterial pathogen was detected, while in the same PCR assay the presence of one or more band at different position revealed the DNA fingerprint of a bacterial pathogen. Development of an effective combined molecular diagnostic and DNA fingerprinting PCR technique for *Xanthomonas oryzae* pv. oryzae (Xoo), *Xanthomonas oryzae* pv. *oryzicola* (Xoc), *Pseudomonas fuscovaginae* (Pf) and *Pseudomonas syringae* pv. *syringae* (Pss) rice pathogens in Africa as revealed by this study offers unique opportunity into early field detection of these pathogens, epidemiology and control (Eisenstein, 1990; Sakthivel *et al*., 2001). The Xoo, Xoc, Pf and Pss specific primers developed in this study have successfully diagnosed and fingerprinted these bacterial pathogens in PCR analysis thus confirming their usefulness and potential. These Xoo, Xoc, Pf and Pss specific primers produced multiple amplified DNA fragments on respective Xoo, Xoc, Pf and Pss isolates thus combining both molecular diagnostic and DNA fingerprinting potential for effective and reliable identification and differentiation of these bacterial pathogens. This makes the present study different from previous studies where bacterial diagnostic primers do not combine both diagnostic and DNA fingerprinting potential as did in this study (Botha *et al*., 2001; Dreo *et al*., 2005; Manceau *et al*., 2005; Fatmi *et al*., 2005).

The application and use of these Xoo, Xoc, Pf and Pss specific primers developed in this study in PCR is reliable and sensitive for diagnosing and fingerprinting seedborne Xoo, Xoc, Pf and Pss pathogens, detecting Xoo, Xoc, Pf and Pss in rice diseased leaf and identification of virulence unique to Xoo, Xoc, Pf and Pss pathogens (Adhikari *et al*., 1999; Sakthivel *et al*., 2001). Besides, the application of these Xoo, Xoc, Pf and Pss specific primers in PCR, because of its speed and sensitivity, holds the promise of making a significant practical impact in rice bacterial disease seed quarantine programs and to monitor germplasm movement within and outside Africa (Adhikari *et al*., 1999; Sakthivel *et al*., 2001). Moreover, these Xoo, Xoc, Pf and Pss specific primers could be useful for identification of Xoo, Xoc, Pf and Pss isolates from culture media thus resolving the lack of consistency and precision often arise from using cultural and morphological techniques (Bonde *et al*., 1993; Sakthivel *et al*., 2001). The convenience of the PCR method makes the application of these Xoo, Xoc, Pf and Pss specific primers highly suitable for analyzing large numbers of samples, allowing for greater efficiency in the study of Xoo, Xoc, Pf and Pss diagnosis, virulence, population structure and movement (Sakthivel *et al*., 2001).

Development of a reliable molecular technique for Xoo, Xoc, Pf and Pss identification and differentiation is a prerequisite into understanding the genetics of Xoo, Xoc, Pf and Pss population structure in Africa and deployment of durable resistance cultivars (Adhikari *et al*., 1995, 1999). In the present study the Xoo specific primer developed has detected Xoo pathogen in 84 out of 95 bacterial isolates tested as well as produced their DNA fingerprints leading to the identification of seven Xoo genotypes (*Xoo-1*, *Xoo-2*, *Xoo-3*, *Xoo-4*, *Xoo-5*, *Xoo-6* and *Xoo-7*) among the 84 Xoo isolates thus revealed it population structure in African countries. This report supports recent isozyme fingerprints of 30 Xoo isolates from 5 countries (Mali, Burkina Faso, Niger, Benin Republic and Nigeria) in West Africa that revealed five genetic groups (Onasanya *et al*., 2007, 2008).

The high distinction pattern of each isolates in this study suggests possible high level of genetic variation and frequent occurrence of mutants in Xoo, Xoc, Pf and Pss isolates in different host cells (Mongkolsuk *et al*., 2000; Innes *et al*., 2001; Onasanya *et al*., 2003). The genetic analyses revealed that all the seven Xoo genotypes (*Xoo-1*, *Xoo-2*, *Xoo-3*, *Xoo-4*, *Xoo-5*, *Xoo-6* and *Xoo-7*) might cover about 88.4% of rice bacterial pathogen population across Niger, Rwanda, Mozambique and Uganda and possibly be responsible for most sporadic cultivars infestation and epidemics in these countries. This was followed by four Xoc genotypes (*Xoc-1*, *Xoc-2*, *Xoc-3* and *Xoc-4*) which possibly covered about 52.6% of rice bacterial pathogen population across Niger, Rwanda, Mozambique and Uganda. Pf and Pss genotypes formed the least with about 20 and 16.8%, respectively of rice bacterial pathogen population across Niger, Rwanda and Uganda. Different Xoo, Xoc, Pf and Pss genotypes were found to exist in Niger, Rwanda, Mozambique and Uganda suggesting possible pathogen migration between these countries and long-term survival (Adhikari *et al*., 1995).

Distinct phenotypes usually consist of isolates that are genetically less related and such identification of isolates using cultural and morphological techniques often lack consistency and precision (Bonde *et al*., 1993). The Xoo, Xoc, Pf and Pss specific primers developed in the study have proven particularly useful in situations where identification of Xoo, Xoc, Pf and Pss isolates using cultural and morphological techniques often lack consistency and precision. For example, in the current study using Xoo, Xoc, Pf and Pss specific primers to screen 95 bacterial isolates only 24 were confirmed pure Xoo isolates and one as pure Xoc isolates while the rest were mixture of Xoo, Xoc, Pf or Pss pathogens.

CONCLUSIONS

It is concluded that the newly developed tool which is a combined molecular diagnostic and DNA fingerprinting PCR technique for *Xanthomonas oryzae* pv. *oryzae* (Xoo), *Xanthomonas oryzae* pv. *oryzicola* (Xoc), *Pseudomonas fuscovaginae* (Pf) and *Pseudomonas syringae* pv. *syringae* (Pss) rice pathogens in Africa, is effective, highly reliable, sensitive and therefore a suitable screening assay for early field detection of these pathogens, their epidemiology, movement, control and estimation of genetic diversity. The method can also serve as a fast and specific identification test for diagnosing and fingerprinting seedborne Xoo, Xoc, Pf and Pss pathogens, virulence unique to Xoo, Xoc, Pf and Pss pathogens and resolving the lack of consistency and precision often arise from using cultural and morphological techniques. It holds the promise of making a significant practical impact in rice bacterial disease seed quarantine programs and to monitor germplasm movement within and outside Africa. Finally, this study developed a reliable molecular technique for Xoo, Xoc, Pf and Pss identification and differentiation which is a prerequisite into understanding the population structure of these pathogens and deployment of durable resistance cultivars into different rice ecologies in African countries.

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