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Short Communication

UP-PCR Analysis of the Seedborne Pathogen *Fusarium fujikuroi* Causing Bakanae Disease in Rice

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ABSTRACT

Twenty isolates of *Fusarium fujikuroi* from Nueva Ecija and Laguna provinces in the Philippines were characterized by Universally Primed Polymerase Chain Reaction (UP-PCR) using AA2M2 and L45 primer. Sixty-six unique banding patterns were generated and a dendrogram was created. High degree of variation was observed among the isolates. At 90% similarity, fifteen clusters were formed, suggesting that sexual reproduction plays a significant role in generating variation of *F. fujikuroi* populations in the field. © 2011 Friends Science Publishers

Key Words: Bakanae; *Fusarium fujikuroi*; *Gibberella fujikuroi*; Genetic structure; Molecular marker; Rice; UP-PCR

INTRODUCTION

Bakanae disease caused by *Gibberella fujikuroi* Sawada Wollenweber (teleomorph) *Fusarium fujikuroi* Nirenberg (anamorph) occurs during seedling and tillering stages of the rice crop. In reality, the disease is caused by one or more *Fusarium* species and is a complex disease causing seedling blight, root and crown rot, stunting and stem elongation (Webster & Gunnell, 1992). Worldwide crop losses caused by the disease may reach up to 20% in epidemic cases. The fungus is seedborne and survives under adverse conditions in infected seeds and other diseased plant parts and soil (IRRI, 1983; Ou, 1985).

Bakanae disease of rice is widely distributed in Asia and was first recognized in Japan in 1828 by Ito and Kimura (1931) and was reported as one of the three major diseases of rice alongside with blast and brown spot by Hori (1898). In the Philippines, the disease was first reported by Reyes in 1939 causing root rot and seedling blight. Recent studies show that the disease incidence of bakanae in Philippine rice fields is increasing (Gergon & Angeles, 2006). The disease is considered as economically important in Asia, the continent contributing almost 92% of the world's rice production. Yield losses accounted were 20% to 50% in Japan, 15% and 3.7% in India and Thailand, respectively (Webster & Gunnell, 1992). Unfortunately, no yield loss data are reported in the Philippines, despite the fact that rice is the staple food of Filipinos.

Knowledge about the pathogen such as population structure, aggressiveness and metabolite production are essential for the development and deployment of efficient control and management strategies. At present there is also little information regarding the population structure of *F. fujikuroi* in rice. Hence, the objective of this study is to determine the genetic diversity of a population of *F. fujikuroi* isolated from rice fields in two Philippine provinces by UP-PCR analysis.

MATERIALS AND METHODS

Sample collection: Bakanae infected rice seed and stem samples from different rice fields in Laguna and Nueva Ecija during the wet 2006 season were collected (Table I). Samples of rice seeds with husks and stem pieces were randomly collected. Individual samples were kept in cold condition before analyses.

Maintenance of cultures: Cultures of *F. fujikuroi* isolated by Aguilar (2007) grown on Potato Dextrose Agar (PDA) and Synthetic Nutrient Agar (SNA) slants were used in this study (Table I). All single-spored strains labeled with a Mycothéque de l'Université Catholique de Louvain (MUCL) prefix were deposited in lyophilized condition in MUCL, Belgium. Identity of cultivars was confirmed by Dr. M.T. Gonzalez-Jacs, University Complutency, Madrid, Spain.

UP-PCR Analysis

Mycelium production and DNA extraction: Genomic DNA of the *Fusarium* isolates was obtained using the protocol described by Cenis (1992). Hyphal threads from fungal cultures were inoculated to 1.5 mL eppendorf tubes containing 500 µL potato dextrose broth (potato

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broth from 250 g potato & 20 g dextrose in 1000 mL distilled water). Incubation followed for 72 h (3 days) at room temperature. The growth or mycelial mat was pelleted by centrifugation at 13,000 rpm for 5 min in the microfuge. The pellet was washed with 500 µL TE buffer (10 mM Tris, 1 mM 0.5 EDTA, pH 8.0 & 800 mL distilled water). Decantation of the TE buffer and the addition of 300 µL of extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) followed. The mycelia were crushed using sterilized toothpick and 150 µL of 3 M sodium acetate, pH 5.2 was added. Then, the eppendorf tubes were placed at -20^{o}C for 10 min. The tubes were again centrifuged and the supernatant removed and transferred to another tube. Then, 450 µL of isopropanol was added and the tubes were placed at room temperature. The precipitated DNA was pelleted using the microfuge. The supernatant was removed and the pellet was washed with 70% ethanol. The pellet was air dried and resuspended in 50 µL of TE buffer. Detection of the presence of extracted DNA was done by running the samples in gel electrophoresis, staining with ethidium bromide and by viewing the result using UV transilluminator.

UP-PCR amplification: Genomic fingerprints of the isolates were generated through UP-PCR. One µL of DNA extract was mixed with 1 µL of 40 ng of AA2M2 primer: 5'- CTGCGACCCAGAGCGG- 3' (Invitrogen), 0.4 µL 2U of i-Taq DNA polymerase (INtRON Biotechnology) and 17.6 µL of reaction buffer. The reaction buffer contained i-Taq 1X PCR buffer (INtRON Biotechnology), 2 mM i-Taq MgCl2, and 0.8 mM deoxynucleoside triphosphate (dNTP) mixture (INtRON Biotechnology). UP-PCR was done in a MyCyclerTM thermal cycler (Bio-Rad Laboratories, California, USA) with the following settings: thirty (30) cycles with DNA denaturation at 92 C for 50 s (first denaturation step at 94 C for 3 min), primer annealing at 56 C for 70 s and primer extension at 72 C for 60 s (final extension step at 72 C for 3 min).

The PCR products were analyzed by electrophoresis. Two agarose gels, of 1.7% (wt/vol) were prepared by mixing 85 g of agarose to 50 mL of 0.7× TBE buffer each. The solution was heated in the microwave oven for 1 min and 15 s, poured into the mould with 15-well comb and allowed to solidify before it was placed in the electrophoresis tank with 0.7x TBE buffer. The PCR products (3 µL of each UP-PCR reaction), with 1 µL of blue juice each, were loaded to individual wells from the third to the twelfth since 10 PCR reactions were electrophoresed per run. Gels were also loaded with 1000-bp ladders (INtRON Biotechnology) as molecular size standards on the second and thirteenth well. Two runs were made to accommodate the 20 PCR reactions. The bands were visualized by staining with ethidium bromide for 10 min.

Cluster analysis: The total number of unique UP-PCR banding profiles was determined. The banding patterns were scored in binary form by coding the presence or absence of bands as 1 or 0, respectively. A dendrogram was generated from the binary data using the numerical taxonomy system (NTSYS-pc) software package (version 2.1, Applied Biostatistics, Inc.). Similarity coefficients for each pair-wise combination of UP-PCR profiles were computed by SIMQUAL (Similarity for qualitative data program) using simple matching coefficient. Cluster analysis was performed based on the similarity coefficients using sequential agglomerative hierarchical nested (SAHN) program with an unweighted pair group arithmetic mean averages (UPGMA) algorithm. The dendrogram form of the tree matrix data was constructed using the TREE (tree dispaly) program.

RESULTS AND DISCUSSION

Genetic variability of the 20 *F. fujikuroi* isolates was evaluated by UP-PCR using AA2M2 and L45 primer. Sixty-six banding patterns were seen (data not shown). These data were used to determine the relatedness or phylogenetic relationship of the isolates among each other by cluster analysis using NTSYS-pc version 2.10.

High degree of variation was observed among the isolates. At 90% similarity, fifteen clusters, designated as I, II...XV, were identified (Fig. 1). Cluster V contains five haplotypes, cluster X contains two haplotypes while the other clusters contain only one haplotypes each. The isolates coming from only one field or geographical origin were found to be genetically different from each other since they belong to different clusters, as seen with PRC isolates, which were from Nueva Ecija and Va isolates from Laguna rice fields. These results are in conformity with the findings of Amoah *et al.* (1995) on the analysis genetic groups of *F. moniliforme* isolates infecting rice, maize and elephant grass. They arrived at the conclusion that population diversity is not related to geographical differences when an isolate from rice from one locality was closely related to the isolates from another area and when diverse genetic groups were found in the same geographic origin. Recently, Wulff *et al.* (2010) showed a high genetic variation of *F. fujikuroi* distributed in four genetic clades.

Genetic variation of fungi may be attributed to different factors. Among these factors, mutation, population size and random genetic drift, gene flow, reproduction/ mating system and selection play a vital role (McDonald & Linde, 2002). Interplay between these various factors results to either high or low variability among and within populations of these pathogens. Cumagun (2007) came up with risk assessment of *Fusarium fujikuroi* based on those factors using the framework proposed by McDonald and Linde (2002), which may explain the pathogen's variability. Intermediate evolutionary risk was established due to high

Table I: List of *Fusarium fujikuroi* isolates analyzed

Isolate Code	MUCL[a]	Origin	Collector
PRC2a	51032	Nueva Ecija	KG Aguilar[b]
PRC3a	51033	Nueva Ecija	KG Aguilar[b]
PRC4a	51034	Nueva Ecija	KG Aguilar[b]
PRC4b	-	Nueva Ecija	KG Aguilar[b]
PRC5b	-	Nueva Ecija	KG Aguilar[b]
PRC6a	51036	Nueva Ecija	KG Aguilar[b]
PRC6b	-	Nueva Ecija	KG Aguilar[b]
PRC7a	51037	Nueva Ecija	KG Aguilar[b]
PRC8a	-	Nueva Ecija	KG Aguilar[b]
PRC9a	51039	Nueva Ecija	KG Aguilar[b]
PRC10a	-	Nueva Ecija	KG Aguilar[b]
PRC11a	51040	Nueva Ecija	KG Aguilar[b]
PRC14b	-	Nueva Ecija	KG Aguilar[b]
PRC17b	-	Nueva Ecija	KG Aguilar[b]
PRC19a	51044	Nueva Ecija	KG Aguilar[b]
PRC19b	-	Nueva Ecija	KG Aguilar[b]
PRC20a	-	Nueva Ecija	KG Aguilar[b]
PRC20b	51045	Nueva Ecija	KG Aguilar[b]
Va2	-	Laguna	CJR Cumagun[c]
Va3	-	Laguna	CJR Cumagun[c]

[a]Code of the strain cultures preserved in MUCL, Belgium
[b]Date of isolation: September 30, 2006
[c]Date of isolation: September 12, 2006

gene/genotype flow and mixed reproduction/mating system (Cumagun, 2007). High gene/genotype flow involves the movement of the propagules, whether sexual or asexual, to other area, which may result to exchange of genes and genotypes and formation of new genotype. Mixed reproduction or mating system also contributed to its diversity. Though the pathogen's reproduction system is mainly asexual, where micro and macroconidia conidia are formed, sexual reproduction, wherein perithecia are produced, also occur due to drastic condition in the field. This mixed system poses the highest risk of evolution since it involves recombination, where the creation of new genotype with high fitness due to the pressure exerted by selection (sexual stage), and maintenance of most fit genotypes through clones, spatial and temporal distribution (asexual stage) (McDonald & Linde, 2002). Mutation rate is still unknown but it may also have big impact to the pathogen's variability.

CONCLUSION

The study analysed the genetic diversity of 20 *F. fujikuroi* isolates originating from two provinces in the Philippines using UP-PCR marker. Results showed high genetic diversity even a small population of *F. fujikuroi* indicating that sexual reproduction may play a role in generating variation in the population in the field.

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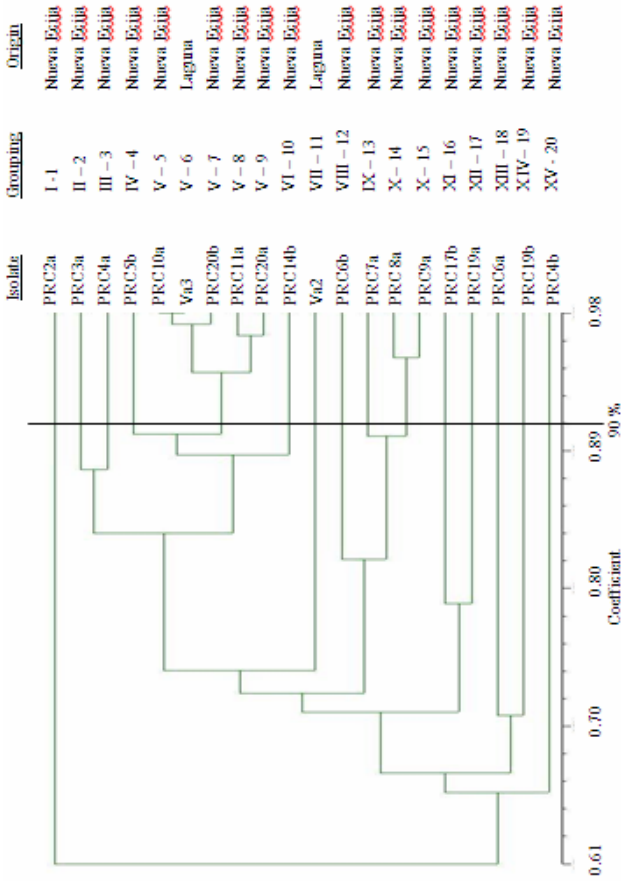
Fig. 1: Relatedness dendrogram derived by the unweighted pair group method, arithmetic mean (UPGMA) showing the similarity relationships among *F. fujikuroi* isolates. Grouping based on dendrogram and geographical origins of the isolates are also indicated



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