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Short Communication: Genetic diversity of walnut blight-associated bacteria from China using rep-PCR

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Abstract. *Zheng C, Zhu J, Yang X, Fu B. 2022. Short Communication: Genetic diversity of walnut blight-associated bacteria from China using rep-PCR. Biodiversitas 23: 5681-5686.* Walnut blight is caused by the bacterium *Xanthomonas arboricola* pv. *juglandis* (*Xaj*), is one of the most serious diseases in walnut production around the world. Understanding the genetic diversity of walnut blight pathogen is of great significance to developing a scientific and reasonable prevention and control strategy. The rep-PCR (Repetitive Sequence Polymerase Chain Reaction) technique is a powerful tool for analyzing plant pathogens. In this paper, using three pairs of rep-PCR universal primers (ERIC, BOX, and REP), a genetic diversity analysis was carried out on walnut blight-associated bacteria mainly isolated from different counties of Hubei Province, China. A study of 60 walnut blight-associated bacteria isolated in 2016 found that they had a high genetic similarity among them. There is no clear correlation between the genetic diversity of these isolates and their geographical or tissue origins. Interestingly, these clusters can also distinguish copper-resistant isolates from sensitive ones. Therefore, rep-PCR is a powerful tool to study the population of walnut pathogenic bacteria.

Keywords: Genetic diversity, plant-associated bacteria, rep-PCR, walnut blight, *Xanthomonas arboricola* pv. *juglandis*

INTRODUCTION

Walnut blight is one of the most serious diseases affecting walnut yield and quality, occurring in all major walnut-growing areas of the world. The disease was first reported in 1901 in California (Pierce 1901) and followed by severe outbreaks in the United States, Italy, France, Iran, Lithuania, Poland, and Korea (Burokiene and Pulawska 2012; Moragrega and Özaktan 2010; Soltani and Aliabadi 2010; Kim et al. 2021). The Gram-negative, rod-shaped bacteria *Xanthomonas arboricola* pv. *juglandis* (*Xaj*) is a causative agent of walnut blight (Romero-Suarez et al. 2012). However, other bacterial species such as *Pseudomonas* sp., *Brenneria nigrifluens*, and *Pantoea agglomerans* have also been associated with the disease (Hajri et al. 2010; Yang et al. 2011; Zou et al. 2019). More recently, a new *Xanthomonas* species *X. euroxanthea* sp. nov. including pathogenic strain CPBF 424T and non-pathogenic strain CPBF 367 was found in Portugal (Martins et al. 2020).

Studying the genetic diversity of plant pathogenic bacteria has great significance to the disease epidemics, variations in virulence of the pathogen, and the emergence of resistance. Repetitive Sequence Polymerase Chain Reaction (rep-PCR), is a DNA fingerprint mapping technique that targets prokaryotes and can reveal differences between genomes by amplifying short repeat sequences widely distributed in bacterial genomes (Ussery and Hallin 2004). In the genomes of most Gram-negative bacteria and several Gram-positive bacteria, there are multiple copies of highly conserved and repetitive DNA sequences occurring in three major types: 35-40 bp Repetitive Extragenic Palindromic (REP) sequences, 124-127 bp Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences (also referred to as intergenic repetitive units), and 154 bp BOX elements that contain various combinations of its subunits (Rademaker et al. 1997). The polymorphisms of these conserved genetic elements can be used to differentiate sequences at a strain, species, or genus level, i.e. *Bacillus pumilus* group classification recently (Husni et al. 2021). Species-specific REPs have been identified in many animal and plant-associated bacteria already (Hossain et al. 2022; Mirzaei et al. 2021). For instance, it has been used on human pathogens (*Escherichia coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa*), plant pathogens (*Agrobacterium tumefaciens* and *Pseudomonas* spp.), and soil bacteria (*Deinococcus radiodurans*, *P. putida*, and *S. meliloti*) (Sheng et al. 2016; Rezene et al. 2018).

Rep-PCR is also very useful for assessing the genetic diversity among strains of genus *Xanthomonas*, especially at the pathovar level, including in some studies on walnut blight pathogens (Ivanović et al. 2015), cabbage black rot (*X. campestris* pv. *campestris*) (Chen et al. 2021). Scortichini et al. (2002) utilized it to analyze the genetic diversity of 31 *Xaj* isolates from multiple locations and, when combined with other biochemical tests, were able to divide those *Xaj* strains into three main groups. Ivanović et

al. (2015) assessed the genetic diversity of 59 *Xaj* isolates in Serbia using rep-PCR, pulse field gel electrophoresis, and DNA gyrase subunit B (*gyr*B) gene sequences. Alternative approaches include multilocus sequence analysis of aconitate hydratase B (*acn*B), glyceraldehyde-3-phosphate dehydrogenase A (*gap*A), *gyr*B, and RNA polymerase sigma factor D (*rpo*D) genes (Marcelletti et al. 2010).

Although many *Xaj* isolates have been studied worldwide, no such analysis of walnut blight-associated strains in China has been reported. Here, a genetic diversity analysis of walnut blight-associated bacteria (including *Xaj* and non-*Xaj*) isolated from multiple host tissues and geographic sites in Hubei Province was conducted using the rep-PCR technique. This research is the first study on the structure and diversity of *Xaj* populations in Central China. It will boost the *Xaj* and walnut-associated bacteria genetic resource for the globe and particularly in Asia. Furthermore, we hope that this will inspire more research on the pathophysiology and molecular epidemiology of walnut blight in the future.

MATERIALS AND METHODS

Bacterial strains

Tissue samples from symptomatic fruits, buds, twigs, and leaves were collected from major walnut-producing areas in Hubei Province, China. Bacterial isolation was conducted according to the method described by Hajri et al. (2010) on the semi-selective growth medium yeast peptone glucose agar (YPGA) at 26°C. The isolates were stored in 30% glycerol at -80°C (Table 1).

Rep-PCR

Bacterial genomic DNA was extracted using an EZ-10 Spin Column Bacterial Genomic DNA Mini-Preps Kit (Sangon Biotech, Shanghai) according to the instructional manual. Rep-PCR was performed according to protocols in Rademaker et al. (1997). PCR reaction in a 25 µL system: 2×Taq PCR Master Mix, 12.5 µL, each primer 0.4 µmol/µL, DNA template 100 ng, added ddH_2O to 25 µL. The thermal cycle follows as 95°C for 5 min, 94°C for 30 s, annealing temperature for 30 sec, 72°C for 30 sec, 30 cycles, and 72°C for 10 min. The annealing temperature of ERIC (ERIC1R and ERIC2), BOX (BOXA1R), and REP (REP1R-I and REP2-I) was 52, 53, and 40°C, respectively. The universal primers ERIC (ERIC1R and ERIC2), BOX (BOXA1R), and REP (REP1R-I and REP2-I) were synthesized by Sangon Biotech, Shanghai (Table 2). Following PCR amplification, conduct electrophoresis 45 min under 5 voltage/cm in 1.8% agarose gel with 0.5×TBE buffer, then imaged by gel image system (Bio-best 135A, SIM, USA).

Data analysis and clustering

Clearly and consistent bands from agarose gel profiles were converted to a binary matrix, where "1" or "0" represent present or absent at the same position with manual checked. The data was established in Excel 95 format file as required by NTSYSpc2.2. The dendrogram was constructed by NTSYSpc2.2 software using the unweighted pair-group method with the arithmetic means (UPGMA) method.

Table 2. Rep-PCR primers were used in this study

Primer	Sequence (5´-3´)
ERIC1R	ATGTAAGCTCCTGGGGATTCAC
ERIC2	AAGTAAGTGACTGGGGTGAGCG
BOXA1R	CTACGGCAAGGCGACGCTGACG
REP1R-I	IIIICGICGICATCIGGC
REP2-I	ICGICTTATCIGGCCTAC

Table 1. Sixty isolates of walnut blight-associated bacteria were used in this study (Fu et al. 2021)

Year	Origin	Tissue	Isolates
2015	Danjiangkou	Fruit (1)	DW3F3
		Twig (1)	DGW1S1
		Leaf (1)	DWW1L1
2016	Danjiangkou	Leaf (4)	DILf3-4, DIIILf4-1, DIIILf4-2, DIIILf4-3
		Bud (10)	DIBd1-1, DIBd1-2, DIBd1-3, DIBd1-4, DIBd3-1, DIBd3-2, DIIIBd2-1, DIIIBd2-2, DIIIBd2-3, DIIIBd2-4
2017	Baokang	Fruit (24)	BKA-1, BKA-2, BKA-3, BKB-1, BKB-2, BKB-3, BKA1-1, BKA1-2, BKA2-1, BKA2-2, BKA3-1, BKA3-2, BKA4-1, BKA4-2, BKA5-1, BKA5-2, BKB1-1, BKB1-2, BKB3-1, BKB3-2, BKB4-1, BKB4-2, BKB5-1, BKB5-2
	Danjiangkou	Fruit (9)	DWQX1-1, DWQX1-2, DWQX1-3, DWQX2-1, DWQX2-2, DWQX2-3, DWQX4-1, DWQX4-2, DWQX4-3
	Suizhou	Fruit (9)	SZY2-1, SZY2-2, SZY2-3, SZG3-1, SZG3-2, SZG3-3, SZG3-4, SZG3-5, SZG3-6
	Beijing	Twig (1)	Xcc-bj

RESULTS AND DISCUSSION

Walnut blight-associated bacterial isolates

One hundred twenty isolates of walnut blight-associated bacteria were isolated from symptomatic diseased tissues, leaves, fruits, buds, and twigs separately (Figure 1). Picking up those colonies that have characteristics consistent with typical *Xaj* morphology, purifying every single representative colony (*Xaj* suspected) from the independent sample several times. Sixty isolates identified as *Xaj* (Fu et al. 2021) were cultured on YPGA medium at 26°C for 3 days, and then stored at -80°C within 30% glycerol for future use.

Xaj rep-PCR fingerprint profiles

The partial electrophoresis gel profiles of the 60 *Xaj* isolates were analyzed using ERIC, BOX, and REP (Figures 2, 3, and 4). A total of 25 polymorphic bands were detected and used for UPGMA cluster analysis (10 ERIC, 6 BOX, 9 REP), they generated fragments length between 250 and 3000 bp. In the samples from Danjiangkou walnut fruits, the bands were unique and distinct from the others in this study (Figure 2).

Clustering analysis of Xaj isolates based on rep-PCR.

A dendrogram tree was constructed based on the concatenated data from three sets of primers using rep-PCR (Figure 5). At a similarity coefficient of 0.89, the 60 *Xaj* isolates can be divided into eight groups (1-8). Groups 1, 7, and 2 were predominant, containing 40, 6, and 4 isolates respectively. Group 1 accounts for 66.7% of all assayed isolates, including all nine isolates isolated from Suizhou, and 91.7% of the isolates in Baokang county. Isolates from this group were also isolated from fruits, leaves, and twigs but differ in that the buds were collected during the winter, potentially indicating a seasonal shift in the *Xaj* community structure. Of the eight identified groups, only 4 and 5 contain a single strain each (DILf3-4 and DWW1L1), both of which were isolated from walnut leaves from Danjiangkou county.

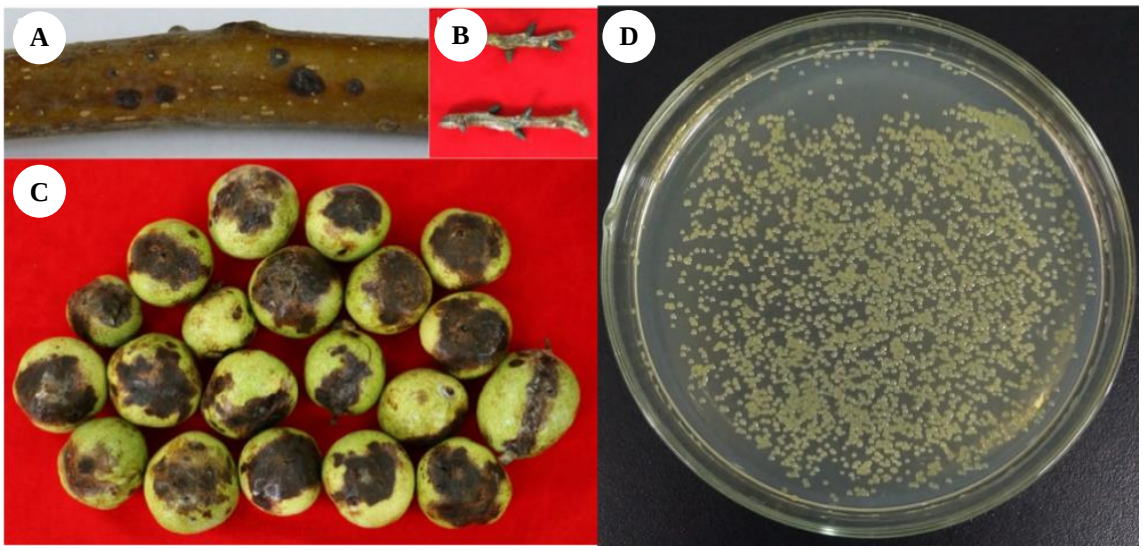

Figure 1. Walnut tissues with symptoms. A. Twig, B. Buds, C. Nuts, D. Isolation on YPGA medium

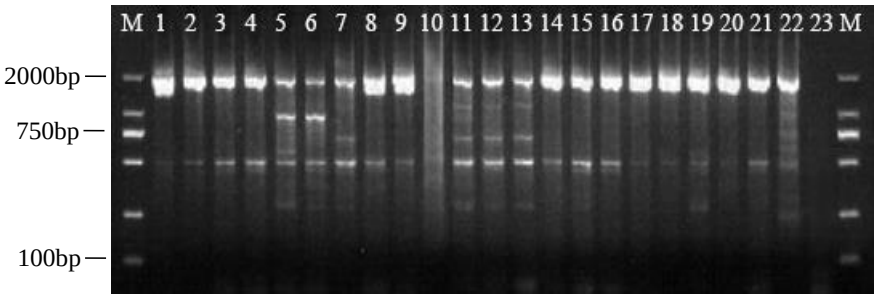

Figure 2. Partial *Xaj* isolates ERIC-PCR electrophoresis agarose gel profile. Note: M: DNA Marker D (100-2000 bp);1-23:DW3F3, BKB4-2, BKB5-1, BKB5-2, DWQX1-1, DWQX1-2, DWQX1-3, DWQX2-1, DWQX2-2, DWQX2-3, DWQX4-1, DWQX4-2, DWQX4-3, SZY2-1, SZY2-2, SZY2-3, SZG3-1, SZG3-2, SZG3-3, SZG3-4, SZG3-5, SZG3-6, H_2O

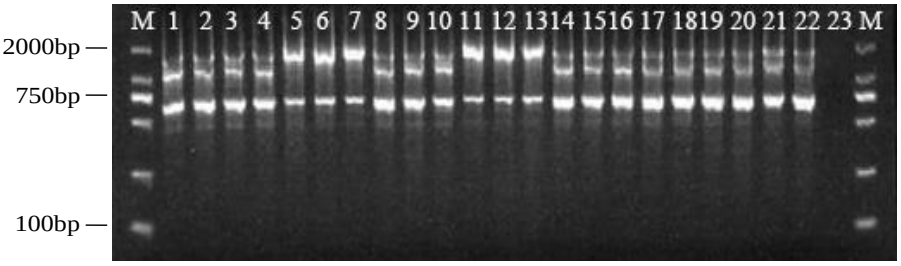

Figure 3. Partial *Xaj* isolates BOX-PCR electrophoresis agarose gel profile. Note: M: DNA Marker D (100-2000 bp);1-23:DW3F3, BKB4-2, BKB5-1, BKB5-2, DWQX1-1, DWQX1-2, DWQX1-3, DWQX2-1, DWQX2-2, DWQX2-3, DWQX4-1, DWQX4-2, DWQX4-3, SZY2-1, SZY2-2, SZY2-3, SZG3-1, SZG3-2, SZG3-3, SZG3-4, SZG3-5, SZG3-6, H_2O

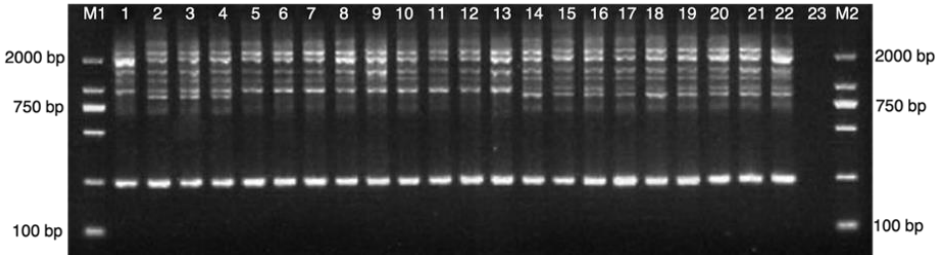

Figure 4. Partial *Xaj* isolates REP-PCR electrophoresis agarose gel profile. Note: M: DNA Marker D (100-2000 bp);1-23:DW3F3, BKB4-2, BKB5-1, BKB5-2, DWQX1-1, DWQX1-2, DWQX1-3, DWQX2-1, DWQX2-2, DWQX2-3, DWQX4-1, DWQX4-2, DWQX4-3, SZY2-1, SZY2-2, SZY2-3, SZG3-1, SZG3-2, SZG3-3, SZG3-4, SZG3-5, SZG3-6, H_2O

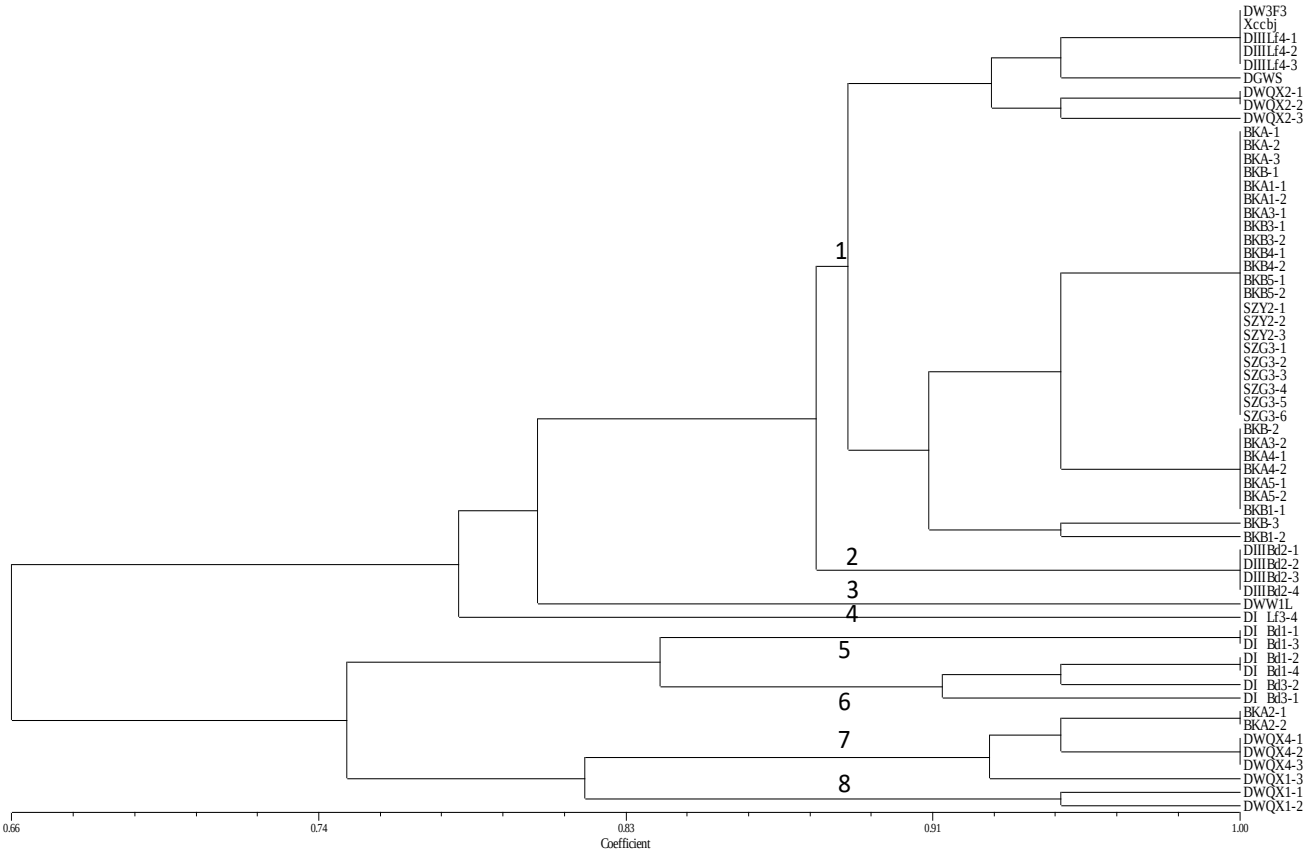

Figure 5. Dendrogram of 60 *Xaj* isolates based on ERIC, BOX, and REP rep-PCR

Discussion

Walnut plant-associated bacteria, from the rhizosphere, have been investigated in recent years. Dar et al. (2018) screened 98 isolates of walnut rhizobacteria and found a few of them with plant growth-promoting attributes, like *B. licheniformis* and *Micrococcus luteus* strains. The diversity and relationship between walnut plant-associated bacteria and the pathogen *Xaj* or close pathovars are still a big concern currently, the knowledge about them might provide clues for disease management in the field (Kaluzna et al. 2021). Microbial diversity particularly in genetic diversity still is an undergoing aspect of current research from bacteria, archaea fungi, and the microbial origin from the rhizosphere, phyllosphere, soil, and plant pathogens. For example, the total *Bacillus* related bacterial species were investigated from different soil environments (Alyousif 2022). Among them, the BOX fingerprint technique was applied to soybean phyllosphere bacteria diversity (Nurcahyanti et al. 2021). Similarly, BOX-PCR fingerprint was carried out to study the genetic diversity of the plant pathogen *Ralstonia solanacearum* from horticultural plants in Indonesia. Twenty-one isolates could be divided into two phylotypes in which most of the isolates clustered according to their original places (Hemela et al. 2019).

Rep-PCR is a conventional and broadly used technique in microbiology and genetic diversity. Such as plant pathogenic bacteria wilt of Solanaceaous vegetables *R. solanacearum* (Hossain et al. 2022), fungal disease strawberry anthracnose (Karimi et al. 2019), and zoonotic disease brucellosis (Amoupour et al. 2019). In this study, using rep-PCR technique, the genetic diversity of 60 walnut bacterial-associated bacteria from different regions was investigated through three sets of primers (ERIC, BOX, and REP). Although these identified isolates have a higher level of similarity, this technique still can cluster them into several groups using a similarity coefficient of 0.89. At this threshold, the eight clusters could still not be differentiated by geographic locations or tissue types. Whereas at a similarity coefficient of 0.74, 60 *Xaj* isolates only divide into two groups.

There have been earlier reports on walnut pathogen genetic diversity analysis using ERIC-PCR, BOX-PCR, and REP-PCR (Hajri et al. 2010; Scortichini et al. 2002). However, the work on genetic diversity could be explained by the geographic origin of samples. The authors explained the clusters based on the geographic origin of the strain but did not mention the significant difference in the origin date (more than 40 years) and seasonal variation. Our result is similar to the work of Ivanović et al. (2015) in that no geographic relationship among the same clusters. Except based on rep- PCR technique, they assessed the genetic diversity of 59 *Xaj* isolates from different locations in Serbia combined with pulse-field gel electrophoresis, PFGE, and *gyr*B sequencing. There was an example of a conclusion different from the above. In some cases, with genetic abundance in the *Xaj* population, the genetic diversity could be correlated to geographic origin and temporal variation (Ivanović et al. 2015).

In this study, the rep-PCR technique was demonstrated as an effective tool to investigate walnut blight-associated bacteria and *Xaj*. However, the three specific primers with different capacities in the study. ERIC and BOX both showed stronger differential ability than REP, both in associated bacteria and the *Xaj* population. The current work exploited and unveiled the most important walnut bacterial disease genetic diversity of the pathogen isolated from China, as well as contributing to the genetic diversity pool of this kind of bacterial pathogen.

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