

Variations among *Xanthomonas axonopodis* pv. *vignicola* isolates in Benue State, Nigeria

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ABSTRACT: *Xanthomonas axonopodis* pv. *vignicola* is the causative agent of bacterial blight, a destructive disease of cowpea in Africa. Laboratory studies were conducted with the objective of detecting variations among *X. axonopodis* pv. *vignicola* isolates which were collected from three different cowpea growing zones (Makurdi, Guma and Gboko Local Government Areas) in Benue state. The isolates were designated MKD388-1, GUM391 and GBK205-8 respectively. Isolates were cultured on Nutrient agar, tested for their Gram-staining reaction and their capability to utilize asparagine as a sole source of carbon and nitrogen. They were also subjected to biochemical tests. The genetic diversity of the isolates was assessed using Single Sequence Repeat (SSR) primers. Four primer combinations selected based on their reproducibility and amplification were used to differentiate the *X. axonopodis* pv. *vignicola* strains. Following these tests, the three isolates showed variations in color and growth character of colonies ranging from yellow to creamy colony color with mucoid growth. Variations to different biochemical tests were also observed among isolates. Primer assay showed genetic variation among the isolates as strains from Makurdi and Guma Local Governments showed high levels of molecular similarity while Gboko strain was distant. This study has led to an understanding of the dynamics of pathogen variability that can be used to develop resistance gene pyramiding or gene deployment strategies. This will prevent selection for new virulence, which are effective against the currently available genetic sources of resistances genes.

KEYWORDS: *Xanthomonas axonopodis* pv. *vignicola*, isolates, strain, variation, primers

1 INTRODUCTION

One of the major biological constraints to increase cowpea production in the smallholder farming sector is attack by fungal, viral and bacterial diseases (7). Besides fungal and viral diseases, bacterial blight and pustles incited by *Xanthomonas axonopodis* pv. *vignicola* (19) formerly *Xanthomonas campestris* pv. *vignicola* (5) is the most important disease of cowpea. Cowpea bacterial blight (CoBB) is prevalent in all major cowpea growing areas of the world (6). The economic gains that can be made by partially or even completely alleviating these constraints are enormous. In the case of most of the fungal and bacterial diseases, the main reason for the all too common "breakdown" of once-effective resistances is the variability that exists in the pathogen population, which necessitates a continual replacement of cultivars due to disease susceptibility. New pathotypes evolve with the introduction of new varieties and hybrids of our crops. Rapid and accurate detection of new virulence will help formulate strategies of deploying resistant cultivars in particular regions and countries. If we develop means to quantify the variability in pathogen populations it will provide a basis of breeding cultivars with durable resistance or designing strategies for the long-term management of major diseases. For example, in the case of pearl millet downy mildew, the information on virulence pattern and sources of resistance will be highly crucial for both public and private sector breeding programs that will ultimately benefit the pearl millet farmers who will not have to suffer yield loss (16). The identification and characterization of *Xanthomonas axonopodis* pv. *vignicola* (Xav) which causes bacterial blight in cowpea crop has been carried out in West Africa. A number of sources of resistance among cowpea genotypes to the several strains of the bacteria causing this disease (CoBB) have been detected (4;17;9;16). Interestingly, limited work has been done to

identify the most useful germplasm for breeding and bacterial blight continues to attack successfully susceptible cultivars, which makes it imperative to develop alternative ways of disease management to overcome the challenges of infection.

The conventional method of identifying the variability in the pathogen has been the use of sets of host differentials and disease rating scales to study the virulence reaction both in the field and greenhouse. Genetic characterization of the pathogen populations based on pathogenicity data will go a long way to develop high yielding cowpea varieties with other important agronomic traits and resistance to bacterial blight (BB). To assess the genetic diversity of *Xanthomonas axonopodis* pv. *vignicola* pathogens, SSR markers were used to study diversity among three *Xav* strains collected from three cowpea growing areas in Benue State.

2 MATERIALS AND METHODS

2.1 MICROSCOPIC EXAMINATION OF SPECIMENS

Diseased plant materials were cut from the boundary between diseased and healthy tissue and placed on a clean slide. A drop of ethylene blue was added and covered with a cover slip. This was examined under the microscope at a magnification of x40 using a light microscope.

2.2 ISOLATION AND PRESERVATION OF *X. AXONOPODIS* PV. *VIGNICOLA*

Diseased plant tissues were cut in bits and suspended in sterile water for two hours to allow sufficient quantity of bacterial cells to ooze out from the tissue. (Nutrient agar was prepared by dissolving 28g of the agar in 1 litre of distilled water and sterilized by autoclaving at 121^oC for 15 minutes). A loopful of the suspension was then streaked on the surface of nutrient agar plates in a zigzag fashion on a quadrant of the plate starting from the circumference (12). The plates were then inverted and incubated at 27^oC for 48-72 hours according to Vauterin *et al.* (19). Bacterial colonies from each plate were further sub-cultured repeatedly until pure colonies were obtained. A loopful of each pure culture was streaked on YPSA plates (Yeast extract 5g; Peptone 10g; Sucrose 20g; Agar 12g in 1 litre of distilled water at a pH of 7.4 and autoclaved at 121^oC for 15 minutes). The plates were incubated at 28^oC for 48 – 72hours. Pure cultures were transferred to YPSA slants incubated at 28^o C for 48 – 72hours and preserved at 4^oC for further work. In both methods, only round, smooth, entire domed and yellowish bacterial colonies were selected as described by Ah-You *et al.* (1).

2.3 MORPHOLOGICAL AND BIOCHEMICAL TESTS

Gram reaction of the isolates was determined following the staining procedure in Vauterin *et al.* (19). First, thinly spread bacterial smear was prepared on a clean slide, air-dried and fixed by passing it over a Bunsen burner flame. The dried smear was flooded with crystal violet solution for one minute and washed in tap water for few seconds. It was then flooded with iodine solution for one minute and washed again in tap water. Thereafter it was decolourised with 95% ethanol by applying drop by drop until no more colour flows from the smear. Finally slides were counter stained for about 10s with safranin, washed in tap water and examined under microscope using oil immersion objective. Gram negative bacteria were selected for further tests.

2.4 GROWTH ON ASPARAGINE MEDIUM

All Gram-negative isolates were allowed to grow on Asparagine medium (Asparagine 0.5g; KH₂PO₄ 0.1g; MgSO₄.7H₂O 0.2g; KNO₃ 0.5g; CaCl₂ 0.1g; NaCl 0.1g and agar 12 – 15g (for plates) in 1 litre distilled water with pH 7 and autoclaved at 121^o C for 15 minutes) at 28^oC for 48 – 72 hours without any other carbon and nitrogen sources (3). This is used as a diagnostic test for *Xanthomonas* because they are not able to grow on it while others like yellow *Enterobacteriaceae* and many *Pseudomonads* can grow on it. The growth of the bacteria on Asparagine agar plates was recorded and those that were unable to grow on the medium were taken for further tests. In all cases uninoculated medium was taken as control.

2.5 GROWTH ON NUTRIENT AGAR WITH 5% GLUCOSE

Isolates were streaked on nutrient agar with 5% glucose (Nutrient agar 28g; 50g glucose in 1 litre distilled water with pH 7 and autoclaved at 121^o C for 15 minutes) and incubated at 28^oC for 48 -72 hours. Mucoïd and yellow colony growth on this medium is one of the characteristics that differentiate *Xanthomonas campestris* from other *Xanthomonas* species (3). Therefore the growth and colony colour were recorded.

2.6 PRESENCE OF XANTHOMONADIN PIGMENT

Following the procedure used by Bobosha (2), each isolate was streaked on nutrient agar and incubated at 28°C for 48 hours. About 2-3 loopful of each bacterial isolate from nutrient agar was transferred to 3ml of methanol in test tubes and placed in boiling water bath until the pigment was removed. The suspension was then centrifuged at 13,000rpm for 15 minutes to remove cell debris. The supernatant was decanted and the methanol allowed to evaporate by keeping the methanol extract in 50-60°C water bath until the optical density of the pigment extract reaches 0.4 at 443nm. Five nanolitre of each extract was spotted on a precoated silica gel plate and a total of 30nl was spotted. The solvent was allowed to move slowly approximately 10cm on the gel plate. A yellow spot was taken as positive for the presence of the pigment as described by Vauterin *et al.* (19).

2.7 CASEIN HYDROLYSIS

The ability of *Xanthomonas axonopodis* pv. *vignicola* to degrade the protein casein by producing proteolytic exo-enzymes was tested by growing the isolates on milk agar plates (Skim milk powder, 100g; Peptone, 5g; Agar, 15g in 1 litre distilled water with pH 7.2 autoclaved at 121°C for 15 minutes). Clear zone around the growth of the isolates was recorded as positive for casein hydrolysis (2).

2.8 STARCH HYDROLYSIS

The isolates were streaked on starch agar medium (soluble starch, 2.0g; Nutrient agar, 28.0g, in 1 litre distilled water with pH 7 and autoclaved at 121°C for 15 minutes) to evaluate their ability to hydrolyze starch (amylase production). The plates were incubated at 28°C for 7 days; starch hydrolysis was observed by flooding the plates with Lugol's iodine solution for 30 seconds. The appearance of yellowish and reddish clear zones around the line of growth of each isolate indicated starch hydrolysis (2).

2.9 SALT TOLERANCE

To test for salt tolerance, isolates were inoculated onto nutrient broth with 0%, 1%, 2%, 3%, 4% and 5% NaCl concentration as described by (2). Inoculated salt free (0%) nutrient broth was used as control. The presence or absence of growth was recorded.

2.10 CATALASE TEST

Few drops (3) of hydrogen peroxide were added on the surface of 48hour old culture of each isolate on YPSA medium. Bubble formation was recorded as positive for catalase activity (8).

2.11 OXIDASE REACTION

One ml of distilled water was added to make 1% solution of 0.01g tetramethylphenylene diamine dihydrochloride. A few drops of the reagent were pipetted on to a filter paper. Using a wire loop, a loopful culture of the test organism was rubbed across the impregnated filter paper. The observation was recorded as positive with purple colour or negative with no purple colour after 60 seconds (8).

2.12 GENETIC CHARACTERIZATION OF THE ISOLATES USING REPETITIVE PCR

2.12.1 DNA EXTRACTION

The DNA extraction was done following the protocol in Vivantis GF-1 Bacterial DNA Extraction Kit for all isolates. In addition, bacterial isolate from soybean bacterial blight infection was included to see whether this causal agent has any similarity with CoBB causal agent. A loopful of each isolate was grown to saturation in 5ml of peptone water overnight. From each of the cultures, 2ml was suspended in eppendorf tube and micro centrifuged at 6,000xg for 2min. The supernatant was decanted completely leaving only the pellet. Pellet was resuspended in 100µl buffer R1 and mixed by repeated pipetting.

The lysate was treated with 10µl lysozyme, mixed thoroughly and incubated at 37°C for 20min after which it was centrifuged at 10,000xg for 3min to pelletize the digested cells and the supernatant decanted completely. Pellet was

resuspended in 180µl of Buffer R2 and 20µl of proteinase K was added, mixed thoroughly and incubated at 65°C for 20min with occasional mixing every 5min. Twenty micro litre of RNAase A was added, mixed and incubated at 37°C for 5min. Four hundred micro litre of Buffer BG was added and mixed thoroughly by inverting tube several times until a homogenous solution was obtained, then it was incubated at 65°C for 10min.

Absolute ethanol of 200µl was added and mixed thoroughly to avoid uneven precipitation of nucleic acid due to high local ethanol concentrations. The sample was transferred to a column assembled in a clean tube and centrifuged at 10,000xg for 1min. The flow through was then discarded. The column was washed with 750µl of wash buffer and centrifuged at 10,000xg for 1min and again centrifuged at 10,000xg for 1min to remove residual ethanol. The column was then placed in a clean microcentrifuge tube and 50µl of pre-heated Elution Buffer was added directly onto column membrane and allowed to stand for 2min; centrifuged at 10,000xg for 1min to elute DNA. DNA was stored at 4°C. The purity of the extracted DNA was evaluated by running on 1% agarose gel.

2.12.2 AGAROSE GEL ELECTROPHORESIS

The gel was prepared using electrophoresis buffer and electrophoresis-grade agarose by melting in a microwave oven, mixing, cooling to 55°C, pouring into a sealed gel casting platform, and inserting the gel comb. After the gel has hardened, the seal was removed from the gel casting platform and the gel comb withdrawn. This was placed into an electrophoresis tank containing sufficient electrophoresis buffer to cover gel approximately 1mm. DNA samples with an appropriate amount of 10x loading dye were prepared and loaded into wells with a pipette and ran at 80volt and 37ampere for 45min and photographed using UV transilluminator

2.12.3 PCR ANALYSIS

The primers used for PCR amplification were SSR primers synthesized in Mike Timko's Laboratory, University of Virginia, USA. PCR analysis was done with 4 primer pairs EX-39, EX-40, EX-41 and EX-43. Accupower PCR premix tube (BIONEER) was used for the PCR reaction to which 1µl of DNA sample, 1µl of primer pair and 18µl of water (Molecular Biology Grade -Lonza) were added. Each PCR premix tube (20µl final volume) contained: 1U Taq DNA polymerase, 250µM dNTP mix, 10mM Tris-HCL (pH 9.0), 30mM KCL, 1.5mM MgCl₂ and stabilizer/tracking dye.

PCR reactions were performed in a heated lid thermal cycler (My Cycler) operated as follows:

1 cycle of initial denaturing at 94°C for 30s,

35 cycles of denaturation at 94°C for 30 sec, followed by annealing at 55°C for 30s and extension at 72°C for 2 min.

A final extension cycle of 10 min at 72°C was added to ensure completion of the final amplification products.

2.12.4 ANALYSIS OF PCR PRODUCT

A 10µl of the final PCR product was electrophoresed on a 2% agarose gel with ethidium bromide staining. The gels were run for approximately 1 h 20 min at 170 voltage in 1 X Tris acetic acid (TAE) buffer (45 mmol L⁻¹ glacial acetic acid, 0.5 mmol l⁻¹ ethylenediaminetetra acetic acid (EDTA), (pH, 8.4). A 1 kb DNA molecular marker ladder was loaded in the first well for band size determination of PCR products. The ethidium bromide-stained gel was visualized on an UV transilluminator and photographed using a Polaroid camera.

2.12.5 DATA ANALYSIS

The bands in each lane were taken for analysis. In the regions where no bands were observed, 0 was used to indicate the absence. The analysis was done using the similarity index $SAB = 2NAB / NA + NB$ (11).

Where SAB = sum of isolate A and B; NA = number of bands for isolate A; NB = number of bands for isolate B.

3 RESULTS

3.1 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

Bacterial cells were seen stained deep blue; while plant tissues appear paler greenish blue. The growth of isolates on nutrient agar with 5% glucose medium showed variation in colour and growth character of colonies. MKD388 -1 isolate

showed smooth, yellow colony colour with high mucoid type of growth. GUM391 appeared smooth, yellow to creamy colony colour with less mucoid growth. Similarly, GBK205-8 was smooth, yellow tiny colony with high mucoid growth. Among the isolates tested for their Xanthomonadin pigment production, MKD388-1 and GBK205-8 isolates produced the pigment while GUM391 isolate did not. Gram reaction was negative for all the isolates while catalase reaction was conversely positive for all. In casein hydrolysis ability, MKD388-1 and GUM391 isolates were able to hydrolyse casein up to 4cm clear zone while GBK205-8 hydrolysed casein up to 2cm. Yellowish clear zones were seen around GUM391 isolate and GBK205-8, while reddish clear zones were observed around the growth of MKD388-1 isolate indicating that starch has been partially hydrolyzed to dextrins. (Table 3.1).

For salt tolerance test, all the isolates tolerated 1% sodium chloride (NaCl) concentration by growing profusely, MKD388-1 and GUM391 isolates also grew profusely on 2% NaCl while GBK205-8 isolate only showed abundant growth. For 3% and 4% NaCl concentrations, all isolates showed abundant and sparse growth respectively. It was only on 5% NaCl that none of the isolates was found tolerant. (Table 3.2).

TABLE 3.1. MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS of *X. axonopodis* pv. *vignicola*

Isolate	MKD388-1	GUM391	GBK205-8
Growth on NA with 5% glucose	Lightyellow colony and Mucoid growth	Creamy colony with less mucoid growth	Yellow tiny colony with high mucoid growth
Xanthomonadin production	+	-	+
Gram reaction	-	-	-
Catalase reaction	+	+	+
Oxidase reaction	-	-	+
Casein hydrolysis	+3	+3	+2
Starch hydrolysis	+1	+3	+3

NB: +3=3-4cm clear zone, +2=1-2cm clear zone, +1 =< 1cm clear zone, + = positive, - = negative

TABLE 3.2. SODIUM CHLORIDE (NaCl) TOLERANCE OF *X. axonopodis* pv. *vignicola* ISOLATES

NaCl concentration(%)	Isolate		
	MKD 388-1	GUM 391	GBK 205-8
1	+++	+++	+++
2	+++	+++	++
3	++	++	++
4	+	+	+
5	-	-	-

+++ = profuse growth, ++ = abundant growth, + = sparse growth, - = no growth

3.2 PCR PRODUCTS

Band pattern of the isolates

The DNA extraction procedure yielded high molecular weight of DNA measuring up to 20ng (Plate 1). Out of the 4 primers screened for amplification of *Xav* DNA, only 1 resulted in non-distinct amplification product, it was therefore discarded and the remaining 3 generated reproducible patterns. Analysis of 3 *Xav* and 1 *Xag* Isolates presented a total of 14 amplicons from the three primer combinations. The highest number of amplicons (9) was produced by primer EX-40 and the lowest number of amplicons (2) by primer EX-39

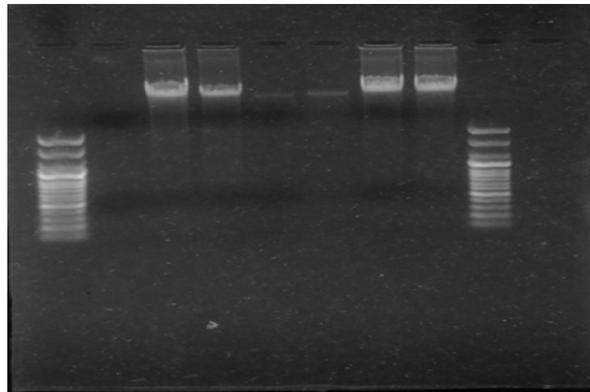
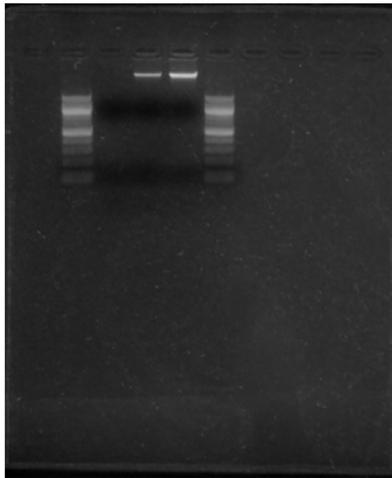
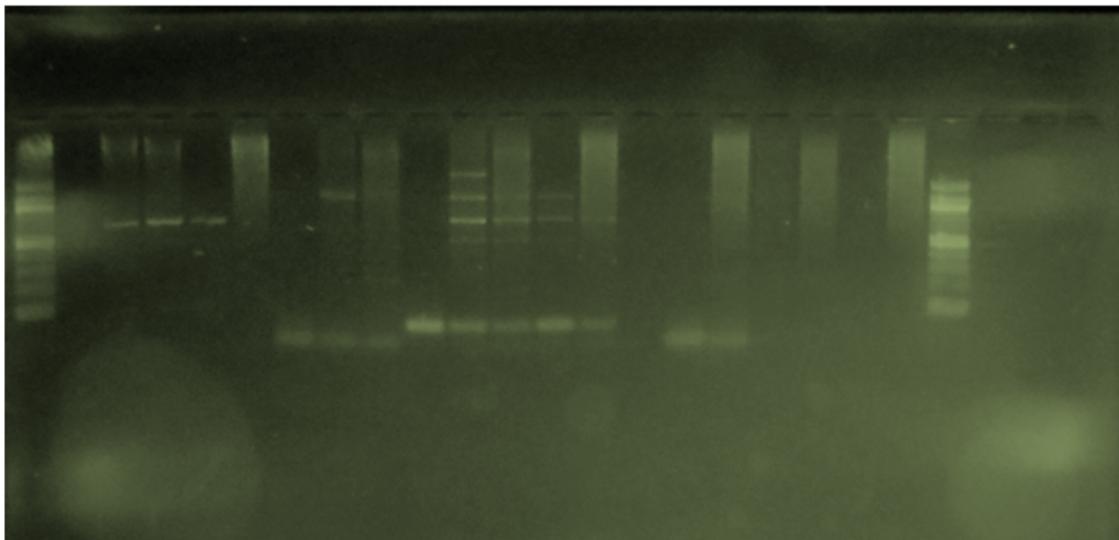


Plate 1: Extracted DNA on 1% agarose

L A1 A2 A3 A4A5 B1 B2 B3 B4 B5 C1 C2 C3 C4 C5 D1 D2 D3 D4 D5 L



N.B: L= Ladder, A=EX-39, B=EX-40, C=EX-41, D=EX-43, 1=Blank, 2=SB, 3=GUM391 4=MKD388-1, 5=GBK205-8

Plate 2: Different band patterns of the isolates stained with Ethidium Bromide

Table 3.3. shows similarity obtained for each pair of isolates. *Xanthomonas* isolate from soybean (SB) showed only 50% similarity to GUM391. There was a very high similarity index of 80% between MKD388-1/GUM391 and SB/MKD388-1. However, no similarity was observed between GBK205-8/MKD388-1, GUM391/GBK205-8 and SB/GBK205-8

**TABLE 3.3. SUMMARY OF GENTIC SIMILARITY AMONG ISOLATES
(SAB=2NAB/NA=NB)**

isolates	% similarity	Remark
SB/GUM391	50	SIMILAR
MKD388-1/GUM391	80	SIMILAR
GBK205-8/MKD388-1	-	NO SIMILARITY
SB/GBK205-8	38	NO SIMILARITY
SB/MKD388-1	80	SIMILAR
GUM391/GBK205-8	-	NO SIMILARITY

4 DISCUSSION AND CONCLUSION

The bacteriological properties of *Xanthomonas axonopodis* pv. *vignicola* observed in Makurdi, Guma and Gboko isolates of Benue State, Nigeria are within the range of variability exhibited by members of the genus *Xanthomonas* (9). In general, morphological and biochemical characteristics of the isolates showed different colours and growth patterns that were characteristics of members of this group of organisms. This confirmed that the isolates were actually *X. axonopodis* pv. *vignicola*. The variation in colour, pigmentation and biochemical characteristics of the isolate colonies could have stem from the different areas where the isolates were obtained and this observation agrees with the findings of (2).

The presence of xanthomonadin pigment is usually taken as a distinct character of the genus *xanthomonas*. However, Bradbury (3) observed that the absence of the pigment does not exclude the isolate from the genus. There could be possibility of losing the pigment during culturing, or on the other hand, the isolates might not carry that character like the white pathovars of *Xanthomonas campestris* as reported by Sugimori and Oliveira (18).

Salt tolerance ability by the isolates reflected what was reported by Khatri-Chhetri (9) and Bobosha (2) but not to the same extent, as 5% NaCl suppressed the growth of all *X. axonopodis* pv. *vignicola* isolates in our study. Bradbury (3) described the characteristics of *Xanthomonas axonopodis* like starch hydrolysis with neither positive nor negative reaction. This indicates the possibility of finding pathovars and strains as positive or negative for that specific test within the species and may account for variations among isolates of a given population. Genetic similarity among *X. axonopodis* pv. *vignicola* isolates have been previously demonstrated through random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and repetitive element-polymerase chain reaction by Ah-You *et al.* (1) and Mhedbi-Hajri *et al.* (13). There was also a relative predominance of a specific group of *X. axonopodis* isolates in East Africa (14) that was found in Spain (10) but not in Americas. In the present study, *X. axonopodis* pv. *vignicola* isolates from Makurdi and Guma Local Governments showed high levels of similarity. The low level of molecular variation may be that the isolates are evolutionary related. While the no similarity of Gboko isolate to any other isolate support the hypothesis that strains from different ecological zones form genetically and evolutionary separate groups; therefore resistance developed to a strain in a particular zone will not be effective on another strain in a different zone. Because of the low similarity observed in soybean isolate and cowpea isolates, the hypothesis that *Xanthomonas* strains from soybean are genetically separate from *Xanthomonas* strains of cowpea is supported. Further investigation of strain variation in bacterial blight strains may be required to develop SSR markers to be specifically used for bacterial blight. It has been demonstrated in this study that SSR markers designed for cowpea may be used in *X. axonopodis* pv. *vignicola* diversity studies and may also facilitate the identification of polymorphisms linked to virulence factors and contribute to the understanding of plant-bacteria interactions at the molecular level.

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