Taxonomic Repositioning of *Xanthomonas campestris* pv. *viticola* (Nayudu 1972) Dye 1978 as *Xanthomonas citri* pv. *viticola* (Nayudu 1972) Dye 1978 comb. nov. and Emendation of the Description of *Xanthomonas citri* pv. *anacardii* to Include Pigmented Isolates Pathogenic to Cashew Plant

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ABSTRACT

Grapevine bacterial canker, which is caused by *Xanthomonas campestris* pv. *viticola*, is one of the most important grapevine diseases in the northeastern region of Brazil. This disease causes severe damage and represents a high potential risk to the development of Brazilian viticulture. In turn, pigmented isolates pathogenic to cashew plant, making cashew fruit unfit for sale, also have been detected in Northeastern Brazil. Given that the taxonomic position of these bacteria is unclear, the multilocus sequence analysis (MLSA) technique, average nucleotide identity (ANI) values and tetranucleotide frequency correlation coefficients (TETRA) were used to analyze their phylogenetic

relationship in relation to other *Xanthomonas* species. *X. campestris* pv. *viticola* was closely related to *X. citri* pv. *mangiferaeindicae* (repetitive-polymerase chain reaction [rep-PCR], MLSA, and ANI) and *X. citri* subsp. *citri* (MLSA and ANI). Pigmented isolates pathogenic to cashew plant were closely related to *X. citri* pv. *anacardii* (rep-PCR, MLSA, ANI, and TETRA). The results obtained in this study support the emendation of the description of *X. citri* pv. *anacardii* to include pigmented isolates of *Xanthomonas* pathogenic to cashew plant. In addition, the reclassification of *X. campestris* pv. *viticola* as *X. citri* pv. *viticola* comb. nov. is suggested.

Brazil is the eighth largest fruit producer worldwide (Food and Agriculture Organization of the United Nations 2018), and the northeastern region is the main tropical fruit producer and exporter (Lopes et al. 2009), accounting for more than 99% of the national grape (*Vitis vinifera* L.) export (Lazzarotto and Fioravanço 2013) and more than 98% of cashew (*Anacardium occidentale* L.) production (Instituto Brasileiro de Geografia e Estatística 2018). In this region, bacterial canker caused by *Xanthomonas campestris* pv. *viticola* (Nayudu) Dye is one of the most important grapevine diseases, responsible for severe damage and representing a serious potential risk to Brazilian viticulture development (Araújo et al. 2005; Rodrigues Neto et al. 2011). This bacterium cause cankers on the twigs, branches, petioles and veins as well as depressed dark lesions on berries. In turn, pigmented isolates of *X. citri* (ex Hasse) Gabriel et al. pathogenic to Anacardiaceae, which apparently are

variants of *X. citri* pv. *anacardii* (Starr and Garces) Constantin et al. were recently detected in the Brazilian northeast, causing angular leaf spots and dark lesions in the leaf veins and surrounding tissues as well as dark necrotic lesions on fruits of cashew tree (Gama et al. 2011; Viana et al. 2006). Considering that young infected fruit results in nuts unfit for sale (Viana et al. 2006), these isolates represent a serious threat to this crop within the region.

The causal agent of grapevine bacterial canker was initially named *Pseudomonas viticola* Nayudu (Nayudu 1972) and subsequently reclassified as *X. campestris* pv. *viticola* (Young et al. 1978), which currently remains the valid nomenclature (Bull et al. 2010). However, since 1995, *X. campestris* has been known to only harbor isolates pathogenic to brassicas (Vauterin et al. 1995). Since that time, studies performed based on the *rpf* (regulator of pathogenicity) region (Takita et al. 2004), the *copA* gene (Marques 2007), the *gyrB* housekeeping gene (Parkinson et al. 2009), comparative analyses of genomic sequences (Takita et al. 2006), and genomic analysis (Bansal et al. 2017; Midha and Patil 2014) have demonstrated a close relationship between *X. campestris* pv. *viticola* and *X. citri* pv. *citri* (ex. Hasse) Constantin et al. (synonymy *X. citri* subsp. *citri*). Contradicting these results, multilocus sequence analyses (MLSA) (*rpoA*, *rpoB*, and spacer region 16S-23S rDNA) and DNA-DNA hybridization have demonstrated that *X. campestris* pv. *viticola* is more closely related to *X. axonopodis* sensu Rademaker et al. (2005) (Ferreira-Tonin 2012; Ferreira-Tonin et al. 2011).

Over the years, the taxonomic positions of the pathovars of *X. citri* pathogenic to Anacardiaceae have been modified numerous times. Originally, these pathovars were named *Bacillus mangiferae*

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(Doidge 1915) and were reclassified as *Pseudomonas mangiferaeindicae* (Patel et al. 1948a, b) and *Erwinia mangiferae* (Breed et al. 1957) until being correctly placed in genus *Xanthomonas*, initially as *X. campestris* pv. *mangiferaeindicae* (Robbs et al. 1974). Next, a new reclassification was performed based on the pathological variation and evolutionary divergence of the genomes observed using amplified fragment length polymorphisms (AFLP), in which *X. campestris* pv. *mangiferaeindicae* was proposed to be separated into three pathovars, which resulted in the following current taxonomic position: *X. axonopodis* pv. *mangiferaeindicae* (Starr and Garces) Ah-You et al. (angular leaf spot of mango), *X. axonopodis* pv. *anacardii* (angular leaf spot of cashew), and *X. axonopodis* pv. *spondiae* (Starr and Garces) Ah-You et al. (angular leaf spot of ambarella) (Ah-You et al. 2007). Based on data from MLSA, DNA-DNA hybridization, average nucleotide identity (ANI) values and Biolog GEN III MicroPlate System, a new reclassification for pvs. *mangiferaeindicae* and *anacardii* was proposed: *X. citri* pv. *anacardii* and *X. citri* pv. *mangiferaeindicae* (Constantin et al. 2016). Interestingly, both pigmented and non-pigmented isolates of *Xanthomonas* have been isolated from cashew trees in Brazil (Gama et al. 2011), however pigmented strains have not been included in the reclassification studies conducted to date (Ah-You et al. 2007, 2009; Constantin et al. 2016).

Because *X. campestris* pv. *viticola* is considered a quarantine pest present in Brazil restricted to the states of Bahia, Ceará, Pernambuco, and Roraima (Regulatory Instruction 59/2013) (Brazil Ministério da Agricultura, Pecuária e Abastecimento 2001) and pathovars of *X. citri* pose higher risk in farming plants of family Anacardiaceae in northeastern Brazil (Gama et al. 2011), the objective of this study was to clarify the taxonomic position of these plant pathogenic bacteria. Thus, we used MLSA, ANI values, tetranucleotide frequency correlation coefficients (TETRA) and Biolog GEN III MicroPlate System to reclassify *X. campestris* pv. *viticola* as *X. citri* pv. *viticola* (Nayudu 1972) Dye 1978 comb. nov. and perform the emendation of the description of *X. citri* pv. *anacardii* to include pigmented strains pathogenic to cashew plant.

MATERIALS AND METHODS

Bacterial isolates, culture conditions, and DNA extraction.

Eighteen isolates of *X. campestris* pv. *viticola* and 10 pigmented isolates pathogenic to cashew plant were selected to determine their taxonomic position (Table 1). The *X. campestris* pv. *viticola* isolates were selected from a collection of 116 isolates (Supplementary Table S1) based on rep-PCR analysis performed in this study and the pigmented isolates pathogenic to cashew plant were selected based on intrapathovar variability described in previous studies (Gama et al. 2011). Four isolates of *X. campestris* pv. *passiflorae*, four isolates of *X. citri* pv. *anacardii* and one isolate each of *X. citri* pv. *mangiferaeindicae*, *X. campestris* pv. *campestris*, *X. citri* subsp. *malvacearum*, *X. vesicatoria*, and *Pseudomonas syringae* pv. *tomato* were included in the metabolic and molecular analyses for comparison.

To determine the taxonomic position of *X. campestris* pv. *viticola* and the pigmented isolates pathogenic to cashew plant, the *gyrB* (PopSet: 188090225), *rpoD* (PopSet: 188090463), *fyuA* (PopSet: 188090027) and *dnaK* (PopSet: 188089823) gene sequences from 46 isolates representing 23 species of genus *Xanthomonas* and subgroups 9.1 through 9.6 of *X. axonopodis sensu* Rademaker et al. (2005) were selected from the GenBank database and included in the phylogenetic analyses.

Unless otherwise noted, the isolates were cultured in NYDA medium (20 g of agar, 10 g of dextrose, 5 g of peptone, 5 g of yeast extract, and 3 g of meat extract, completing the volume with sterile distilled water [SDW] to 1,000 ml) for 36 h at 29°C (±0.5°C).

Molecular characterization. DNA extraction from the isolates used in this study was performed using the MiniPrep kit for bacterial genomic DNA extraction (Axygen Biosciences, MA) following the manufacturer's recommendations. The genomic DNA was quantified as previously described (Gama et al. 2011).

The repetitive element palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and BOX element (BOX)-polymerase chain reaction (PCR) analyses were performed according to Louws et al. (1994), with some modifications: 1× PCR Master Mix 2× (0.05 U/μl of *Taq* DNA polymerase, reaction buffer, 4 mM MgCl₂, 0.4 mM of each dNTP), 2 μM of each primer, and 200 ng of DNA. The samples were amplified in a thermocycler, model PTC-100 (MJ Research, MA). Negative controls (DNA-free reactions) were included in all of the experiments to test for the presence of contaminants. Twelve microliters of each amplification reaction was mixed with 3 μl of loading buffer and 2.5 μl of Safe SYBER, applying a total volume of 17.5 μl in each well of the gel. The amplified fragments were visualized using a 1.5% agarose gel. The electrophoretic run was performed for 3 h at 80 V in 0.5× Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) buffer using the GenRuler 100-bp DNA Ladder (Fermentas Life Sciences, Ontario, Canada) and GenRuler 1-kb DNA Ladder (Fermentas Life Sciences) markers. Next, the gel was photographed. The analyses were performed two times.

The amplification profiles generated with the REP-, ERIC-, and BOX-PCR primers were visually analyzed according to the presence (1) or absence (0) of 100- to 3,000-bp bands, and only reproducible bands (present in both analyses) were recorded. The data generated with each primer were analyzed separately and together using the FreeTree program (Pavlicek et al. 1999). The profiles of the strains of *X. campestris* pv. *viticola* and the species/pathovars of *Xanthomonas* described on Table 1 were analyzed separately. The Jaccard similarity coefficient was used to determine the genetic relationships between the isolates, and a distance tree was constructed using the unweighted pair group method with arithmetic mean (UPGMA) method. Bootstrap analyses with 1,000 replicates were conducted to evaluate the internal consistency of the branches (Hampl et al. 2001).

Phylogenetic characterization. Amplification and sequencing. Primers were used that have been developed to amplify partial sequences of the genes *gyrB* housekeeping (DNA gyrase beta subunit), *rpoD* (RNA polymerase sigma subunit), *fyuA* (tonB-dependent receptor), *dnaK* (chaperone protein dnaK) (Young et al. 2008), and *hrpB* for the pathogenicity and hypersensitive response cluster (Leite et al. 1994). The reactions comprised the following: 1× PCR Master Mix, 0.5 μM of each primer, and 200 ng of DNA. The samples were amplified in a thermocycler, model PTC-100, and subjected to the following conditions: *gyrB* and *rpoD*—94°C for 3 min, followed by 30 cycles of 30 s at 94°C for denaturation, 30 s at 54°C for annealing of the primers, and 1 min at 72°C for extension of the new strand; *hrpB*—95°C for 3 min, followed by 30 cycles of 30 s at 95°C for denaturation, 30 s at 52°C for annealing of the primers, and 1 min at 72°C for extension of the new strand. A 10-min cycle at 72°C was performed for the final extension of the sequences of the three genes. Negative controls (DNA-free reactions) were included in all of the experiments to test for the presence of contaminants. The amplified fragments were visualized by 1% agarose gel and an electrophoretic run performed for 1.5 h at 80 V in 0.5× TBE buffer using the GenRuler 100-bp DNA Ladder marker. The PCR products were purified using a Clean-up kit (Axygen Biosciences), and the forward and reverse strands for each marker were sequenced by Macrogen (Seoul, South Korea). Quality analysis of the nucleotide sequences and contig assembly were performed using the Staden Package (Staden et al. 1998).

Phylogenetic analyses. The nucleotide sequences were aligned using Muscle (Edgar 2004), available in Molecular Evolutionary Genetic Analysis (MEGA) v. 5.20 (Tamura et al. 2011), and manually adjusted to allow for maximum similarity of the sequences. The ends of the sequences were trimmed, resulting in 865-, 873-, 698-, 940-, and 731-nucleotide sequences for *gyrB*, *rpoD*, *fyuA*, *dnaK*, and *hrpB*, respectively.

Two approaches were performed for MLSA. In the first approach, analyses were performed with the sequences (Supplementary Table S2) of the *Xanthomonas* isolates used in this study (Table 1) together with sequences of 23 species of *Xanthomonas* collected from the GenBank

database using the partial sequences of the *gyrB*, *rpoD*, *fyuA*, and *dnaK* genes alone as well as the concatenated sequence. In the second approach, analyses were performed aiming for more precise infra-specific delineation from the partial sequences of the *gyrB*, *rpoD*, *fyuA*, *dnaK*, and *hrpB* genes alone as well as from the concatenated sequence. In this second approach, only the isolates listed in Table 1 were analyzed. The inconsistency among the sequences of the concatenated genes in both approaches was evaluated with the phylogenetic analysis using parsimony (PAUP*) v. 4.0b10 software (Swofford 2002) using the partition homogeneity test based on an analysis of the incongruence length difference (ILD) with 100 pseudoreplicates (bootstrap).

The individual and concatenated sequences were compared using maximum likelihood and Bayesian inference. The analyses were performed using models of nucleotide substitution selected by Akaike information criterion (AIC) using Mr. ModelTest 2.3 (Nylander 2004). The maximum likelihood analyses were performed via bootstrap of 1000 replicates using the PhyML 3.1 program (Guindon and Gascuel 2003). The Bayesian inference analyses were performed using the Mr. Bayes v. 3.2.6 (Ronquist et al. 2012) that is implemented in CIPRES server (<https://www.phylo.org/portal2/home.action>), with

100,000,000 generations of the Monte Carlo Markov chain, where sampling was performed every 1,000 generations, starting the process from a random tree.

Whole-genome DNA sequencing, assembly, calculation of ANI values, and TETRA coefficients. Standard DNA library preparation and genome sequencing was performed using the Illumina HiSeq 2500 platform at the Functional Genomics Center of the University of São Paulo. A paired-end (PE) DNA library was constructed with the Illumina Nextera XT DNA Library Prep Kit v4, and sequencing was performed on a HiSeq Flow Cell v4, with the HiSeq SBS Kit v4 and paired-reads 100 bp (2×). Initially, quality reads were analyzed by FastQC (Andrews 2010) and then the reads were treated by removing the adapters using FASTX-Toolkit (v. 0.0.13) (Hannon Lab 2014). Then, de novo assembly was performed with the trimmed reads data set using the SPAdes software (v. 1.10) (Bankevich et al. 2012).

The whole-genomes sequences of 117 strains belonging to different species of *Xanthomonas* genus available in the NCBI database were downloaded and analyzed along to the draft genomes of the strains LMG965 (=IBSBF1967^{PT}) (accession numbers CBZT010000001 to

TABLE 1. Description of the *Xanthomonas* isolates used in this study

Code ^x	Species/pathovar	Host	Year/origin ^y
IBSBF1967 ^{PT}	<i>Xanthomonas campestris</i> pv. <i>viticola</i>	<i>Vitis vinifera</i>	1972 India
CCRMXCV8	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2008 Brazil (PE)
CCRMXCV90	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2009 Brazil (BA)
CCRMXCV79	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2009 Brazil (PE)
CCRMXCV76	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2009 Brazil (PE)
CCRMXCV68	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2009 Brazil (BA)
CCRMXCV55	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2009 Brazil (BA)
CCRMXCV18	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2009 Brazil (PE)
CCRMXCV13	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2009 Brazil (PE)
CCRMXCV17	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2009 Brazil (PE)
CCRMXCV80	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2009 Brazil (PE)
CCRMXCV117	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2009 Brazil (PE)
CCRMXCV108	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2009 Brazil (PE)
CCRMXCV156	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2010 Brazil (PE)
CCRMXCV178	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2011 Brazil (BA)
CCRMXCV142	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2010 Brazil (BA)
CCRMXCV202	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2011 Brazil (PE)
CCRMXCV229	<i>X. campestris</i> pv. <i>viticola</i>	<i>V. vinifera</i>	2012 Brazil (BA)
CCRMCAST2	<i>Xanthomonas</i> ^z	<i>Anacardium occidentale</i>	2008 Brazil (PI)
CCRMCCP76	<i>Xanthomonas</i> ^z	<i>A. occidentale</i>	2006 Brazil (CE)
CCRMATAQ3	<i>Xanthomonas</i> ^z	<i>A. occidentale</i>	2009 Brazil (SP)
CCRMATAQ9	<i>Xanthomonas</i> ^z	<i>A. occidentale</i>	2009 Brazil (SP)
CCRMATAQ10	<i>Xanthomonas</i> ^z	<i>A. occidentale</i>	2009 Brazil (SP)
CCRMATAQ13	<i>Xanthomonas</i> ^z	<i>A. occidentale</i>	2009 Brazil (SP)
CCRMATAQ18	<i>Xanthomonas</i> ^z	<i>A. occidentale</i>	2009 Brazil (SP)
CCRMXCMF1	<i>Xanthomonas</i> ^z	<i>A. occidentale</i>	2008 Brazil (PI)
CCRMXCMF2	<i>Xanthomonas</i> ^z	<i>A. occidentale</i>	2008 Brazil (PI)
CCRMPC2	<i>Xanthomonas</i> ^z	<i>A. occidentale</i>	2009 Brazil (PI)
IBSBF2579 ^{PT}	<i>X. citri</i> pv. <i>anacardii</i>	<i>A. occidentale</i>	Brazil
IBSBF1508	<i>X. citri</i> pv. <i>anacardii</i>	<i>A. occidentale</i>	Brazil
IBSBF1971	<i>X. citri</i> pv. <i>anacardii</i>	<i>A. occidentale</i>	Brazil
IBSBF657	<i>X. citri</i> pv. <i>anacardii</i>	<i>Mangifera indica</i>	1988 Brazil
IBSBF2586 ^{PT}	<i>X. citri</i> pv. <i>mangiferaeindicae</i>	<i>M. indica</i>	India
Xap1163	<i>X. axonopodis</i> pv. <i>passiflorae</i>	<i>Passiflora edulis</i>	Brazil
IBSBF960	<i>X. axonopodis</i> pv. <i>passiflorae</i>	<i>P. edulis</i>	1992 Brazil
IBSBF1007	<i>X. axonopodis</i> pv. <i>passiflorae</i>	<i>P. edulis</i>	1993 Brazil
IBSBF1343 ^{PT}	<i>X. axonopodis</i> pv. <i>passiflorae</i>	<i>P. edulis</i>	1967 Brazil
IBSBF959	<i>X. campestris</i> pv. <i>campestris</i>	<i>Brassica napus</i>	1992 Brazil
UnB824	<i>X. campestris</i> pv. <i>vesicatoria</i>	<i>Solanum lycopersicum</i>	1991 Brazil
IBSBF1733 ^{PT}	<i>X. citri</i> subsp. <i>malvacearum</i>	<i>Gossypium</i> sp.	1958 Sudan
CCRMPSST	<i>Pseudomonas syringae</i> pv. <i>tomato</i>	<i>S. lycopersicum</i>	1982 USA

^x IBSBF1967^{PT} (pathotype isolate) = ICMP3867, LMG965, and NCPPB2475; IBSBF2579^{PT} = ICMP4088; IBSBF2586^{PT} = ICMP5740, LMG941, NCPPB490, and ATCC11637; IBSBF959 = ICMP12188; IBSBF960 = ICMP12189; IBSBF1343^{PT} = ICMP3151, LMG810, and NCPPB2346; IBSBF1733^{PT} = ICMP5739 and LMG761; UnB824 = IBSBF905. IBSBF: Phytopathogenic Culture Collection of Instituto Biológico (São Paulo, Brazil); ICMP: International Collection of Microorganisms from Plants (Auckland, New Zealand); LMG: Belgian Coordinated Collections of Micro-Organisms (Ghent, Belgium); NCPPB: National Collection of Plant Pathogenic Bacteria (York, United Kingdom); UnB: Phytopathogenic Bacteria Collection of the Department of Phytopathology of the University of Brasília (Federal District, Brazil). CCRM = isolates from the Laboratory of Phytobacteriology (LAFIBAC) Culture Collection of Federal Rural University of Pernambuco-UFRPE (Pernambuco, Brazil).

^y BA = Bahia state, CE = Ceará state, PE = Pernambuco state, PI = Piauí state, and SP = São Paulo state.

^z Pigmented isolates pathogenic to cashew plant.

CBZT010000050) (Midha and Patil 2014) and CCRMXCV80 (accession number NWTJ01000000) (Lima et al. 2017) of *X. campestris* pv. *viticola*, the nonpigmented isolates CFBP2913 (accession number CP024057.1) and IBSBF2579^{PT} (Silva et al. 2018) of *X. citri* pv. *anacardii*, and the pigmented isolates pathogenic to cashew plant, CCRMTAQ13 and CCRMTAQ18, sequenced for this study and deposited at DDBJ/ENA/GenBank under the accession number PESG01000000 and PESH01000000, respectively. The list of the complete genomes downloaded from the NCBI and the draft genome analyzed, and extra details is provided in the Supplementary Table S3. The sequences were used for ANI (ANIm) and TETRA calculations (Richter and Rosselló-Móra 2009) using the pyani 0.2.7 Python3 module (<https://github.com/widowquinn/pyani>) (Pritchard et al. 2016) obtained by whole-genome alignments through NUCmer (Kurtz et al. 2004).

Metabolic characterization. Metabolic characterization was performed using a Biolog System, Inc. (Hayward, CA) using Biolog GenIII microplates (71 carbon sources and 23 inhibitory substances) according to the manufacturer's recommendations. The isolates were cultured in BUG medium (Biolog Universal Growth) at 29°C (±0.5°C) for 36 h, and the suspensions were prepared in IF-A inoculation fluid, adjusting the transmittance to 95%. Next, 150 µl of the suspension was added to the microplate wells, which were incubated at 33°C for 36 h. Visual evaluations were performed to observe the wells change color. The experiment was repeated to verify the generated profiles.

Pathological characterization. In separate experiments, five isolates of *X. campestris* pv. *viticola* (IBSBF1967^{PT}, CCRMXCV17, CCRMXCV80, CCRMXCV117, and CCRMXCV178), five pigmented isolates pathogenic to cashew plant (CCRMTAQ3, CCRMTAQ13, CCRMTAQ18, CCRMXCMF1, and CCRMXCMF2), three isolates of *X. citri* pv. *anacardii* (IBSBF657, IBSBF1508, and IBSBF2579^{PT}), and one isolate of *X. citri* pv. *mangiferaeindicae* (IBSBF2586^{PT}) were inoculated on the following: (i) grape seedling leaves (*V. vinifera* 'Red Globe'), and (ii) cashew leaves (clone CCP76). The concentrations of the bacterial suspensions were adjusted to 10⁸ CFU/ml using a spectrophotometer (Analyzer 500 M, São Paulo, Brazil) (Gama et al. 2011). The inoculation was performed into four points of a leaf blade (the first eight leaves of each seedling were used, starting at the apex) by infiltration of 100 µl (±10⁷ CFU) of the suspensions with the aid of a needleless hypodermic syringe (Ah-You et al. 2007). Leaves inoculated with SDW alone comprised the negative control. The experiment was completely randomized with four replicates such that each replicate comprised one leaf with four inoculation points per isolate. After the inoculations, the seedlings were incubated in a greenhouse and evaluated daily for 12 days to determine the incubation period, which is the number of days from inoculation until the onset of the first symptoms. Disease severity was evaluated at 12 days after inoculation, estimating the extent of damage in diametrically opposite directions. The population sizes in leaf lesions from grape and cashew were estimated 13 days after inoculation for isolates CCRMXCV80, CCRMXCMF1, IBSBF2579^{PT}, and IBSBF2586^{PT} as previously described (Ah-You et al. 2007). Four leaf fragments (1 cm²) were excised from the leaf blade, surface-sterilized and individually homogenized in 1 ml of SDW. Aliquots of the homogenized suspensions and of 10-fold dilutions obtained from the suspensions were plated on NYDA media. Bacterial population sizes were based on enumeration of isolates colonies. The experiment was performed twice.

Considering that no significant ($P \leq 0.05$) differences were observed regarding variance between the two experiments, the data were evaluated as replicates in time. The assumptions of the analysis of variance were verified by Shapiro-Wilk and Levene tests using the Statistix 9 software (Tallahassee, FL). The means were compared by the Scott-Knott test ($P \leq 0.05$) using the SAEG program version 9.0 (Federal University of Viçosa-UFV, Viçosa, Minas Gerais, Brazil) and by the least significant difference test using the Statistix 9 program.

RESULTS

Molecular characterization. The grouping analysis of the 116 isolates of *X. campestris* pv. *viticola* performed by rep-PCR data (REP, ERIC, and BOX-PCR) enabled the formation of a single group comprising all of the *X. campestris* pv. *viticola* isolates at the 88% similarity level and bootstrap values of 100% (Supplementary Fig. S1). Seven subgroups were defined at a 90% similarity level: subgroup I comprised 81% of the isolates, including the pathotype isolate IBSBF1967^{PT} and isolates collected in the states of Ceará (IBSBF2647), state unidentified (UnB1318), Paraná (4779-B), Roraima (BHV482), and São Paulo (IBSBF2738 and IBSBF2739); subgroup II consisted of isolates CCRMXCV13, CCRMXCV52, CCRMXCV178, and CCRMXCV208; subgroup III consisted of isolates CCRMXCV225, CCRMXCV79, CCRMXCV116, CCRMXCV156, and CCRMXCV206; subgroup IV consisted of isolates CCRMXCV229, CCRMXCV232, CCRMXCV228, and CCRMXCV205; subgroup V consisted of isolates CCRMXCV142 and CCRMXCV38; subgroup VI consisted of isolate CCRMXCV55; and subgroup VII consisted of isolates CCRMXCV171, CCRMXCV39, CCRMXCV5, CCRMXCV8, CCRMXCV117, and CCRMXCV68. No relationships were identified between the rep-PCR subgroups and the year, origin, organ, or cultivar from which the isolates were obtained.

Cluster analyses of the *Xanthomonas* species/pathovars listed in Table 1 using REP, ERIC, and BOX data alone (data not shown) linearly corroborated the analysis of the combined data, allowing for the formation of a single group containing all of the *Xanthomonas* isolates and the differentiation of *P. syringae* pv. *tomato* at 58% similarity and 100% bootstrap (Fig. 1). The following six groups were formed at 64% similarity: group I, comprising isolates of *X. citri* pv. *mangiferaeindicae*, *X. campestris* pv. *viticola*, and *X. citri* subsp. *malvacearum*; group II, comprising pigmented isolates pathogenic to cashew plant and *X. citri* pv. *anacardii*; group III, comprising *X. vesicatoria*; group IV, comprising isolates of *X. axonopodis* pv. *passiflorae*; group V, comprising *X. campestris* pv. *campestris*; and group VI, comprising *P. syringae* pv. *tomato*. Additionally, the groups I, II, and III found in this study corresponded with the groups 9.5, 9.6, and 9.2 of *X. axonopodis sensu* Rademaker et al. (2005), respectively.

Phylogenetic characterization. In the first approach performed with the *Xanthomonas* isolates used in this study (Table 1), together with 23 *Xanthomonas* species retrieved from the GenBank database, the phylogenetic trees constructed based on the partial sequences of the *gyrB*, *rpoD*, *fyuA*, and *dnaK* genes analyzed separately exhibited similar structures using the maximum likelihood and Bayesian inference methods. The ILD analysis demonstrated that those trees generated with individual sequences of the *gyrB*, *rpoD*, *fyuA*, and *dnaK* genes were highly consistent with the trees generated with the concatenated sequence ($P = 0.01$). The phylogenetic trees generated with the concatenated sequence (3,376 nucleotides) using the two methods cited also exhibited similar structures. A phylogenetic tree obtained using Bayesian inference with the general time reversible (GTR) model with gamma variation (G) and proportions of invariable sites (I) provided one true reproduction of the current taxonomic positions of the species and pathovars of *Xanthomonas* (Fig. 2). Two groups were formed as previously described by Hauben et al. (1997) and Young et al. (2008). Additionally, subgroups 9.1 to 9.6 of *X. axonopodis sensu* Rademaker et al. (2005) were satisfactorily reproduced.

The isolates of *X. campestris* pv. *viticola* (CCRMXCV17, CCRMXCV80, CCRMXCV117, and IBSBF1967^{PT}) and pigmented isolates pathogenic to cashew plant (CCRMTAQ13, CCRMTAQ18, and IBSBF2579^{PT}) clustered in the groups 9.5 and 9.6 of *X. axonopodis sensu* Rademaker et al. (2005), respectively, and within phylogenetic group I (PGI), subgroup 2 *sensu* Constantin et al. (2016).

In the second approach performed with the *Xanthomonas* isolates used in this study (Table 1), the phylogenetic trees constructed

The ILD analysis demonstrated that the sequences of the *gyrB*, *rpoD*, *fyuA*, *dnaK*, and *hrpB* genes (4,107 nucleotides) were highly inconsistent with the concatenated sequence ($P = 0.35$). The phylogenetic tree generated with the partial sequence of the *hrpB* gene by Bayesian inference with the GTR model and proportions of invariable sites (I) placed the isolates of *X. campestris* pv. *viticola* in subgroup 9.5 and the pigmented isolates pathogenic to cashew plant

in subgroup 9.6 (Fig. 3). However, in subgroup 9.5, all of the isolates of *X. campestris* pv. *viticola* grouped separately from the isolates of *X. citri* pv. *mangiferaeindicae* (IBSBF2586^{PT}) and *X. citri* subsp. *malvacearum* (IBSBF1733^{PT}) with values above 99% posterior probability. Similarly, in subgroup 9.6, all of the pigmented isolates pathogenic to cashew plant (CCRMCAST2, CCRMCCP76, CCRMTAQ3, CCRMTAQ9, CCRMTAQ10, CCRMTAQ13,

CCRMATAQ18, CCRMXCMF1, CCRMXCMF2, and CCRMPC2) grouped separately from the isolates of *X. citri* pv. *anacardii* with values above 99% posterior probability, indicating the branch's high reliability.

Whole genome sequence analysis. The ANI and TETRA data showed that isolates of *X. campestris* pv. *viticola* should not be classified as *X. campestris*. ANI values between the isolates of

Fig. 2. Multilocus sequence analysis performed by Bayesian inference from the partial sequences of the *gyrB*, *rpoD*, *fyuA*, and *dnaK* genes. The bar indicates the number of substitutions per site. The positions of *Xanthomonas campestris* pv. *viticola* and the pigmented isolates pathogenic to cashew plant in subgroups 9.5 and 9.6 of *Xanthomonas axonopodis* sensu Rademaker et al. (2005) are shown. Posterior probability values are shown on the branches and indicated by arrows. ^z indicates isolates reclassified by Constantin et al. (2016).

X. campestris pv. *viticola* [LMG965^{PT} = IBSBF1967^{PT}) and CCRMXCV80] and the type isolate of *X. campestris* pv. *campestris* (ATCC 33913^{PT}) were below 88% (data not shown). On the other hand, ANI values and TETRA coefficients between the isolates of *X. campestris* pv. *viticola* and *X. citri* pathovars were always above 96% and 0.99, respectively. We observed ANI values of 98.9 and 98.9% and TETRA coefficients of 0.999 and 0.995 between the isolates of *X. campestris* pv. *viticola* (IBSBF1967^{PT} and CCRMXCV80) and the pathotype isolate of *X. citri* pv. *mangiferaeindicae* (LMG 941^{PT} = IBSBF2586^{PT}), respectively. ANI values of 98.9 and 98.9% and TETRA coefficients of 0.999 and 0.996 were observed between the isolates of *X. campestris* pv. *viticola* (IBSBF1967^{PT} and CCRMXCV80) and the type isolate of *X. citri* subsp. *citri* (LMG 9322^T), respectively. In turn, ANI values of 96.3 and 96.3% and TETRA coefficients of 0.997 and 0.992 were detected between *X. campestris* pv. *viticola* (IBSBF1967^{PT} and CCRMXCV80) and *X. citri* pv. *aurantifolii* (strain 1566), respectively.

ANI values and TETRA coefficients between pigmented isolates pathogenic to cashew plant (CCRMQA13 and CCRMQA18) and *X. citri* pathovars were always above 96% and 0.99, respectively (data not shown). We observed ANI values of 99.5 and 99.5% and TETRA coefficients of 0.999 and 0.999 between the pigmented isolates pathogenic to cashew plant (CCRMQA13 and CCRMQA18) and the pathotype isolate of *X. citri* pv. *anacardii* (IBSBF2579^{PT}), while ANI values of 96.3 and 96.4% and TETRA coefficients of 0.994 and 0.997 were detected between the pigmented isolates pathogenic to cashew plant and the pathotype isolate of *X. citri* pv. *mangiferaeindicae* (IBSBF2586^{PT}).

Metabolic characterization. The reactions of *Xanthomonas* isolates analyzed in this study are represented by the percentage of isolates with positive, negative or variable reactions for each of the 94 biochemical tests contained in the Biolog Gen III microplates system (Supplementary Table S4). All isolates of *X. campestris* pv. *viticola* showed 100% positive reactions for L-malic acid, citric acid, α -keto-glutaric acid, D-cellobiose, D-fructose, D-galactose, D-mannose, D-trehalose, gentiobiose, L-fucose, N-acetyl-D-glucosamine, 1% NaCl, pectin, pH 6, sucrose, Tween 40, and α -D-glucose and negative reactions for D-aspartic acid, D-malic acid, formic acid, fusidic acid,

L-galactonic acid lactone, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, γ -amino-butyric acid, sodium bromate, sodium butyrate, lithium chlorite, D-arabitol, D-mannitol, D-serine, L-serine, D-sorbitol, α -D-lactose, guanidine HCl, minocycline, myo-inositol, 4% NaCl, 8% NaCl, Niaproof 4, potassium tellurite, troleandomycin, and β -methyl-D-glucoside.

All pigmented isolates pathogenic to cashew plant and isolates of *X. citri* pv. *anacardii* showed 100% positive reactions for L-malic acid, acetic acid, citric acid, L-glutamic, D-cellobiose, dextrin, D-fructose, D-galactose, D-trehalose, L-fucose, N-acetyl-D-glucosamine, pectin, pH 6, sucrose, and α -D-glucose and negative reactions for D-aspartic acid, D-malic acid, fusidic acid, L-galactonic acid lactone, L-pyrogutamic, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, nalidixic acid, p-hydroxy-phenylacetic acid, quinic acid, β -hydroxy- D,L-butiric acid, γ -amino-butyric acid, sodium bromate, sodium butyrate, lithium chlorite, D-mannitol, D-serine, L-serine, D-sorbitol, stachyose, guanidine HCl, L-arginine, L-histidine, minocycline, myo-inositol, 4% NaCl, 8% NaCl, Niaproof 4, potassium tellurite, troleandomycin, vancomycin, and β -methyl-D-glucoside. These isolates showed the same reaction in approximately 65% of substrates analyzed.

Pathological characterization. Only isolates of *X. campestris* pv. *viticola* were able to cause damage to grape leaves of cultivar Red Globe. However, no significant ($P < 0.05$) differences were observed among the isolates for the variables analyzed (data not shown). Population density of isolate CCRMXCV108 on grape cultivar Red Globe was 5.3×10^6 CFU per lesion (data not shown).

All of the isolates were able to cause damage to the cashew leaves. The cluster analysis performed with the epidemiologic data allowed for identification of two groups (A and B) based on the incubation period: group A, comprising two isolates of *X. campestris* pv. *viticola* and one isolate of *X. citri* pv. *anacardii* with an incubation period of 4.0 to 4.3 days, and group B, comprising the other isolates with an incubation period of 2.5 to 3.5 days (Table 2). Six groups were identified (A to F) based on the disease severity data: group A, comprising two pigmented isolates pathogenic to cashew plant with a severity of 6.9 to 7.4 mm; group B, comprising one pigmented isolated pathogenic to cashew plant with a severity of 5.8 mm; group

Fig. 3. Phylogenetic tree constructed by Bayesian inference from the partial sequence of the *hrpB* gene. The positions of *Xanthomonas campestris* pv. *viticola* and the pigmented isolates pathogenic to cashew plant in subgroups 9.5 and 9.6 *Xanthomonas axonopodis* sensu Rademaker et al. (2005) and separation among the pigmented isolates and *X. citri* pv. *anacardii* are shown. The bar indicates the number of substitutions per site. Posterior probability values are shown on the branches.

C, comprising one isolate of *X. citri* pv. *anacardii* with a severity of 5.2 mm; group D, comprising one pigmented isolate pathogenic to cashew plant with a severity of 4.9 mm; group E, comprising four isolates of *X. campestris* pv. *viticola*, two isolates of *X. citri* pv. *anacardii*, one pigmented isolate pathogenic to cashew plant and one isolate of *X. citri* pv. *mangiferaeindicae* with a severity of 3.5 to 4.3 mm; and group F, comprising the pathotype isolate of *X. campestris* pv. *viticola* (IBSBF1967) with a severity of 1.5 mm.

On cashew tree seedlings of clone CCP76 no significant ($P < 0.05$) differences were observed among population density of the pigmented isolates pathogenic to cashew plant (9.4×10^7 CFU per lesion) and *X. citri* pv. *anacardii* (8.3×10^7 CFU per lesion) (Table 3). The population density of these isolates was significantly larger than the population density of the isolates of *X. citri* pv. *mangiferaeindicae* (5.0×10^7 CFU per lesion) and *X. campestris* pv. *viticola* (9.87×10^2 CFU per lesion).

DISCUSSION

The genetic structure of the 116 *X. campestris* pv. *viticola* isolates was analyzed using the rep-PCR technique. The UPGMA analysis performed based on the rep-PCR data grouped all *X. campestris* pv. *viticola* isolates at the 88% similarity level and a 100% bootstrap value, demonstrating the high reliability of the branch. Based on the similarity matrix, 18 isolates representatives of the seven subgroups defined at a 90% similarity level were selected for additional studies. Similar results were obtained with an earlier collection (1998 to 2001) of 41 isolates that showed an overall homogeneous pattern sharing multiple bands, regardless of host cultivar, year, or location (Trindade et al. 2005).

Using the isolates listed in Table 1, cluster analysis based on rep-PCR data were able to reproduce subgroups 9.2, 9.5, and 9.6 of *X. axonopodis* sensu Rademaker et al. (2005). The isolates of *X. campestris* pv. *viticola*, *X. citri* pv. *mangiferaeindicae*, and *X. citri* subsp. *malvacearum* exhibited high genetic similarity, forming a single group at 64% similarity. Subgroup 9.5 of *X. axonopodis* was originally formed by isolates of *X. citri* subsp.

TABLE 2. Pathological characterization of isolates of *Xanthomonas campestris* pv. *viticola*, *X. citri* pv. *mangiferaeindicae*, *X. citri* pv. *anacardii*, and pigmented isolates pathogenic to cashew plant using artificial inoculation in cashew plants clone CCP76

Isolate ^v	Incubation period (days) ^w	Severity (mm) ^x
IBSBF1967 ^{PT}	3.0 b ^y	1.5 f
CCRMXCV17	4.0 a	3.8 e
CCRMXCV 80	3.0 b	4.0 e
CCRMXCV 117	4.0 a	3.7 e
CCRMXCV 178	3.0 b	3.5 e
IBSBF2586 ^{PT}	2.8 b	4.0 e
IBSBF657	3.4 b	4.2 e
IBSBF1508	4.3 a	4.0 e
IBSBF2579 ^{PT}	2.6 b	5.2 c
CCRMQAQ 3	3.0 b	4.9 d
CCRMQAQ13	2.5 b	5.8 b
CCRMQAQ18	2.5 b	6.9 a
CCRMXCMF1	2.6 b	7.4 a
CCRMXCMF2	3.2 b	4.3 e
CV (%) ^z	16.8	9.5

^v IBSBF1967^{PT}, CCRMXCV 17, CCRMXCV 80, CCRMXCV 117, and CCRMXCV 178 = isolates of *X. campestris* pv. *viticola*; IBSBF2586^{PT} = *X. citri* pv. *mangiferaeindicae*; IBSBF657, IBSBF1508, and IBSBF2579^{PT} = *X. citri* pv. *anacardii*; and CCRMQAQ3, CCRMQAQ13, CCRMQAQ18, CCRMXCMF1, and CCRMXCMF2 = pigmented isolates pathogenic to cashew plant.

^w Incubation period: number of days from inoculation until the appearance of the first symptom.

^x Severity: evaluated by measuring the damage at 23 days after inoculation.

^y Mean of eight replicates. Means followed by the same letter in the column did not exhibit significant differences by the Scott-Knott test ($P \leq 0.05$).

^z CV: coefficient of variation.

citri (synonymy *X. axonopodis* pv. *citri*) and *X. citri* subsp. *malvacearum* (*X. axonopodis* pv. *malvacearum*), among other plant pathogenic bacteria (Rademaker et al. 2005), and more recently by *X. citri* pv. *mangiferaeindicae* (Ah-You et al. 2007, 2009). This indicates a close relationship of *X. campestris* pv. *viticola* with this subgroup and, consequently, with *X. citri*, as described in other studies (Bansal et al. 2017; Marques 2007; Parkinson et al. 2009; Takita et al. 2004, 2006) (M. A. S. V. Ferreira, S. Bonneau, M. Briand, S. Cesbron, P. Portier, A. Darrasse, M. A. S. Gama, M. A. G. Barbosa, R. L. R. Mariano, E. B. Souza, and M. A. Jacques, unpublished data). In turn, the pigmented isolates pathogenic to cashew plant and the isolates of *X. citri* pv. *anacardii* were closely related, forming a single group with more than 70% similarity, according to previous studies (Gama et al. 2011).

According to Young et al. (2008), the MLSA technique using concatenated partial sequences of the *gyrB*, *rpoD*, *fyuA*, and *dnaK* genes is a robust tool for differentiating species of *Xanthomonas* spp. because it generates groups similar to the groups formed by DNA-DNA hybridization (Vauterin et al. 1995), AFLP (Rademaker et al. 2000), 16S rDNA region sequencing (Hauben et al. 1997), and rep-PCR (Rademaker et al. 2000, 2005). In the present study, the MLSA technique was successfully used to reconstruct the taxonomy of the group *Xanthomonas* and to show the position of *X. campestris* pv. *viticola* and pigmented isolates associated with the cashew plant. Previous studies performed with the MLSA technique and genus *Xanthomonas* demonstrated that the partial sequences of the *gyrB*, *rpoD*, *fyuA*, and *dnaK* genes were highly consistent with the concatenated sequence, suggesting that more robust taxonomic delineation is achieved using sequences with high levels of congruence rather than multiple sequences with low congruence (Young et al. 2008). In the present study, the partial sequences of the *gyrB*, *rpoD*, *fyuA*, and *dnaK* genes were highly consistent with the concatenated sequence ($P = 0.01$). However, it was not possible to concatenate the sequences of the *gyrB*, *rpoD*, *fyuA*, *dnaK*, and *hrpB* genes, which exhibited high levels of incongruence ($P = 0.35$) with the concatenated sequence.

The interactions between plant pathogenic bacteria and their hosts are strongly affected by ecological niches, the evolution of bacterial genetic components, and resistance proteins (R proteins) present in the hosts, which can detect the presence of plant pathogenic bacteria (Castillo and Greenberg 2007). The evolutionary forces acting on certain plant pathogenic bacteria and their populations can be further understood by studying housekeeping genes, which are less subject to horizontal transfer events. High levels of differentiation may be achieved using a combination of housekeeping genes with pathogenicity or virulence genes, which exhibit higher variation (Perez-Losada et al. 2005). However, considering that there was no consistency among the sequences of the housekeeping genes (*gyrB*, *rpoD*, *fyuA*, and *dnaK*) and the sequence of the pathogenicity gene (*hrpB*) used in this study, the intrapathovar variability of *X. campestris* pv. *viticola* and the variability among the pigmented isolates associated with the

TABLE 3. Population density recorded from leaf lesions 13 days after inoculation with *Xanthomonas campestris* pv. *viticola*, pigmented isolates pathogenic to cashew plant, *X. citri* pv. *anacardii*, and *X. citri* pv. *mangiferaeindicae* in cashew plants clone CCP 76

Isolate ^x	Log-transformed CFU ml ⁻¹
CCRMXCV80	2.90 c ^y
CCRMXCMF1	7.97 a
IBSBF2579 ^{PT}	7.90 a
IBSBF2586 ^{PT}	7.68 b
CV (%) ^z	11.23

^x CCRMXCV80 = *X. campestris* pv. *viticola*; IBSBF2586^{PT} = *X. citri* pv. *mangiferaeindicae*; IBSBF2579^{PT} = *X. citri* pv. *anacardii*; and CCRMXCMF1 = pigmented isolate pathogenic to cashew plant.

^y Mean of eight replicates. Means followed by the same letter in the column did not exhibit significant differences by the least significant difference test ($P \leq 0.05$).

^z CV: coefficient of variation.

cashew plant and *X. citri* pv. *anacardii* were analyzed based on the partial sequence of the *hrpB* gene alone. The resulting phylogenetic tree generated by Bayesian inference allowed for satisfactory separation among the pigmented isolates pathogenic to cashew plant and the isolates of *X. citri* pv. *anacardii*. These results suggest substantial differences with respect to host range or aggressiveness against different hosts. However, considering that previous studies have demonstrated that pigmented isolates pathogenic to cashew plant and *X. citri* pv. *anacardii* are equally aggressive to the cashew plant (Gama et al. 2011), it is most likely that these differences can only be detected via epidemiologic studies conducted with a broad host range.

To confirm the significance of the MLSA tree in the evolutionary relationship of the species of the genus *Xanthomonas*, we used drafts and complete available genomes of the *Xanthomonas* genus. The ANI data and the TETRA correlation coefficients showed that strains of *X. campestris* pv. *viticola* should be classified as a pathovar of *X. citri* because the ANI values and TETRA correlation coefficients between these isolates were always above 96% and 0.99, and the ANI value and TETRA correlation coefficients used for species delineation cut-off is below of 95% (Goris et al. 2007) and 0.99 (Richter and Rosselló-Móra 2009), respectively.

ANI and TETRA correlation coefficients values between pigmented isolates pathogenic to cashew plant (CCRMATQ13 and CCRMATQ18) and *X. citri* pv. *anacardii* (CFBP 2913 and IBSBF 2579) and within these isolates were always above 99% and 0.99. Thus, these data confirmed the result obtained by MLSA, which showed a close relationship among the pigmented isolates pathogenic to cashew plant and *X. citri* pv. *anacardii*. These data indicate that the pigmented isolates pathogenic to cashew plant should also be classified as *X. citri* pv. *anacardii*.

Biolog Microplate System has been successfully used to characterize metabolic diversity between the species of genus *Xanthomonas* (Vauterin et al. 1995) and within species/pathovars of this genus (Constantin et al. 2016; Stoyanova et al. 2014; Verniere et al. 1993). In general, the analysis of *Xanthomonas* isolates by Biolog Microplate System show that some substrates can be useful as markers to differentiate species/pathovars for the purposes of the routine identification. For example, Verniere et al. (1993) reported that the substrates L-fucose, D-galactose, and alaninamide were useful to differentiate isolates of *X. citri* pathogenic to citrus. Despite the high metabolic similarity observed among the *Xanthomonas* isolates analyzed in this study, some tests exhibited high discriminatory power, and thus, combining them may be used in differentiating the species/pathovars. All of the pigmented isolates pathogenic to cashew plant and the isolate of *X. citri* pv. *mangiferaeindicae* exhibited a positive reaction for L-aspartic acid and a negative reaction for Rifamycin SV while all isolates of *X. citri* pv. *anacardii* showed a negative reaction for L-aspartic acid and a positive reaction for Rifamycin SV. As for using γ -amino-butyric acid as a carbon source, the pigmented isolates pathogenic to cashew plant and *X. citri* pv. *anacardii* exhibited a negative reaction while *X. citri* pv. *mangiferaeindicae* showed a positive reaction. Similar results were reported by Pruvost et al. (1998), who observed that isolates of *X. citri* pathogenic to Anacardiaceae exhibited a positive reaction for γ -amino-butyric acid and Rifamycin SV. However, in contrast to the results obtained in this study, Pruvost et al. (1998) detected variations among isolates for L-aspartic acid.

All the isolates of *X. campestris* pv. *viticola* and isolates pathogenic to cashew plant were pathogenic to the cashew plant clone CCP76. The five groups (A to E) formed by pigmented isolates pathogenic to cashew plant and *X. citri* pv. *anacardii*, identified in previous studies based on the severity of leaf damage caused by artificial inoculations (Gama et al. 2011), were confirmed. All isolates of *X. campestris* pv. *viticola* were inside the less aggressive groups (groups E and F of severity) and the population density of the isolate CCRMXCV80 was significantly lower than those obtained for pigmented isolate pathogenic to cashew plant,

X. citri pv. *anacardii* and *X. citri* pv. *mangiferaeindicae*, indicating low aggressiveness of these isolates to the cashew plant. In turn, the pigmented isolates pathogenic to cashew plant exhibited shorter incubation periods, higher severity values and higher population density, indicating higher aggressiveness of these isolates against the cashew plant, as previously demonstrated (Gama et al. 2011). In contrast, only isolates of *X. campestris* pv. *viticola* were pathogenic on the leaves of grapevine cultivar Red Globe, showing high population density (5.3×10^6 CFU per lesion). This information is important for differentiating and characterizing these isolates because *X. campestris* pv. *viticola*, pigmented isolates pathogenic to cashew plant, *X. citri* pv. *anacardii* and *X. citri* pv. *mangiferaeindicae* were pathogenic to cashew plants, but only *X. campestris* pv. *viticola* was able to cause lesions on the grapevine leaves.

It was clearly demonstrated that *X. campestris* pv. *viticola* was placed in subgroup 9.5 of *X. axonopodis sensu* Rademaker et al. (2005) and within PGI, subgroup 2, *sensu* Constantin et al. (2016), which are currently composed by *X. citri* pathovars. *X. campestris* pv. *viticola* was closely related to *X. citri* pv. *mangiferaeindicae* (rep-PCR, MLSA, ANI, and TETRA) and *X. citri* subsp. *citri* (MLSA, ANI, and TETRA). Consequently, it is suggested that *X. campestris* pv. *viticola* be reclassified as *X. citri* pv. *viticola* comb. nov. In turn, pigmented isolates pathogenic to cashew plant were closely related to *X. citri* pv. *anacardii* (rep-PCR, MLSA, ANI, TETRA, and pathological characteristics). Thus, pigmented isolates pathogenic to cashew plant should also be classified as *X. citri* pv. *anacardii*.

Description of *X. citri* pv. *viticola* (Nayudu 1972) Dye 1978 comb. nov. Based on rep-PCR, MLSA using sequences of the housekeeping genes *gyrB*, *rpoD*, *fyuA*, and *dnaK*; sequencing and phylogenetic analysis of the *hrpB* gene for the pathogenicity and hypersensitive response; and ANI values and TETRA correlation coefficients, the bacterium is genetically related to pathovars of *X. citri sensu* Constantin (Constantin et al. 2016). Isolates of *X. citri* pv. *viticola* can be readily distinguished from another pathovars of *X. citri* by rep-PCR analysis, MLSA, ANI values and TETRA coefficient. Its genome contains G-C content of 63.84% and approximately 4,427 genes (Lima et al. 2017). Isolates of *X. citri* pv. *viticola* produce nonpigmented (creamy white) colonies when cultivated on NYDA medium, and on Biolog GEN III MicroPlate System show 100% positive reaction for L-malic acid, citric acid, α -keto-glutaric acid, D-cellobiose, D-fructose, D-galactose, D-mannose, D-trehalose, gentiobiose, L-fucose, N-acetyl-D-glucosamine, 1% NaCl, pectin, pH 6, sucrose, Tween 40, and α -D-glucose and negative reaction for D-aspartic acid, D-malic acid, formic acid, fusidic acid, L-galactonic acid lactone, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, γ -amino-butyric acid, sodium bromate, sodium butyrate, lithium chlorite, D-arabitol, D-mannitol, D-serine, D-serine, D-sorbitol, α -D-lactose, guanidine HCl, minocycline, myo-inositol, 4% NaCl, 8% NaCl, Niaproof 4, potassium tellurite, troleandomycin, and β -methyl- D-glucoside. *X. citri* pv. *viticola* affects all aerial parts of the grapevine plant. The most common symptoms of disease are cankers on the twigs, branches, petioles and veins. Leaf symptoms begin as small water-soaked spots delineated by veins, becoming dark lesions, with or without a chlorotic halo. Sometimes the veins and surrounding tissues can exhibit dark spots. Berries symptoms appear as several depressed dark lesions. In some cases, an exudation can be observed over the lesions. Elevated population sizes ($\pm 5 \times 10^6$ CFU per lesion) can be recovered from grapevine leaf lesions. The pathotype isolate is IBSBF 1967^{PT} (Phytopathogenic Culture Collection of Instituto Biológico, Brazil) = LMG 965^{PT} (Belgian Coordinated Collections of Microorganisms, University of Ghent, Belgium) = NCPPB 2475^{PT} (National Collection of Plant Pathogenic Bacteria, UK) = CFBP 7660^{PT} (Collection Française de Bactéries associées aux Plantes/CIRM-CFBP, France).

Emended description of *X. citri* pv. *anacardii* (Ah-You et al. 2007) emend. (Constantin et al. 2016). Description of

Ah-You et al. (2007) and Constantin et al. (2016) extended to include yellow-pigmented isolates pathogenic to cashew plant (this study). Based on Biolog GEN III MicroPlate System, yellow-pigmented isolates show reaction positive for L-aspartic acid and negative reaction for Rifamycin SV, while nonpigmented isolates show negative reaction for L-aspartic acid and positive reaction for Rifamycin SV. The pathotype isolate is IBSBF2579^{PT} (Phytopathogenic Culture Collection of Instituto Biológico, Brazil) = CFBP 2913^{PT} (Collection Française de Bactéries associées aux Plantes, France) = ICMP 4088^{PT} (International Collection of Micro-organisms from Plants, New Zealand).

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