Detection and identification of *Xanthomonas* pathotypes associated with citrus diseases using comparative genomics and multiplex PCR

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Background: In *Citrus* cultures, three species of *Xanthomonas* are known to cause distinct diseases. *X. citri* subsp. *citri* patothype A, *X. fuscans* subsp. *aurantifolii* pathotypes B and C, and *X. alfalfae* subsp. *citrumelonis*, are the causative agents of cancrosis A, B, C, and citrus bacterial spots, respectively. Although these species exhibit different levels of virulence and aggressiveness, only limited alternatives are currently available for proper and early detection of these diseases in the fields. The present study aimed to develop a new molecular diagnostic method based on genomic sequences derived from the four species of *Xanthomonas*. Results: Using comparative genomics approaches, primers were synthesized for the identification of the four causative agents of citrus diseases. These primers were validated for their specificity to their target DNA by both conventional and multiplex PCR. Upon evaluation, their sensitivity was found to be 0.02 ng/µl *in vitro* and

1.5×10^4 CFU ml^{-1} in infected leaves. Additionally, none of the primers were able to generate amplicons in 19 other genomes of *Xanthomonas* not associated with *Citrus* and one species of *Xylella*, the causal agent of citrus variegated chlorosis (CVC). This denotes strong specificity of the primers for the different species of *Xanthomonas* investigated in this study. Conclusions: We demonstrated that these markers can be used as potential candidates for performing *in vivo* molecular diagnosis exclusively for citrus-associated *Xanthomonas*. The bioinformatics pipeline developed in this study to design specific genomic regions is capable of generating specific primers. It is freely available and can be

utilized for any other model organism.

1 **Detection and identification of *Xanthomonas* pathotypes associated with citrus**
2 **diseases using comparative genomics and multiplex PCR**

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31   Short Title

32   Molecular diagnostic of bacterial citrus diseases

34   Abstract

35   Background:

36   In citrus cultures, three species of *Xanthomonas* are known to cause distinct diseases. *X. citri* subsp.
37   *citri* patothype A, *X. fuscans* subsp. *aurantifolii* pathotypes B and C, and *X. alfalfae* subsp.
38   *citrumelonis*, are the causative agents of cancrosis A, B, C, and citrus bacterial spots, respectively.
39   These species exhibit different levels of virulence and aggressiveness, and only limited alternatives
40   are currently available for proper and early detection of these diseases in the field. The present
41   study aimed to develop a new molecular diagnostic method based on genomic sequences derived
42   from the four pathotypes of *Xanthomonas*.

43   Results:

44   Using comparative genomics approaches, primers were synthesized for the identification of the
45   four causative agents of citrus diseases. These primers were validated for their specificity to their
46   target DNA by both conventional and multiplex PCR. Upon evaluation, their sensitivity was found
47   to be 0.02 ng/μl *in vitro* and 1.5×10^4 CFU ml^{-1} in infected leaves. Additionally, none of the primers
48   were able to generate amplicons in 19 other genomes of *Xanthomonas* not associated with citrus
49   and one species of *Xylella*, the causal agent of citrus variegated chlorosis (CVC). This denotes
50   strong specificity of the primers for the different species of *Xanthomonas* investigated in this study.

51   Conclusions:

52   We demonstrated that these markers can be used as potential candidates for performing *in vivo*
53   molecular diagnosis exclusively for citrus-associated *Xanthomonas*. The bioinformatics pipeline
54   developed in this study to design specific genomic regions is capable of generating specific
55   primers. It is freely available at https://git.facom.ufms.br/bioinfo/specific-primers and can be
56   utilized for any other organism.

57

58   Keywords: Multiplex PCR; molecular diagnostic; citrus canker; *Xanthomonas*; comparative
59   genomics analysis.

60

Introduction

Xanthomonas is a genus of phytopathogenic Gram-negative bacteria that cause a variety of diseases in several agricultural commodities, including citrus [1]. These diseases cause huge economic losses to the agricultural sector. Consequently, bacteria from this genus have been targeted as an essential model of study in different pathosystems; for instance, *X. oryzae* in *Oryza* spp. (rice), *X. campestris* in *Brassica* spp. (cabbage), *X. vesicatoria* in *Solanum* spp. (potato), and *X. citri* in *Citrus* spp. (orange) [2–6]. Since *Xanthomonas* are quarantine pests in all these pathosystems and controlling them with agrochemicals is very expensive, early detection is of paramount importance for the control and management of the diseases caused by these bacteria.

The *Xanthomonas* and citrus pathosystem is composed mainly of the following three species: *X. citri* subsp. *citri* pathotype A (*XccA*), *X. fuscans* subsp. *aurantifolii* (*Xau*), and *X. alfalfae* subsp. *citrumelonis* (*Xacm*). These bacteria damage crops and consequently cause losses [7,8] (Table 1). Although the pathosystem as here defined contains only three species, control and eradication efforts are complicated by the genetic diversity of the pathotypes, with varying degrees of aggressiveness and virulence [9,10]. *XccA*, the causative agent of Asian citrus canker, is the most aggressive and virulent among the pathotypes. It infects all cultivated varieties of citrus hosts [11]. In addition to the pathotype A, two other variants, A* (*XccA**) and A^W (*XccA^W*) have been reported in previous studies [12,13]. Although they are genetically close to type A, these variants are associated with a smaller set of compatible hosts (Table 1) [9,13]. *Xau* is represented by two pathotypes known as B (*XauB*) and C (*XauC*). Pathotype B causes false citrus canker or cancrosis B, a less aggressive disease than citrus canker that affects sour lemons (*Citrus limon*) severely [10,14]. Pathotype C is the cause of cancrosis C, which is characterized by induction of hypersensitivity reaction in its hosts as described in 'Galego' acid lime (*C. aurantifolia*) [10] and 'Swingle' citrumelo (*Citrus paradisi* × *Poncirus trifoliata*) [15]. *Xacm*, or pathotype E, is the causative agent of citrus bacterial spot. It is not classified as cancrosis since it does not cause typical corticosteroid lesions. However, citrus bacterial spot may be confused with cancrosis, and pathotype E is pathogenic in 'Swingle' citrumelo [16–19].

Due to this considerable diversity in the characteristics of infection and disease spread, these bacteria are one of the main phytosanitary problems that affect and decimate Brazilian orange orchards. As they are cosmopolitan pathogens, they not only harm the economy of Brazil, a major citrus producer, but also the world [20]. Since the management of the plants during cultivation and export are different for each infecting strain, correct diagnosis of the pathogen may reduce damages [9].

Molecular diagnosis is a cost-effective option for identifying pathogens with great genetic proximity. Molecular biology techniques have been extensively used for identifying various species of the genus *Xanthomonas* [21–30]. The genetic diversity of the *Xanthomonas* pathotypes that infect citus makes their correct identification a challenging task. The low sensitivity and false negative cases of serological tests [13, 31], and false positive amplifications of the molecular tests,

99   demonstrate the need for new detection tests that are specific and that discriminate the different
100   variants of this class of plant pathogens [32]. Thus, this study aimed to develop a molecular
101   detection method from unique genomic sequences, identified by comparative genomics, which can
102   distinguish different *Xanthomonas* pathotypes infecting *Citrus* spp. These sequences are intended
103   to be used only for detecting *Xanthomonas* isolates obtained from *Citrus* spp..

104

Materials and methods

106   For a better understanding of the methodological steps involved in this study, a flowchart of actions
107   was established (Fig. 1).

108

109   *Comparative Genomics for identification of pathotype-specific regions*

110   In order to identify the pathotype-specific regions, we have built a very simple 4-step pipeline. The
111   input for the method is the set of nucleotide FASTA sequences from the four genomes of the
112   genus *Xanthomonas,* belonging to each of the disease-causing-pathotypes in citrus obtained from
113   NCBI (Table 2). The first step is to pre-process sequences, to concatenate all contigs of each
114   genome, in case of being in a draft condition, and also to concatenate all FASTA genome files in
115   a unique one. This file is the input for the second step, where mauveAligner, from the Mauve
116   package [33], is used to build a multiple sequence alignment. MauveAligner outputs unique
117   regions defined as regions that are present in one genome but absent from at least one of the other
118   genomes. We call these unique regions 'Mauve islands', and they are the input to a custom Perl
119   script to pick out specific regions from each genome. The third step is to post-process data just to
120   separate all specific regions in distinct FASTA files. The candidate regions found at this point are
121   double-checked in the fourth step, using BLASTn [34]. BLASTn is then performed on each
122   candidate region from genome *G* against the set of all genomes except *G*. The final output of this
123   workflow consists of the list of all unique regions with no hits in this BLASTn run. This
124   comparative genomics pipeline is freely available at https://git.facom.ufms.br/bioinfo/specific-
125   primers, including a short and simple tutorial for installation and using, and can be used for any
126   set of genomes in a regular Linux system.

127   Other specific regions finders are available in the literature, for instance Insignia [35]. Insignia is
128   a web application where the user can find specific regions from a genomic sequence, but only
129   against sequences available currently in the previously populated database by the authors. On the
130   other hand, our tool is a very easy-to-install and easy-to-use stand-alone tool where the user can
131   find specific regions against any set of genomes (available in databases or not), even draft ones.

132

133   *Selection of specific sequences and production of primers*

134   The sets of unique regions determined for each genome were used as a reference for the selection
135   of the primers using Primer-BLAST tool [36]. To verify the specificity of each primer to their
136   target DNA, each generated primer was subjected to local alignment against *Xanthomonas*
137   pathotypes that infect citrus using BLASTn. Primers that demonstrated specificity and ability to
138   form amplicons of different sizes among the analyzed pathotype were selected for *in vitro*
139   empirical verification.

140

141   *In silico* evaluation of possible false positive amplifications

142   To verify false positive amplifications from other *Xanthomonas* species or other plant-associated
143   microorganisms whose DNA could be extracted from infected plant tissue, the primers were
144   compared against all sequences available at GenBank (using BLASTn against NT database).

145

146   *Bacterial strain and growth conditions*

147   For the biological assays, 16 strains belonging to the pathotype *XccA* (7 strains), *XauB* (2 strains),
148   *XauC* (5 strains), and *Xacm* (2 strains) were selected. These strains came from culture collections
149   maintained by the Agronomy School of the University of São Paulo (ESALQ) and by the Faculty
150   of Agrarian and Veterinary Sciences of the State University of São Paulo (Campus Jaboticabal),
151   both in the state of São Paulo, Brazil. The isolates were reactivated in nutrient agar medium - NA
152   (5 g/l peptone, 3 g/l beef extract, 15 g/l agar, pH 6.8-7.0) and incubated at 28°C for 48 hours for
153   *XccA* and *Xacm* strains and 96 hours for *XauB* and *XauC* strains. Then, a colony was grown in
154   nutrient broth medium (NB; NA without agar) under agitation at 250 rpm at 28 ºC for 12 hours.

155   In addition to the citrus-pathogenic *Xanthomonas* isolates, the markers have also been tested
156   against other *Xanthomonas* strains that are not known to infect citrus: *X. axonopodis* pv. *vignicola*
157   FDC 1701 (IBSBF 1739), *X. axonopodis* pv. *vesicatoria* 1387 (IBSBF 1387), *X. axonopodis* pv.
158   *vesicatoria* FDC 1689 (IBSBF 331), *X. axonopodis* pv. *allii* 1770 (IBSBF 1770), *X. axonopodis*
159   pv. *phaseoli* IAC 11280, *X. alfafae* subsp. *alfalfae* FDC 1711 (IBSBF 3174), *X. axonopodis* pv.
160   *passiflorae* M89, *X. axonopodis* pv. *patelli* FDC 1727 (IBSBF 3181), *X. axonopodis* pv. *coracanae*
161   FDC 1729 (IBSBF 3187), *X. axonopodis* pv. *manihotis* 1954 (IBSBF 1954), *X. axonopodis* pv.
162   *phaseoli* var. *fuscans* IAC13755, *X. citri* pv. *rhynchosiae* FDC 1721 (IBSBF 3184), *X. axonopodis*
163   *vasculorum* 2008 (IBSBF 2008), *X. axonopodis* pv. *axonopodis* FDC 1696 (IBSBF 1444), *X.*
164   *axonopodis* pv. *dieffenbachiae* 1478 (IBSBF 1478), *X. axonopodis* pv. *glycines* FDC 1694 (IBSBF
165   333), *X. citri* pv. *cajani* FDC 1718 (IBSBF 3180), *X. citri* pv. *sesbaniae* FDC 1719 (IBSBF 3179),
166   and against *Xylella fastidiosa* strain 9a5c [37], a phytopathogenic bacterium that is disseminated
167   by insects among citrus.

168

169 *Extraction of DNA from isolates*

170 The DNA of the isolates was extracted with the *Spin Tissue™ Core Kit* (*Qiagen Biosciences, MD,*
171 *USA*) following the manufacturer's recommendations.

172

173 *Inoculation of the isolates in the leaves and genomic DNA extraction from bacterial cells in*
174 *exudate*

175 The bacteria were inoculated into Hamlin-type orange leaves with a needleless syringe. The
176 syringe was pressed lightly into the abaxial part of the leaf to allow entry of the phytopathogen
177 into the intercellular space of the leaf. A leaf from the same plant was inoculated likewise with
178 ultrapure water as a negative control. Immediately after the inoculation, the inoculated area was
179 cut into small pieces, and bacterial exudation was carried out.

180 To mimic the condition of naturally infected plants, spray inoculation methods were also used.
181 Spray inoculation was perform in 'Pera-rio' orange leaves according to the methods described by
182 Yan and Wang [38]. After 30 days of inoculation, leaves containing lesions were detached from
183 the plants and each lesion was cut into small pieces to get the exudate with bacteria. The inoculated
184 area/lesions were submerged in 1 mL of ultrapure water for 30 min and agitated for 5 min to
185 facilitate bacterial exudation from the leaf. After agitation, genomic DNA extraction from bacterial
186 cells in the exudate was performed with a rapid manual DNA extraction protocol. The exudate was
187 centrifuged at 16,000 x g for 4 min at 4 °C and washed twice with an isotonic solution (33 mM
188 KH_2PO_4, 60 mM K_2HPO_4, 7.6 mM $(NH_4)_2SO_4$, 3.3 mM sodium citrate, and 4.0 mM $MgSO_4$). The
189 pellet was resuspended in lysis buffer (10 mM Tris-HCl 1 M, pH 8.0; 100 mM EDTA 0.5 M, pH
190 8.0; 10 mM NaCl 5 M; 0.5% of 10% SDS), and isotonic solution with subsequent extraction with
191 phenol and incubation with RNAse (5 mg/ml) for 1 hour at 37 °C. After further extraction with
192 phenol, the DNA was precipitated with ethanol and sodium acetate (7.5 M, pH 7.4) and then diluted
193 in Tris–EDTA buffer 10:0.1.

194

195 *PCR Conditions*

196 PCR amplifications were carried out in a final volume of 25μl. The final concentrations of the
197 reagents in the reaction were: 2 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM of each primer (Foward and
198 Reverse), 1 unit of Taq DNA polymerase™ (*CellCo, SP, Brazil*) and 0.4 ng of the DNA sample
199 from the isolates grown in medium or 1 μl of the total DNA extracted from the exudates. The initial
200 denaturation temperature was 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for
201 45 seconds, annealing at 59 °C for 45 seconds and extension at 72 °C for 45 seconds, followed by
202 a final extension at 72 °C for 5 min. Verification of PCR amplicons was established by applying
203 10 μl of the PCR product in 3.0% (w/v) agarose gel.

204

205 *Multiplex PCR*

206 Multiplex PCR amplifications (mPCR) were also performed in final volumes of 25 μl. The final
207 concentration of the reagents in the reaction was the same as on the conventional PCR, except for
208 increasing the concentration of dNTPs to 400 μM and using 0.2 μM of each primer (Forward and
209 Reverse) of all four primer pairs (XccAm, XauBm, XauCm, and Xacmm) in the same reaction.

210

211 *Sensitivity of PCR*

212 To verify the sensitivity of the PCR reaction *in vitro*, progressive dilutions of DNA from the
213 isolates were performed until the amplification checked the minimal DNA concentration
214 detectable by PCR. The isolates used in this test were *XccA* strain 306 (*XccA*306), *XauB* strain
215 FDC 1561 (*XauB*1561), *XauC* strain FDC 1559 (*XauC*1559), and *Xacm* strain 1510 (*Xacm*1510).
216 For leaf testing, sensitivity was found from minimal inoculations of leaf bacteria (1.5×10^7,
217 1.5×10^6, 1.5×10^5, 1.5×10^4, 1.5×10^3 CFU ml^{-1}) with consequent lower extraction of DNA from the
218 exudate.

219

220 *In-silico PCR*

221 The *in-silico* PCR was performed using the tool '*In silico* PCR amplification' [39] available at
222 http://insilico.ehu.eus/.

223

224 Results

225 Specific sequences and selection of primers

226 Mauve found 5,217 islands. From these, 173 candidates for specific regions of *XccA*306, 59 of
227 *XauB*1561, 55 of *XauC*1559 and 38 of *Xacm*1510 were identified. After the BLAST refinement
228 step, we found 135 specific regions of *XccA*306, 23 of *XauB*1561, 12 of *XauC*1559, and 33 of
229 *Xacm*1510. From the sequences, a pair of primers were developed for each *Xanthomonas*
230 pathotype. Each marker was developed using a different amplicon size, allowing its differentiation
231 from the other pathotypes. The primer sequences and their respective amplicons are described in
232 Table 3. Primer XccAm appears to amplify part of a hypothetical gene and a transcriptional
233 activator FtrA, generating a 509bp amplicon. Primer XauBm amplifies an intergenic region
234 (434bp); primer XauCm amplifies part of a hypothetical gene (160bp) and primer Xacmm
235 amplifies part of a gene encoding a D-alanyl-D-alanine synthetase A (115bp). Due to the

236   intraspecific genetic proximity between *Xanthomonas*, a reference genome was used for each
237   target pathotype.

238   BLASTn analysis of the primers against NCBI's non-redundant sequence database NT revealed
239   high sequence specificity (100% identity) for the citrus-associated *Xanthomonas* pathothypes in
240   all cases, and also for other non-citrus-associated *Xanthomonas* species (80 to 100% identity).
241   However, *in silico* analysis of the possible amplicons generated in the non-citrus-associated
242   species indicated different sizes from those expected for the defined targets. Only one pair of
243   primers (*XauC*) indicated possible amplification in the case of *Stenotrophomonas maltophilia*, a
244   bacterium that lives in aqueous environments, soil, and plants. However, the coverage of the
245   XauCm forward and reverse primers on the genome of *Stenotrophomonas maltophilia* is not
246   complete and the size of *in silico* amplicon is not similar to that of previously predicted amplicon
247   for *XauC*.

248

249   Specificity of molecular markers and sensitivity of PCR

250   The specificity for citrus-associated *Xanthomonas* verified *in silico* was confirmed as shown in
251   Fig. 2. Primer XccAm generated an amplicon of size 509 bp, primer XauBm generated an amplicon
252   of 434 bp, primer XauCm generated an amplicon of 160 bp, and primer Xacmm generated an
253   amplicon of 115 bp for their corresponding pathotypes. All markers generated unique amplicons
254   for their respective target DNAs without cross-amplifications for other investigated strains.
255   Progressive dilutions of the target DNA verified the *in vitro* sensitivity. The minimum
256   concentrations that allowed PCR amplification were 0.02 ng/µl for *XccA* (~70 cells), 0.03 ng/µl
257   for *XauB* (~100 cells), 0.12 ng/µl for *XauC* (~400 cells), and 0.07 ng/µl *Xacm* (~ 200 cells) (Fig.
258   3).

259   These results were reproducible when the markers were subject to multiplex PCR with their
260   respective target DNAs and with the DNAs of the four pathotypes (Fig. 4). Despite the findings *in*
261   *silico*, when tested with the other 18 species of *Xanthomonas* that do not infect citrus, the markers
262   developed for *XccA*, *XauB*, and *XauC* did not demonstrate amplification with any of these isolates
263   (Fig. 5). In contrast, Xacmm amplified a weak fragment for five of the investigated strains (*X.*
264   *axonopodis* pv. *vesicatoria* 1689, *X. axonopodis* pv. *passiflorae* M89, *X. axonopodis* pv. *manihotis*
265   IBSBF1954, *X. axonopodis* pv. *axonopodis* 1696, and *X. axonopodis* pv. *glycines* 1694), but with
266   a different amplicon size (~125 bp) without correspondence to the established standards for citrus-
267   associated xanthomonads. Although now empirically shown, this result does not affect detection
268   since none of these strains with false positive amplification infects citrus. Finally, no amplicons
269   were generated due to cross-amplification with DNA from *Xylella*, a plant pathogen that also
270   infects citrus.

271   *In vivo* assays replicated the results obtained *in vitro*. The markers were specific for the inoculated
272   bacterium (Fig. 6) and citrus canker lesions (Fig. 7) without cross-reaction with any other DNA

273   that may have been extracted from exudate, including endophytic DNA. Additionally, the plants
274   inoculated with water did not amplify with any marker tested, confirming the specificity for
275   bacteria of the genus *Xanthomonas* (Fig. 6). For the exudate, the minimum concentrations that
276   allowed PCR amplification were approximately 1.5×10^4 CFU mL^{-1} for *XccA*, 1.5×10^5 CFU mL^{-1}
277   for *XauB*, 1.5×10^5 CFU mL^{-1} for *XauC,* and 1.5×10^4 CFU mL^{-1} for *Xacm* (Fig. 6).

278

Discussion

280   Currently, the citrus disease of greatest concern to citrus growers worldwide is greening, also
281   known as Huanglongbin (HLB) [40]. However, citrus canker still is a problem for the citrus
282   industry due to its virulence and rapid propagation characteristics [41]. Coupled with deficiencies
283   in maintaining legislation and inspecting contaminated orchards, citrus canker causes great losses
284   to farmers worldwide [42]. Thus, the correct identification of the pathogen is crucial to restrain
285   this disease, given that the management of plants during cultivation and export may differ with
286   each infecting strain [9].

287   Due to its high virulence and host range, the development of diagnostics is aimed towards the
288   detection of *XccA* strains, the causative agent of Asian citrus canker. Currently, field detection is
289   performed through rapid tests based on the immune-identification of its proteins as Xcc
290   ImmunoStrip® Test, Agdia, Inc. (Catalog number: STX 92200), but there are several studies that
291   have developed serological diagnoses for *XccA* [14,28,43–47]. However, these tests have low
292   sensitivity, and in some cases, false negative results have already been reported in phenotypically
293   different strains of *XccA* [13,31]. Additionally, its high cost per sample is a significant
294   disadvantage when compared to methods based on molecular biology techniques.

295   Some studies have already demonstrated the potential of molecular diagnosis for *Xanthomonas*,
296   more specifically for *XccA* strains, based on several techniques such as conventional PCR,
297   multiplex PCR, real-time PCR (qPCR), nested-PCR, loop-mediated isothermal amplification
298   (LAMP-PCR), droplet digital polymerase chain reaction (ddPCR), BOX-PCR, and FISH [22-
299   27,29,30,48-53]. The wide variety of techniques used in the molecular diagnostics developed for
300   citrus canker solved the problem of cost and low sensitivity presented by serological methods.
301   However, the false-positive amplifications already highlighted among strains of genetically close
302   variants, such as *XccA* and *Xau*, once again reveal the need for new detection methods that are
303   specific and that distinguish the different pathotypes of these plant pathogens [32]. Thus, to the
304   best of our knowledge, this is the first study that presents a single method that simultaneously
305   distinguishes the four pathotypes of *Xanthomonas* that infect citrus. In this method, each of these
306   four pathotypes will have its own distinct amplicon.

307   It is important to emphasize that the method presented here is not a general method for identifying
308   the *Xanthomonas* strains discussed here with respect to other bacterial isolates. The application
309   setting for which the detection tool was designed is for strains isolated from citrus trees only.

310 The specificity verified in the results enables the adoption of individualized management for each
311 pathotype, which could reduce cultivation losses. Although BLASTn analysis of primer sequences
312 against genomes deposited in NCBI has shown that non-specific binding to other *Xanthomonas*
313 genomes may occur, two factors must be considered with respect to false positives: a) a possible
314 PCR product generated for other species of *Xanthomonas* would likely have a different size from
315 that observed for citrus pathotypes; b) as far as we know, the only members of the *Xanthomonas*
316 genus that infect citrus are the ones considered here. Therefore we think that the probability of
317 amplification of false positives in samples from diseased trees is small, which is corroborated by
318 the analyses presented in Fig. 5.

319 The sensitivity of the PCR *in vivo* tests shows the potential of its usage as a preventive detection
320 tool since it can detect as little as 10^4 CFU of bacteria, allowing the pathogen to be screened during
321 the colonization and survival phase of its life cycle, avoiding its later dissemination and infection
322 of healthy plants. Remaining canker lesions under favorable conditions have a concentration of up
323 to 10^7-10^8 CFU of viable bacteria for a new infection [54,55].

324 Another potential benefit of the correct detection of *Xanthomonas* strains presented here is its use
325 on seedlings that will be distributed to rural producers, since the introduction of infected seedlings
326 in orchards is one of the mechanisms of dissemination of the bacteria to healthy plants [10,19,56].
327 Additionally, our method can be used for a biogeographic survey of the pathotypes, to trace the
328 distribution profile among citrus orchards throughout the world. This becomes important for
329 epidemiological surveillance of this phytopathogen, as this type of analysis is typically hampered
330 by the lack of adequate molecular typing techniques for surveillance and outbreak investigation
331 [25].

332 Our approach is based on conventional PCR because it is an accessible and inexpensive technique,
333 without compromising the quality of the analysis. Furthermore, the present study has established
334 the detection of all pathotypes in the same PCR reaction, as demonstrated in the results of multiplex
335 PCR. This technique increases the importance of the detection method, since the same sample can
336 be submitted to a PCR reaction with all the primers, reducing the time for obtaining the result.
337 Another technique used in our study, which could enhance the speed of detection, was the
338 extraction of total DNA from the exudate of the leaves. This extraction takes less than four hours
339 to be carried out; thus, there is no need to wait for several days for bacterial growth from its lesion
340 isolation.

341 Collectively, both experimental procedures make the methodology feasible on a large scale and at
342 a reduced cost. It is important to highlight that the variable size of the PCR products for each
343 pathotype was established to facilitate its identification, and the use of PCR-RFLP would only be
344 justifiable to verify genetic diversity within species, which is not the objective of this study.
345 Moreover, although we did not use the TaqMan system to verify the potential of these molecular
346 markers in the identification of these pathogens, it is likely that the use of this system would only
347 make detection sensitivity higher than that observed by conventional PCR, as described for other

348 organisms [57–59]. However, the cost of analysis would be higher, which could significantly
349 burden large-scale analyses.

350 Thus, our results not only allow the correct identification of the four pathotypes of *Xanthomonas*
351 that infect citrus, but they also point to a possible bioinformatics pipeline for the production of
352 species-specific molecular markers for any set of genomes defined by the user. The high specificity
353 and sensitivity obtained demonstrated the effectiveness of our bioinformatics analysis.

354

Conclusions

356 In summary, the identification of unique sequences from different genomes, using comparative
357 genomics, allowed the development of specific molecular markers. This is the first conventional
358 or multiplex PCR-based method that discriminates four different citrus-associated *Xanthomonas*
359 pathotypes. This study further describes an effective pipeline for the generation of species-specific
360 molecular markers.

361

Author´s contributions

363 NFA, GPT, DA, AMV, JCS, and LMM designed and implemented the comparative genomics
364 workflow.

365 LMM, JAF and JBJ select the strains.

366 NFA, DA, NPF, and LMM designed and selected the pathotypes specific primers.

367 NPF, EBF, WLC, ABS, IFC, and RABA performed *in vitro* assays.

368 FMSC, CGLB, NFP performed *in vivo* assays.

369 NPF, NFA, and LMM analyzed the results and drafted an initial version of the manuscript.

370 AMV, RABA, GPT, NFA, JCS, and LMM wrote the final version of the manuscript.

371

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378

Competing interests

380   The authors declare that they have no competing interests.

381

Availability of data and materials

383   Data sharing not applicable to this article as no datasets were generated or analyzed during the
384   current study.

385

Consent for publication

387   Not applicable.

388

Ethics approval and consent to participate

390   This study did not involve human participants or animals.

391

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399

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424   and *X. alfalfae* subsp. *citrumelo* (ex Riker and Jones) Gabriel et al., 1989 sp. nov. nom. rev.
425   comb. nov.; *X. campestris* pv. *malvacearum* (ex smith 1901) Dye 1978 as *X. smithii* subsp.
426   *smithii* nov. comb. nov. nom. nov.; *X. campestris* pv. *alfalfae* (ex Riker and Jones, 1935)
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621

623   Tables

624

625   **Table 1:** Relation of compatible host and causal agents

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628

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630

631   **Figure 1: Experimental design.** Stages and procedures from the development of the *in silico*
632   stages to the validation of the molecular results of the plant diagnosis.

633

634   **Figure 2: Specificity of molecular markers *in vitro*.** Conventional PCR electrophoresis using
635   Xcc-specific oligonucleotides for *Xanthomonas citri* subsp. *citri* pathotypes A (*XccA*) and A*
636   (*XccA**) with a 509 bp amplicon, (A) *Xanthomonas fuscans* subsp. *aurantifolii* pathotype B (*XauB*)
637   with 434 bp amplicon, (B) *Xanthomonas fuscans* subsp. *aurantifolii* pathotype C (*XauC*) with 160
638   bp amplicon, (C) *Xanthomonas alfalfae* subsp. *citrumelonis* (*Xacm*) with an amplicon of 115 bp,
639   and (D) MW: molecular weight (ladder 100 bp).

640

641   **Figure 3: Progressive DNA dilutions demonstrating the sensitivity of PCR.** (A) *XccA* (0.02
642   ng/µl, ~70 cells), (B) *XauB* (0.03 ng/µl, ~100 cells), (C) *XauC* (0.12 ng/µl, ~400 cells), and (D)
643   *Xacm* (0.07 ng/µl, ~200 cells). MW: molecular weight (ladder 100 bp).

644

645   **Figure 4: Multiplex PCR of *Xanthomonas* that infect citrus.** (A) Multiplex PCR electrophoresis
646   containing the combination of the DNA of one species with all pairs of specific primers generating
647   only their amplicon (lines 2 to 5). MP - Combination of DNAs of all pathotypes with all primers
648   (line 6). MW: molecular weight (ladder 100 bp) and (B) Theoretical electrophoresis of multiplex
649   PCR.

650

651   **Figure 5: Multiplex PCR electrophoresis of *Xanthomonas* spp. that do not infect citrus.**
652   (1701) - *X. axonopodis* pv. *vignicola* strain 1701; (1387) - *X. axonopodis* pv. *vesicatoria* strain
653   IBSBF1387; (1689) - *X. axonopodis* pv. *vesicatoria* strain 1689; (1770) - *X. axonopodis* pv. *allii*
654   strain IBSBF1770; (11280) - *X. axonopodis* pv. *phaseoli* strain IAC11280; (1711) - *X. alfafae*
655   subsp. *alfalfae* strain 1711; (M89) - *X. axonopodis* pv. *passiflorae* strain M89; (1727) - *X.
656   axonopodis* pv. *patelli* strain 1727; (1729) - *X. axonopodis* pv. *coracanae* strain strain 1729; (1954)
657   - *X. axonopodis* pv. *manihotis* strain IBSBF 1954; (13755) - *X. axonopodis* pv. *phaseoli* var.
658   *fuscans* strain IAC13755; (1721) - *X. citri* pv. *rhynchosiae* strain 1721; (2008) - *X. axonopodis* pv.
659   *vasculorum* strain IBSBF 2008; (1696) - *X. axonopodis* pv. *axonopodis* strain 1696; (1478) - *X.
660   axonopodis* pv. *dieffenbachiae* strain IBSBF 1478; (1694) - *X. axonopodis* pv. *glycines* strain 1694;
661   (1718) - *X. citri* pv. *cajani* strain 1718; (1719) - *X. citri* pv. *sesbaniae* strain 1719; (Xf) *Xylella*
662   *fastidiosa* strain 9a5c. MW: molecular weight (ladder 100 bp).

663

Figure 6: Specificity and sensitivity of amplification by *in vivo* PCR. Conventional PCR of bacterial exudate using the specific markers for *XccA*, *XauB*, *XauC*, and *Xacm*. The minimum concentration detected by PCR (A) for *XccA* was 1.5×10^4 CFU ml^{-1}, (B) for *XauB* was 1.5×10^5 CFU ml^{-1}, (C) for *XauC* was 1.5×10^5 CFU ml^{-1}, and (D) for *Xacm* 1.5×10^4 CFU ml^{-1}. PC: positive control. NC: negative control of leaves inoculated with water. MW: molecular weight (ladder 100 bp).

670

Figure 7: Specificity of the markers in exudate samples from leaves inoculated by spraying with *XccA* strain. PC: Positive control *in vitro* DNA extraction; *XccA, XauB, XauC*, and *Xacm* PCR using XccAm, XauBm, XauCm, and Xacmm specific markers respectively, for exudate samples of citrus canker lesions induced by *XccA*306. MW: molecular weight (ladder 100 bp).

Relation of compatible host and causal agents

Source: (Gottwald et al. 1991; Gottwald et al. 1988; Graham et al. 1990; Jaciani 2012; Schoulties et al. 1987; Schubert et al. 2001; Sun et al. 2004; Vernière et al. 1998). *The results presented in this table are means of several reports of the disease in plantations and studies of pathogenicity tests. +++ very pathogenic to host ++ moderately pathogenic to host + little pathogenic to the host

1 **Table 1** Relation of compatible host and causal agents

Host	Pathogen					
	XccA	*XccA**	*XccA*ʷ	*XauB*	*XauC*	*Xacm*
Citrus sinensis	+++	-	-	+	±	+
Citrus aurantium	+++	-	-	+	-	+
Citrus limon	++	-	-	++	+	-
Citrus limonia	+++	-	-	-	+	+
Citrus latifolia	++	-	-	-	+	+
Citrus aurantiifolia	+++	+	+	+	+++	+
Citrus macrophylla	+	-	+	-	-	-
Citrus paradisi × *Poncirus trifoliata*	+++	-	-	-	++	+
Citrus reticulata (Tangerina 'Ponkan')	+	-	-	-	+	-
Citrus reticulata (Tangerina 'Cravo')	++	-	-	-	+	+
Citrus reshni	+	-	-	-	+	-
Citrus paradisi	+++	-	-	-	-	+
Citrus maxima	+	-	-	+	-	-

3 *The results presented in this table are means of several reports of the disease in plantations and studies of
4 pathogenicity tests [10, 12, 13, 16-20].
5 +++ very pathogenic to host
6 ++ moderately pathogenic to host
7 + little pathogenic to the host

General characteristics of the reference genomes

[1]WDCM (World Data Centre for Microorganisms)

[2]IBSBF - Instituto Biológico de São Paulo

[3]*Xanthomonas euvesicatoria* pv. *citrumelonis* strain 1510 is the same as *X. alfalfae* subsp*. citrumelonis* strain 1510 (https://www.ncbi.nlm.nih.gov/nuccore/NZ_LAUO00000000.1)

1   **Table 2** General characteristics of the reference genomes

Organism	WDCM[1] (Accession number)	Abbreviations	Genome	NCBI Reference Sequence	CDSs	Ref
X. citri subsp. *citri* pathotype A strain 306	IBSBF[2] (1594)	*XccA306*	Complete	NC_003919.1	4,687	(Da Silva et al. 2002)
Xanthomonas citri subsp. *aurantifolii* pathotype B strain 1561	IBSBF (409)	*XauB1561*	Complete	CP011250.1	4,397	Unpublished
Xanthomonas citri subsp. *aurantifolii* pathotype C strain 1559	IBSBF (381)	*XauC1559*	Complete	CP011160.1	4,733	Unpublished
X. alfalfae subsp. *citrumelonis* strain 1510	IBSBF (1925)	*Xacm1510*	Draft	GCF_005059785.1[3]	4,017	Unpublished

3   [1]WDCM (World Data Centre for Microorganisms)
4   [2]IBSBF - Instituto Biológico de São Paulo
5   [3]*Xanthomonas euvesicatoria* pv. *citrumelonis* strain 1510 is the same as *X. alfalfae* subsp. *citrumelonis* strain 1510
6   (https://www.ncbi.nlm.nih.gov/nuccore/NZ_LAUO00000000.1)

Molecular markers selected for the assays.

The oligonucleotides and amplicons generated by PCR areprotected by the patent application registration under process number BR 10 2018 067332 7.

1   **Table 3** Molecular markers selected for the assays

Pathotypes	Markers	Forward	Reverse	Amplicon size (bp)	Tm (°C)
XccA	**XccAm**	ATGCTGAGCAA GCCTTCGAT	AGCTGGGAACGA TGATGGTG	509	59
XauB	**XauBm**	TCGATCGCACG GACTACTTG	AAAATGCGGCTC TCCCTCTC	434	59
XauC	**XauCm**	CACTGGAGGCA GGAGTCGAG	CCACCCTCAAGTT CAGCAACA	160	59
Xacm	**Xacmm**	ACCAACACCTT GTGGTCGTA	TGTTCGTCAAACC GGCCA	115	59

3   The oligonucleotides and amplicons generated by PCR areprotected by the patent application registration under
4   process number BR 10 2018 067332 7.

Figure 1

Experimental design.

Stages and procedures from the development of the *in silico* stages to the validation of the molecular results of the plant diagnosis.

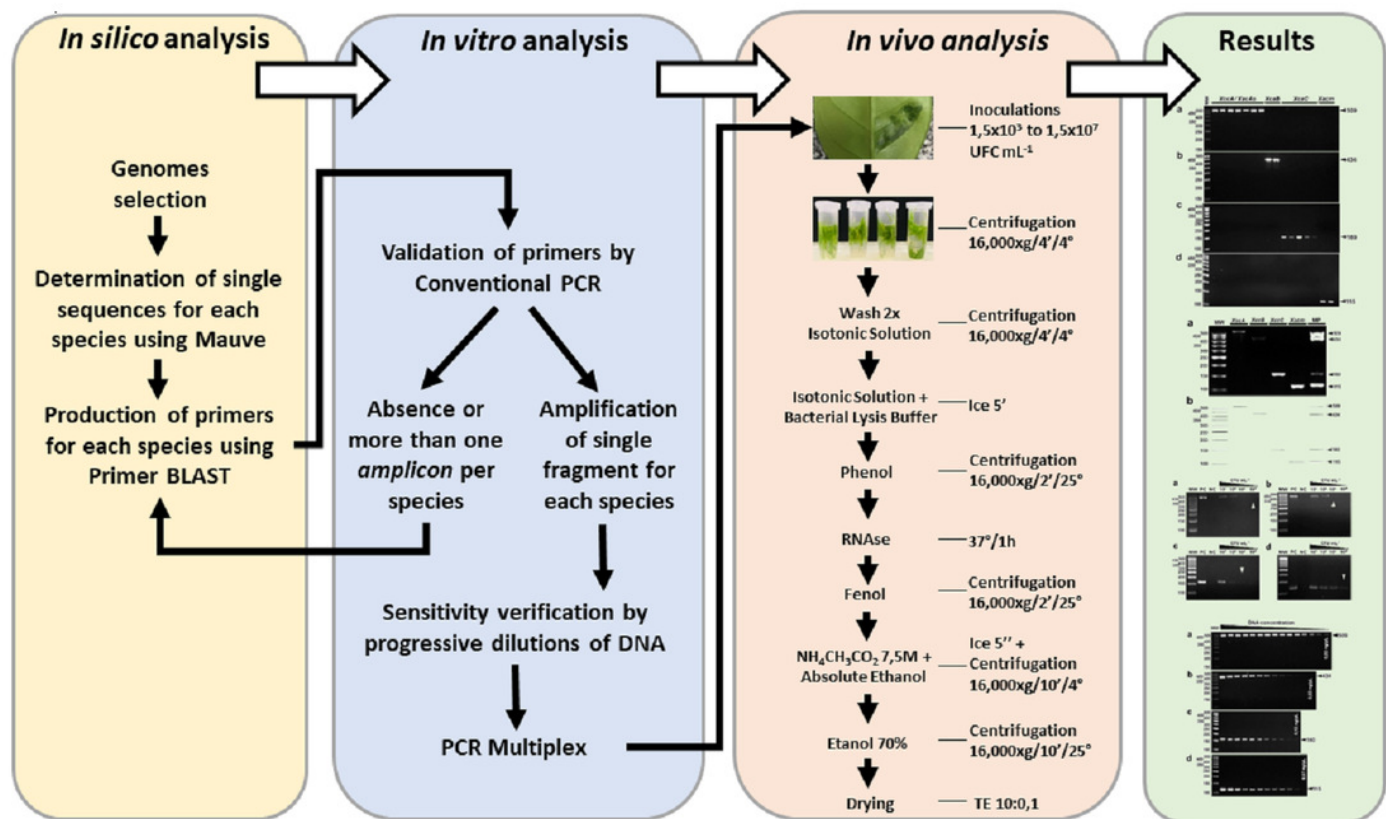



Figure 2

Specificity of molecular markers *in vitro*.

Conventional PCR electrophoresis using Xcc-specific oligonucleotides for *Xanthomonas citri* subsp*. citri* pathotypes A (*XacA*) and A* (*XacA**) with a 509 bp amplicon, (A) *Xanthomonas fuscans* subsp*. aurantifolii* pathotype B (*XauB*) with 434 bp amplicon, (B) *Xanthomonas fuscans* subsp*. aurantifolii* pathotype C (*XauC*) with 160 bp amplicon, (C) *Xanthomonas alfalfae* subsp*. citrumelonis* (*Xacm*) with an amplicon of 115 bp, and (D) MW: molecular weight (ladder 100 bp).

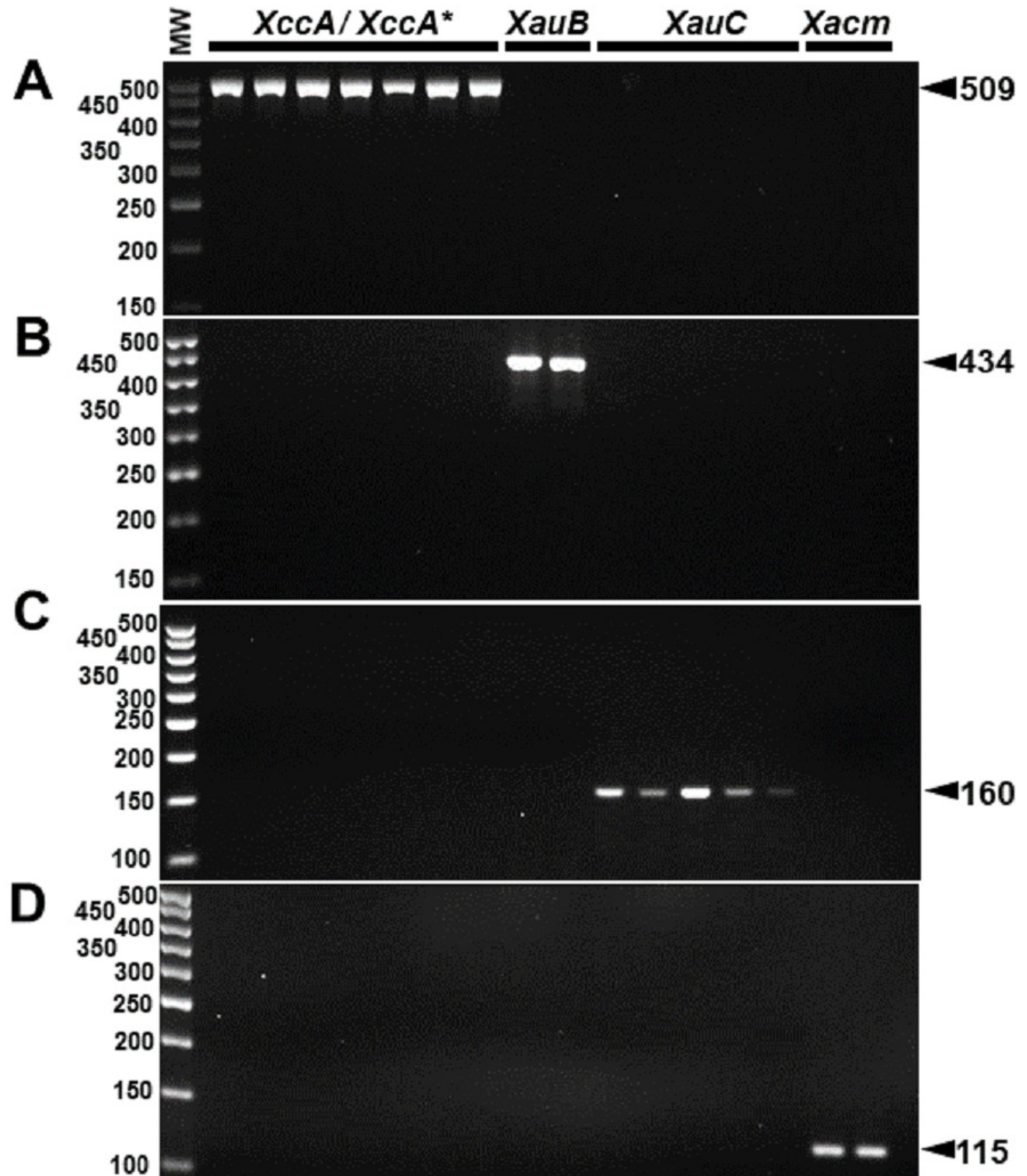



Figure 3

Progressive DNA dilutions demonstrating the sensitivity of PCR.

(A) *XccA* (0.02 ng/μl, ~70 cells), (B) *XauB* (0.03 ng/μl, ~100 cells), (C) *XauC* (0.12 ng/μl, ~400 cells), and (D) *Xacm* (0.07 ng/μl, ~200 cells). MW: molecular weight (ladder 100 bp).

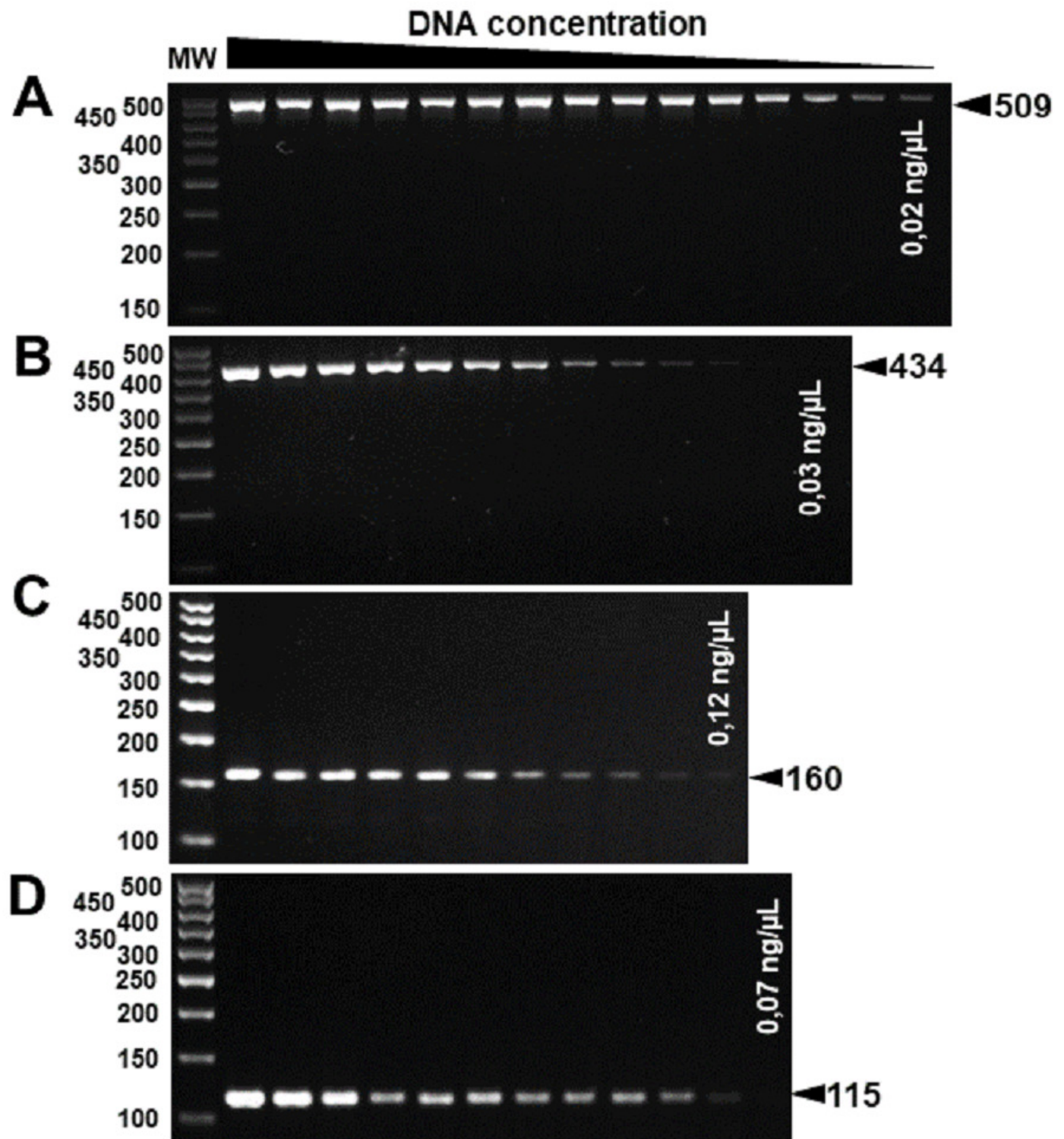



Figure 4

Multiplex PCR of *Xanthomonas* that infect citrus.

(A) Multiplex PCR electrophoresis containing the combination of the DNA of one species with all pairs of specific primers generating only their amplicon (lines 2 to 5). MP - Combination of DNAs of all pathotypes with all primers (line 6). MW: molecular weight (ladder 100 bp) and (B) Theoretical electrophoresis of multiplex PCR.

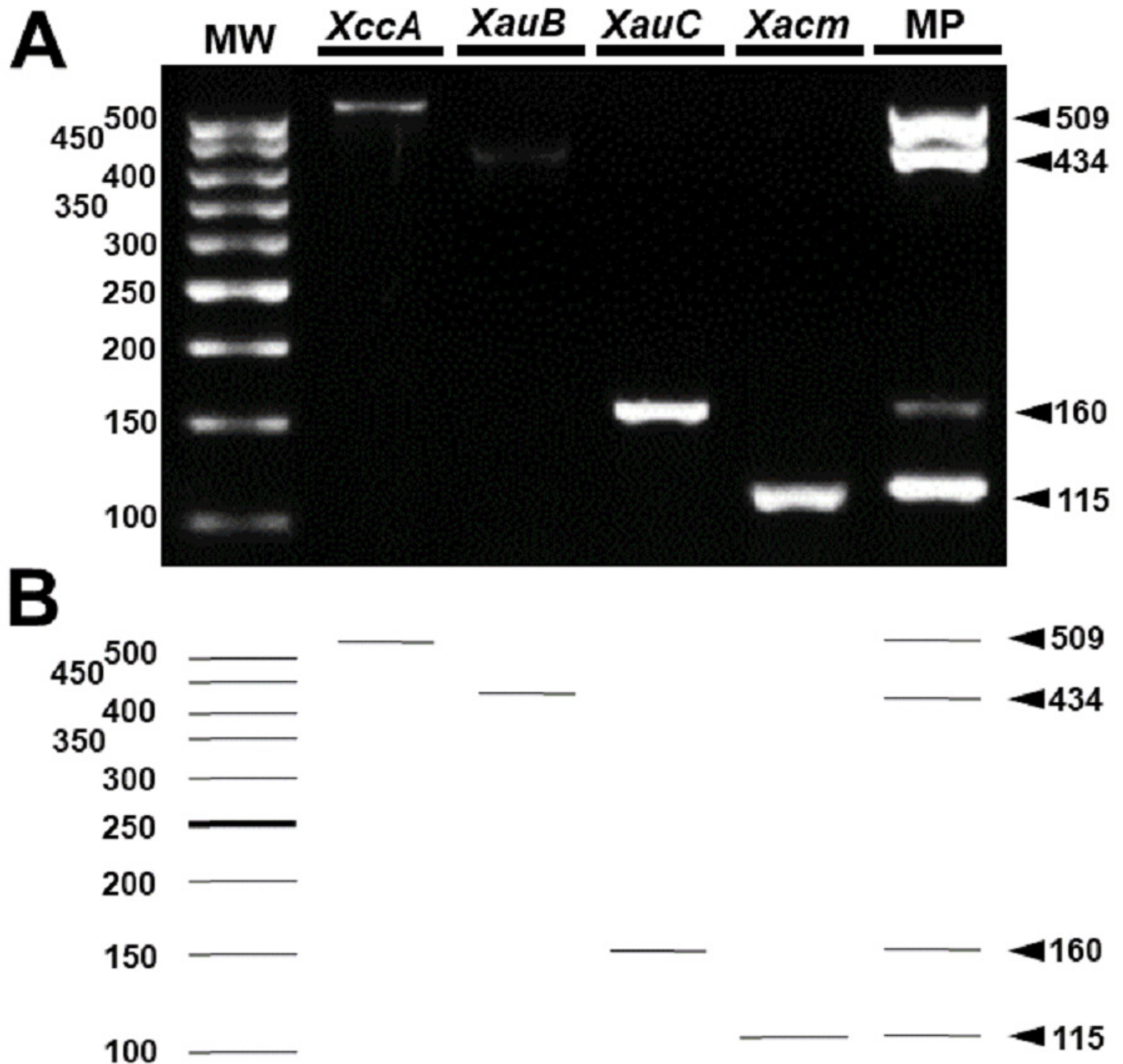
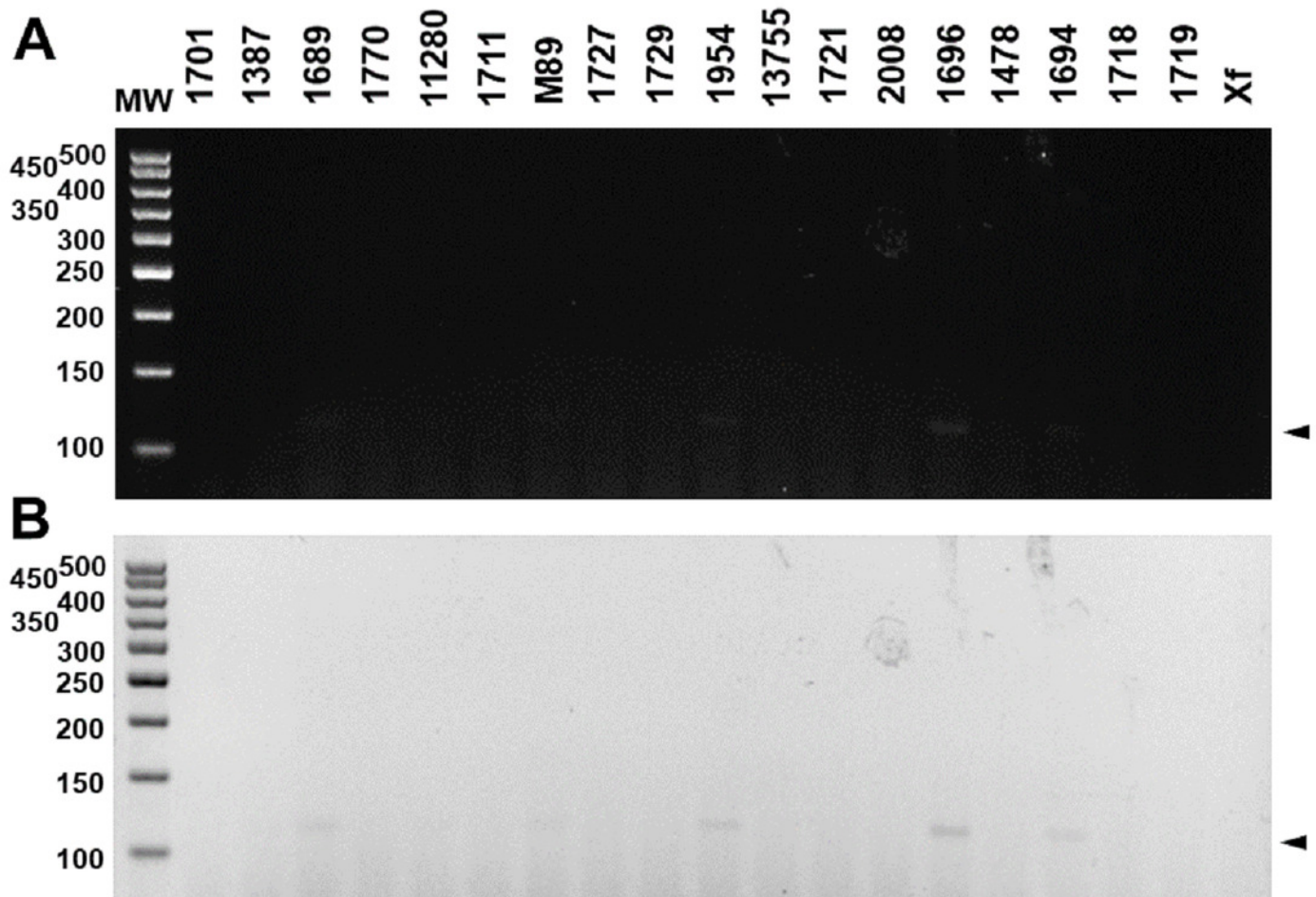



Figure 5

Multiplex PCR electrophoresis of *Xanthomonas* sp. that do not infect citrus.

(1701) - *X. axonopodis* pv*. vignicola* strain 1701; (1387) - *X. axonopodis* pv. *vesicatoria* strain IBSBF1387; (1689) - *X. axonopodis* pv. *vesicatoria* strain 1689; (1770) - *X. axonopodis* pv. *allii* strain IBSBF1770; (11280) - *X. axonopodis* pv. *phaseoli* strain IAC11280; (1711) - *X. alfafae* subsp*. alfalfae* strain 1711; (M89) - *X. axonopodis* pv*. passiflorae* strain M89; (1727) - *X. axonopodis* pv*. patelli* strain 1727; (1729) - *X. axonopodis* pv*. coracanae* strain strain 1729; (1954) - *X. axonopodis* pv*. manihotis* strain IBSBF 1954; (13755) - *X. axonopodis* pv*. phaseoli* var*. fuscans* strain IAC13755; (1721) - *X. citri* pv*. rhynchosiae* strain 1721; (2008) - *X. axonopodis* pv*. vasculorum* strain IBSBF 2008; (1696) - *X. axonopodis* pv*. axonopodis* strain 1696; (1478) - *X. axonopodis* pv*. dieffenbachiae* strain IBSBF 1478; (1694) - *X. axonopodis* pv*. glycines* strain 1694; (1718) - *X. citri* pv*. cajani* strain 1718; (1719) - *X. citri* pv*. sesbaniae* strain 1719; (Xf) *Xylella fastidiosa* strain 9a5c. MW: molecular weight (ladder 100 bp).

A

MW 1701 1387 1689 1770 11280 1711 M89 1727 1729 1954 13755 1721 2008 1696 1478 1694 1718 1719 Xf

450 500
400
350
300
250

200

150

100 ◀

B

450 500
400
350
300
250

200

150

100 ◀

Figure 6

Specificity and sensitivity of amplification by *in vivo* PCR.

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