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2 **Characterization, genetic diversity and distribution of *Xanthomonas***

3 ***campestris* pv. *campestris* races causing black rot disease in**

4 **cruciferous crops of India**

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10

11 Black rot, caused by *Xanthomonas campestris* pv. *campestris* (Xcc) is a disease of crucifer

12 crops. The objective of this study was to characterize races of Xcc, their distribution and

13 genetic diversity in India. Two hundred and seventeen isolates of bacteria were obtained from

14 12 different black rot-infected crucifer crops from 19 states of India; these were identified as

15 Xcc based on morphology, *hrp* gene and 16S rRNA gene based molecular markers and

16 pathogenicity tests. Characterization of races was performed by using a set of seven

17 differential crucifer hosts, comprising two cultivars of turnip (*Brassica rapa* var. *rapa*) and

18 cultivars of Indian mustard (*B. juncea*), Ethiopian mustard (*B. carinata*), rapeseed mustard

19 (*B. napus*), cauliflower (*B. oleracea*) and cabbage (*B. oleracea* var. *sabauda*). Races 1, 4 and

20 6 of Xcc were identified and, among these races, race 1 followed by race 4 dominated most

21 of the states of India. Genetic diversity of the Indian isolates of Xcc was analysed using

22 repetitive sequence-based PCR (rep-PCR) including primers for REP (repetitive extragenic

23 palindromic), ERIC (enterobacterial repetitive intergenic consensus) and BOX (amplifying

24   with BOX A1 R primer) repetitive elements. This method of fingerprinting grouped the

25   isolates into 56 different DNA types (clusters) with a 75% similarity coefficient. Among

26   these clusters, DNA types 22 and 53 contained two different races 1 and 4, whereas DNA

27   type 12 contained races 1, 4 and 6. However, no clear relationship was observed between

28   fingerprints and races, hosts or geographical origin.

29   *Keywords: Brassica*, crucifers, genetic diversity, races, rep-PCR, *Xanthomonas campestris*

30   pv. *campestris*

31

32   Introduction

33   Black rot disease of crucifers, caused by *Xanthomonas campestris* pv. *campestris* (Xcc), is an

34   important disease across the world. Economically, the most important host species in the

35   Brassicaceae family is *Brassica oleracea*; this species includes cole crops, e.g. cauliflower

36   (*Brassica oleracea* var. *botrytis*), cabbage (*B. oleracea* var. *capitata*), kohlrabi (*B. oleracea*

37   var. *gongylodes*), broccoli (*B. oleracea* var. *italica*), Brussels sprouts (*B. oleracea* var.

38   *gemmifera*), kale (*B. oleracea* var. *acephala*), radish (*Raphanus sativus*), turnip (*B. rapa* var.

39   *rapa*), Indian mustard (*B. juncea*), vegetable mustard (*B. juncea*) and black mustard (*B.*

40   *nigra*), and other cruciferous crops such as weeds, and ornamentals can also be attacked. The

41   bacterium enters the plant system through infected seeds, wounds and hydathodes. The

42   disease is characterized by developing V-shaped yellow sectors with blackened veins along

43   the margin of leaves; later these sectors enlarge, become brown, necrotic and papery, and

44   may cover the whole leaf. For control of black rot disease, the best management strategy is

45   planting disease resistant varieties. However, black rot resistance, particularly in cole crops

46   (*B. oleracea*), is usually race-specific (Vicente *et al*., 2001), meaning that resistance breeding

47   programmes should be locally targeted and germplasm should be screened against locally

48   prevalent races of Xcc.

49   For characterization of the bacteria at species and pathovar level, classical methods

50   (Schaad *et al.*, 2001) and advanced molecular techniques, especially those based on PCR, are

51   very powerful (Massimo *et al*., 2007; Singh & Dhar, 2011). In the PCR-based technique, a set

52   of primers is developed from different conserved genes of bacteria, which is specific to a

53   particular group of bacteria and identifies the bacteria at species or pathovar level (Vincell &

54   Tisserat, 2008; Singh & Dhar, 2011). Previous studies have investigated the potential for

55   specific amplification of the *hrp* (hypersensitive response and pathogenicity) genes to

56   identify and detect *X. axonopodis* pv. *vesicatoria* in tomatoes and capsicums and *X.*

57   *campestris* pathovars in crucifers (Berg *et al*., 2006; Singh & Dhar, 2011). The *hrp* gene

58   cluster is crucial for the interaction between plant pathogenic bacteria and their hosts,

59   resulting in disease in susceptible plants or the hypersensitive response in resistant plants

60   (Walton, 1997). The *hrp* gene clusters are largely conserved among phytopathogenic bacteria,

61   where they encode type III secretion systems that deliver pathogenicity factors, elicitors and

62   avirulence proteins to the plant cell (Bonas, 1994; Hueck, 1998).

63   Pathovars of *X. campestris* can be subdivided into races on the basis of differential

64   responses caused by pathogen on various crucifer hosts. A postulated gene-for-gene model to

65   explain the relationship between races and cultivars of crucifer was previously described by

66   Vicente *et al*. (2001) within Xcc. The model presented was the simplest hypothesis involving

67   the smallest number of genes necessary to explain the observed interactions. However, nine

68   races of Xcc have been identified based on the interaction between differentials with *R* genes

69   and avirulence genes of the bacterial pathogen across the world. Races 1 and 4 predominate

70   worldwide, whereas other races 2, 3 and 5 are rare, and race 6 has been reported only in *B.*

71   *rapa* (Vicente *et al.*, 2006).

72   Assessment of the genetic variability of a bacterium can facilitate both its detection

73   and the investigation of its taxonomy and epidemiology. Genomic fingerprinting by PCR

74   amplification, with primers specific to highly conserved, repetitive elements such as the 35–

75   40 bp repetitive extragenic palindromic (REP) sequence, the 124–127 bp enterobacterial

76   repetitive intergenic consensus (ERIC) and the 154 bp BOX element, has been used

77   successfully to characterize a large number of bacteria and also differentiates closely related

78   strains of bacteria (Versalovic *et al*., 1991). Repetitive sequence-based PCR (rep-PCR) is a

79   rapid, low-cost, and reliable method that has been extensively used to assess the genetic

80   diversity of Xcc strains (Lema *et al*., 2012; Mulema *et al*., 2012).

81   *Xanthomonas campestris* pv. *campestris* is found widely in the tropics, subtropics and

82   temperate climatic conditions in India (Singh *et al*., 2011), but no information is available on

83   the races of Xcc and their distribution in different agroclimatic regions. Understanding local

84   bacterial pathogen races and their genetic diversity is the first step to successful plant

85   breeding and integrated disease management programmes. The purpose of this study was to

86   characterize the races of Xcc isolates collected from different crucifer crops from different

87   agroclimatic conditions and investigate their genetic diversity and geographic distribution.

88   The resulting distribution and race profiling of the pathogen will be used to optimize a

89   breeding programme for black rot resistance, particularly in cole crops, for Indian farmers.

90

Materials and methods

Isolation and characterization of Xcc

93   Leaf samples from one or two plants with black rot disease (V-shaped lesions with blackened

94   veins) were collected per field from 12 crucifer crops: cauliflower, cabbage, kohlrabi,

95   broccoli, Brussels sprouts, kale, radish, turnip, Indian mustard, Ethiopian mustard, vegetable

96   mustard, black mustard. Black rot disease samples were collected from 12 of the 15

97   agroclimatic regions of India, covering 19 major states of the country, where the majority of

98   crucifer crops are grown (Table S1). The diseased samples were dried between sheets of

99   paper at room temperature before isolation of Xcc. A loopful of suspension was streaked on

100 nutrient sucrose agar medium containing 23 g nutrient agar, 20 g sucrose and 5 g agar powder

101 per litre and incubated at 28 °C for 48 h (Schaad *et al*., 2001). A typical Xcc colony (pale

102 yellow, raised, mucoid) from each plate was subcultured on YGCA slants containing 10 g

103 yeast extract, 10 g D-glucose anhydrous, 20 g calcium carbonate and 20 g agar powder in 1 L

104 water (Schaad *et al*., 2001). The cultures were routinely grown on YGCA medium and stored

105 at –80 °C in a mixture of nutrient broth and glycerol (100%) in a ratio of 1:1 for further

106 study. Representative isolates of races 1, 4 and 6 were deposited in the Indian Type Culture

107 Collection (ITCC) New Delhi, India with accession numbers Xcc-C102 (ITCC-BH-0009)

108 and Xcc-C12 (ITCC-BH-0010) for race 1, Xcc-C30 (ITCC-BH-0011) and Xcc-C106 (ITCC-

109 BH-0013) for race 4 and Xcc-C278 (ITCC-BH-0012) for race 6.

110

111 **Pathogenicity test**

112 The suspected isolates of Xcc were tested for their pathogenicity on susceptible cauliflower

113 cv. Pusa Sharad seedlings, which had been grown in the field at the Division of Plant

114 Pathology, Indian Agricultural Research Institute, New Delhi. The three youngest leaves of

115 30-day-old plants were inoculated using small scissors that had been dipped in a suspension

116 from a 48-hour-old culture of Xcc grown on NSA medium at 28°C (Singh *et al*., 2011). The

117 black rot disease reaction was recorded 15 days after pathogen inoculation.

118

119   **Race characterization**

120   Race characterization of 217 isolates of Xcc isolated from different agroclimatic regions of

121   India was performed using a set of seven cultivars of *Brassica* species: turnip (*B. rapa* var.

122   *rapa*) 'Just Right' F_1 and 'Seven Top' turnip, Indian mustard (*B. juncea*) 'Florida Broad

123   Leaf', Ethiopian mustard (*B. carinata*) PI 199947, rapeseed mustard (*B. napus*) 'Cobra' line

124   14R, cauliflower (*B. oleracea*) 'Miracle' F_1 and cabbage (*B. oleracea* var. *sabauda*) 'Wirosa'

125   F_1. The cultivars and accessions of *Brassica* spp. used for race typing were as described by

126   Vicente *et al*. (2001, 2006). The seeds of these differential lines were obtained from

127   University of Warwick, UK and from Otis S. Twilley Seed Co. Inc. (*B. juncea*, *B. rapa* var.

128   *rapa* (EC732033 to EC732035)). A 48-h-old culture of each isolate of Xcc was pelleted and

129   resuspended in sterilized distilled water to give an OD of 0.1 at 600 nm using a

130   spectrophotometer. The suspensions were then used to inoculate 35-day-old plants of Just

131   Right turnip F_1, Seven Top turnip, Florida Broad Leaf, PI 199947 and Cobra 14R and 30-day-

132   old plants of Miracle F_1 and Wirosa F_1 plants. Leaves were inoculated by clipping secondary

133   veins, near the margins, with small scissors dipped in the bacterial suspension. Ten points of

134   inoculation were made in the youngest leaves on each plant, with three replications. The

135   number of infections per leaf and the severity of symptoms were assessed 15 and 30 days

136   after inoculation. Symptoms were rated on a scale of 0–9, based on the relative size of the

137   largest lesion on the leaf, as described by Vicente *et al*. (2001).

138

139   **DNA extraction and molecular characterization by PCR of a *hrp* gene**

140   The isolates of Xcc were grown in nutrient broth for 24 h at 28 °C. The total genomic DNA

141   of the bacteria was extracted by the CTAB method (Murray & Thompson, 1980). Molecular

142   characterization of 217 isolates of Xcc was performed using PCR of a *hrp* gene, as described

143   by Singh *et al*. (2014). Two primers, designed to amplify the 3′ end region of the *hrpF* gene

144   locus 3521496 to 3522264 with product size 769 bp were used: Dhrp_Xcc_F 5′-

145   GTGGCCATGTCGTCGACTC-3′ and Dhrp_Xcc_R 5′-GAATAAACTGTTTCCCCAATG-

146   3′. Twenty-five microlitres of PCR reaction mixture containing 1 x *Taq* buffer, 0.2 mM

147   dNTPs (0.5 μL), 1.5mM MgCl$_2$, 200 nM forward and reverse primers, 1.2 U GoTaq Flexi

148   DNA polymerase (Promega) and 100ng DNA was used for PCR amplification. The PCR was

149   carried out under the following conditions using a C-1000 gradient thermocycler (Bio-Rad):

150   94 °C for 3 min; 40 cycles of 95 °C for 40 s, 60 °C for 40 s and 72 °C for 40 s; and 72 °C for

151   5 min. Electrophoresis was carried out using 1.2% agarose gel containing ethidium bromide

152   at 60 V for 1.5 h. Products were visualized on a Gel Doc XR+ gel documentation system

153   (Bio-Rad) under UV light (300 nm) and photographed using IMAGELAB v. 2.0.1 software

154   (Bio-Rad) for gel analysis.

155

156   **Molecular characterization by PCR of the 16S rRNA gene**

157   Molecular characterization of Xcc isolates by PCR of the 16S rRNA gene was also

158   performed. Two primers were designed to amplify the 3′ end region of the 16S rRNA gene

159   locus 4561337 to 4562295 of Xcc B100 (accession no. AM920689) to give a product size of

160   959 bp: Xcc 16S_ F: 5′-GCAAGCGTTACTCGGAATTA-3′ and Xcc16S_R: 5′-

161   TACGACTTCACCCCAGTCAT-3′. The primers were designed using PRIMER3

162   (www.frodo.wi.nit.edu) and checked for specificity *in silico* using www.insilico.ehu.es. The

163   primers were validated for their universality across *Xanthomonas* and related bacteria by

164   primer BLAST using www.ncbi.nlm.nih.gov. Twenty-five microlitres of PCR reaction mixture

165   containing 5× *Taq* buffer (5 μL), 10 mM dNTPs (0.5 μL), 25 mM MgCl$_2$ (1.5 μL), 10 μM

166   forward and reverse primers (0.5 μL each), *Taq* DNA polymerase (0.24 μL), molecular grade

167   water and 1 μL DNA (100 ng) was used for PCR amplification. PCR was carried out under

168   the following conditions using a C-1000 gradient thermocycler: 95 °C for 2 min; 30 cycles of

169   95 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min; and 72 °C for 5 min. The PCR product

170   was mixed with 1 μL loading dye. A 1.2% agarose gel, prepared in $1 \times$ Tris-acetate-EDTA

171   (TAE) buffer containing ethidium bromide (0.5 μg mL^{-1}), was used for electrophoresis,

172   which was carried out at 60 V for 1.5 h. Products were visualized on a Gel Doc XR+ gel

173   documentation system under UV light (300 nm) and photographed using IMAGELAB v. 2.0.1

174   for gel analysis. Out of 217 isolates, the PCR products of five isolates, representing different

175   races, were purified: Xcc-C102 (race 1, cabbage, Laxminagar, Delhi), Xcc-C12 (race 1, black

176   mustard, Laxminagar, Delhi), Xcc-C30 (race 4, cabbage, Karnataka), Xcc-C 106 (race 4,

177   vegetable mustard, Laxminagar, Delhi) and Xcc-C278 (race 6, cabbage, Laxminagar, Delhi).

178   Purification was performed using a Gel and PCR Clean-Up system (Promega) kit.

179   Sequencing of these purified products was performed by Sanger's method (Applied

180   Biosystem Machine-3130; Chromas Biotech, Bangalore, India). The 16S rRNA gene

181   sequences of these isolates were submitted to NCBI GenBank. The accession number of

182   isolates Xcc-C102, Xcc-C12, Xcc-C30, Xcc-C106 and Xcc-C278 were KM458092,

183   KM458093, KM458094, KM458095 and KR061873, respectively. The sequences obtained

184   were aligned pairwise. Multiple alignments compared to those of the type/reference strains

185   were performed with CLUSTALW v. 1.7 software (Thompson *et al.*, 1997). Phylogenetic trees

186   were generated using MEGA v. 6.0 (Tamura *et al.*, 2013) with default parameters, K2P

187   distance model (Kimura, 1980) and the neighbour-joining algorithm (Saitou & Nei, 1987).

188   Statistical support for tree nodes was evaluated by bootstrap (Felsenstein, 1985) analyses

189   with 1000 samplings.

190

191 **Genetic diversity by rep-PCR**

192 Genetic diversity of 217 Xcc isolates was assessed by repetitive sequence-based PCR (rep-

193 PCR) with the BOX-PCR (BOXA1R: 5′-CTACGGCAAGGCGACGCTGACG-3′), ERIC-

194 PCR (ERIC-1R: 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and ERIC-2: 5′-

195 AAGTAAGTGACTGGGGTGAGCG-3′), and REP-PCR (REP1R-I 5′-

196 IIIICGICGICATCIGGC-3′ and REP2-I 5′-ICGICTTATCIGGCCTAC-3′) using conditions

197 described by Schaad *et al*. (2001). For REP-, ERIC- and BOX-PCR amplifications were

198 carried out in a final volume of 25 μL. PCR products were analysed by gel electrophoresis in

199 a 1.5% agarose gel, stained with ethidium bromide, documented in Gel Doc XR+ gel

200 documentation system under UV light (300 nm) and photographed using IMAGELAB v. 2.0.1

201 as described by Singh *et al*. (2011). The positions of bands were assessed visually. The

202 normalized data generated from PCR (BOX-, ERIC- and REP-PCR) fingerprinting profiles

203 were used either separately or combined together for generating a similarity matrix by using

204 SIMQUAL module for the NTSYSPC v. 2.02e. The similarity matrix thus generated was used

205 for cluster analysis by the unweighted pair group method of arithmetic average (UPGMA)

206 using sequential, agglomerative, hierarchical, nested clustering module of NTSYSPC v. 2.02e.

207 The output data were graphically presented as a phylogenetic tree (Rohlf, 1998).

208

209 Results

210 **Characterization of bacteria**

211 Two hundred and seventeen isolates of Xcc were isolated from 12 crucifer crops from 12

212 agroclimatic regions ranging from temperate to subtropical climates, in 19 states of India

213 (Table S1). These isolates produced yellow, translucent, raised, mucoid colonies on NSA

214 medium. They were Gram-negative, rod shaped, aerobic and with a monotrichous flagellum.

215 All the isolates were pathogenic on cauliflower cv. Pusa Sharad and produced typical black

216 rot symptoms including blackening of veins within 15 days after inoculation. These isolates

217 were considered to be Xcc on the basis of morphological and pathogenicity tests.

218 Amplification of DNA from the isolates using primers Dhrp_Xcc_F and Dhrp_Xcc_R, which

219 are specific to Xcc, confirmed the identification. Amplification was not observed from any

220 isolates belonging to other *Xanthomonas* species such as *X. oryzae* pv. *oryzae* or *X.*

221 *axonopodis* pv. *punicae* (data not shown).

222   The nucleotide sequences of the 16S rRNA gene of three races, race 1 (Xcc-C102,

223 Xcc-C12), race 4 (Xcc-C30, Xcc-C106) and race 6 (Xcc-C278) of Xcc were analysed. Four

224 isolates had a more than 99% similarity index to each other and also to Xcc isolates B100

225 (race 1, China) and 8004 (race 9, cauliflower, UK; Fig. 1). In contrast, Xcc-C106 (race 4),

226 isolated from vegetable mustard cv. Pusa Sag 1 from Delhi, showed a 99% similarity index

227 with Xcc ATCC 33913 (race 3, Brussels sprout, UK), *X. arboricola*, Xcc DBRU and *X.*

228 *campestris* LMG 5793, *Pseudomonas* sp. DTPF-3 and *Xylella fastidiosa* were used as the

229 out-groups and these showed an 85% similarity index with *Xanthomonas* spp.

230

231 **Race characterization of Xcc**

232 The Indian isolates belong to three races, i e. races 1, 4 and 6. *Xanthomonas campestris* pv.

233 *campestris* isolates that showed a positive disease reaction in 5 differential cultivars (Wirosa

234 F_1, Just Right turnip, line 14R of Cobra, PI 199947, Florida Broad Leaf Mustard) were

235 designated as race 1 isolates. Race 4 showed positive disease reactions only in 2 cultivars i.e.

236 Wirosa F_1 and Miracle F_1, whereas race 6 showed positive reactions in all seven cultivars

237 (Table 1). Out of 217 isolates of Xcc, 119 isolates belonged to race 1 (54.83%), 97 isolates

238   belonged to race 4 (44.70%) and a single isolate, Xcc-C278 (from Cabbage, Delhi), belonged

239   to race 6 (0.47%). All three races of Xcc were isolated from cabbage, whereas only race 1

240   and race 4 infected cauliflower, turnip, broccoli, kohlrabi, Indian mustard, radish, vegetable

241   mustard and black mustard. It was also noted that race 1 also infected kale and race 4 infected

242   Brussels sprouts. Race 1 dominated in all 12 agroclimatic regions of India compared to race 4

243   and 6. Race 1 was found in most of the states of India except Andhra Pradesh and Orissa,

244   whereas race 4 was found in all the states of India except Meghalaya, Manipur, Goa (Fig. 2);

245   race 6 was rare and found only in cabbage from Delhi.

246

247   **Genetic diversity by rep-PCR**

248   The genetic diversity of races of *X. campestris* pv. *campestris* was assessed by cluster

249   analysis of the genomic fingerprint patterns obtained from rep-PCR. Three different sets of

250   primers were used and DNA fragments of 300 bp to 6.0 kb were amplified by BOX-PCR,

251   400 bp to 8.0 kb by ERIC-PCR and 300 bp to 6.0 kb by REP-PCR. The highest number of

252   amplicons was obtained by BOX-PCR (29 amplicons), while 27 amplicons were obtained

253   with ERIC-PCR and 18 amplicons with REP-PCR. The amplicons obtained varied between

254   isolates with each method of PCR (Fig. 3) and all 217 isolates of Xcc clustered into 56 DNA

255   types (clusters) at 75% similarity coefficient. Representative isolates of each cluster are given

256   in Figure 4; among them, DNA type 22 contained the maximum of 57 isolates that were

257   obtained from different agroclimatic conditions and different crucifer hosts such as

258   cauliflower, cabbage, broccoli Indian mustard and black mustard cultivars. The second

259   largest cluster was DNA type 53 (21 isolates), followed by DNA type 12 (16 isolates). DNA

260   types 22 and 53 both consisted of two different races, 1 and 4, whereas DNA type 12

261   contained races 1, 4 and 6 and was the most genetically diverse group of Xcc isolates (Fig. 4).

262

Discussion

264   Black rot disease is widely distributed in all agroclimatic regions of India. Until now, no

265   exhaustive collection, isolation, identification and race characterization of Indian Xcc isolates

266   has been accomplished. In the present study, isolates of Xcc causing black rot were

267   characterized by classical methods (cultural and morphological), pathogenicity tests and

268   molecular techniques including PCR of a *hrp* gene (Berg *et al*., 2006; Singh & Dhar, 2011),

269   which is highly conserved and enables differentiation of pathovars (Singh *et al*., 2014).

270   However, the PCR primers Dhrp_Xcc_F and Dhrp_Xcc_R, based on *hrp* gene sequences,

271   could not differentiate races of Xcc. In addition, nucleotide sequence analysis of the 16S

272   rRNA gene of representative isolates of three races i.e. race 1 (Xcc-C102, Xcc-C12), race 4

273   (Xcc-C30, Xcc-C106) and race 6 (Xcc-C278) of Xcc, using MEGA 6 software did not

274   distinguish between races. Based on rRNA gene sequence analysis, the isolates of Xcc in this

275   study were very close to isolate B100 from Italy, and Xcc8004 from the UK, apart from

276   isolate Xcc-C106, which was very close to ATCC33913 from China, as reported earlier by

277   Popovic *et al.* (2013). It might be speculated that the Indian strains may have migrated from

278   these countries through seeds. This finding indicates that 16S rRNA gene sequence analysis

279   is a useful tool to characterize the bacteria and also show diversity within the isolates, but

280   cannot be used to identify races.

281   Worldwide, nine races of Xcc have been identified, based on their interaction with

282   differential cultivars (Kamoun *et al.*, 1992; Ignatov *et al*., 1999; Vicente *et al.*, 2001; Taylor

283   *et al*., 2002; Fargier & Manceau, 2007; Jensen *et al*., 2010). In this study, the three races 1, 4

284   and 6, were found in *B. oleracea* crops in India . However races 1 and 4 dominated in most of

285   the agroclimatic regions of India having tropical, subtropical and temperate climates,

286   indicating that distribution of races is not affected by climatic conditions. This is in

287   agreement with the study of Vicente *et al.* (2001), which reported that races 1 and 4 were

288   predominant worldwide and that the other races, 2, 3, 5 and 6 were rare. Although, in this

289   study, 217 isolates of Xcc were taken from diverse climatic conditions and hosts, races 2, 3

290   and 5 were not found. However, race 6 was found on cabbage from Delhi, which is in the

291   Trans-Gangatic plains region. Previously, race 6 has only been reported in *B. rapa* and found

292   rarely (Vicente *et al.*, 2006). Jensen *et al.* (2010) reported that races 1, 4 and 6 are the most

293   common in cabbage; blight-like symptoms were produced by a race 7 isolate and by some

294   isolates of races 1, 5 and 6 but no blight symptoms were produced by isolates of race 4. This

295   was contrary to the result of the present study, where all isolates of Xcc produced typical V-

296   shaped black rot disease symptoms on their respective hosts. Hence, for development of

297   varieties resistant to black rot disease, particularly in *B. oleracea* (cole crops), testing with

298   both races 1 and 4, is a minimum requirement to evaluate the germplasm of crops (Soengas *et*

299   *al.*, 2007).

300   Analysis of the genetic diversity of Xcc clearly indicates that the different DNA types

301   did not originate from the same host and agroclimatic conditions. In addition, polymorphisms

302   resulting from repetitive sequences in bacterial genomes may be used to define differences

303   between species and pathovars. A similar level of separation was observed in previous rep-

304   PCR studies of *Xanthomonas* (Vicente *et al.*, 2006; Jensen *et al.*, 2010; Singh *et al.*, 2011).

305   The data obtained from pathogenicity and genetic variability analysis in this study confirm

306   previous findings describing the heterogeneity within Xcc (Ignatov *et al.*, 1999). The isolates

307   within the DNA types revealed complex but polymorphic bands resulting in groups of closely

308   related isolates based on their fingerprint pattern. Isolates recovered from leaf samples from

309   the same host and states/agroclimatic regions with the same race did not fall into one macro-

310   but microclusters spread over the dendrogram, although previous studies had demonstrated a

311   correlation with races (Jensen *et al*., 2010) or geographic origin (Massimo *et al.*, 2007). In the

312   present study, no correlation was obtained between races and genetic grouping. Thus, a

313   relationship between rep-PCR and races of a given group of isolates may not occur in all

314   investigations, and may depend on the repetitive sequences chosen for the analysis. This

315   study revealed the existence of variability within the Indian isolates of the Xcc population

316   and, among the three races, races 1 and 4 were distributed in most of the agroclimatic

317   conditions in different states of India. This information will be used in future breeding

318   programmes to develop varieties resistant to black rot disease, particularly in cole crops.

319

326

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404

Supporting Information

406 Additional Supporting Information may be found in the online version of this article at the

407 publisher's web-site.

408 **Table S1** List of *Xanthomonas campestris* pv. *campestris* isolates obtained from crucifer

409 crops grown in different states of India, year of collection, races, *hrp* gene-based PCR

410 reaction and DNA typing.

411

412 **Figure legends**

413 **Figure 1** Phylogenetic tree of 16S rRNA gene sequences of five Indian isolates of

414 *Xanthomonas campestris* pv. *campestris* (indicated with *) and 24 nucleotide sequences

415 obtained from the NCBI database. The evolutionary history was inferred using the maximum

416 likelihood phylogenetic tree method using MEGA v. 6.0.

417 **Figure 2** Distribution of races of *Xanthomonas campestris* pv. *campestris* in different states

418 of India.

419   **Figure 3** Fingerprinting of isolates of *Xanthomonas campestris* pv. *campestris* collected

420   from different hosts of crucifer by using BOX- (a), ERIC- (b) and REP- (c) PCRs. Lane M:

421   1.0 kb DNA ladder, lanes 1-56 Xcc-C3, Xcc-C7, Xcc-C20, Xcc-C23, Xcc-C26, Xcc-C98,

422   Xcc-C99, Xcc-C100, Xcc-C105, Xcc-C113, Xcc-C114, Xcc-C118, Xcc-C128, Xcc-C130,

423   Xcc-C132, Xcc-C133, Xcc-C135, Xcc-C137, Xcc-C138, Xcc-C140, Xcc-C141, Xcc-C142,

424   Xcc-C143, Xcc-C144, Xcc-C147, Xcc-C148, Xcc-C150, Xcc-C155, Xcc-C157, Xcc-C158,

425   Xcc-C160, Xcc-C163, Xcc-C167, Xcc-C169, Xcc-C170, Xcc-C173, Xcc-C181, Xcc-C188,

426   Xcc-C196, Xcc-C215, Xcc-C222, Xcc-C230, Xcc-C261, Xcc-C266, Xcc-C268, Xcc-C270,

427   Xcc-C271, Xcc-C272, Xcc-C274, Xcc-C276, Xcc-C279, Xcc-C281, Xcc-C285, Xcc-C286.

428   **Figure 4** Phylogenetic analysis of 217 isolates of *Xanthomonas campestris* pv. *campestris*

429   collected from different hosts of *Brassica* spp. The normalized data from BOX-, REP- and

430   ERIC-PCR of the isolates were used to generate a similarity matrix used for cluster analysis

431   by the unweighted pair group method of arithmetic average (UPGMA), and the results are

432   presented as a phylogenetic tree. The numbers in parentheses indicate the number of isolates

433   in each DNA type. Details of DNA types are given in Table S1.