

Allelic profile of Serbian *Xanthomonas campestris* pv. *campestris* isolates from cabbage

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SUMMARY

Xanthomonas campestris pv. *campestris* (Xcc), the causal agent of black rot disease of cabbage (*Brassica oleracea* var. *capitata* L.), is one of the most important bacteria which affect proper cabbage growth, leading to head weight and quality losses and thereby drastically reducing its marketing value. The pathogen is genetically diverse, which is evident from the presence of eleven races worldwide and more than thirty combinations of allelic profiles. Therefore, this study aimed to determine the allelic profiles of Serbian cabbage Xcc strains obtained in 2014. The analysis was done on three selected Xcc strains whose DNA was first amplified using polymerase chain reaction (PCR) with four housekeeping genes – P-X-*dnaK*, *fyuA*, *gyrB*, and *rpoD*, then sequenced, and the obtained sequences were finally used to determine allelic profiles. Allelic profiles were determined by comparison with 33 Xcc strains obtained from different hosts and regions, whose allelic profiles had been determined previously. A non-redundant database (NRDB) from the pubMLST was used for allelic profile determination and PhyloViz software for constructing a minimum spanning tree. The obtained allelic profile of all Serbian Xcc cabbage strains was 1, 3, 1, 1 for the P-X-*dnaK*, *fyuA*, *gyrB* and *rpoD* genes, respectively. This profile is assigned as sequence type 2 (ST2) and it coincides with a Portuguese *B. oleracea* Xcc strain, CPBF 213, originating from *B. oleracea* var. *costata*. No connection between sequence type (ST) and the host was detected.

Keywords: *Xanthomonas campestris*, black rot, cabbage, genetic diversity, allelic profile

INTRODUCTION

Yields and quality of cruciferous vegetables (fam. *Brassicaceae*), such as cabbage (*Brassica oleracea* var. *capitata* L.), cauliflower (*B. oleracea* var. *botrytis* L.),

broccoli (*B. oleracea* var. *italica* L.), kale (*B. oleracea* var. *sabellica* L.), etc. are widely affected by various pathogens (Neik et al., 2017). Their high nutritional value, extensive use in human diet, and annual production rate of about 250.000 hg/ha (www.fao.org/)

faostat/en) in Serbia strongly indicate the importance of these vegetables in agricultural production of this country. Cabbage is one of the most important crucifers worldwide, especially because of its richness in beneficial phytochemicals which are an important part of human nutrition (Singh et al., 2006).

Cabbage yields may be reduced by the activity of many plant pests and pathogens, and *Xanthomonas campestris* pv. *campestris* (*Xcc*), the causal agent of black rot disease, is one of the most important pathogens (Massomo et al., 2004; Popović et al., 2013, 2019; Afrin et al., 2019; da Silva et al., 2020). This pathogen affects cabbage growth, leading to head weight and quality losses, and thereby drastically reducing its economic value. Under favorable weather conditions, losses caused by *Xcc* on cabbage can exceed 50% (Williams, 1980). The main source of infection is the infected seed, but the pathogen can also spread via raindrops, wind, irrigation, non-sterile tools, etc., thus entering the host plant through leaf openings such as stomata, hydathodes or some artificially created wounds (Hugouvieux et al., 1998; Afrin et al., 2019). Disease symptoms can be easily noticed in the field as the blackening of leaf veins, which is one of the first indicators of pathogen presence and multiplication inside plant vascular vessels. Also, symptoms caused by *Xcc* can be noticed in the form of V-shaped chlorotic to necrotic lesions on leaf margins, which further expand towards leaf base. The presence of these symptoms contributes to stopping growth, plant wilting, and finally rotting (Peňázová et al., 2015).

Considering the great yield loss rate and a resulting need for fast detection and suppression of *Xcc*, many molecular, PCR-based techniques for fast and sensitive detection of this pathogen in seeds and plants have been developed. Pathovar-specific primers are mainly designed to target genes involved in pathogen virulence, such as zinc uptake regulator (*Zur*) and genes belonging to the hypersensitive reaction and pathogenicity (*hrpF*) cluster (Eichmeier et al., 2019; Rubel et al., 2019). The multilocus sequence typing (MLST) approach, based on PCR amplifications of various housekeeping genes is becoming one of the main techniques for more precise identification and characterization (Young et al., 2008; Almeida et al., 2010). Such techniques provide useful information, especially now that globally accessible databases are available, such as the National Center for Biotechnology Information (NCBI),

and Plant Associated and Environmental Microbes Database (PAMDB). Usage of these databases is of great importance because they allow fast comparison of test strains with all previously deposited strains of certain species, isolated from various hosts and regions/countries. Multilocus sequence analysis (MLSA) is also widely used for establishing phylogenetic relatedness among test strains, allowing us to provide information about recombination events, the presence/absence of genetic diversity among species and their evolution (Fargier et al., 2011). Besides the nucleotide-based approach for analyzing MLST data, a new allelic-based approach has been recently developed (Feil et al., 2004). That approach is based on globally optimized implementation of the eBURST algorithm (goeBURST), which is applied for analyzing sequences of a set of typically seven selected housekeeping genes which are assumed to be under moderate to strong purifying selection (Feil et al., 2004).

This study aimed to apply a new technique for allelic profiles determination of Serbian cabbage *Xcc* strains and thus provide more information about the genetic structure of the cabbage *Xcc* population.

MATERIAL AND METHODS

Bacterial strains

Three representative *Xcc* cabbage strains isolated in Serbia in 2014, coded as Xc40, Xc48, and Xc75, were selected for allelic profile determination out of a total of 44 strains previously characterized by Popović et al. (2019). The three strains were representative considering the high homology among *Xcc* populations from cabbage determined by the MLSA with ten housekeeping genes.

DNA extraction

Total genomic DNA of the test *Xcc* strains was extracted using a modified CTAB protocol proposed by Le Marrec et al. (2000). Single bacterial colonies of each strain, grown on YDC agar for 48 h, were resuspended in 500 µl of sterile distilled water and centrifuged at 13000 rpm for 5 min. The obtained pellet was resuspended in a mix of 567 µl of Tris-EDTA (TE) buffer (pH7, 10mM TRIS, 1mM EDTA), 30 µl of 10% (*w/v*) sodium dodecyl sulfate (SDS) and 20 mg ml⁻¹

proteinase K, and incubated at 37 °C for 30 min. The samples were then treated with 100 µl of 5M NaCl and 250 µl of 3% hexadecyltrimethylammonium bromide (CTAB, pH 8.0). The mix was then heated at 65 °C for 20 min. After the incubation period, the DNA was purified with 750 µl of chloroform and centrifuged for 10 min at 13000 rpm. The obtained upper phase was transferred to clean tubes and 1/10 of 3M sodium-acetate (pH5.0) was added to precipitate DNA. The next step was the addition of ice-cold isopropanol and centrifugation at 13000 rpm for 15 min. The final phase consisted of washing the obtained pellet with 1 ml of 96% of ice-cold ethanol and centrifugation for 10 min at 13000 rpm. The obtained DNA was dried at room temperature for 30 min, dissolved in 50 µl TE buffer, and stored at -20 °C until use.

Multilocus sequence typing (MLST)

The extracted DNA of *Xcc* strains was amplified using P-X-*dnaK* primer pair (forward: 5'-GGTATTGACCTCGGCACCAC-3'; reverse: 5'-ACCTTCGGCATAACGGGTCT-3') constructed based on a partial sequence of the gene encoding 70-kDa heat shock protein (Hsp70) (Fargier et al., 2011).

PCR amplification was performed in 25 µl mixture made with 12.5 µl of 2 × PCR TaqNova-RED Master Mix, 9.5 µl of PCR water, 1 µl of each primer and 1 µl of total sample DNA. PCR conditions were set

according to a protocol given by Fargier et al., (2011) with an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation (94 °C for 50 sec), hybridization (62 °C for 50 sec) and elongation (72 °C for 1 min). The final elongation step was performed at 72 °C for 7 min.

The obtained PCR products were visually checked for band presence on the searched position (540 bp) in comparison to 200-10000 bp SmartLadder MW-1700-10 DNA Ladder (Eugentec) on 1% agarose gel stained with ethidium bromide. Before sending them for sequencing in the MacroGen Sequence service (Amsterdam, the Netherlands), the PCR products were purified with QIAquick PCR Purification Kit (Qiagen) according to the protocol proposed by the manufacturer.

To confirm the identity of the strains, the obtained sequences were checked for their quality and compared with sequences deposited in the NCBI database using the nucleotide BLAST (BLAST_n) option. The obtained sequences were deposited into the NCBI database to obtain accession numbers (Table 1).

Sequences of the other three genes (*fyuA*, *gyrB*, and *rpoD*) were retrieved from the GenBank, where they had been deposited earlier by Popović et al. (2019) (Table 1) and used for allelic profile determination.

Table 1. NCBI database accession numbers of the Serbian cabbage *Xcc* strains

Strain code	Accession number			
	P-X- <i>dnaK</i>	<i>fyuA</i>	<i>gyrB</i>	<i>rpoD</i>
Xc40	MT106770	MH972101	MH972155	MH972137
Xc48	MT106771	MH972102	MH972156	MH972138
Xc75	MT106772	MH972103	MH972157	MH972139

Allelic-profile determination

Allelic profiles of the Serbian cabbage *Xcc* strains were determined based on sequences of four housekeeping genes (P-X-*dnaK*, *fyuA*, *gyrB*, and *rpoD*). The genetic structure and position of test strains were determined in relation to 33 *Xcc* strains selected to represent each of the 33 *Xcc* sequence types (STs) described so far by Cruz et al. (2018) and Bella et al. (2019). A list of strains used for comparison, hosts, and countries of their isolation are presented in Table 2. Sequences of all strains included in the comparison for each of the four genes were retrieved from the GenBank, aligned using the ClustalW segment of BioEdit v. 7.0.5 and trimmed to the same size (P-X-*dnaK*-470 nt, *fyuA*-538 nt, *gyrB*-418 nt, *rpoD*-422 nt). Allelic profiles for each gene were determined using a Non-redundant PubMLST database (<https://pubmlst.org/analysis/nrdb.shtml>). Each DNA sequence that differed from all other sequences of the same gene in one or more nucleotides was marked with a different allele number. The combination of the obtained allelic profiles for each strain was considered as ST.

To represent graphically the position of Serbian cabbage *Xcc* strains in relation to the other 33 *Xcc* strains from the database used for comparison, a Minimum Spanning (MS) tree was constructed using the Phyloviz software (<http://www.phyloviz.net/goeburst/>) with implemented global optimal eBURST (goeBURST) algorithm (Feil et al., 2004). Each nodule on the MS tree represents one ST and its size is determined based on the number of isolates comprising it. The links connecting nodules represent single (SLVs), double (DLVs) and triple (TLVs) locus variants, meaning that the tested genotype differs from the founder genotype at only one, two or three out of four MLST loci, respectively. Different colors of the links connecting nodules (STs) represent SLVs (blue), DLVs (green), TLVs (light grey), and edges without ties (black).

RESULTS AND DISCUSSION

The allelic profiles and STs obtained for the tested Serbian cabbage *Xcc* strains (Xc40, Xc48, and Xc75) and comparative *Xcc* strains from diverse hosts are presented in Table 2. All three Serbian strains had the same allelic profile: 1, 3, 1, 1, for the genes P-X-

dnaK, *fyuA*, *gyrB*, and *rpoD*, respectively, indicating their affiliation to the ST2. The same allelic profile combination was obtained for the strain CPBF 213 isolated from *B. oleracea* var. *costata* in Portugal (Table 2). The obtained results indicate the absence of genetic diversity among the Serbian *Xcc* strains originating from cabbage, but also highlight ST2 as a new allelic profile found for the cabbage *Xcc* strains. Conversely, genetic heterogeneity was confirmed among eight strains of different cabbage types (*B. oleracea* var. *capitata*, *B. oleracea* convar. *capitata* var. *alba* and *B. oleracea* convar. *capitata* var. *sabauda*) used for comparison – CPBF 1135, CFBP 6865, CPBF 1136, CFBP 1712, CPBF 604, ISPaVe 1032, DAPP-PG 249, and LMG 8001, which were proved to belong to the ST5, ST9, ST10, ST20, ST29, ST31, ST32, and ST33, respectively.

The obtained MS tree shown in Figure 1 represents the distribution of STs among hosts. No strict connection between a host of isolation and ST affiliation was noticed, meaning that the *Xcc* isolates obtained from the same host can be genetically different. The central position of ST5 on the obtained tree indicates that it is the founder genotype, and diversification of the remaining 32 STs (ST1-ST4, ST6-ST33) included in comparison is shown. The ST2 differs from the founder genotype ST5 in one (*rpoD*) locus out of four tested.

A recent MLST-based study performed with the same four genes (P-X-*dnaK*, *fyuA*, *gyrB*, and *rpoD*) revealed the presence of as many as five (ST3, ST5, ST9, ST26, and ST47) different STs in the collection of fifteen Serbian *Xcc* strains obtained from winter oilseed rape (*Brassica napus*) over a nine-year period (2010-2018) (Jelušić et al., 2020). In another study on *Xcc* strains from Serbia, performed by Popović et al. (2019), which was based on the phylogenetic analysis of ten housekeeping genes (*dnaK*, *fusA*, *fyuA*, *rpoD*, *gapA*, *gyrB1*, *gyrB2*, *gluA*, *lacF*, and *lepA*), it was noticed that strains that originated from broccoli, cauliflower, collard greens, kohlrabi and kale, along with cabbage, were genetically homogenous. The absence of genetic diversity among the strains obtained in 2014 indicates that the long tradition of brassica crops cultivation in Serbia has not caused an evolution of different *Xcc* populations. Based on DNA-fingerprinting profiles obtained in 2010 after performing repetitive element palindromic PCR (rep-PCR) with the primers GTG₅ and ERIC, Serbian cabbage *Xcc* strains were distinguished from *Xcc* strains isolated from kale

Table 2. The list of Serbian cabbage *Xcc* strains used for allelic profile determination and strains used for comparison. Strains from cabbage and different cabbage types are bolded

Strain	Host	Country	Sequence type	Allelic profile				Reference
				<i>dnaK</i>	<i>fyuA</i>	<i>gyrB</i>	<i>rpoD</i>	
Xc40	<i>B. oleracea</i> var. <i>capitata</i>	Serbia	ST2	1	3	1	1	Popović et al., 2019
Xc48	<i>B. oleracea</i> var. <i>capitata</i>	Serbia	ST2	1	3	1	1	
Xc75	<i>B. oleracea</i> var. <i>capitata</i>	Serbia	ST2	1	3	1	1	
Xcc147	<i>B. oleracea</i>	-	ST1	1	1	1	1	Cruz et al., 2018
CPBF 213	<i>B. oleracea</i> var. <i>costata</i>	Portugal	ST2	1	3	1	1	
CFBP 4956	<i>B. oleracea</i> var. <i>botrytis</i>	Belgium	ST3	1	3	1	4	
CFBP 1124	<i>B. oleracea</i> var. <i>botrytis</i>	France	ST4	1	3	1	9	
CPBF 1135	<i>B. oleracea</i> convar. <i>capitata</i> var. <i>alba</i>	Portugal	ST5	1	3	1	11	
CPBF 46	<i>B. oleracea</i> convar. <i>acephala</i> var. <i>sabellica</i>	Portugal	ST6	1	3	1	14	
CPBF 140	<i>B. oleracea</i> var. <i>costata</i>	Portugal	ST7	1	3	1	16	
CPBF 278	<i>B. oleracea</i>	Portugal	ST8	1	3	1	21	
CFBP 6865	<i>B. oleracea</i> var. <i>capitata</i>	Australia	ST9	1	3	3	11	
CPBF 1136	<i>B. oleracea</i> convar. <i>capitata</i> var. <i>alba</i>	Portugal	ST10	1	4	1	4	
CPBF 210	<i>B. oleracea</i> convar. <i>botrytis</i> var. <i>botrytis</i>	Portugal	ST11	1	4	1	11	
CPBF 1175	<i>B. oleracea</i> convar. <i>botrytis</i> var. <i>botrytis</i>	Portugal	ST12	1	4	1	12	
CPBF 329	<i>B. oleracea</i> var. <i>costata</i>	Portugal	ST13	1	4	1	22	
CPBF 1176	<i>B. oleracea</i> convar. <i>botrytis</i> var. <i>botrytis</i>	Portugal	ST14	1	4	1	31	
CFBP 3838	<i>Iberis</i> sp.	Tanzania	ST15	1	10	1	12	
CPBF 824	<i>B. oleracea</i>	Portugal	ST16	1	23	1	29	
CFBP 5814	<i>B. oleracea</i> var. <i>botrytis</i>	Spain	ST17 ^a	3	2	6	7	
CFBP 5241	<i>B. oleracea</i> var. <i>gemmifera</i>	UK	ST18 ^a	9	3	1	4	
CFBP 1121	<i>B. oleracea</i> convar. <i>bullata</i> var. <i>gemmifera</i>	France	ST19 ^a	9	3	1	11	
CFBP 1712	<i>B. oleracea</i> var. <i>capitata</i>	France	ST20 ^a	9	13	1	11	
CPBF 208	<i>B. oleracea</i> var. <i>costata</i>	Portugal	ST21 ^a	15	18	10	19	
CPBF 211	<i>B. oleracea</i> convar. <i>botrytis</i> var. <i>botrytis</i>	Portugal	ST22 ^a	17	4	1	16	
CPBF 212	<i>B. oleracea</i> var. <i>costata</i>	Portugal	ST23 ^a	18	3	12	12	
CPBF 216	<i>B. oleracea</i> convar. <i>botrytis</i> var. <i>botrytis</i>	Portugal	ST24 ^a	19	20	1	20	
CPBF 330	<i>B. oleracea</i> var. <i>costata</i>	Portugal	ST25 ^a	20	3	1	23	
CPBF 332	<i>B. oleracea</i> var. <i>costata</i>	Portugal	ST26 ^a	21	3	1	24	
CPBF 489	<i>B. oleracea</i> var. <i>costata</i>	Portugal	ST27 ^a	24	4	14	26	
CPBF 589	<i>B. oleracea</i> convar. <i>botrytis</i> var. <i>botrytis</i>	Portugal	ST28 ^a	25	3	1	27	
CPBF 604	<i>B. oleracea</i> convar. <i>capitata</i> var. <i>sabauda</i>	Portugal	ST29 ^a	26	3	1	1	
CPBF 668	<i>B. oleracea</i>	Portugal	ST30 ^a	27	22	1	28	
ISPaVe 1032	<i>B. oleracea</i> var. <i>capitata</i>	Italy	ST31 ^b	1	13	1	4	Bella et al., 2019
DAPP-PG 249	<i>B. oleracea</i> var. <i>capitata</i>	Italy	ST32 ^b	27	4	1	11	
LMG 8001	<i>B. oleracea</i> var. <i>capitata</i>	UK	ST33 ^b	1	3	1	N3	

^a ST designations ST17–ST30 correspond to the following designations provided by Cruz et al. (2018): ST17=ST18; ST18=ST25; ST19=ST26; ST20=ST27; ST21=ST34; ST22=ST36; ST23=ST37; ST24=ST38; ST25=ST39; ST26=ST40; ST27=ST43; ST28=ST44; ST29=ST45; ST30=ST46.

^b ST designations ST31–ST33 correspond to the following designations provided by Bella et al. (2019): ST31=AP8; ST32=AP3; ST33=AP15.

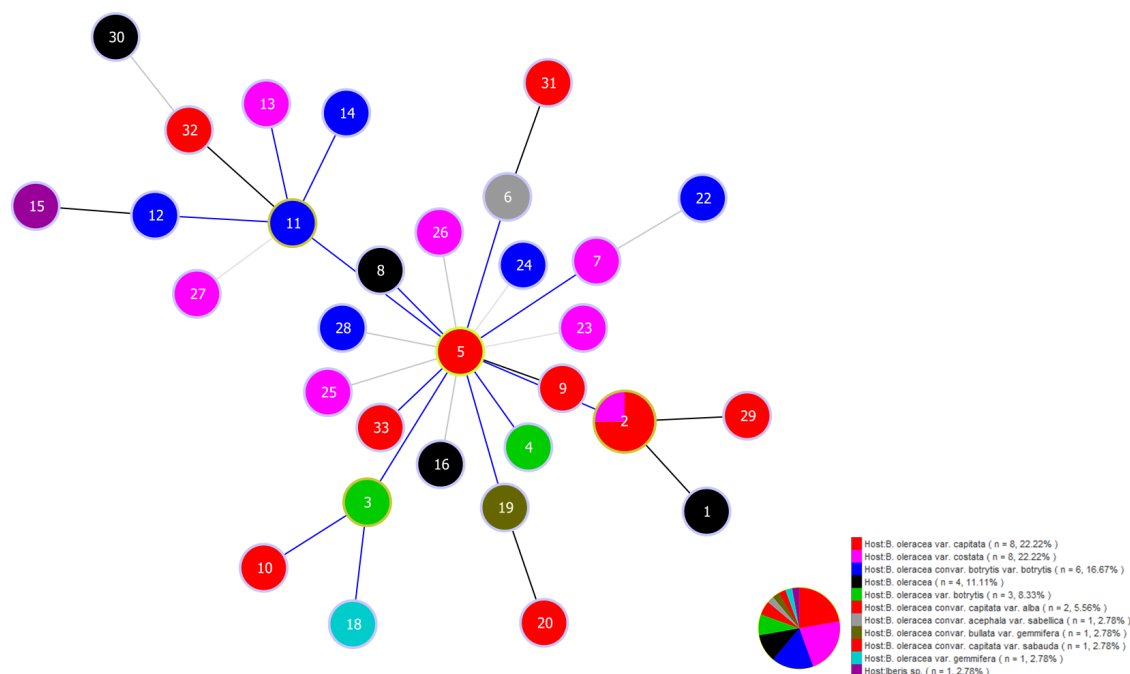


Figure 1. The MS tree showing distribution of 33 *Xcc* STs obtained for four genes (*P-X-dnaK*, *fyuA*, *gyrB*, and *rpoD*) in Serbian cabbage *Xcc* isolates and 33 comparative *Xcc* strains from different hosts and countries

and broccoli in the same year (Popović et al., 2013). An allelic-profile determination study performed with seven genes (*atpD*, *dnaK*, *efp*, *fyuA*, *glnA*, *gyrB*, and *rpoD*) for a collection of 31 Italian *Xcc* strains from various cruciferous crops (broccoli, cabbage, cauliflower, kale, kohlrabi, Savoy cabbage, seakale and rutabaga) showed the presence of 14 STs (Bella et al., 2019). In the same study, five strains from cabbage (ISPaVe 1032; PVCT 189.1.1; ISCI 88; OMP-BO 588/90; DAPP-PG 249) had four different allelic profiles (AP1=ST5, AP3=ST32, AP4=ST10, and AP8=ST31).

In conclusion, the present study challenges our earlier knowledge about the genetic diversity of *Xcc* within specific plant species, and confirmed genetic homogeneity of cabbage *Xcc* strains, revealing the presence of only one ST (ST2), which was additionally found to be a new *Xcc* ST on cabbage.

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Alelski profil izolata *Xanthomonas campestris* pv. *campestris* sa kupusa u Srbiji

REZIME

Xanthomonas campestris pv. *campestris* (Xcc), prouzrokovatelj crne truleži kupusa (*Brassica oleracea* var. *capitata* L.) svrstava se među najvažnije fitopatogene bakterije koje utiču na pravilno razviće kupusa, dovodeći do gubitka težine glavice i njenog kvaliteta i na taj način drastično smanjuje njegovu ekonomsku vrednost. Ovaj patogen je genetički heterogen, što se ogleda kroz prisustvo dokazanih jedanaest rasa i više od trideset kombinacija alelskih profila širom sveta. Zbog svega navedenog, cilj ovog rada je determinacija alelskih profila Xcc sojeva poreklom sa kupusa prikupljenih 2014. godine. Analiza je vršena kod tri reprezentativna Xcc soja čija je DNK amplifikovana primenom lančane reakcije polimeraze (PCR) sa četiri konzervativna gena - *dnaK*, *fyuA*, *gyrB* i *rpoD*, a zatim sekvencirana i korišćena za determinaciju alelskih profila. Alelski profili su određivani poređenjem sa 33 Xcc soja izolovana sa različitih domaćina i regiona, čiji su alelski profili prethodno utvrđeni. Non-redundant baza podataka (NRDB) od pubMLST je korišćena za determinaciju alelskih profila, a Phyloz softver za konstrukciju Minimum Spanning stabla. Dobijeni alelski profil za sve Xcc sojeve sa kupusa iz Srbije je 1, 3, 1, 1 za gene *dnaK*, *fyuA*, *gyrB* i *rpoD*, redom. Ovaj profil je označen kao tip sekvence 2 (ST2) i podudara se sa portugalskim *B. oleracea* Xcc sojem CPBF 213 poreklom sa *B. oleracea* var. *costata*. Veza između tipa sekvence (ST) i biljke domaćina nije pronađena.

Ključne reči: *Xanthomonas campestris*, crna trulež, kupus, genetska raznovrsnost, alelski profil