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Characterization of *Xanthomonas arboricola* pv. *pruni* from *Prunus* spp. orchards in Western Balkans

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Abstract

The present study provides a new insight into the existing *Xanthomonas arboricola* pv. *pruni* (Xap) bacterial population originating from peach and apricot in two Western Balkans countries (Serbia and Montenegro). Multilocus sequence typing and analysis of the sequences of nine housekeeping genes revealed homology between the tested Xap strains as well as with the European population of this bacterium. The tested strains share the same haplotype (Haplotype I) with the Xap strains from Italy, France, Spain, United States, Australia and Brazil. The revealed single nucleotide change (G ↔ C) in the sequences of the *gyrB1* gene differentiates Haplotype I from Haplotype II (Xap from South Korea, New Zealand, Argentina and Uruguay). The detached-leaf bioassay results confirmed differences in virulence between strains originating from peach and apricot towards *Prunus armeniaca* (apricot), indicating host specialization of the apricot strain towards this host. For the first time, immunity of *P. fruticosa* (European ground cherry) to Xap was established. According to the AUDPC, PCA and cluster analysis, other *Prunus* spp. were classified as having low susceptibility (*P. mahaleb*, *P. cerasus* and *P. avium*), as susceptible (*P. domestica*) and as highly susceptible (*P. persica*, *P. dulcis*, *P. cerasifera* and *P. spinosa*). Xap strains were also found to be susceptible to 10 tested antibiotics. This study provides valuable knowledge on the Xap population from stone fruit grown in the Western Balkans region as well as the source of immunity, which could serve as a starting point for breeding *Prunus* cultivars and could be used as the main control strategy.

KEYWORDS
bacterial spot, genetic characterization, pathogenicity, stone fruits

1 | INTRODUCTION

The *Xanthomonas arboricola* complex includes phytopathogenic bacteria responsible for emerging diseases worldwide (Lamichhane, 2014). Nine pathovars, based on the host range of the species—*arracaciae*, *celebensis*, *corylina*, *fragariae*, *guizotiae*, *juglandis*, *populi*, *pruni* and *zantesdeschiae*—were included in this complex (Fischer-Le Saux et al., 2015), with *corylina*, *juglandis* and *pruni* as the most virulent and economically important for cultivated crops (Morales et al., 2015; Young et al., 2008). Bacterium *Xanthomonas arboricola* pv. *pruni* (Xap) is an emerging threat for cultivated and ornamental *Prunus* species. Due to the damage it causes, Xap is classified as a regulated nonquarantine organism in Europe (EU_2019_2072_AnnexIV_RNQP; Anonymous, 2019), while in Serbia it is on the IA List, Part I (Anonymous, 2015). Among the cultivated stone fruits, peach (*Prunus persica*), nectarine (*P. persica* var. *nucipersica*), apricot (*P. armeniaca*), almond (*P. dulcis*) and plum (*P. domestica*) are considered as most susceptible to Xap infections. Occasionally, Xap infects sweet cherry (*P. avium*) and sour cherry (*P. cerasus*) (Garita-Cambronero et al., 2018), and other hosts such as

Japanese apricot (*P. mume*), Chinese wild peach (*P. davidiana*), *P. buergeriana*, *P. crassipes* and *P. donarium* (EPPO, 2006, 2021). Recently, Xap was reported as a causal agent of shot-hole disease on plumcot, an interspecific hybrid product between Japanese plums and apricots grown in Korea (Dumin et al., 2021). Among the ornamental *Prunus* species, Xap has been identified on *P. laurocerasus* (Tjou-Tam-Sin et al., 2012).

Bacterial spot on leaves and fruits is the most characteristic disease symptom caused by Xap, but can be accompanied by cankers and dieback of twigs and branches (EPPO, 2006, 2021). Symptoms on leaves appear along leaf midribs or margins in the form of brown to black spots, with a pale green to yellow halo, evident on both leaf surfaces (Iličić & Popović, 2020, 2021). The diseased area drops out, giving a shot-hole appearance, and affected leaves turn yellow and fall prematurely. Bacterial spots on fruit take the form of water-soaked or dark brown sunken lesions (Haack et al., 2020; Iličić & Popović, 2021). Severe defoliation and spots on the fruit reduce its commercial value, while prematurely defoliated trees become more susceptible to low winter temperatures. Under optimal climatic conditions for Xap development, bacterial spot in stone fruit cannot be controlled.

The presence of Xap has been reported on all five continents (Rosellò et al., 2012). In Europe, disease outbreaks caused by this bacterium have become more frequent in recent years, with reports from Spain, France, Italy, Bulgaria, Romania, Ukraine, Turkey, Switzerland, Slovenia, Hungary, Montenegro, Serbia, Moldova, Netherlands and Russia (EPPO, 2021; Gerin et al., 2019; Iličić & Popović, 2020, 2021; Marchi et al., 2011; Nagy et al., 2020; Palacio-Bielsa et al., 2010; Schwarczinger et al., 2017, 2018; Tjou-Tam-Sin et al., 2012).

The aim of this work was to characterize the Xap population recently identified in Serbia and Montenegro by using molecular-based techniques, to contribute to a better understanding of Xap introduction in Western Balkans countries and to evaluate its virulence potential for 10 *Prunus* species, with the goal of predicting future pathogen spread to new hosts.

2 | MATERIALS AND METHODS

2.1 | Strains used in the study

Strains of the bacterium Xap used for this study were previously identified by Iličić and Popović (2020, 2021). They were collected in 2019 from peach leaves showing bacterial spot symptoms in orchards located in Irig (Vojvodina, Serbia; strain coded as Xp219) and in the Podgorica region (Montenegro; strains coded as Xp1, Xp4, Xp1 and Xp10), as well as strains collected in 2020 from apricot fruits showing bacterial spot symptoms in the locality of Bešenovo (Vojvodina, Serbia; strain coded as Xp320). All analyses were performed on strains grown on yeast extract–dextrose–calcium carbonate agar (YDC) for 3 days at 26°C and subsequently stored at −20°C in Luria-Bertani broth (LB medium) with 20% glycerol.

2.2 | Molecular analysis

2.2.1 | DNA extraction

Genomic DNA from all strains obtained from peach and apricot in Serbia and Montenegro in both isolation years (2019 and 2020) was extracted using the hexadecyltrimethyl ammonium bromide (CTAB) extraction protocol. Single bacterial colonies of strains grown on YDC agar for 48 h at 26°C were transferred to nutrient broth (NB) and incubated for 48 h. The obtained bacterial cultures were centrifuged at 10,000 × *g* for 10 min. The resulting pellet was resuspended in Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0) before being incubated at 37°C for 30 min with a mix of 10% (wt/vol) sodium dodecyl sulphate (SDS) and 20 mg/ml proteinase K. After incubation, strains were treated with 5 M NaCl and 3% CTAB (pH 8.0) and heated at 65°C for 20 min. The DNA was purified with chloroform and centrifuged for 10 min at 10,000 × *g*. The upper phase was recovered by adding ice-cold isopropanol and centrifugation at 10,000 × *g* for 15 min. Finally, the obtained pellet was washed with 96% ice-cold ethanol, centrifuged at 10,000 × *g* for 10 min, and dried at room temperature for 30 min. The pelleted DNA was dissolved in 50 µl of TE buffer and stored at −20°C.

2.2.2 | Multilocus sequence typing and analysis

Strains were characterized using the multilocus sequence typing (MLST) technique based on the two schemes proposed by Young et al. (2008) and Almeida et al. (2010). The two proposed schemes for *Xanthomonas* spp. identification are based on sequencing of the following nine housekeeping genes: *fusA* (elongation factor 4), *gapA* (glyceraldehyde-3-phosphate dehydrogenase A), *gltA* (citrate synthase), *gyrB* (DNA gyrase subunit B), *lepA* (elongation factor 4), *lacF* (PTS system lactose-specific EIIA component), *fyuA* (tonB-dependent receptor), *dnaK* (molecular chaperone DnaK) and *rpoD* (RNA polymerase sigma factor RpoD). Additionally, another primer pair (P-X-dnaK-F/P-X-dnaK-R) targeting molecular chaperone DnaK was included in the study (Fargier et al., 2011). Nucleotide sequences of the used primers, their annealing temperatures and amplicon sizes are provided in Table S1. All PCRs were performed in a total mixture volume of 25 µl consisting of 12.5 µl of 2× PCR TaqNova-RED Master Mix (Blirt), 9.5 µl of PCR-grade water, 1 µl of each of the primers (forward/reverse) and 1 µl of sample DNA. The obtained PCR products were visualized on 1.5% agarose gel focusing on the 200–10,000 bp DNA ladder (SmartLadder MW-1700-10; Eurogentec) and purified using the QIAquick PCR Purification Kit (QIAGEN). Purified PCR products were sent for sequencing to the Macrogen Sequencing Service. To get an indication of the species affiliation, the obtained sequences were manually checked for quality and compared with the strains available in the National Center for Biotechnology Information database (NCBI) using the nucleotide BLAST (BLASTn) function. Sequences were deposited in GenBank to obtain accession numbers.

Multilocus sequence analysis (MLSA) was further performed to properly identify strains and determine their phylogenetic position. For generating the phylogenetic tree, sequences of all nine housekeeping genes for all strains were aligned using the ClustalW function of the BioEdit v. 7.0.5 program and trimmed to the same size (*dnaK*, 678 nucleotides [nt]; *fyuA*, 597 nt; *gyrB1*, 735 nt; *rpoD*, 609 nt; *fusA*, 697 nt; *gapA*, 722 nt; *gltA*, 738 nt; *gyrB2*, 580 nt; *lepA*, 853 nt; and *lacF*, 446 nt). Neighbour-joining (NJ) phylogenetic trees were produced for each gene separately, as well as with concatenated sequences comprising four genes according to the scheme proposed by Young et al. (2008) (2619 nt) and six genes according to the scheme of Almeida et al. (2010) (4804 nt). NJ trees were created in MEGA X software to compare six peach and apricot strains obtained in Serbia (Xp219 and Xp320) and Montenegro (Xp1, Xp4, Xp7 and Xp10) in 2019 and 2020 with the strains of *X. arboricola* and different *X. arboricola* pathovars (*pruni*, *corylina*, *juglandis*, *celebensis*, *populi* and *fragariae*) from GenBank (Table S2). Trees were rooted with *X. campestris* pv. *campestris* strain 3811, also retrieved from GenBank (Table S2). Genetic distances were computed using the Kimura two-parameter nucleotide substitution model (Kimura, 1980). Sequences of the genes *rpoD*, *gyrB1* and *fyuA* of the peach strains Xp1, Xp4, Xp7 and Xp10 were retrieved from a previous study by Iličić and Popović (2020) (Table 1).

2.2.3 | Haplotype network

Genetic and phylogeographic relatedness between six Xap strains from this study (Xp219, Xp320, Xp1, Xp4, Xp7 and Xp10) and 17 Xap strains isolated in different regions of the world (Europe [Eastern – Italy, Western – France and Southern – Spain], America [Northern – United States; Southern – Brazil, Argentina and Uruguay], East Asia [South Korea] and Australasia [Australia and New Zealand]) and from different *Prunus* spp. hosts (*P. persica*, *P. japonica*, *P. domestica*, *P. dulcis*, *P. armeniaca* and *P. salicina*) were assessed using the haplotype network. The Templeton, Crandall and Sing's (TCS; Templeton et al., 1992) haplotype network was constructed based on the concatenated sequences (2558 nt) comprising four housekeeping genes, *fyuA*, *gyrB1* and *rpoD* according to Young et al. (2008) and *dnaK* according to Fargier et al. (2011). Sequences of the comparative strains previously identified by Fischer-Le Saux et al. (2015) were retrieved from GenBank. Information for the comparative strains and accession numbers for the genes of interest for this investigation are listed in Table S3. TCS haplotype network was generated based on the concatenated sequences using the TCS algorithm (Clement et al., 2002) implemented in PopART v. 1.7 (Leigh & Bryant, 2015), whereby each circle on the TCS haplotype network presents one haplotype, different colours are used to differentiate the countries from which the compared strains originate, and the circle size reflects the number of strains (*n*) grouped within it. Black dots between two haplotypes represent the number of nucleotide differences between them.

TABLE 1 GenBank accession numbers of *Xanthomonas arboricola* pv. *pruni* isolates from this study

Isolate code	*rpoD*	*gyrB1*	*fyuA*	*dnaK*	*fusA*	*gapA*	*gltA*	*gyrB2*	*lacF*	*lepA*	P-X-*dnaK*
Xp1	MN520627[a]	MN520631[a]	MN520623[a]	MZ301308	MZ301270	MZ301276	MZ301282	MN520619	MZ301290	MZ301296	OK500108
Xp4	MN520628[a]	MN520632[a]	MN520624[a]	MZ301309	MZ301271	MZ301277	MZ301283	MN520620	MZ301291	MZ301297	OK500109
Xp7	MN520629[a]	MN520633[a]	MN520625[a]	MZ301310	MZ301272	MZ301278	MZ301284	MN520621	MZ301292	MZ301298	OK500110
Xp10	MN520630[a]	MN520634[a]	MN520626[a]	MZ301311	MZ301273	MZ301279	MZ301285	MN520622	MZ301293	MZ301299	OK500111
Xp219	MZ301302	MZ301304	MZ301306	MZ301312	MZ301274	MZ301280	MZ301286	MZ301288	MZ301294	MZ301300	OK500112
Xp320	MZ301303	MZ301305	MZ301307	MZ301313	MZ301275	MZ301281	MZ301287	MZ301289	MZ301295	MZ301301	OK500113

[a]Sequences retrieved from previous work (Iličić & Popović, 2020) and used for the multilocus sequence analysis in this paper.

2.3 | Host range

The host range of three selected Xap strains (Xp219, Xp320 and Xp1) originating from Serbia and Montenegro, each presenting host or locality from which they were isolated, was evaluated by performing the pathogenicity test on the leaves of 10 *Prunus* species, namely sweet cherry (*P. avium*, genotype 3/82–93), sour cherry (*P. cerasus*, genotype OV5/NC), peach (*P. persica*, genotype 2/5–15), plum (*P. domestica*, genotype 5/59), apricot (*P. armeniaca*, genotype RŠ10/12), almond (*P. dulcis*, genotype RŠ4/05), myrobalan plum (*P. cerasifera*, genotype 5/66), blackthorn (*P. spinosa*, genotype 5/57), mahaleb cherry (*P. mahaleb*, genotype 5/26–34) and European ground cherry (*P. fruticosa*, genotype SV11BJ). All *Prunus* genotypes originated from the Department of fruit growing, viticulture, horticulture and landscape architecture (Faculty of Agriculture University in Novi Sad).

Young detached leaves were inoculated using a hypodermic syringe without a needle according to the method described by Randhawa and Civerolo (1985). Bacterial suspension was prepared in sterile distilled water (SDW) to a final concentration of 1.5×10^7 cfu/ml. Three replications (with 10 leaves in each) were used for each Xap strain. Leaves inoculated with SDW served as negative controls and assays were independently performed twice. The inoculated leaves were maintained on wet wool in plastic boxes with high relative humidity at 24 ± 2°C for 10 days. Disease symptom progress was initially recorded 2 days after inoculation (DAI) and at 2-day intervals thereafter, by measuring spot-necrosis diameters (expressed in mm).

Spot-necrosis reactions were compared among the *Prunus* species based on the area under the disease (mean severity) progress curve (AUDPC) estimated using the following expression:

$$\text{AUDPC} = \sum_{i=1}^{n-1} \left(y_i + y_{i+1}/2\right)\left(t_{i+1} - t_i\right), \quad (1)$$

where y_i = disease progress assessment at the i^{th} observation, t_i = time (days) at the i^{th} observation and n = total number of observations.

AUDPC values were subjected to analysis of variance (ANOVA) and were compared by conducting Duncan's multiple range test, with $p < 0.05$ considered statistically significant (Statistics 32-bit. Ink). According to the findings yielded by statistical analyses, *Prunus* spp. were classified into five susceptibility categories: immune, low susceptible, susceptible, highly susceptible and transitionally susceptible (intermediate).

Multivariate analyses involved principal component analysis (PCA) and cluster analysis using combined data (10 hosts × 3 Xap strains × rating per days) and were performed using the freeware software package Minitab (Minitab Inc., 2021).

2.4 | Antibiogram

The sensitivity of three selected Xap strains (Xp219, Xp1 and Xp320) to 10 antibiotics—amikacin (30 ppm), erythromycin (15 ppm), penicillin (6 ppm), kanamycin (30 ppm), vancomycin (30 ppm), chloramphenicol (30 ppm), neomycin (30 ppm), gentamycin (30 ppm), tetracycline (30 ppm) and streptomycin (10, 25, 300 and 500 ppm)—was examined using the disc diffusion method. For this purpose, 100 µl of the bacterial suspensions (adjusted to 10^7 cfu/ml) were spread with a sterile L-shaped glass rod onto a nutrient agar surface on Petri plates and then incubated for 15 min. Afterwards, the agar plates were impregnated with the antibiotic discs (Torlak) and were incubated at 26°C for 48 h.

Following the incubation period, the visible zone of inhibition was evaluated and the results were interpreted qualitatively as resistant or susceptible.

3 | RESULTS

3.1 | Molecular characterization

MLST analysis of the sequences of nine housekeeping genes (*dnaK*, *fyuA*, *gyrB* [*gyrB1* and *gyrB2*], *rpoD*, *fusA*, *gapA*, *gltA*, *lacF* and *lepA*) revealed 100% identity of six tested peach and apricot strains (Xp1, Xp4, Xp7, Xp10, Xp219 and Xp320) with different Xap strains from GenBank. The obtained GenBank accession numbers of the tested strains are listed in Table 1.

The concatenated NJ phylogenetic trees with genes, constructed according to the schemes proposed by Young et al. (2008) and Almeida et al. (2010), are presented in Figure 1 and Figure S1, respectively. Both NJ trees exhibited genetic homogeneity among all six tested strains irrespective of the sequenced gene, host (peach and apricot), country (Serbia and Montenegro) or isolation year (2019 and 2020). In both trees, all six peach and apricot isolates were clustered together with the adopted reference Xap strain(s). However, although the six tested strains were shown to be genetically homogenous, the phylogenetic tree with genes concatenated according to Young et al. (2008) indicated the existence of heterogeneity between them and reference strains ICMP 51 and 15–088 (Figure 1). The prominent divergence is due to a single nucleotide change (G ↔ C) on the partial sequence of the *gyrB1* gene (Figure S2). Separate trees for the three remaining genes (*dnaK*, *fyuA* and *rpoD*) generated according to Young et al. (2008) are presented in Figure S3, while separate trees with six genes generated according to Almeida et al. (2010) are presented in Figure S4. While no differences between the tested and reference Xap strains were noted, they proved to be diverse enough to clearly distinguish the tested strains from the reference strains of different closely related *X. arboricola* pathovars (*corylina*, *juglandis*, *celebensis*, *populi* and *fragariae*). *X. campestris* pv. *campestris* 3811 was positioned on a separate tree branch as an outgroup in all NJ trees.

Figure 2 shows the TCS haplotype network presenting phylogeographic relatedness between the Xap strains from this study and strains of the same species originating from different countries and hosts. According to the obtained results, the strains from Serbia and Montenegro share the same haplotype (Haplotype I) with the Xap strains from Italy (CFBP 3893 and CFBP 3921), France (CFBP 5580

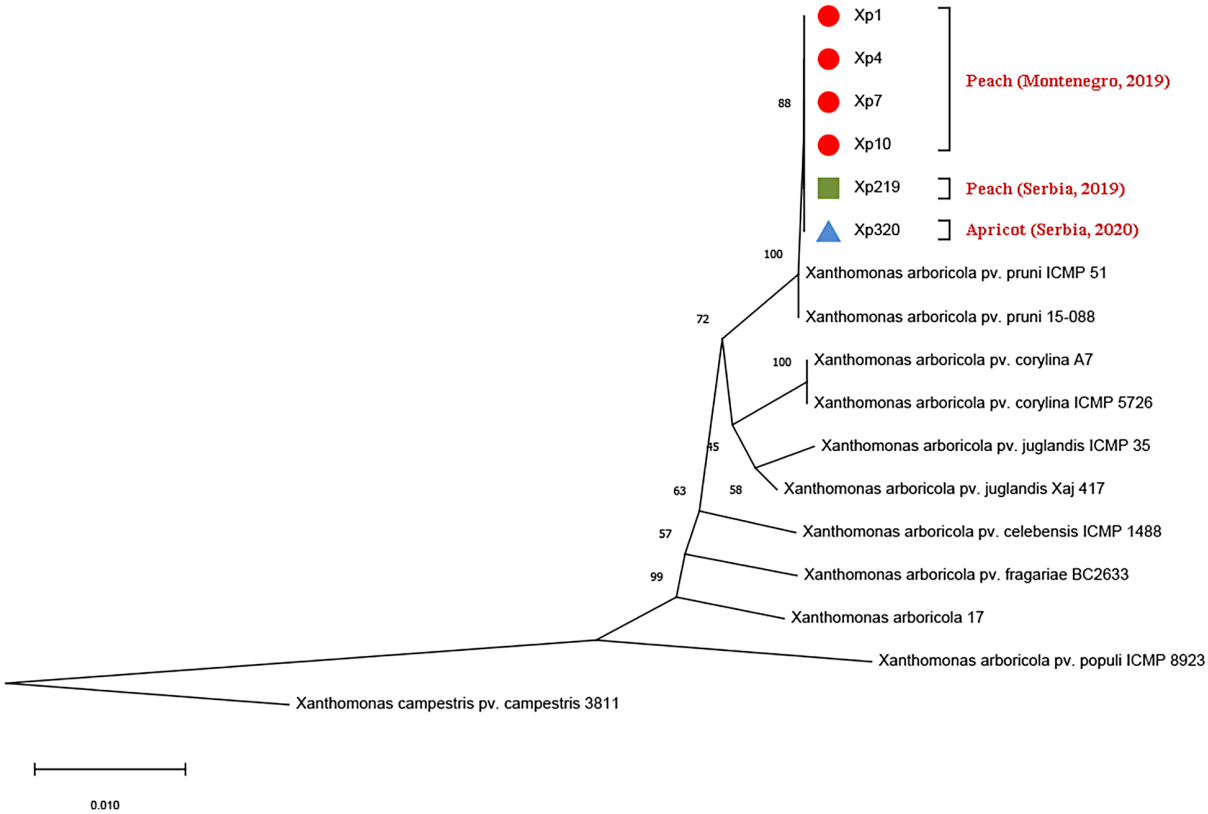


FIGURE 1 Neighbour-joining phylogenetic tree based on the concatenated sequences of four housekeeping genes (*dnaK*, *fyuA*, *gyrB1* and *rpoD*) according to Young et al. (2008) for six *Xanthomonas arboricola* pv. *pruni* strains from this study obtained from peach and apricot in Serbia (isolation 2019 ■ and 2020 ▲) and Montenegro (isolation 2019 ●), and 10 strains of *X. arboricola* and different *X. arboricola* pathovars (*pruni*, *corylina*, *juglandis*, *celebensis*, *populi* and *fragariae*) retrieved from GenBank. The tree was rooted with *X. campestris* pv. *campestris* strain 3811 as an outgroup. [Colour figure can be viewed at wileyonlinelibrary.com]

and CFBP 6653), Spain (CFBP 7098, CFBP 7099 and CFBP 7100), United States (CFBP 411, CFBP 3898, CFBP 3900 and CFBP 3901), Australia (CFBP 5529) and Brazil (CFBP 5722). The revealed single nucleotide change (G ↔ C) found in the sequences of the *gyrB1* gene (Young et al., 2008) differentiates Haplotype I from Haplotype II, shared by the four Xap strains from South Korea (15–088), New Zealand (ICMP 51), Argentina (CFBP 5229) and Uruguay (CFBP 5723) (Figure S5).

3.2 | Host range

Eight of the 10 tested *Prunus* spp. (*P. avium*, *P. cerasus*, *P. persica*, *P. domestica*, *P. armeniaca*, *P. dulcis*, *P. cerasifera* and *P. spinosa*) developed first symptoms (spot-necrosis with mean diameter of 2.0–3.8 mm) 2 DAI when the Montenegrin peach strain Xp1 was used. On *P. mahaleb*, necrotic spots were noticed 6 DAI (2.0 mm), while *P. fruticosa* remained symptomless for the duration of the experiment (Figure S6). Serbian peach strain Xp219 caused first symptoms in the form of spot-necrosis (mean diameter 2.0–2.1 mm) 2 DAI on three tested hosts (*P. persica*, *P. domestica* and *P. spinosa*), while five hosts (*P. avium*, *P. cerasus*, *P. dulcis*, *P. cerasifera* and *P. mahaleb*) and one host (*P. armeniaca*) developed first symptoms 4 and 6 DAI, respectively (mean diameter 1.5–2.7 mm). On the other hand, *P. fruticosa* remained symptomless until the end of the experiment (Figure S6). When Serbian apricot strain Xp320 was used for inoculation, first symptoms appeared 2 DAI on five hosts (*P. persica*, *P. armeniaca*, *P. spinosa*, *P. cerasifera* and *P. dulcis*), 4 DAI on *P. domestica* and 6 DAI on three hosts (*P. avium*, *P. cerasus* and *P. mahaleb*) with the mean diameter ranging from 1.5 to 3.2 mm. Only *P. fruticosa* remained negative throughout the experiment (Figure S6).

During the final rating performed 10 DAI, differences in spot-necrosis mean diameters were noted depending on the tested *Prunus* spp., allowing hosts to be separated into two groups. Specifically, for Montenegrin peach strain Xp1, the mean diameters ranged from 2.0–4.7 mm (*P. mahaleb*, *P. armeniaca*, *P. cerasus*, *P. avium* and *P. domestica*) to 9.1–12.9 mm (*P. dulcis*, *P. persica*, *P. spinosa* and *P. cerasifera*), whereas for Serbian peach strain Xp219, the mean diameters ranged from 2.5–3.4 mm (*P. cerasus*, *P. armeniaca* and *P. avium*) to 5.0–8.4 mm (*P. mahaleb*, *P. domestica*, *P. spinosa*, *P. persica*, *P. cerasifera* and *P. dulcis*) and for Serbian apricot strain Xp320, the mean diameters ranged from 2.0–3.5 mm (*P. mahaleb*, *P. cerasus*, *P. avium* and *P. domestica*) to 9.9–12.2 mm (*P. dulcis*, *P. cerasifera*, *P. armeniaca*, *P. spinosa* and *P. persica*). No symptoms were found in the leaves that served as negative controls for the duration of the experiment.

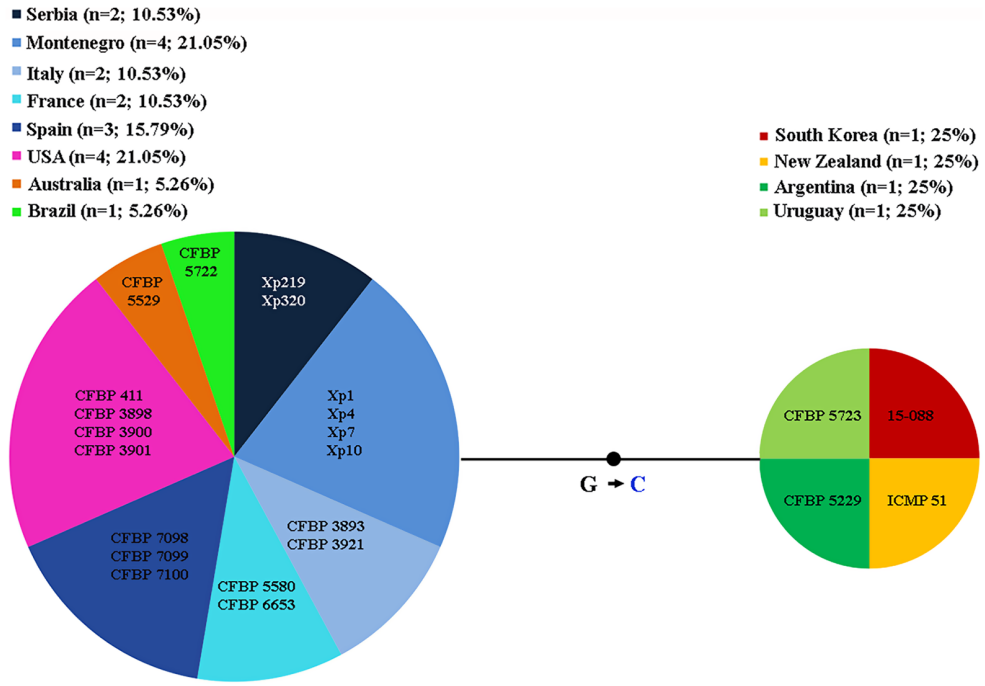


FIGURE 2 Templeton, Crandall and Sing's (TCS; Templeton et al., 1992) haplotype network presenting the phylogeographic position of the six tested peach and apricot *Xanthomonas arboricola* pv. *pruni* strains from this study and 17 *X. arboricola* pv. *pruni* strains from different countries and hosts. Different colours on the haplotype network represent countries from which the compared strains were obtained. The size of each pie slice reflects the number of strains (*n*) grouped within it. The black dot represents one nucleotide difference between the obtained haplotypes. [Colour figure can be viewed at wileyonlinelibrary.com]

The AUDPC values ranged from 0 (*P. fruticosa*) for all three Xap strains to 39.9 (*P. persica*), 63.6 (*P. cerasifera*) and 60.4 (*P. persica*) for Xp219, Xp1 and Xp320, respectively (Figure 3). According to the AUDPC values, the 10 tested *Prunus* spp. were classified into five susceptibility groups: (i) immune: *P. fruticosa* (AUDPC 0); (ii) having low susceptibility: *P. mahaleb* (AUDPC 8.0–23.0), *P. cerasus* (AUDPC 8.0–24.0) and *P. avium* (AUDPC 10.5–28.3); (iii) susceptible: *P. domestica* (AUDPC 14.9–37.4); (iv) highly susceptible: *P. persica* (AUDPC 39.9–60.4), *P. dulcis* (AUDPC 30.9–49.5), *P. cerasifera* (AUDPC 30.2–63.6) and *P. spinosa* (AUDPC 36.5–60.7); and (v) transitionally susceptible (intermediate): *P. armeniaca* (apricot) because it showed a highly susceptible reaction towards the apricot strain Xp320 (AUDPC 59.9), but low susceptible reaction to peach strains Xp219 and Xp1 (AUDPC 10.6 and 21.4, respectively) (Figure S7).

When PCA was used to analyse the set of combined data (10 host ×3 Xap strains × rating per days), two principal components (PC1 and PC2) were selected that jointly accounted for 85.6% of the total variance (69.1% and 16.5%, respectively), as shown in Figure 4. According to the PCA biplot, *Prunus* spp. could be segregated into four groups, denoted as A1, A2, B1 and B2, with Groups A1 and B2 being most distinct (Figure 4a). Group A1 comprised closely related hosts—*P. fruticosa*, *P. cerasus* and *P. mahaleb*—even though the latter two were more similar. Group B2 comprised *P. cerasifera*, *P. spinosa*, *P. persica* and *P. dulcis* and was clearly delineated from Group B1, with apricot (*P. armeniaca*) being the only species within the Group. Species *P. avium* and *P. domestica* formed Group A2 but were quite distant.

Cluster analysis allowed two distinct groups of hosts (a and b) to be distinguished in terms of increasing spot-necrosis susceptibility (Figure 4b). In Group a, *P. fruticosa* was clustered alone, and while *P. avium*, *P. cerasus* and *P. mahaleb* were closely related, *P. domestica* was less related. Group b consisted of *P. spinosa* and *P. persica*, with which *P. dulcis* was less related, and *P. cerasifera* and *P. armeniaca* were assigned to a separate subcluster.

3.3 | Antibiogram

Due to producing clear zones of inhibition, Xap strains Xp1, Xp219 and Xp320 were classified as susceptible to all examined antibiotics—amikacin, erythromycin, penicillin, kanamycin, vancomycin, chloramphenicol, neomycin, gentamycin, tetracycline and streptomycin—in the applied concentrations.

4 | DISCUSSION

Plant-pathogenic bacterium Xap causes bacterial spot on stone fruit, resulting in severe yield losses in *Prunus* production worldwide. By 2019, in the countries neighbouring Serbia, the presence of Xap was confirmed on different *Prunus* species (Marchi et al., 2011; Nagy et al., 2020; Palacio-Bielsa et al., 2010; Rosellò et al., 2012; Schwarczinger et al., 2017, 2018; Scortichini, 1990; Tjou-Tam-Sin et al., 2012). In Montenegro, Xap was first detected

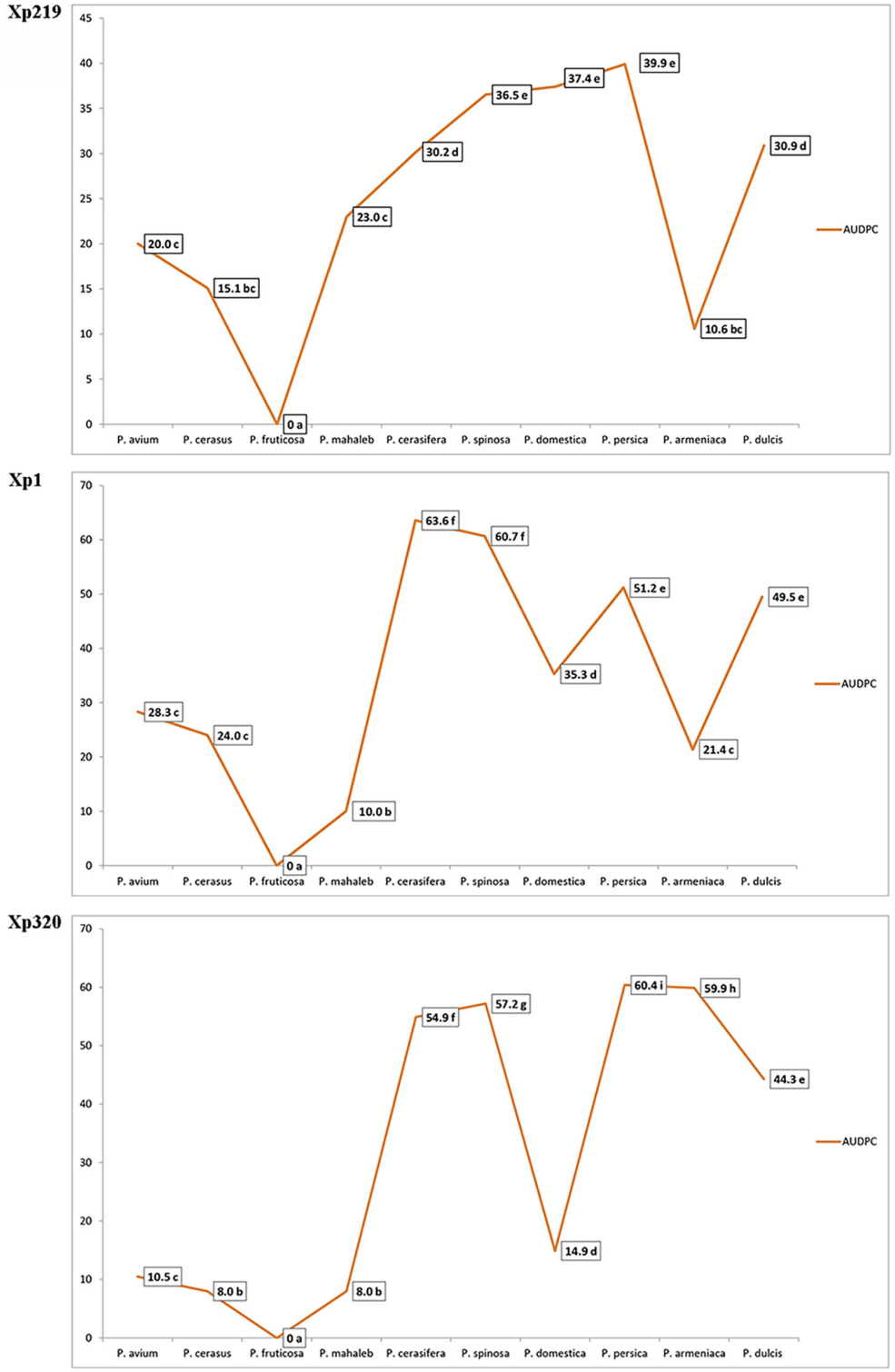


FIGURE 3 Disease progression, as area under the disease progress curve (AUDPC) from serial spot-necrosis ratings, resulting from inoculation with three *Xanthomonas arboricola* pv. *pruni* strains. Different letters represent significant statistical differences. [Colour figure can be viewed at wileyonlinelibrary.com]

in 1994 in commercial almond orchards near the city of Podgorica (Panić et al., 1998). In subsequent years, the bacterium was isolated again, albeit from a young peach orchard consisting of four cultivars, Sugar Time, Britney Lane, Royal Bell and Royal Time (Iličić & Popović, 2020). Despite frequent monitoring and trade restrictions in Serbia, in this country, the presence of Xap was experimentally confirmed for the first time in 2019 on peach, and then in 2020 on apricot cvs NS4, NS Rodna and Roxana in the restricted area in Fruška Gora (Iličić & Popović, 2021). These findings indicate that Xap is spreading through the Western Balkans region. The findings of

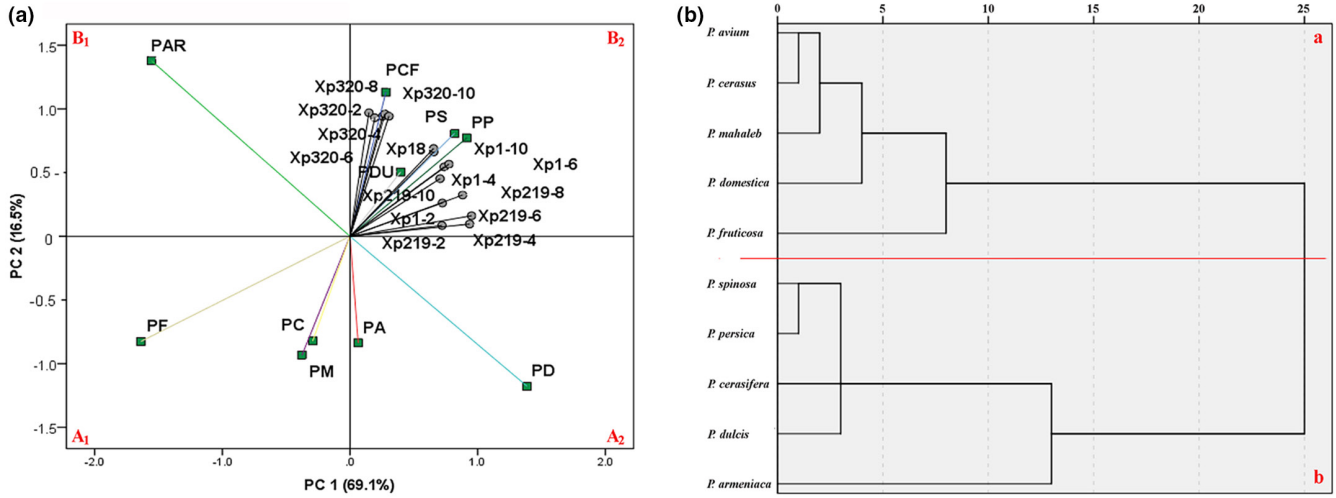


FIGURE 4 (a) Principal component analysis of *Xanthomonas arboricola* pv. *pruni* strains based on their virulence on 10 different *Prunus* spp. rated 2, 4, 6, 8 and 10 days after inoculation (biplot of the first and second principal components). PAR: *P. armeniaca*, PA: *P. avium*, PCF: *P. cerasifera*, PC: *P. cerasus*, PD: *P. domestica*, PDU: *P. dulcis*, PF: *P. fruticosa*, PM: *P. mahaleb*, PP: *P. persica*, PS: *P. spinosa*; ■: tested *Prunus* host, ●: tested *X. arboricola* pv. *pruni* strain; – day of rating. (b) Dendrogram generated by multivariate statistical analysis on the basis of the virulence of *X. arboricola* pv. *pruni* strains for 10 different *Prunus* spp. rated 2, 4, 6, 8 and 10 days after inoculation. Red line differentiates two *Prunus* groups according to their susceptibility to *X. arboricola* pv. *pruni* strains. The numbers present the percentage of *Prunus* relatedness regarding to their susceptibility to *X. arboricola* pv. *pruni* strains. [Colour figure can be viewed at wileyonlinelibrary.com]

the current study indicate that the population is genetically homogeneous irrespective of the host (peach and apricot), country (Serbia and Montenegro) or year of isolation (2019 and 2020). Moreover, the identified population is genetically identical to the European population (Haplotype I).

This study highlights the potential of using nine housekeeping genes (*dnaK*, *fyuA*, *gyrB* [*gyrB1* and *gyrB2*], *rpoD*, *fusA*, *gapA*, *gltA*, *lacF* and *lepA*) sequenced according to the schemes proposed by Young et al. (2008) and Almeida et al. (2010) to properly identify tested Xap strains originating from peach and apricot, as well as to distinguish them from the closely related *X. arboricola* pathovars (*pruni*, *corylina*, *juglandis*, *celebensis*, *populi* and *fragariae*). To the best of our knowledge, this is the first attempt to adopt the MLST based on partial sequences of the six genes according to Almeida et al. (2010) to identify and characterize Xap strains. However, the MLST scheme developed by Young et al. (2008) was previously used either completely, with all four genes (Garita-Cambronero et al., 2017), or partially, in combination with different or the same genes targeting other gene regions (Fischer-Le Saux et al., 2015). The Plant Associated and Environmental Microbes Database (PAMDB) provides three MLST schemes commonly used for *Xanthomonas* spp. identification: the scheme developed by Young et al. (2008) based on four genes (*dnaK*, *fyuA*, *gyrB* and *rpoD*); the scheme proposed by Almeida et al. (2010) based on six genes (*fusA*, *gapA*, *gltA*, *gyrB*, *lacF* and *lepA*); and the scheme published by Fischer-Le Saux et al. (2015) based on seven genes (*atpD*, *dnaK*, *efp*, *fyuA*, *glnA*, *gyrB* and *rpoD*). The two schemes adopted in this study did not reveal genetic differences among the tested Xap strains from Serbia (peach and apricot) and Montenegro (peach). Genetic homogeneity among 64 Xap stains from the United States, France, Italy, New Zealand, Brazil and Uruguay with genes *atpD*, *dnaK*, *efp* and *glnA* was previously reported by Boudon et al. (2005). However, although strains from Serbia were homogenous with those from Montenegro, they differed from the Xap populations from South Korea, New Zealand, Argentina and Uruguay in a single nucleotide (point mutation – transition G ↔ C) of the *gyrB1* sequence (Young et al., 2008). Identification of this change enabled the separation of Xap strains into two haplotypes (Haplotypes I and II) on the TCS network. Based the obtained results, our study points to the potential use of the *gyrB1* gene for the characterization of Xap strains. The high discriminatory potential of the *gyrB* gene was also demonstrated by Parkinson et al. (2007), where this gene enabled accurate reconstruction of xanthomonad phylogeny. Due to its proven discriminatory value, this gene should be considered as a reference gene for phylogenetic species identification within the genus (Fischer-Le Saux et al., 2015). In summary, due to the above-mentioned and proven discriminatory power of the *gyrB1* gene alone for distinguishing between various Xap strains from different hosts and countries, as well as the obtained genetic homogeneity with the other eight sequenced genes, the use of the MLST scheme based on just four genes proposed by Young et al. (2008) could be sufficient and recommended for future studies of this pathogen. However, even though the tested peach and apricot Xap strains from this study were characterized as genetically homogenous, they differed based on virulence potential on different *Prunus* spp. Therefore, some future studies should be directed toward whole genome sequencing for possible detection of differences in virulence genes that could point to the existence of host specialization. The host range specificity results further indicate that the existing Xap bacterial population originating from

peach and apricot in Serbia and Montenegro presents a threat to the nine tested *Prunus* species (*P. avium*, *P. cerasus*, *P. persica*, *P. domestica*, *P. armeniaca*, *P. dulcis*, *P. cerasifera*, *P. spinosa* and *P. mahaleb*), while European ground cherry (*P. fruticosa*) was found to be immune. Considering that no immune *Prunus* cultivars have been detected to date (Garita-Cambronero et al., 2018; Suesada et al., 2019), our investigation provides the first evidence of immunity of *P. fruticosa* to Xap. Furthermore, there are no literature data related to *P. fruticosa* susceptibility to bacterial pathogens prior to this research. This finding can be valuable for future breeding programmes based on interspecific hybridization with the aim of creating new cultivars that are resistant or less susceptible to this pathogen. Repeated backcrosses of such immune species and cultivars/selections having high fruit quality are recommended as an effective method for developing new *Prunus* cultivars combining bacterial spot resistance and fruit quality and productivity. Such backcrosses using Brazilian cultivars/selections with Japanese cultivars/selections having high fruit quality is proposed by Suesada et al. (2019). Socquet-Juglard et al. (2012) stated that the availability of inoculation techniques that allow the degree of susceptibility of cultivars to be evaluated in a standardized quantitative manner may foster the selection or identification of resistant or tolerant cultivars. With the exception of *P. armeniaca* (apricot), the three Xap strains tested exhibited similar virulence. A highly significant host–pathogen interaction occurred between apricot host and Xap apricot strain Xp320 (highly susceptible reaction) while peach strains Xp219 and Xp1 induced reactions indicative of low susceptibility. These results point to the host specialization (apricot strain Xp320 to apricot host). In the case of the *P. persica* (peach) host, there was no difference in strain virulence, as a highly susceptible reaction was noted for all three Xap strains. Moreover, PCA and cluster analysis results supported the AUDPC findings. Although the apricot strain was better adapted, further strains and host plants need to be tested to make a solid conclusion. According to the antibiogram, Xap strains were susceptible to all tested antibiotics (amikacin, erythromycin, penicillin, kanamycin, vancomycin, chloramphenicol, neomycin, gentamycin, tetracycline and streptomycin), which might be expected, as antibiotics are not approved for use in the control of plant bacterial diseases in Europe. Control of Xap is difficult and is restricted to preventive measures, such as prevention of the entry and spread of Xap via trade or through movement of plants for planting, along with chemical treatments in the orchards and use of forecast models (Garita-Cambronero et al., 2018). The forecasting model developed by Morales et al. (2018) could be adopted in warning systems for determining the most optimal timing of copper sprays for bacterial spot disease control, as well as for early detection of disease outbreaks in quarantine and surveillance strategies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that all data underlying the findings are fully available from the corresponding author without any restrictions.

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