



Bacterial, Fungal and Virus Molecular Biology

Molecular methods for diversity assessment among xanthomonads of Bulgarian and Macedonian pepper



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ARTICLE INFO

Article history:

Received 13 December 2016

Accepted 23 August 2017

Available online 3 May 2018

Associate Editor: Welington Araújo

Keywords:

Bacterial spot

RFLP

RAPD

REP-PCR

Heterogeneity

ABSTRACT

Bacterial spot is an important disease of pepper in Bulgaria and Macedonia. For characterization of *Xanthomonas* species associated with bacterial spot, 161 strains were collected from various field pepper-growing regions. Among them, 131 strains were identified as *Xanthomonas euvesicatoria* and 30 as *Xanthomonas vesicatoria* using species-specific primers and polymerase chain reaction followed by restriction fragment length polymorphism analysis. To assess the genetic diversity of the strains, two methods (Random Amplified Polymorphic DNA and Repetitive Element Palindromic-Polymerase Chain Reaction) were applied. Discriminatory index was calculated and analysis of molecular variance was carried out.

Combined random amplified polymorphic DNA analysis of the *X. euvesicatoria* strains with primers CUGEA-4 and CUGEA-6 had greater discriminative power (0.60) than repetitive element palindromic-polymerase chain reaction with ERIC and BOX A1R primers, which makes this method applicable for strain diversity evaluation. Discrimination among the *X. vesicatoria* strains was achieved by the use of ERIC primers and only for the Bulgarian strains. The results demonstrated that *X. euvesicatoria* was more diverse than *X. vesicatoria* and heterogeneity was observed mainly in the Bulgarian populations. According to the analysis of molecular variance, genetic variations in *X. euvesicatoria* were observed among and within populations from different regions, while the differences between the two countries were minor. Following the principal coordinates analysis, a relation between the climatic conditions of the regions and a genetic distance of the populations may be suggested.

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<https://doi.org/10.1016/j.bjm.2017.08.011>

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Introduction

Bacterial spot is one of the most serious diseases of pepper (*Capsicum annum* L.) and tomato (*Solanum lycopersicum* L.) plants worldwide. In areas with warm and humid weather conditions, the disease can be destructive to pepper and tomato seedlings and can result in total crop loss. Plant debris and contaminated seeds are the most common source of primary infection.¹ To date, three genetically and phenotypically distinct pathogens have been defined as causative agents of bacterial spot: *Xanthomonas euvesicatoria* (including the former *Xanthomonas perforans* species), *Xanthomonas vesicatoria*, and *Xanthomonas gardneri*.^{2,3} *X. euvesicatoria* and *X. gardneri* have been isolated from both symptomatic tomato and pepper, *X. vesicatoria* primarily from tomato and *X. perforans*, until recently, only from tomato.^{4,5} The *X. euvesicatoria* strains were reported as more aggressive on pepper plants and in several countries as the prevalent pathogen.^{4–8}

In Bulgaria and Macedonia, bacterial spot is a common disease on pepper plants and was first described in 1963 in Bulgaria and in 1999 in Macedonia.^{9,10} Control of the disease mostly relies on sanitation, cultural practices, including the use of pathogen-free seeds and chemical control by using copper and streptomycin sprays. Characterization of the population structure, diversity, and evolution are the main factors for understanding the pathogen biology and providing information necessary for the development of effective means for disease control. Families of repetitive DNA sequences found in all prokaryotes, such as repetitive extragenic palindromic sequences (rep), BOX elements, and enterobacterial repetitive intergenic consensus (ERIC), as well as random amplified polymorphic DNA (RAPD) analysis have been used for determination of variability within the species of several genera.^{11–15} The amplification reaction using random oligomeric primers (RAPD-PCR) has been employed for analysis of genetic variations in different *Xanthomonas* species.^{16–19}

Even though bacterial spot pathogens have been classified as A2 quarantine organisms by the EPPO (European and Mediterranean Plant Protection Organization) and the disease has been reported in many countries, there is not much data available on the structure and diversity of the pathogen populations.

This study aimed to investigate the heterogeneity in the populations of the species causing bacterial spot on pepper in Bulgaria and Macedonia, and the application of different methods for evaluation of diversity.

Materials and methods

Strains

The bacteria evaluated in this work (161 pathogenic strains) were collected from field grown pepper originating from Bulgaria and Macedonia in the period 1999–2013 (Table 1). The strains were isolated from leaves, petioles, fruits, and flowers with symptoms of bacterial spot (single small necrotic spots or large water-soaked necrotic spots on leaves, ring necrosis on petioles, necrotic scabs on fruits, and brown

necrotic flowers with bacterial exudates). The type cultures *X. vesicatoria* NBIMCC 2427 (DSM-22252), *X. euvesicatoria* NBIMCC 8731 (DSM-19128), *X. perforans* NBIMCC 8729 (DSM-18975) and *X. gardneri* NBIMCC 8730 (DSM-19127) were used as references.

DNA extraction

DNA was extracted from bacterial suspensions with OD₆₀₀ = 1 with DNeasy Blood & Tissue Purification Kit (Qiagen). Control of yield and purity of the obtained DNA was performed by measuring absorbance at 230 nm, 260 nm, 280 nm, and 320 nm with a spectrophotometer Nanodrop 2000 (Thermo Scientific).

PCR with species-specific primers

Five species-specific primers were used for identification of the strains: Xeu 2.4/Xeu 2.5 and Bs-XeF/Bs-XeR for *X. euvesicatoria*, XvF/Bs-XvR for *X. vesicatoria*, Bs-XgF/Bs-XgR for *X. gardneri* and Bs-XpF/Bs-XpR for *X. perforans* (Table 2).

PCRs with all primer sets were performed in a total volume of 25 µL, containing (final concentrations): 0.5× Red Taq DNA polymerase MasterMix (VWR Int.), 4 pmol of each primer, and 100 ng of template DNA. The amplification with primers Bs-XeF/Bs-XeR, Bs-XvF/Bs-XvR, Bs-XgF/Bs-XgR, and Bs-XpF/Bs-XpR was carried out as described by Koenraadt et al.²⁰ and with primers Xeu 2.4/Xeu 2.5 as described by Moretti et al.²¹

PCR-RFLP

Restriction of the 16S-23S ITS region, amplified with primers 16S-p2/23S-p7 (Table 2), with HpaII was performed as a second method for identification.²³ Amplification was carried out in a total volume of 50 µL containing (final concentrations) 1× PCR buffer (STS); 1.5 mM MgCl₂; 0.15 mM dNTPs; 0.4 U Taq DNA polymerase (STS); 10 pmol of each primer; 100 ng of template DNA, under the following reaction conditions: a denaturation step at 95 °C for 5 min, followed by 30 cycles at 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 45 s, and a final step at 72 °C for 7 min.

RAPD-PCR

The amplification program was designed according to Momol et al.,²⁴ using four random primers: CUGEA-3, CUGEA-4, CUGEA-5, and CUGEA-6 (Table 2). Amplification was carried out in a final volume of 25 µL, containing (final concentrations) 1× buffer, 2.5 mM MgCl₂, 50 pmol of each primer, 0.1 mM dNTPs, 0.5 U Taq polymerase, and 100 ng of DNA.

REP-PCR

Three primers were used: BOX A1R, ERIC1R, and ERIC2 (Table 2). PCR mix contained (final concentrations) 1× buffer, 2.5 mM MgCl₂, 50 pmol of each primer, 0.1 mM dNTPs, 0.5 U Taq polymerase, and 100 ng of DNA in a total volume of 25 µL. PCR amplification consisted of an initial denaturing step (94 °C for 7 min); followed by 35 cycles of denaturation (94 °C for 1 min),

Table 1 – Strain designation, year of isolation, location and host variety for *Xanthomonas* isolates from Bulgaria and Macedonia.

Strain designation	Year of isolation	Location	Host variety
1b,2b	1999	Lovech, Bulgaria (NB)	Kapiya
3b	1999	Lovech, Bulgaria (NB)	Hot pepper
4b,5b	2000	Institute of Genetics, Sofia, Bulgaria ^a (WB)	Sortiment
6b,7b	2001	Institute of Genetics, Sofia, Bulgaria ^a (WB)	Genetic material
8b	2002	Lovech, Bulgaria (NB)	Kapiya
9b	2002	Trebich, Bulgaria (WB)	cv. Chorbadzhiyska
10b	2002	Pazardzhik, Bulgaria (SB)	Kapiya
11b	2003	IPP, Kostinbrod, Bulgaria ^b	cv. California wonder
12b	2003	Petrich, Bulgaria (WB)	Kapiya
13b	2004	IPP, Kostinbrod, Bulgaria ^b (WB)	cv. California wonder
14b,15b	2005	Institute of Genetics, Sofia, Bulgaria (WB)	Ornamental
16b	2006	Kavarna, Bulgaria (NEB)	Kapiya
17b	2006	IPP, Kostinbrod, Bulgaria ^b (WB)	Kapiya
18b,19b	2006	IPP, Kostinbrod, Bulgaria ^b (WB)	cv. Golden medal
22b	2006	IG, Sofia, Bulgaria ^a (WB)	L No. 82
23b,24b	2006	IG, Sofia, Bulgaria ^a (WB)	cv. OK
25b	2006	IG, Sofia, Bulgaria ^a (WB)	L 80×67
26b	2006	IG, Sofia, Bulgaria ^a (WB)	cv. Rama
27b	2006	IG, Sofia, Bulgaria ^a (WB)	L B2/13
28b,29b	2006	IG, Sofia, Bulgaria ^a (WB)	Selection material
30b	2006	IG, Sofia, Bulgaria ^a (WB)	L 206
31b	2006	IG, Sofia, Bulgaria ^a (WB)	Hot pepper
32b,33b	2007	Haskovo, Bulgaria (SB)	Kapiya-red
34b,35b	2007	Stara Zagora, Bulgaria (SB)	Kapiya-red
36b,37b	2008	IPP, Kostinbrod, Bulgaria ^b (WB)	cv. Golden medal
38b	2008	IG, Sofia, Bulgaria ^a (WB)	Orange kapiya
39b,40b	2009	Kostinbrod, Bulgaria (WB)	Kapiya
41b,42b	2009	Veliko Tarnovo, Bulgaria (NB)	Kapiya
43b	2009	Veliko Tarnovo, Bulgaria (NB)	Hot pepper
44b	2010	MVCRI, Plovdiv, Bulgaria ^c (SB)	Kapiya
45b	2010	MVCRI, Plovdiv, Bulgaria ^c (SB)	Kurtovska kapiya
47b	2010	MVCRI, Plovdiv, Bulgaria ^c (SB)	Kapiya
48b	2010	MVCRI, Plovdiv, Bulgaria ^c (SB)	Hot pepper
49b,50b	2010	MVCRI, Plovdiv, Bulgaria ^c (SB)	Shipka
51b,52b	2010	MVCRI, Plovdiv, Bulgaria ^c (SB)	Hot pepper
53b	2011	Novi Iskar, Bulgaria (WB)	Kapiya
54b	2011	IPP, Bulgaria ^b (WB)	Siwriya
55b,56b	2011	Kostinbrod, Bulgaria (WB)	Kapiya
57b,58b	2011	MVCRI, Plovdiv, Bulgaria ^c (SB)	White kapiya
59b	2011	MVCRI, Plovdiv, Bulgaria ^c (SB)	White kapiya
60b,61b,62b,63b,64b,65b	2011	IG, Sofia, Bulgaria ^a (WB)	White kapiya
66b,67b,68b,69b	2012	Pavlikeni, Bulgaria (NB)	Kambi
70b,71b,72b	2012	Pavlikeni, Bulgaria (NB)	Kapiya-red
73b,74b,75b,76b	2012	Pavlikeni, Bulgaria (NB)	cv. Shipka
77b,78b,79b	2012	Durankulak, Bulgaria (NEB)	cv. Chorbadzhiyska
80b,81b,82b	2012	Shabla, Bulgaria (NEB)	Kapiya
83b,84b,85b	2012	Tyulenowo, Bulgaria (NEB)	Kambi
86b,87b,88b	2012	Kavarna, Bulgaria (NEB)	Kapiya-red
89b,90b,91b,92b	2013	Kostinbrod, Bulgaria (WB)	Kapiya
93b,94b,95b	2013	Sadowo, Bulgaria (SB)	Kapiya
96b,97b	2013	Byala Cherkva, Bulgaria (NB)	Siwriya
98b,99b,100b	2013	Byala Cherkva, Bulgaria (NB)	Kapiya-red
101b,102b,103b	2013	Byala Cherkva, Bulgaria (NB)	Kambi
105b	2013	Byala Cherkva, Bulgaria (NB)	cv. Chorbadzhiyska
106b	2013	Shabla, Bulgaria (NEB)	cv. Chorbadzhiyska
107b	2013	Kavarna, Bulgaria (NEB)	Kambi
108b,109b,110b,111b,112b, 113b,114b,115b	2013	Kavarna, Bulgaria (NEB)	Kapiya-red
1M,2M,5M,7M,11M	2005	Strumitza, Macedonia (M)	cv. Kurtovska kapiya

Table 1 – (Continued)

Strain designation	Year of isolation	Location	Host variety
15M,21M,25M	2005	Kochani, Macedonia (M)	cv. Kurtovska kapiya
28M,31M,35M,37M,38M	2005	Belasitza, Macedonia (M)	cv. Kurtovska kapiya
44M,50M,53M	2005	Strumitza, Macedonia (M)	cv. Kurtovska kapiya
54M,55M,56M,57M,58M,59M, 60M,61M,62M,63M,64M,65M, 66M,67M,68M,69M,70M	2012	Lazhani, Macedonia (M)	Sivriya
71M,72M,73M,74M,75M	2013	Radovish, Macedonia (M)	cv. Kurtovska kapiya
76M,77M,78M	2013	Belasitza, Macedonia (M)	cv. Golden medal
79M,80M,81M	2013	Kochani, Macedonia (M)	Hot pepper
82M,83M,84M	2013	Strumitza, Macedonia (M)	Kapiya
85M,86M,87M	2013	Lazhani, Macedonia (M)	cv. Babura

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^b Institute of Plant Protection (part of Institute of Soil Science, Agrotechnologies and Plant Protection ‘Nikola Poushkarov’, Sofia, Bulgaria, since 2012).

^c Maritsa Vegetable Crops Research Institute.

WB, Western Bulgaria; NB, Northern Bulgaria; NEB, North-Eastern Bulgaria; SB, Southern Bulgaria; M, Macedonia.

Bold font-strains identified as *X. vesicatoria*; regular font-strains identified as *X. euvesicatoria*.

Table 2 – Sequences of oligonucleotide primers used in PCR amplifications.

Primer	Oligonucleotide sequence (5'→3')	Reference
Bs-XeF	CAT GAA GAA CTC GGC GTA TCG	20
Bs-XeR	GTC GGA CAT AGT GGA CAC ATA C	20
Xeu 2.4	CTG GGA AAC TCA TTC GCA GT	21
Xeu 2.5	TTG TGG CGC TCT TAT TTC CT	21
Bs-XvF	CCA TGT GCC GTT GAA ATA CTT G	20
Bs-XvR	ACA AGA GAT GTT GCT ATG ATT TGC	20
Bs-XgF	TCA GTG CTT AGT TCC TCA TTG TC	20
Bs-XgR	TGA CCG ATA AAG ACT GCG AAA	20
Bs-XpF	GTC GTG TTG ATG GAG CGT TC	20
Bs-XpR	GTG CGA GTC AAT TAT CAG AAT GTG G	20
16S-p2	CTT GTA CAC ACC GCC CGT C	22
23S-p7	GGT ACT TAG ATG TTT CAG TTC	22
CUGEA-3	GCG GTA CCC G	24
CUGEA-4	GCG AAT TCC G	24
CUGEA-5	CGA TCG ATGC	24
CUGEA-6	GGA AGC TTC G	24
BOX A1R	CTA CGG CAA GGC GAC GCT GAC G	25
ERIC1R	ATG TAA GCT CCT GGG GAT TCA C	26
ERIC2	AAG TAA GTG ACT GGG GTG AGC G	26

annealing (56.5 °C for 1 min for BOX-PCR and 54 °C for 1 min for ERIC-PCR) and extension (72 °C for 5 min); followed by a final extension cycle (65 °C for 15 min). The PCR program was performed in a thermocycler Biocycler[®] (Applied Biosystems).

RAPD-PCRs and REP-PCRs were carried out in duplicates and only the main products were taken into consideration.

Electrophoresis

The PCR and restriction products were separated electrophoretically in 1.5% agarose gel in Tris-borate-EDTA (TBE) buffer for 30 min at 100 V, stained with ethidium bromide (EtBr) and visualized under UV light. GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific) was used. The gels were analyzed by GenoSoft Capture and GenoSoft Imaging software (VWR Int.).

Discriminatory index

The Discriminatory power of the methods was calculated using the Hunter and Gaston²⁷ discriminatory index (*D*):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^s x_j(x_j - 1)$$

where *D* is the index of discriminatory power, *N* is the number of unrelated strains tested, *S* is the number of different types, and *x_j* is the number of strains belonging to the *j*th type.

Data analysis

Genetic diversity among the strains of *Xanthomonas* species was estimated by an analysis of molecular variance (AMOVA). The strains were grouped according their geographic location into two countries (Bulgaria and Macedonia) and five regions (populations): Northern Bulgaria (22 strains), Southern Bulgaria (12 strains), North-Eastern Bulgaria (23 strains), Western Bulgaria (27 strains), and Macedonia (47 strains). Binary matrices of presence/absence of bands at specific positions were prepared from the molecular data. The results were reported by standard AMOVA table including degree of freedom (d.f.), sums of squares, variance components, percentage of variation, φ statistics and *p* value. The significance was examined with 999 random permutations. The genetic differences between the strains from the investigated regions were assessed by means of Nei's unbiased genetic distance.²⁸ Principal coordinates analysis (PCoA) was performed for visualizing the patterns of relationship via the genetic distance matrix. GenAlEx 6.5 software²⁹ was used for data analysis.

Results

Identification

Two bacterial spot pathogens were identified among the analyzed strains: *X. vesicatoria* and *X. euvesicatoria*. The majority

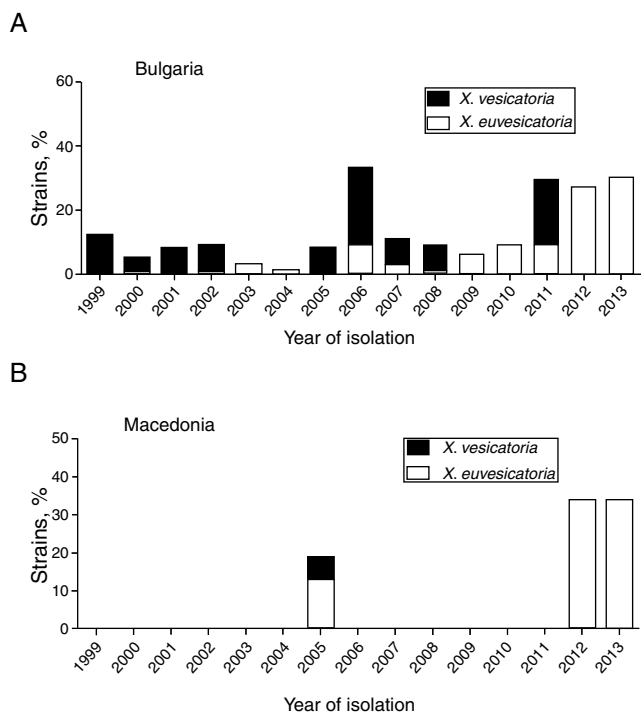


Fig. 1 – Incidence of *X. vesicatoria* and *X. euvesicatoria* in Bulgaria (A) and Macedonia (B) through the years (as % from the total number of strains from each species and country).

of the strains belonged to the species *X. euvesicatoria* (84 from Bulgaria and 47 from Macedonia), which was confirmed by both PCR amplification with species-specific primer pairs and the PCR-RFLP analysis. Only three Macedonian strains isolated in 2005 and 27 Bulgarian strains were identified as *X. vesicatoria*. The distribution of the two species during the study is presented in Fig. 1.

RAPD-PCR

In the course of RAPD-PCR analysis, 16 amplification products were obtained for the *X. euvesicatoria* and 7 for the *X. vesicatoria* strains. The primers chosen to evaluate polymorphism generated bands in a wide size range: from 330 bp to 2443 bp for *X. euvesicatoria* and from 550 bp to 2500 bp for *X. vesicatoria*. Amplification with CUGEA-5 was not applicable for analysis and comparison due to the large number of indistinguishable products. In the presence of the CUGEA-3 primer, only one product for each species was obtained – around 650 bp and 1100 bp for *X. euvesicatoria* and *X. vesicatoria*, respectively. After the amplification with the CUGEA-6 primer, three profiles were revealed for *X. euvesicatoria* strains – profile I, II, and III, respectively. Profile I consisted of five products and was formed by only 8% of the Bulgarian strains, all isolated in 2012.⁸ The rest of the strains generated profile II which was characterized by five products. The type culture of *X. euvesicatoria* formed the third profile. With CUGEA-6, no intraspecies diversity was observed for the isolated *X. vesicatoria* strains: they all formed a profile of six products (about 1100 bp, 1400 bp, 1500 bp, 1800 bp, 2100 bp, and 3000 bp). Only the type *X. vesicatoria* strain showed a different pattern,

Table 3 – Profiles generated by amplification with CUGEA-4 of *X. euvesicatoria* strains.

Strains	Profiles				
	I	II	III	IV	V
Bulgarian strains	10%	1%	45%	4%	40%
Macedonian strains	0	0	89%	9%	2%

consisting of two amplification products (around 1100 bp and 1800 bp). The amplification of the *X. euvesicatoria* strains with CUGEA-4 generated five profiles (Fig. 2, Table 3). Most of the analyzed strains formed profile III. Profiles I and II were exclusive to Bulgarian strains. Profile V comprised a great part of the Bulgarian (40%) and only 2% of the Macedonian strains (Table 3). *X. vesicatoria* strains formed only one RAPD-pattern with CUGEA-4 (550 bp, 650 bp, 800 bp, 1000 bp, 1300 bp and 1600 bp) with the exception of the type strain which lacked the product of 1300 bp, and had additional one of 2500 bp. The discriminatory index of RAPD-PCR with CUGEA-6 and CUGEA-4 for *X. euvesicatoria* was 0.14 and 0.55, respectively, whereas the combined analysis had D of 0.60.

REP-PCR

The REP-PCR analysis with BOX A1R generated 11 well defined amplification products with lengths of 250–2100 bp and revealed four profiles among the population of the *X. euvesicatoria* strains (Figs. 3 and 4). The majority of the strains (97%) formed profile I which was also characteristic for the type strain, *X. euvesicatoria* NBIMCC 8731. All Macedonian strains were grouped together in profile I. Profiles II and III united only Bulgarian strains (11b, 38b, and 28b) isolated from Western Bulgaria. Only one strain (69b), isolated from Northern Bulgaria, characterized profile IV. The discriminatory index was 0.06.

All *X. vesicatoria* strains were grouped in profile I with five amplification products (Fig. 5). The type strain, *X. vesicatoria* NBIMCC 2427 formed a separate pattern of six products (Fig. 5).

ERIC-PCR analysis revealed two profiles among *X. euvesicatoria* strains. Eight well defined amplification products with length 370–1565 bp were observed (Fig. 6). All the Macedonian and most of the Bulgarian strains were grouped into profile I. Only two Bulgarian strains (25b and 27b) formed profile II. One of these strains (25b) was separated also with the analysis with BOX A1R primer, where it formed profile III. The discriminatory index of REP-PCR was 0.03 which makes this analysis less usable for evaluation of genetic diversity of *X. euvesicatoria* than RAPD-PCR. Among *X. vesicatoria* strains two different profiles were defined. Most of the Bulgarian strains (70%) were grouped together with all the Macedonian strains in profile I (Fig. 7). Diversity among the Bulgarian strains of the species was achieved only by the use of ERIC primers.

Genetic variance

Two- and three level AMOVA were conducted for the both types of molecular primers to reveal the genetic differentiation of *Xanthomonas* strains. The summary statistics for *X. euvesicatoria* are presented in Table 4. The partitioning

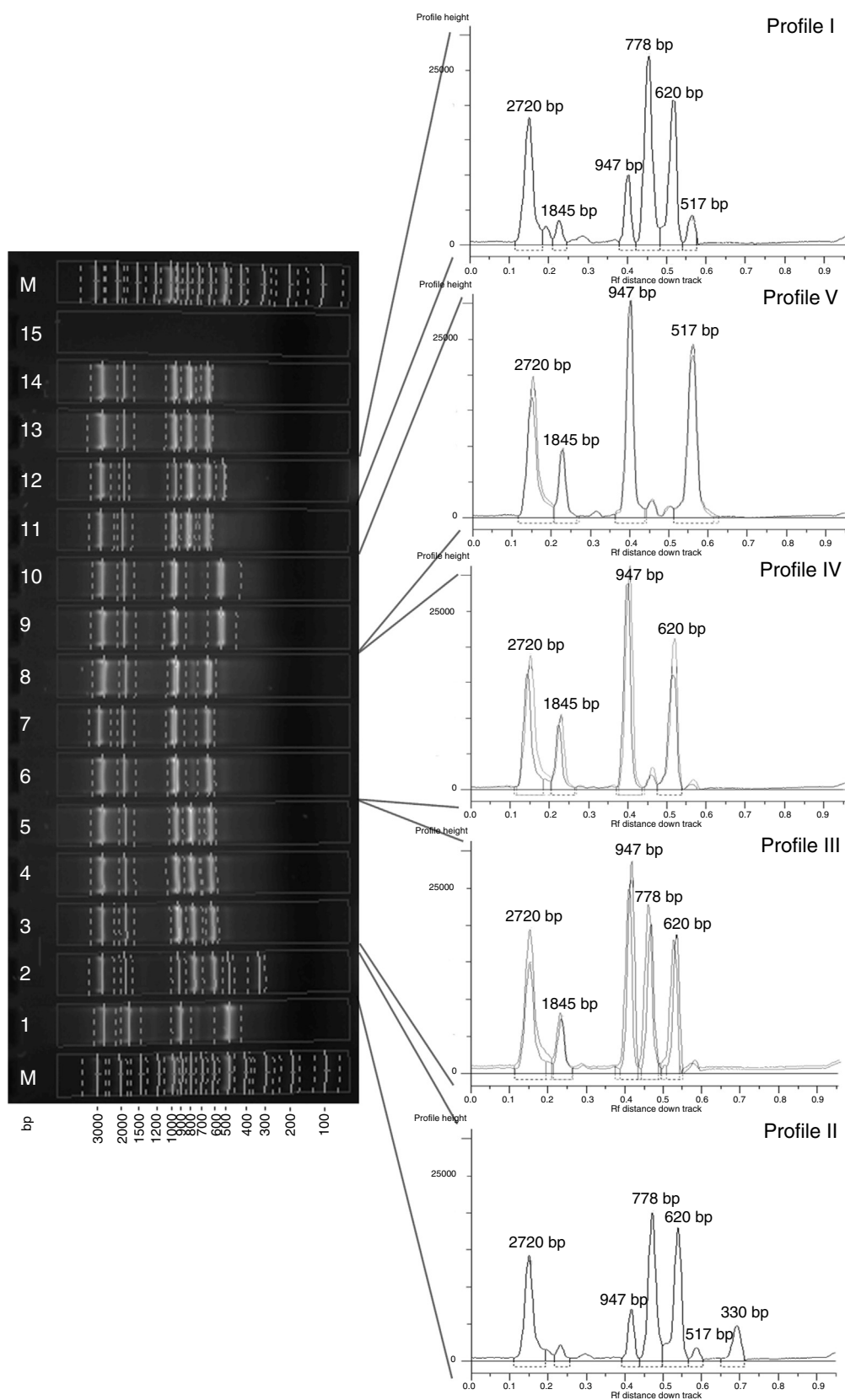


Fig. 2 – RAPD-PCR amplification of *X. euvesicatoria* strains with the CUGEA-4 primer. On the left: M-DNA ladder; lane 12 – representative *X. euvesicatoria* strain forming profile I; lane 2 – representative *X. euvesicatoria* strain forming profile II; lanes 3–5, 11, 13, 14 – representative *X. euvesicatoria* strains forming profile III; lanes 6–8, 18 – representative *X. euvesicatoria* strains forming profile IV; lanes 1, 9, 10 – representative *X. euvesicatoria* strains forming profile V; lane 15 – PCR mix. On the right: graphs of the five profiles. The numbers at the tops of the peaks correspond to the lengths of the amplicons.

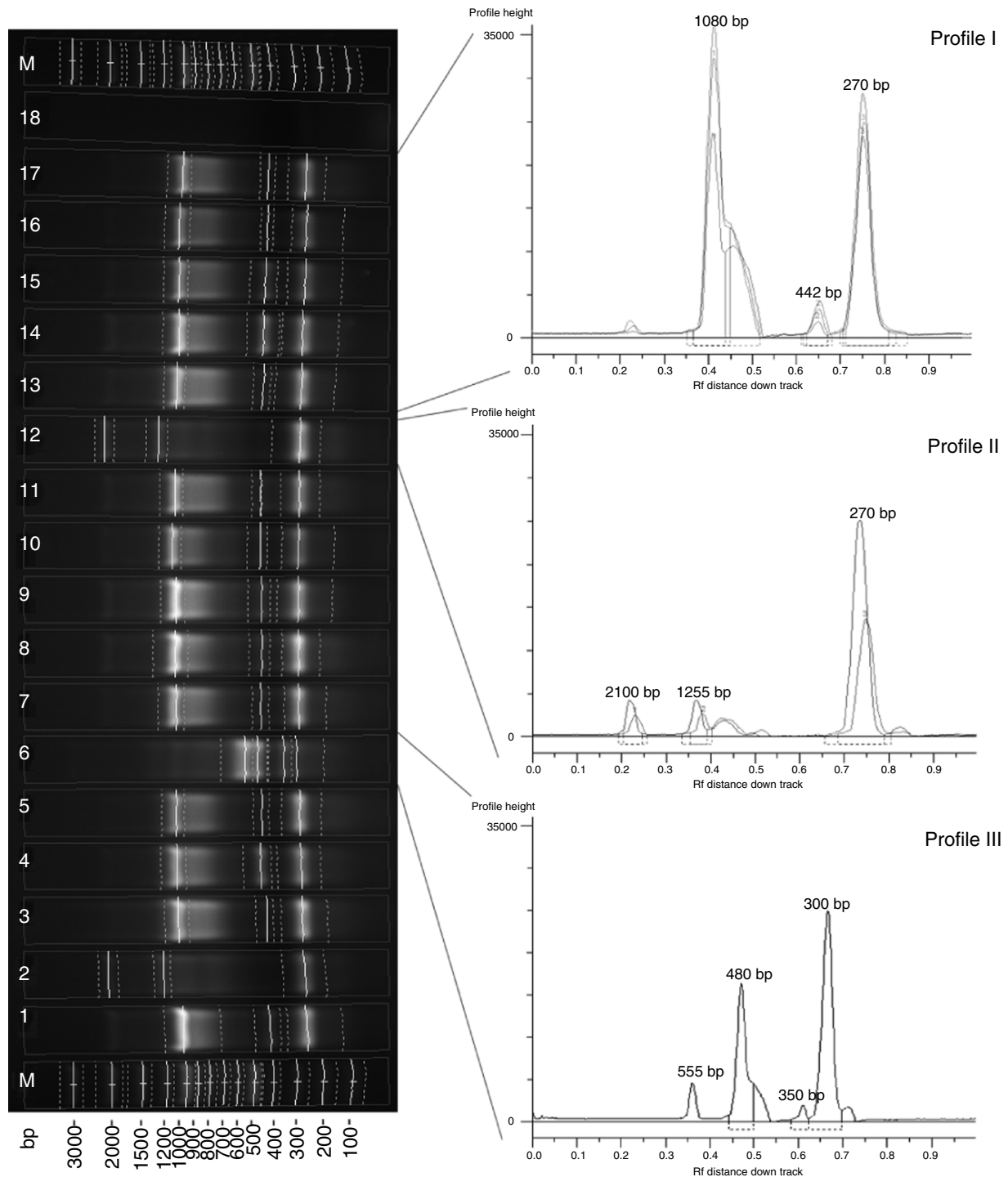


Fig. 3 – REP-PCR amplification of *X. euvesicatoria* strains with the BOX A1R primer – Profiles I–III. On the left: M-DNA ladder; lanes 1, 3–5, 7–11, 13–17 – representative *X. euvesicatoria* strains forming profile I; lanes 2, 12 – representative *X. euvesicatoria* strains forming profile II; lane 6 – representative *X. euvesicatoria* strain forming profile III; lane 18 – PCR mix. On the right: graphs of the three profiles. The numbers at the tops of the peaks correspond to the lengths of the amplicons.

of genetic variation between the strains showed a larger share within the regions and was accounted for 67–68% for RAPD-PCR and 95–97% for REP-PCR respectively. In AMOVA based on RAPD-PCR, the percentages of variation attributed to among regions were 32% (two-level analysis) and 28% (three-level analysis). It was established that the difference between

the countries (Bulgaria and Macedonia) was minor (5%). The contributions of all variance components were statistically significant ($p=0.01$ for within- and among-region levels and $p<0.05$ for among-country level). Furthermore, pairwise ϕ matrix showed significant differences between all regions excluding Macedonian and North-Eastern Bulgarian strains.

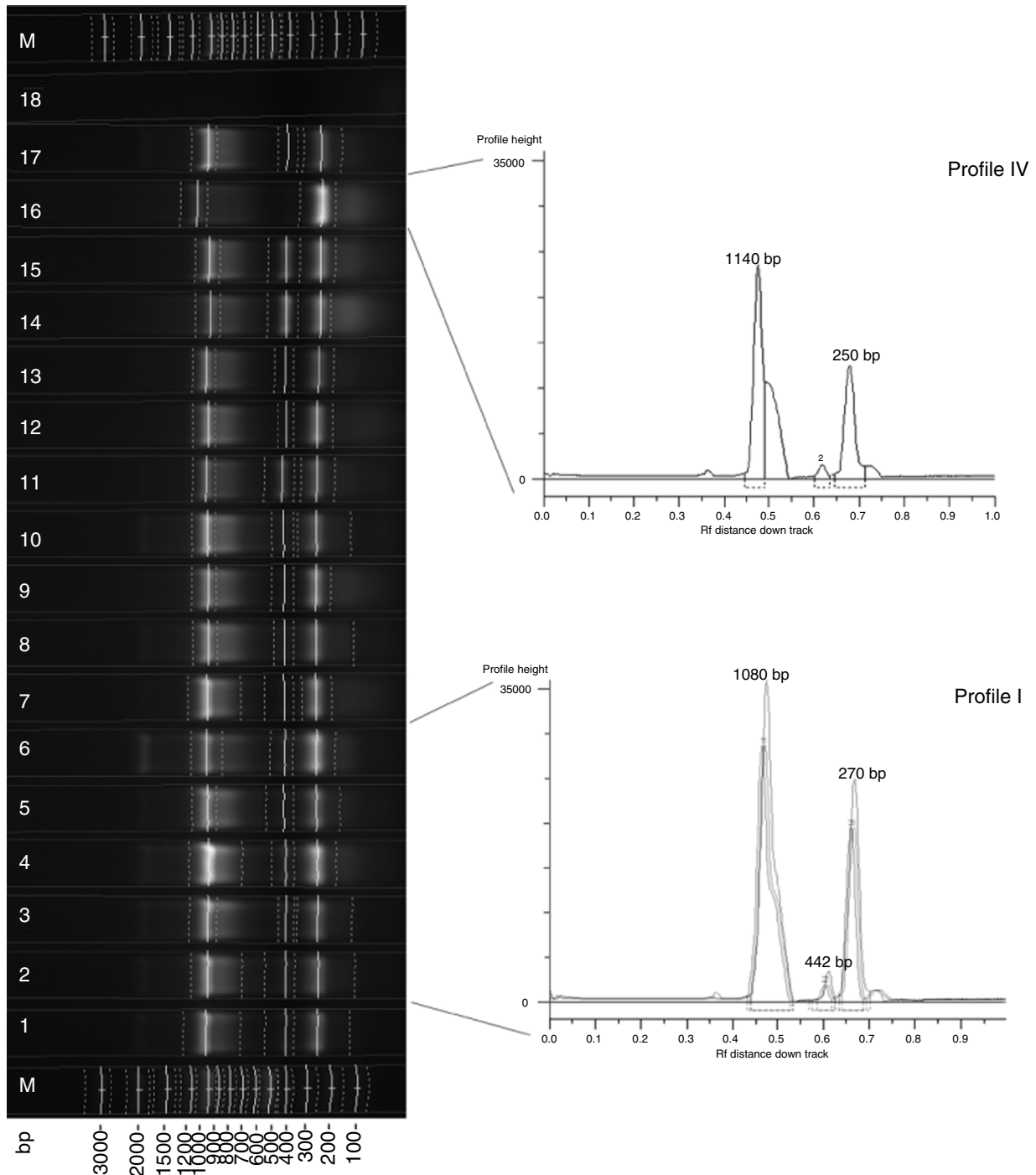


Fig. 4 – REP-PCR amplification of *X. euvesicatoria* strains with BOX A1R primer – Profiles I, IV. On the left: M-DNA ladder; lanes 1–15, 17 – representative *X. euvesicatoria* strains forming profile I; lane 16 – representative *X. euvesicatoria* strain forming profile IV; lane 18 – PCR mix. On the right: graphs of the two profiles. The numbers at the tops of the peaks correspond to the lengths of the amplicons.

Only 3–5% of the total variance was due to the variation among regions on the base of REP-PCR. No differences were recorded between countries. The AMOVA results for *X. vesicatoria* strains were not reported because of non-significant partitioning of the genetic variation.

Additionally, Nei's unbiased genetic distance between all pairs of regions was calculated using RAPD-PCR data for *X.*

euvesicatoria (Table 5). The largest distances were obtained between Macedonian strains and those from Northern (0.154) and Southern Bulgaria (0.134), and the smallest distances – between the strains originated from North-Eastern Bulgaria and those from Macedonia (0.001) and Western Bulgaria (0.015). Principal Components Analysis (PCoA) based on the genetic distance matrix was carried out (Fig. 8). According to

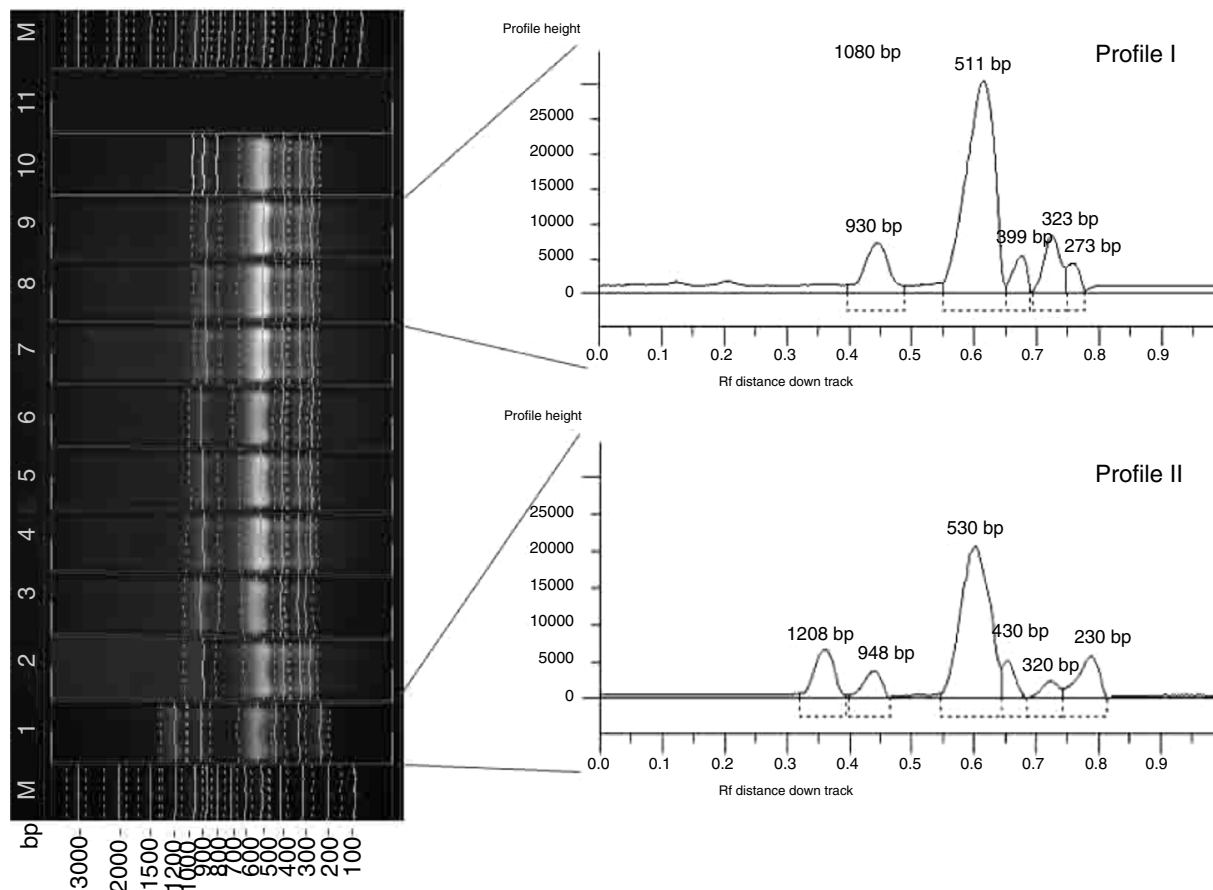


Fig. 5 – REP-PCR amplification of *X. vesicatoria* strains with the BOX A1R primer. On the left: M-DNA ladder; lane 1 – type strain *X. vesicatoria* forming profile II; lanes 2–10 – representative *X. vesicatoria* strains forming profile I; lane 11 – PCR mix. On the right: graphs of the two profiles. The numbers at the tops of the peaks correspond to the lengths of the amplicons.

the results, the first two coordinates explained 86% and 13% of the total molecular variation, respectively. Scatter PCoA plot displayed the close relationship between the three regions mentioned above – Macedonia (M), North-Eastern Bulgaria (NEB) and Western Bulgaria (WB) (Fig. 8).

Discussion

The distribution of *X. vesicatoria* and *X. euvesicatoria* varied through the years. Interestingly, in the last two years of the study, *X. vesicatoria* was not detected. The shift between the populations of *X. euvesicatoria* and *X. vesicatoria*, which we observed in 2012 and 2013, can hardly be explained. Bulgaria and Macedonia are neighboring countries with similar climate. A possible reason for the shift may be local adaptation to climate conditions of the respective years and the more narrow specialization of *X. euvesicatoria* to pepper compared to *X. vesicatoria*.

Analysis with CUGEA-6 revealed diversity only among the *X. euvesicatoria* Bulgarian strains. A greater diversity was observed by amplification with CUGEA-4 of *X. euvesicatoria* strains from both countries. The combined RAPD-PCR analysis with the two random primers CUGEA-4 and CUGEA-6 enabled a more comprehensive study of the genome and could be used

for investigation of the genetic diversity in populations. However, according to our results, these primers were not suitable for seeking of intraspecies diversity within the species *X. vesicatoria* as only the type strain formed a profile different from the other strains. Investigations of more strains are needed to confirm this statement. Heterogeneity for the population of *X. euvesicatoria* was also detected by BOX-PCR and for *X. vesicatoria* – by ERIC-PCR. Diversity was observed mainly in the Bulgarian populations of both species. The Macedonian *X. euvesicatoria* strains were relatively homogenous when analyzed by RAPD-PCR with CUGEA-6, ERIC-PCR, and BOX-PCR and the *X. vesicatoria* strains also grouped together by all applied methods.

RAPD-PCR analysis with the two primers CUGEA-4 and CUGEA-6 had greater discriminative power (0.60) than BOX- and ERIC-PCR for *X. euvesicatoria*, which makes this method applicable for strain diversity evaluation. However, the genetic patterns obtained by the different methods used in this study did not show correlation in the grouping of the strains – only one Bulgarian *X. euvesicatoria* strain formed a different profile than the majority of the strains determined by both BOX- and ERIC-PCR analyses.

To evaluate the diversity of the strains *X. euvesicatoria* according to their place of isolation, AMOVA based on the regions and countries was carried out. Differences between

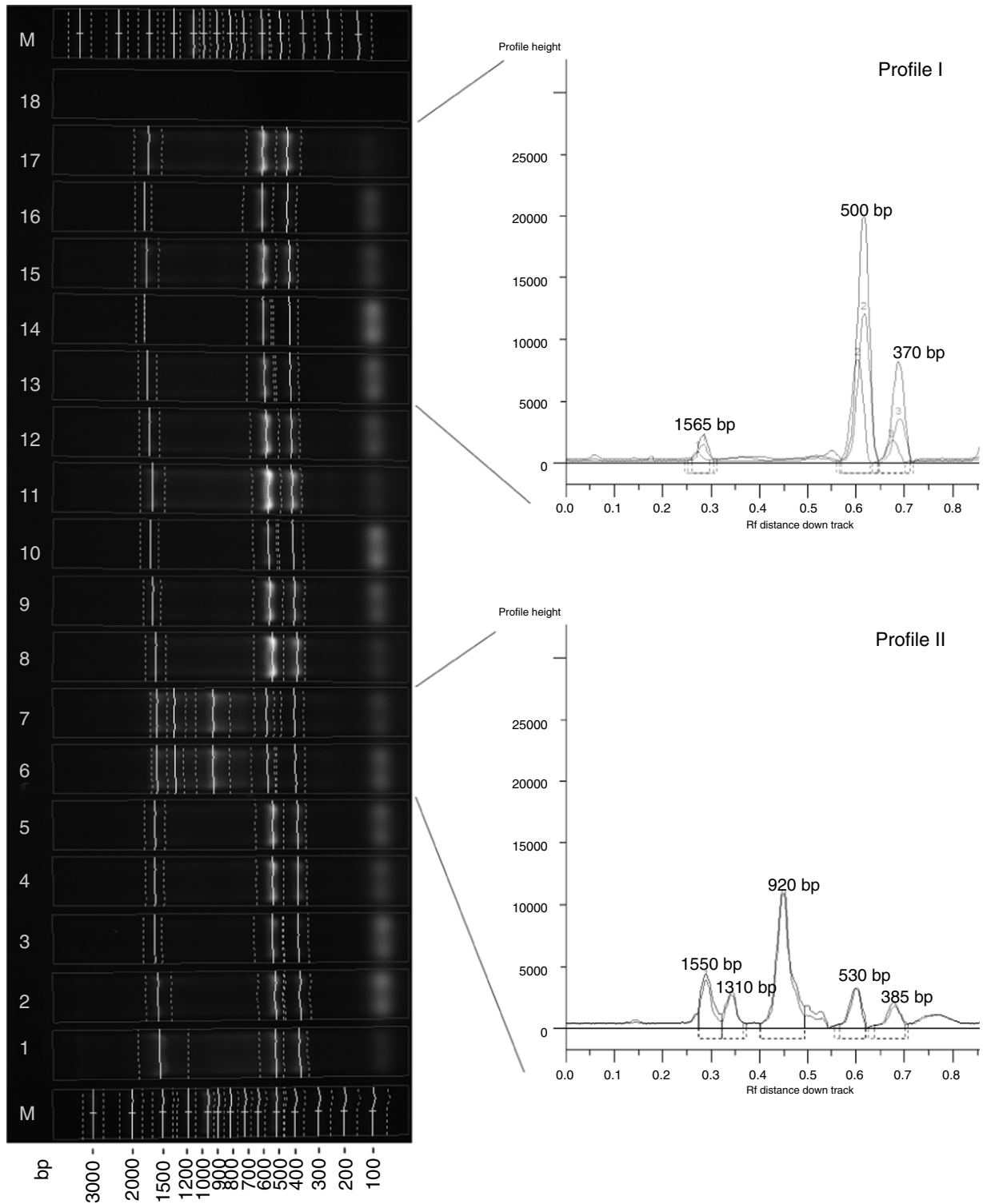


Fig. 6 – REP-PCR amplification of *X. euvesicatoria* strains with ERIC primers. On the left: M-DNA ladder; lanes 1-5, 8-17 – representative *X. euvesicatoria* strains forming profile I; lanes 6, 7 – representative *X. euvesicatoria* strains forming profile II; lane 18 – PCR mix. On the right: graphs of the two profiles. The numbers at the tops of the peaks correspond to the lengths of the amplicons.

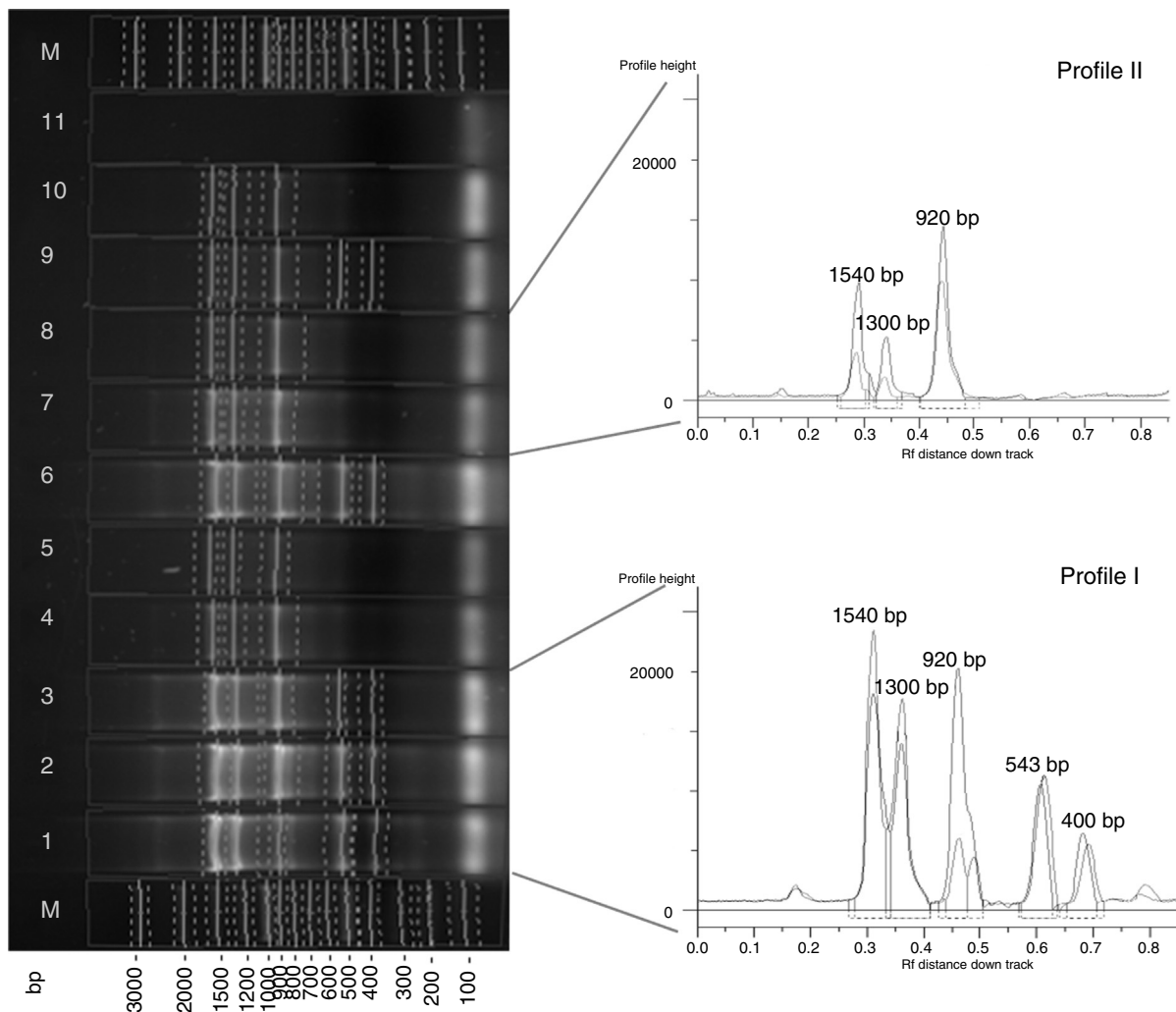


Fig. 7 – REP-PCR amplification of *X. vesicatoria* strains with ERIC primers. On the left: M-DNA ladder; lanes 1–3, 6, 9 – representative *X. vesicatoria* strains forming profile I; lanes 4, 5, 7, 8, 10 – representative *X. vesicatoria* strains forming profile II; lane 11 – PCR mix. On the right: graphs of the two profiles. The numbers at the tops of the peaks correspond to the lengths of the amplicons.

the Bulgarian and Macedonian strains were minor with RAPD-PCR and insignificant with REP-PCR. Variations were observed among populations originating from different regions – WB, NB, NEB, SB, and M, which may be related to the specific soil and climate conditions of each region. However, much greater variation existed among the strains irrespective of their origin (67–68% vs. 28–32%). The strains in this study have been isolated during a period of 13 years which could explain these results. Similarities between the regions M and WB may be due to the closest location to each other compared to the others, while M and NEB, which have least genetic distance, are geographically the two most distant regions. However, NEB is the only region alongside the Black Sea coast, which is characterized with the mildest climate of all investigated regions, and Macedonia (M) has milder climate than SB, NB, and WB due to its most southern location.

RAPD-PCR and REP-PCR have been successfully used for the characterization of populations of different xanthomonads.^{16,30–35} To our knowledge, our recent and

previous study⁷ are the first analyses of populations of the causative agents of bacterial spot of pepper using these methods. According to the obtained data, the Bulgarian population of *X. euvesicatoria* is more diverse and prevalent than the population of *X. vesicatoria*. The domination of one genotype among the xanthomonads in Bulgaria and Macedonia could be due to a common source of infection or origin. Trade of seeds and seedlings between the neighboring countries and the different regions is probable. The great homogeneity among the strains of certain species could be a result of being in an isolated and restricted area. The distribution of the pathogens in different regions is a key to the development of genetic diversity. The high genetic identity among strains, isolated from geographically close areas with nearly similar climatic conditions, is commonly observed and is crucial for the adaptation capabilities of the pathogens.

There is evidence for a relationship between the regions of isolation and the grouping of strains according to their

Table 4 – Analysis of molecular variance of *X. euvesicatoria* strains based on RAPD-PCR (CUGEA-4 and CUGEA-6) and REP-PCR (with primers BOX A1R and ERIC).

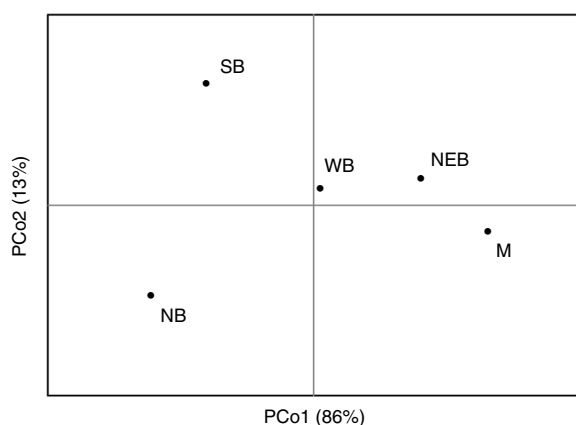
Source of variation	df	Sums of squares	Variance components	Percentage of variation	φ statistics	p value
RAPD-PCR						
A.						
Among regions	4	42.4	0.391	32%	0.316	0.001
Within regions	126	106.7	0.847	68%		
B.						
Among countries	1	18.0	0.061	5%	0.048	0.025
Among regions	3	24.3	0.354	28%	0.295	0.001
Within regions	126	106.7	0.847	67%	0.329	0.001
REP-PCR						
A.						
Among regions	4	1.92	0.009	3%	0.032	0.039
Within regions	126	33.4	0.265	97%		
B.						
Among countries	1	0.35	0.000	0%	-0.024	0.888
Among regions	3	1.57	0.013	5%	0.045	0.054
Within regions	126	33.4	0.265	95%	0.022	0.028

A. Two level AMOVA: the strains were grouped into regions according their geographical locations; B. Three level AMOVA: the regions from Bulgaria and Macedonia were separated.

Table 5 – Pairwise matrix of Nei's unbiased genetic distance for *X. euvesicatoria* strains.

Regions ^a	M	NB	SB	NEB
NB	0.154			
SB	0.134	0.070		
NEB	0.001	0.124	0.100	
WB	0.033	0.047	0.033	0.015

^a NB, Northern Bulgaria; SB, Southern Bulgaria; NEB, North-Eastern Bulgaria; WB, Western Bulgaria; M, Macedonia.

**Fig. 8 – Scatter PCoA plot of *X. euvesicatoria* strains.**

REP-PCR patterns for other *Xanthomonas* species.^{31-33,35} Relationships between the particular strain pattern groups and the regions of isolation in this study were not recorded, however, based on the PCoA, a relation between the climatic conditions of the regions and the genetic distance of the populations may be suggested. Correspondence between metabolic clusters of *X. euvesicatoria* and the climatic characteristics of the regions

was detected in a previous study for strains isolated in a single year (2012). In this year, the Bulgarian strains from North-Eastern Bulgaria were also closest to Macedonian strains and more distant from the strains isolated from other parts of Bulgaria.⁸

The occupation of specialized niches could influence the organization of the genome and the distribution of repetitive elements in the bacterial genome. This could have altered the genetic profile and the emergence of new characteristic profiles for certain species or strains. The pepper varieties, at this stage of the studies, seem not to be related to the profiles formed by the repetitive elements. For example, some strains, isolated from the same local pepper varieties in Bulgaria, were separated as different and genetically heterogeneous. The population of the two pathogens, *X. euvesicatoria* and *X. vesicatoria*, in Macedonia is more homogenic. Cv. Kurtovska kapyia is the main pepper variety grown in Macedonia and adaptation to this host could not be a factor which defines the genetic diversity within the population. A comparison of a large number of strains from different pepper varieties from a single region in a single year may show some relatedness, however, based on the overall picture, the variety of the host seems to have much less significance compared to the region of isolation.

In conclusion, we evaluated the genetic diversity based on repetitive elements in the two bacterial species as an initial step to understanding the population structure of the pathogens identified as causative agents of bacterial spot of pepper in Bulgaria and Macedonia. This study may serve as a platform study for extended investigations in this area and refined characterization of the relations region – climate adaptation – host variety adaptation – genetic diversity of the pathogens. Our results also showed that the RAPD primers rather than ERIC- and BOX-primers were efficient in differentiating strains. Additional samples and yearly comparisons are needed to fully understand the population structure.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgement

This study was supported by project DFNI B02/4 by the National Science Fund of Bulgaria.

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