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Genetic relationships among strains of *Xanthomonas campestris* pv. *campestris* revealed by novel rep-PCR primers*

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Abstract

Novel primers for rep-PCR were developed with the original software and based on 'ancient diverged periodical sequences'. Rep-PCR with these primers was applied to study genetic relationships among 51 *Xanthomonas campestris* strains. The strains were collected from different countries including Russia, Japan, UK, Germany and Hungary. Reference strains of three *X. campestris* pathovars and five other *Xanthomonas* species were included. Based on qualitative differences in amplification profiles, the strains were divided into four major groups. Two subgroups recognised within *X. campestris* population were similar to RFLP haplotypes. The third subgroup included strains of two other pathovariants and Japanese isolates of *X. campestris* pv. *campestris* while the fourth group comprised the other species of *Xanthomonas*. The analysis of the diversity within *X. campestris* resulted in the conclusion that isolates belong to distinct clonal populations (subgroups). The differences between the subgroups of *X. campestris* were only slightly smaller than between species of *Xanthomonas*. A PCR fragment about 600 bp amplified by primer KRPN2 was found in nearly all tested strains of *X. campestris*. SCAR primers designed for this marker produced a single specific band for strains of *X. campestris*, but not for other *Xanthomonas*, *Pseudomonas* and *Erwinia* strains tested. Application of the new primer set for rep-PCR offers a rapid, simple and reproducible method for identification of bacterial strains. The *X. campestris*-specific SCAR primers may be used in diagnostics of this important plant pathogen.

Introduction

Incidence of black rot caused by proteobacterium *Xanthomonas campestris* pv. *campestris* (*Xcc*) on *Brassica* crops is recognised worldwide (Williams, 1980). Since the 1990s the disease has become more harmful in cabbage production fields in Russia and other countries. International trade of infected seeds is generally blamed for the spread of highly virulent strains of the black rot pathogen, but there are no effective ways to trace the origin of infection. The clonal structure of *Xcc* populations was revealed by evaluation of serological properties (Franken et al., 1992; Alvarez et al., 1994; Ignatov et al., 1998) and RFLP patterns (Alvarez et al., 1994). A number of races of the pathogen have recently been identified based on interaction of the isolates with differential varieties of several *Brassica* species (Kamoun et al., 1992; Ignatov et al., 1998, 1999a; Vicente et al., 2001). Thus, the variation within *Xcc* must be determined in order to design effective control strategies, especially seed health tests.

PCR-based DNA-fingerprinting is a fast, reliable and an inexpensive method to study genetic diversity, but its effectiveness depends on primers chosen for analysis. There are many highly

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conserved, repetitive DNA sequences, present in the genomes of Gram-negative bacteria. Three families of repetitive sequences including repetitive extragenic palindromic (REP) sequences (Higgins et al., 1982), enterobacterial repetitive intergenic consensus (ERIC) sequences (Sharples and Lloyd, 1990), and BOX element (Martin et al., 1992) were identified. Several methods for assessment of genetic diversity of bacteria employing PCR with different primers homologous to repetitive sequences were named in general as rep-PCR (De Bruijn, 1992). The function of these repetitive sequences as intercistronic regulatory element of prokaryotic operons (Sharples and Lloyd, 1990) lead to a hypothesis about their biased distribution across different genomic regions, confirmed in analysis of complete sequences genomes of *Xcc* and *X. axonopodis* pv. *citri* (Ignatov et al., in press).

Other repetitive sequences referred as latent periodical ones were found *in silica* in many sequenced genomes, including bacterial ones (Korotkov et al., 1999). These sequences appear to be located inside protein-encoding genes. Their use as primers for PCR leads to selective amplification of distinct genomic regions including highly polymorphic intergenic regions. This is a major difference between 'ancient diverged periodical sequences' and previously employed repetitive intergenic sequences.

Xanthomonas campestris possesses interspecies variation on the level of pathovars, haplotypes and serogroups and races (Kamoun et al., 1992; Alvarez et al., 1994; Vauterin et al., 1995). Here, we report the utility of a novel class of rep-PCR primers for genomic fingerprinting of phytopathogenic bacteria.

Materials and methods

Bacterial strains. Reference strains of *Xanthomonas* species and the isolates of *Xcc* recovered from diseased cabbage, broccoli, and turnip plants in Russian Federation, UK, Japan, Germany, and Hungary are given in the Table 1. All were previously tested for pathogenicity on brassicas, and some for serotype and haplotype (Nachtigall., 1998; Ignatov et al., 1998, 1999a; A.N. Ignatov, unpubl.). Isolates of other plant pathogenic and epiphytic bacteria were obtained from the Laboratory of Bacterial Plant Diseases at the Russian Institute of Phytopathology. The strains were cultured on King's B agar plates at 28 °C.

DNA isolation and PCR analysis. DNA was extracted as described (Boulygina et al., 2002) and for PCR analysis, primers that amplify the 'ancient diverged periodical sequences' were calculated by original algorithms (Korotkov et al., 1999). Among suggested primers, the following were used in this work: KRPN2 5′-CGCCIGGIGGAT-3′; KRP2 5′-CAGGAAGAAG-3′; and KRP8 5′-GA AGTTCAGG-3′. Optimisation of amplification conditions was done according to the modified method of Taguchi and Wu (1980). The final PCR mixtures (25 μl) had the following content: 1× PCR buffer (17 mM $(NH_4)SO_4$, 6 mM Tris–HCl, pH 8.8, 2.5 mM $MgCl_2$), 7.5 mM dNTP, 50 ng of DNA, 10 pM of primer and 1.25 units of BioTaq DNA Polymerase ('Dialat Ltd', Russia).

DNA amplification was performed in a 'Cetus 480' thermocycler ('Perkin Elmer', Sweden) with the following reaction profile: first cycle – 94 °C × 30 s, 30 °C × 30 s and 72 °C × 1 min 30 s; next 43 cycles – 94 °C × 5 s, 30 °C × 30 s and 72 °C × 1 min 30 s, with a final extension step of 72 °C for 7 min. Reproducibility of the PCR protocol was verified on two other PCR machines: 'Tpersonal' ('Biometra GmbH', Germany) and 'Genius' ('Techne (Cambridge) Ltd', UK). PCR products were analysed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and documented in 'BioDoc Analyze System' ('Biometra', Germany). The positions of bands were assessed visually. Genetic distances (*D*) were calculated according to Nei (1987), UPGMA and Neighbour-joining (NJ) trees were built using 'Treecon W 1.6' software (Van de Peer et al., 1994).

PCR fragment cloning and sequencing. Purified *Xcc*-specific PCR fragments were cloned in the *Hinc*11 site of the pGEM-3Zf(+) vector polylinker ('Promega' USA) using competent cells of *E. coli* DH5α for transformation. Plasmid DNA was extracted and purified using a 'Wizard MiniPrep' kit ('Promega', USA) according to the recommendations of the manufacturer. Sequencing was performed by the Sanger's method (1977) using the 'Silver Sequencing' kit

Table 1. Origin of *Xanthomonas* spp. strains and other plant-associated bacteria

Strains	Species, pathovar	Year, country, crop	Race	S/H[a]
Group[b] *9*				
NCPPB633^T	*X. axonopodis* pv. *malvacearum*	1958, Sudan, cotton	–	–
HRI924a	*X. axonopodis* pv. *phaseoli*	1976, UK, beans	–	–
Group[b] *10*				
NCPPB3002^T	*X. oryza* pv. *oryzae*	1965, India, rice	–	–
NCPPB1585^T	*X. oryzae* pv. *oryzicola*	1964, Malaysia, rice	–	–
Group[b] *14*				
NCPPB422^T	*X. vesicatoria* pv. *vesicatoria*	1955, New Zealand, tomato	–	–
Group[b] *15*				
NCPPB1946^T	*X. campestris* pv. *raphani*	1966, USA, radish	–	–
NCPPB2986^T	*X. campestris* pv. *aberrans*	1975, Australia, cabbage	1	1/1
NCPPB347^T	*X. campestris* pv. *armoraciae*	1954, Tanzania, horse-radish	1	1/1
NCPPB528^T	*X. campestris* pv. *campestris*	1974, UK, cabbage	1	3/3
NCPPB1711	*Xcc*	1965, Canada, rape	4	3/3
HRI1279a	*Xcc*	1986, UK, broccoli	4	3/3
PHW231	*Xcc*	USA, cabbage	1	1/1
PHW117	*Xcc*	USA	1	1/1
2D520	*Xcc*	USA, cabbage	2	1/1
Ru1	*Xcc*	1998, Russia, cabbage	4	NT
Ru2	*Xcc*	1999, Russia, broccoli	4	NT
Ru3	*Xcc*	2000, Russia, cabbage	4	NT
Ru4	*Xcc*	–	4	NT
Ru5	*Xcc*	–	1	NT
D1	*Xcc*	1994, Germany, cauliflower	1	1/1
D4	*Xcc*	1994, Germany, cabbage	4	Xa[c]
D7	*Xcc*	1996, Germany, cauliflower	4	1/1
D16	*Xcc*	1995, Germany, red cabbage	1	3/3
H1	*Xcc*	1999, Hungary, broccoli	1	NT
H2	*Xcc*	1999, Hungary, cauliflower	1	NT
H11	*Xcc*	1999, Hungary, cabbage	4	NT
UK1–UK14	*Xcc*	1995, UK, cabbage	1	1
J19a	*Xcc*	1997, Japan, cabbage	1	NT
J20	*Xcc*	1997, Japan, kale	1	1/NT
J41	*Xcc*	1997, Japan, cabbage	4	3/NT
PS132	*Pseudomonas syringae* pv. *maculicola*	1995, UK, cauliflower		
PS32	pv. *syringae*	1992, Russia, soil		
R11	*Ralstonia solanacearum*	1999, Russia, potato		
PA3	*Pantoae aglomerance*	1998, Russia, oats		
ER1	*Erwinia carotovora*	1994, Russia, cabbage		
HRI5231	*Erwinia chrisantemi*	–, UK		
PCH7	*Pseudomonas cichori*	1994, Russia, lettuce		

[a] Serotype/haplotype were evaluated according to Alvarez et al. (1994).
[b] DNA–DNA hybridisation groups according to Vauterin et al. (1995).
[c] Serotype similar to *X. campestris* pv. *armoraciae*.
Races of *Xcc* are given according to Kamoun et al. (1994).

('Promega', USA) in accordance with the recommendations of the manufacturer (with minor modification). Electrophoresis was run on 'Macrophore' ('Pharmacia', Sweden) and SQ3 Sequencer ('Hoefer', USA) in 0.19 mm-thick polyacrylamide gels. The universal plasmid primers, SP6 and T7, were used for sequencing in both directions.

SCAR-PCR analysis. For the *Xcc*-specific region amplification the following PCR mixtures (25 µl) were used: 1× PCR buffer with $(NH_4)SO_4$ ('Fer-

mentas Co.', Lietuva) with 1.5 mM $MgCl_2$, 7.5 nM dNTP, 50 ng of DNA, 10 pM of each primer and 1.25 units of BioTaq DNA Polymerase ('Dialat Ltd', Russia). DNA amplification was performed in the 'Cetus 480' thermocycler using the following reaction profile: initial denaturation – 94 °C, 3 min, following 26 cycles of denaturation at 94 °C, 30 s, annealing at 68 °C 30 s and elongation at 72 °C, 1 min. PCR products were detected by electrophoresis in 1% agarose gel and documented in 'BioDoc Analyze System'.

Results

Rep-PCR fingerprinting. To ensure reliable PCR results, the primers were tested on three different PCR machines with isolates of the *Xcc* type strain NCPPB528^T. Similar banding patterns for those isolates were obtained with both primer sets in three independent PCR reactions (data not shown) on every PCR machine. Rep-PCR analysis of 51 *Xanthomonas* strains with two chosen primers sets revealed 60 polymorphic bands in total (Figure 1). Most bands generated with *Xcc* strains were unique compared to those of other *Xanthomonas* sp.. The majority of the *Xcc* strains were identified as members of three distantly related subgroups. Genetic distance values (*D*) between the subgroups reached 0.9, demonstrating almost the same range as those revealed for different species of *Xanthomonas* (Figures 2 and 3). The dendrograms derived from the calculated distance matrix demonstrated that the bacteria isolated from one field, as UK11–UK13 and UK4–UK7, normally exhibited no significant divergence (Figures 2 and 3). The groups could be visualised quantitatively on the basis of a few major bands with molecular weight ranging from 1600 to 150 bp (Figure 1).

The 46 strains of *Xcc* including strains of *X. campestris* pv. *raphani* and *X. campestris* pv. *armoraciae* were clustered into three large subgroups by both UPGMA and NJ (Figures 2 and 3). The first subgroup includes strains of worldwide origin and belonged to 1st serotype and 1st haplotype according to Alvarez et al. (1994), and consisted of cluster A (isolates of European and Japanese origin), and cluster B (US and European strains) (Figure 2). All Japanese isolates of subgroup I were obtained from plants grown from black rot contaminated seeds imported to Japan.

The subgroup 2 was formed by isolates from UK, Russia, Germany, Hungary and Canada, and belonged to serogroup 3 (Alwarez et al., 1994), including the type strain *Xcc* NCPPB528^T. The type strains of other *Xanthomonas* species were clearly separated from *Xcc*. Subgroup 3 was represented by isolates of Japanese origin from the

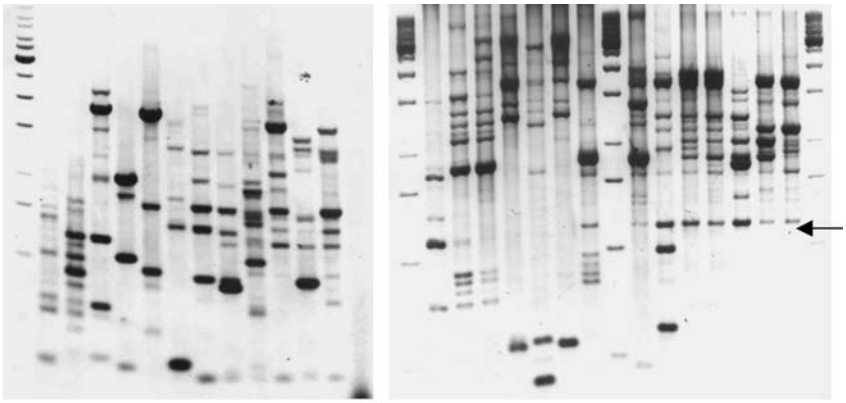

Figure 1. Rep-PCR fingerprint obtained in PCR with primers KRP2 + KRP8 (a) and KRPN2 (b) for *Xanthomonas* species and *Xcc* reference strains. 1, *Erwinia carotovora*, ER1; 2, *X. axonopodis* pv. *malvacearum* NCPPB633^T; 3, *X. oryzae* pv. *oryzicola* NCPPB3002^T; 4, *X. oryzae* pv. *oryzicila* NCPPB1585^T; 5, *X. vesicatoria* pv. *vesicatoria* NCPPB422^T; 6, *X. axonopodis* pv. *phaseoli* HRI924a; 7, *X. campestris* pv. *raphani* NCPPB1946^T; 8, *X. campestris* pv. *armoraciae* NCPPB347^T; 9, *X. campestris* pv. *armoraciae* Xa5; 10 and 13 *Xcc* NCPPB528^T; 11, *Xcc* PHW117; 12, *Xcc* HRI1279a 14, *Xcc* HRI1279b. Arrow indicates the KRPN2-600 marker characteristic for *X. campestris* strains.

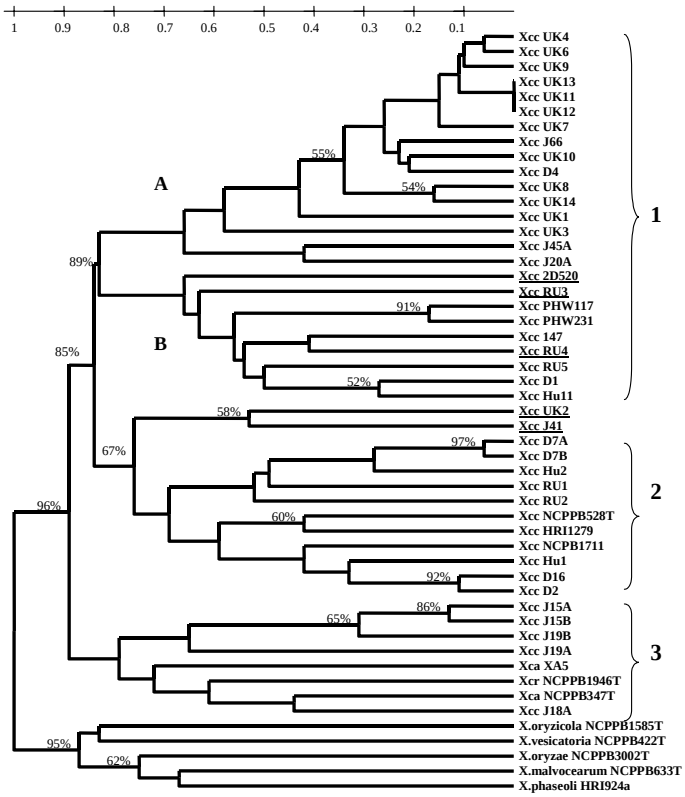

Figure 2. Dendrogram of genetic relatedness of *X. campestris* strains based on rep-PCR. The tree was constructed by the UPGMA method from 60 polymorphic loci. The scale indicates the rate of differences. Numbers and letters correspond to the subgroups of *X. campestris* described in text. The strains with unstable UPGMA and NJ grouping are underlined. Bootstrep values were calculated for 500 replications and given in percents of row value.

highlands of Gufu and Tsumagoy Prefectures. Reference strains of *X. campestris* pv. *raphani* NCPPB1946^T, *X. campestris* pv. *armoraciae* NCPPB347^T and XA5 were also placed in this cluster.

Nevertheless, some strains isolated from brassicas (2D520, UK2, RU4, RU3 and J41) represented rare pathogenic groups and were clustered differently by NJ and UPGMA algorithms (Figures 2 and 3). Out-grouping of those strains was confirmed by further serological and virulence analysis. The strains UK2, RU4, RU3 and J41 produced leaf spots besides the black rot symptoms on cauliflower and broccoli plants, while strain 2D520 was avirulent on all tested cabbage and oilseed rape varieties (Ignatov et al., 1998, 1999a).

Development of SCAR primers for Xanthomonas campestris. The PCR fragment KRPN2-600 found in nearly all tested strains of *X. campestris* (Figure 1b) was cloned and sequenced. The sequence was deposited to GeneBank under accession number AY221954. The fragment length was equal to 586 bp. BLAST search through the GeneBank accessions revealed very high similarity (>99%) of KRPN2-600 sequence to part of clone AE012093.1 (*Xcc* ATCC 33913, complete genome) (Figure 4); clone Z95386.1 (*Xcc exbD1*, *exbD2*, *exbB* and *tonB* genes) and AF527951.1 (*Xcc orf268*, *orf398*, *tonB*, *exbB*, *exbD1*, and *exbD2*); and high similarity (>89%) with GeneBank accessions AY055110.1 (*X. oryzae* pv. *oryzae* KACC10331 BAC 4K15), AE011623.1 (*X. axonopodis* pv. *citri* 306, complete genome). The 5′ end fragment KRPN2-600 was homologous to putative protein gene *XC007* sequence position 800. The 3′ end of PCR fragment KRPN2-600 was identical to *tonB* gene sequence, position 27 (AE012093.1).

Specific primers were designed from most polymorphic region between the compared sequences:

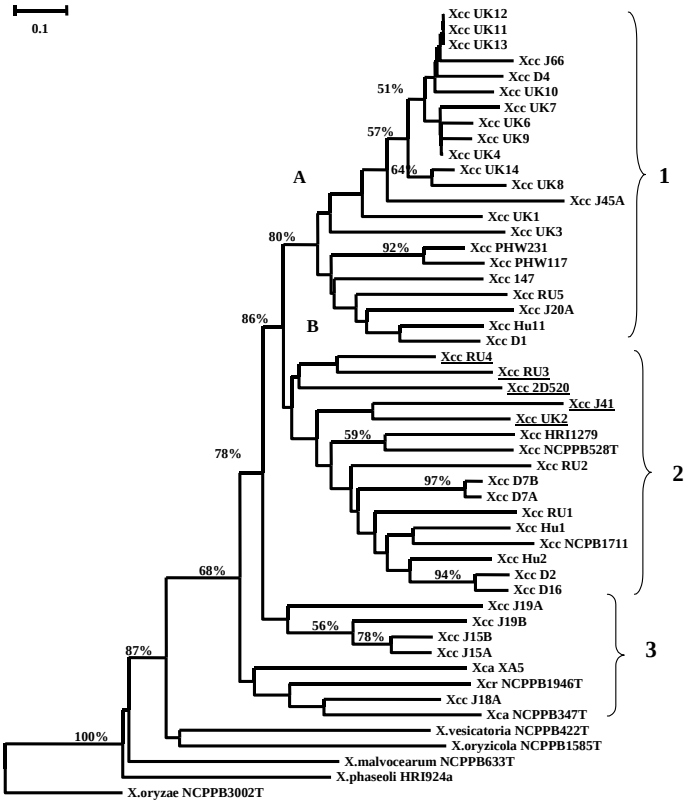

Figure 3. Dendrogram of genetic relatedness of *Xanthomonas* strains based on rep-PCR. The tree was constructed by the neighbor-joining method from 60 polymorphic loci. The scale indicates the Genetic Difference equal 0.1. Numbers correspond to subgroups of *X. campestris* described in text. The strains with unstable UPGMA and NJ grouping are underlined. Bootstrep values were calculated for 500 replications and given in percents of row value.

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KRPN2-600       cgccgg ggggatggac aagctgcgcg cctccggtca gctgaccgaa gagaaggaat
ae012093.1  aggcggccgg ggtgatggac aagctgcgcg cctccggtca gctgaccgaa gagaaggaat

KRPN2-600   acaagcagct gtactcgatc tacgc.aaca ccgagaacaa ggaaaaggac gtcatcgccg
ae012093.1  acaagcagct gtactcgatc tacgccaaca ccgagaacaa ggaaaaggac gtcatcgccg

KRPN2-600   tcatcaccga gggcatgcag aagggcattc tcaagcctga ctaccagacc tacctcgcgt
ae012093.1  tcatcaccga gggcatgcag aagggcattc tcaagcctga ctaccagacc tacctcgcgt

KRPN2-600   tggcgcagtc gtactactac agcgaagacg tgccgaag.c gatcgagaat tggcagaagg
ae012093.1  tggcgcagtc gtactactac agcgaagacg tgccgaaggc gatcgagaat tggcagaagg

KRPN2-600   ccgccccccct ctccaaggac ggtgagacct atctgaatct ggccaaggtg ctgcattcgg
ae012093.1  ccgccccccct ctccaaggac ggtgagacct atctgaatct ggccaaggtg ctgcattcgg

KRPN2-600   aaggacgcat ccccgaggcc aagcaggccg cacagcaggc gatcgccaag ggcgttaaaa
ae012093.1  aaggacgcat ccccgaggcc aagcaggccg cacagcaggc gatcgccaag ggtgttaaaa

KRPN2-600   aacccgaaga cgccaagaag atcatcaatc tgaagtaaaa aaaggaaata aacccccgaa
ae012093.1  aacccgaaga cgccaagaag atcatcaatc tgaagtaaaa aaagaaaata aacccccgaa

KRPN2-600   atccctgtgt ttttagtggt tacaggactc gggattggta taagcttggg agttc.tgcg
ae012093.1  atccctgtgt ttttagtggt tacaggactc gggattggta taagcttggg agttcctgcg

KRPN2-600   gtgtcaaagc cgtccgaaaa cctggggatc atcacagtga tccgggccc ccactgaaag
ae012093.1  gtgtcaaagc cgtccgaaaa cctggggatc atcacagtga tccgggccc ccactgaaag

KRPN2-600   agccattggc gcatgacgga acaactcgtt atccccccggc g
ae012093.1  agccattggc gcatgacgga acaactcgtt atccacaggc atgactatga tgccgggaac

ae012093.1  cagggtctga gctgggcccg cattatcggc attgctttcg taattgccct gcaccttactgc
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Figure 4. Sequence alignment of PCR fragment KRPN2-600 (GeneBank accession AY221954) and homologue fragment of GeneBank accession AE012093.1 (*Xanthomonas campestris* pv. *campestris* ATCC 33913, complete genome). SCAR primers for KRPN2-600 are underlined.

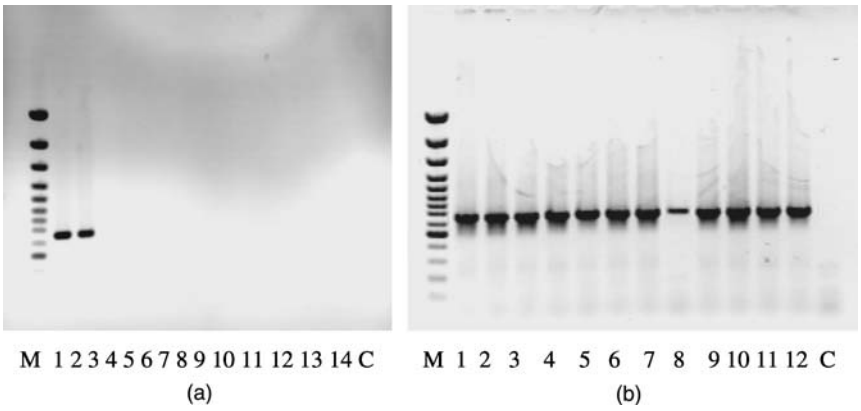

Figure 5. PCR amplification with SCAR primers for *Xcc*. (a) 1, *Xcc* NCPPB528^{T}; 2, *Xcc* HRI1279a; 3, *X. axonopodis* pv. *malvacearum* NCPPB633$^{T;}$; 4, *X.oryza* pv. *oryzae* NCPPB3002$^{T;}$; 5, *X.vesicatoria* pv. *vesicatora* NCPPB422^{T}; 6, *X. axonopodis* pv. *phaseoli* var. *fuscans* HRI924a; 7, *Erwinia chrisantemi*. HRI5231; 8, *Erwinia carotovora ER1*; 9, *Ralstonia solanacearum R11*; 10, *Ps. cichori PCH7*; 11, *Pseudomonas syringae* pv. *syringae* PS32; 12, *Pantoea aglomerance PA3*; 13, *Pseudomonas syringae* pv. *maculicola* PS132; (b) *Xcc* strains: 1, H11, 2, D16; 3, D2; 4, RU1; 5, RU2; 6, RU3; 7, RU4; 8, J45a; 9, J15a; 10, J19b; 11, J66;, 12, J41a. C, control without template DNA; M, DNA 100bp® ladder ('Fermentas', Lituenia).

804F (5′-GGCCGGGGTAATGGACAAGC-3′) and 87R (5′-GCAGTAAGGTGCA-GGGCAAT-TACG-3′). The reverse primer was designed for *tonB* gene fragment flanking KRPN-600 from 3′ side according to the published sequences. PCR with the primers resulted in a single specific 639 bp band in the strains of *X. campestris*. This band did not appear in PCR in other *Xanthomonas* species (data not shown), or on strains of other common plant-associated bacteria including *Pseudomonas* sp., *Ralstonia solanacearum*, *Erwinia carotovora*, and *Pantoea aglomerance* strains (Figure 5). It should be noted that under less stringent PCR conditions (Ta < 60 °C) this band appeared in strains of other *Xanthomonas* species.

Discussion

The black rot pathogen belongs to the genus *Xanthomonas* that causes diseases on many agricultural and wild plant species. This genus comprises 20 DNA homology groups, which were considered as genomic species. Sixteen of them consist of former *X. campestris* pathovars (Vauterin et al., 1995). Although the relationship of *Xcc* to other members of the genus *Xanthomonas* (Vauterin et al., 1991, 1995; Rademaker et al., 1999) and its intraspecies DNA variation has been examined (Alvarez et al., 1994), no extensive analysis with of strains belonging to *Xcc* from remote geographic areas has been performed previously.

The conservation of epitopes and DNA fragments within different strains of *Xanthomonas* obscured the apparent distinction between some pathovars that were based on pathogenicity testing (Alvarez et al., 1994). Three pathovars of the same genomic species *X. campestris*: pv. *campestris*, pv. *armoraciae* and pv. *raphani* infect the same cultivated *Brassica* plants, but symptoms are different. It is known that the symptoms are dependent on environment conditions, particular host plants, or specific genes (Chen et al., 1994; Ignatov et al., 1999b; N.W. Schaad, USDA, USA, pers. comm.).

Genetic characterisation of the pathogen would be valuable for tracing the source of the pathogen and to study its bacteria distribution. Biochemical and molecular techniques have been applied to examine variability within bacteria (Franken et al., 1992; Ignatov, 1992; Alvarez et al., 1994). REP, BOX, and ERIC primers were used to generate genomic fingerprints that differentiate *Xanthomonas* pathovars and species (Louws et al., 1994; Pooler et al., 1996). A high correlation between different PCR methods and DNA–DNA hybridisation was observed, suggesting that genomic fingerprinting techniques reveal taxonomic relationships between organisms (Rademaker et al., 1999). However, certain genomic regions may have less or no copies of particular repeated element than others and cannot be characterised

by this method. For instance, the DNA fragments similar to the BOX, RIC and REP primers had strongly biased localisation across the genome of *Xcc* strain ATCC 33913 (GeneBank accession NC_003902.1) and *X. axonopodis* pv. *citri* strain 306 (GeneBank accession NC_003919.1) with 3–6 peaks over the genomes regions about 20,000 bp in length (Ignatov et al., in press). It can be speculated, that the intergenic repetitive sequences with particular regulatory function (Sharples and Lloyd, 1990; Martin et al., 1992) are abundant in some certain genomic regions and rare in others.

Recently, a novel class of repetitive elements in protein-encoding genes, so-called 'ancient diverged periodical sequences', was discovered *in silica* across the sequenced genes and genomes (Korotkov et al., 1999). A high degree of conservation and abundance in bacterial genomes provided utility of those elements as primers for rep-PCR analysis. The DNA repeats homologous to primers KRP2 and KRPN2 were distributed monotonously. In contrast, the KRP8-like repeats were over-represented in first half of the complete xanthomonads' genomes (Ignatov et al., in press).

Unlike others, these primers were developed on the basis of ancient diverged repetitive regions and generate polymorphic banding profiles, specific for species and strains. Being sequenced, the novel rep-PCR bands clearly reveal their intergenic origin (B.B. Kuznetsov, unpubl.). Amplification of highly variable intergenic regions is a major advantage of the reported primers comparing to previously employed REP, ERIC and BOX primers based on intergenic repetitive sequences.

The majority of the studied *Xcc* isolates belonging to subgroups I and II agreed with serotypes identified by antigenic properties of exopolysaccharide (Franken et al., 1992; Alvarez et al. 1994; Ignatov et al., 1998), and with haplotypes previously recognised by RFLP (Alvarez et al., 1994). The Japanese isolates, which were not associated with imported contaminated seeds, were grouped together with reference strains *X. campestris* pv. *armoraciae* and *X. campestris* pv. *raphani*. Alvarez et al. (1994) have separated *X. campestris* pv. *armoraciae* from serotypes and haplotypes of *Xcc*, but Nachtigall (1998) has recently reported a positive reaction of several German *Xcc* strains with the monoclonal antibodies specific for *X. campestris* pv. *armoraciae*. Several strains, including 2D520, the only representative of race 2 (Kamoun et al., 1992), could not be placed into any of group derived by both UPGMA and NJ analysis.

Thus, the new rep-PCR primers proved to give results consistent with previous information about polymorphisms within *Xcc* population, and may aid for ecological and epidemiological studies. Despite the short length of the primers and low annealing temperature, amplification patterns were repeatable between three or four independent replications of rep-PCR analysis.

The results support the previous conclusions about a high degree of diversity in the population of *Xcc* strains (Alvarez et al., 1994; Ignatov et al., 1998). It may be hypothesised that the groups of *X. campestris* evolved from the same ancestral population towards better adaptation to a particular climatic regime (Ignatov et al., 1998) or other selective factors. Comparison of 16s–23s rRNA ITS regions made for representative strains of the groups 1, 2 and 3 (A.N. Ignatov, unpubl.) favours the last hypothesis.

Several specific genome regions, including *hrp* (Leite et al., 1994) and 16s–23s rRNA ITS region (Barry et al., 1991), have been used as targets for diagnostics of *Xanthomonas* species. However, some species defined by DNA–DNA similarities could not be characterised by primary sequences of these regions (Moore et al., 1997). Finding the high homogeneity of the *XCC007-tonB* region within *X. campestris* and its variability on interspecies level for other *Xanthomonas* species facilitates the design of an effective diagnostic PCR primers for those phytopathogenic bacteria.

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