

ASSESSMENT OF GENETIC DIVERSITY AMONG DIFFERENT INDIGENOUS *XANTHOMONAS* ISOLATES VIA RAPD AND ISSR.

SABIN FATIMA¹, RUKHSANA BAJWA¹, TEHMINA ANJUM¹ and ZAFAR SALEEM²

¹*Institute of Plant Pathology, Quaid-e-Azam Campus, University of the Punjab, Lahore, Pakistan*

²*Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan*

Abstract - The genetic diversity among seven *Xanthomonas* isolates representing four species was assessed using RAPD and ISSR PCR-based techniques. Both techniques revealed high degrees of polymorphisms among the studied isolates. A cluster dendrogram based on the combined data of RAPD and ISSR showed that genetic diversity exists in local isolates of *Xanthomonas*. In terms of percentage similarity values, the genomic variation was found to be in the range of 29.29% - 100% among the isolates. *X. campestris* (*Mangifera indica*) remained unclustered in cluster dendrogram and revealed a unique genomic profile compared to other isolates used in this study.

Key words: *Xanthomonas*, Genetic diversity, RAPD, ISSR.

INTRODUCTION

The genus *Xanthomonas* in the gamma subdivision of Proteobacteria consists of 27 plant-associated species, many of which cause important diseases of crops and ornamentals (Abdo-Hasan et al., 2008). Cells have the appearance of straight rods, usually 0.4-0.7 wide x 0.7-1.8 um long. They are gram negative, aerobic and motile by a single polar flagellum (Bradbury, 1984). Collectively, members of the genus cause disease on at least 124 monocot species and 268 dicot species, including fruit and nut trees, solanaceous and brassicaceous plants, and cereals (Hayward, 1993). Variability within species has been determined by means of genetic techniques such as random amplified polymorphic DNA (RAPD) (Pooler et al., 1996), restriction fragment length polymorphism (RFLP) (Roberts et al., 1998) and repetitive-sequence PCR (rep-PCR) (Gillings et al., 1998). In recent years the RAPD-PCR approach has been shown to be useful in classifying a number of microbial strains and species (Williams et al., 1990)

including various *Xanthomonas* spp. such as *X. albilineas* (Permaul et al., 1996), *X. fragariae* (Pooler et al., 1996), *X. maltophilia* (Yao et al., 1995) and *X. campestris* pv. *pelargonii* (Manulis et al., 1994). ISSRs are semi-arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Such amplification does not require genome sequence information and leads to multi-locus and highly polymorphous patterns (Nagaoka et al., 1997). Each band corresponds to a DNA sequence delimited by two inverted microsatellites. Like RAPDs, ISSR markers are quick and easy to handle, but they seem to have the reproducibility of SSR markers because of the longer length of their primers. Therefore, analysis of genomic DNA using RAPD and ISSR is a suitable typing method since both techniques have proved to be fast, sensitive, and reliable for determining genetic relationships among *Xanthomonas* isolates (Abdo-Hasan et al., 2008).

Results are discussed in the light of previous findings regarding their genetic diversity, generally

attributed to geographical distribution and/or pathogenicity of various *Xanthomonas* species/pathovars. The present research sheds light on the characterization of some indigenous *Xanthomonas* species and inter/intraspecific variations among them.

MATERIALS AND METHODS

Procurement of bacterial cultures

Seven isolates of *Xanthomonas* representing four different species including *X. campestris*, *X. maltophilia*, *X. axonopodis* and *X. nematophilus*, were procured from the First Fungal Culture Bank of Pakistan (FCBP), Institute of Plant Pathology, University of the Punjab, Lahore. A list of these isolates along with their substrate sources is given in Table 1.

Isolation of genomic DNA

DNA was extracted from bacterial cells following the method described by Herrick et al. (1996) with slight modifications. A single colony from a fresh LBA (Luria-Bertani agar) plate was used to inoculate 10 ml LB broth that was grown to saturation. An aliquot of 1.5 ml of the culture was centrifuged at maximum speed (14000 rpm for 5 min), the supernatant was discarded and pellets were re-suspended by vigorous vortexing in 500 µl TE buffer to which 60 µl 10% SDS and 60 µl proteinase K (20 mg/ml) were added. After 1 h of incubation at 37°C, DNA was extracted twice with phenol/chloroform (1:1) and twice with chloroform. Nucleic acids were precipitated using absolute ethanol and sodium chloride. The resulting pellet was resuspended in TE buffer containing RNase A and incubated at 37°C for 30 min. DNA was finally precipitated with 5M ammonium acetate and isopropanol. After centrifugation, the pellet was washed twice with 70% ethanol, air dried and dissolved in TE buffer.

Randomly Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSRs) analyses

Seven selected isolates of *Xanthomonas* were tested against twenty-eight primers from Gene Link™ and

two primers from Operon Technologies (USA) for RAPD analysis (Table 2) according to the method described by Williams et al. (1990). Three ISSR primers (Table 3) were used for the analysis according to Bornet and Branchard (2001).

All PCR reactions were performed in a total volume of 50 µl containing 10 X PCR buffer + 25 mM Mg, 1 mM dNTPs, 10p mole primer, 2.5 units of *Taq* polymerase (Eppendorf, USA) and 1 µg genomic DNA. Using a Master cycler gradient PCR (TECHNE TC-412), these reactions were subjected to initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 33°C for 1.3 min, and primer extension at 72°C for 1.3 min. Final extension was set at 72°C for 7 min. The reaction was terminated at 4°C in 2-3 h. Amplification products were stored at 4°C until use. Amplified products were run on 2% agarose gel and stained in ethidium bromide. Electrophoresis was carried out at 100 V for 45 min at room temperature. A 100 bp ladder (Fermentas, USA) was used to estimate the approximate molecular weight of amplification products. The bands were visualized through UV transilluminator (WiseDoc MUV-M20) and photographed on the Gel Documentation System.

RESULTS

Genomic DNA extraction of Xanthomonas isolates

Seven isolates of *Xanthomonas* representing four species were subjected to DNA extraction and the samples were run on 0.8 % agarose gel. Results of all these isolates are shown in Plate 1.

RAPD analysis

Primer screening for RAPD analysis

Thirty different available decamers were screened out as described in Table 2. After examining their nanomole values (nm), these were diluted up to 10 pMole concentrations. RAPD results were achieved only with two primers from the Operon series that were specifically designed for *Xanthomonas*, i.e.

OPR-02 and OPR-20. Different melting temperature conditions were checked across the set of thirty decamers used to screen the genome of different *Xanthomonas* species/isolates, i.e. 20°C, 25°C, 28°C, 30°C, 33°C, 34°C, 35°C and 38°C; the melting temperature was optimized at 33°C for the decamers used in the study.

*Analysis of amplified DNA fragments
with different decamers*

Seven isolates of *Xanthomonas* species were subjected to RAPD analysis along with a negative control and DNA marker (100 bp) by using two primers from the Operon series. In negative control, sterile distilled water was replaced by the template DNA so that no amplification was observed, indicating the validity of RAPD assay. The dendrograms (gene trees) were constructed from the amplification patterns of each primer by using the software MINITAB.

*Amplification and dendrogram observed
by decamer OPR-02*

Genetic variation among the different isolates of *Xanthomonas* species was assessed through RAPD analysis by using decamer OPR-02 (Plate 2). The result showed that all the isolates exhibited a common band at 450 bp. *X. maltophilia* (V) and *X. nematophilus* (VII) shared two common bands at 200 bp and 1000 bp. Isolates of *X. axonopodis* (I & II) showed two similar bands at 300 bp and 450 bp, whereas the polymorphic band produced by *X. axonopodis* (I) was at 950 bp and by *X. axonopodis* (II) at 800 bp. *X. campestris* (III) exhibited four bands at 200 bp, 250 bp, 450 bp and 550 bp, whereas five bands were produced by *X. maltophilia* (IV) at 200 bp, 300 bp, 450 bp, 550 bp and 800 bp. However, two bands were shared by both isolates at 200 bp and 550 bp.

The dendrogram constructed based on amplification with primer OPR-02 was comprised of two main clusters (Fig 1). Strains falling into cluster 1 include *X. axonopodis* (II), *X. maltophilia* (IV) and *X. nematophilus* (VI). A genomic homology of 59.18% was observed among *X. axonopodis* (II) and *X. nematophilus* (VI), whereas *X. maltophilia* (IV) showed 42.26% similarity with other members representing cluster 1. Cluster 2 was composed of *X. maltophilia* (V) and *X. nematophilus* (VII) strains representing 100% genetic similarity. *X. axonopodis* (I) and *X. campestris* (III) showed unique behavior in terms of genetic profile variation, indicating 29.29% genetic similarity with other isolates.

Amplification and dendrogram observed by OPR-20

The amplification results obtained from primer OPR-20 showed that two bands were shared by *X. axonopodis* (II) and *X. maltophilia* (III) strains at 550 bp and 1000 bp (Plate 5). *X. axonopodis* (I) exhibited two bands of 350 bp and 450 bp, respectively. *X. campestris* (III) represented a single band at 450 bp, whereas the rest of the isolates displayed no amplification and revealed that they are more genomically diverse than the other isolates.

Amplification and dendrogram observed by OPR-20

The dendrogram constructed on the amplifications observed by decamer OPR-20 showed two main clusters (Fig. 2). The first cluster displayed 50% similarities between *X. axonopodis* (I) and *X. campestris* (III), whereas the second cluster indicated maximum similarity (100%) among *X. axonopodis* (II) and *X. maltophilia* (IV) isolates. Both clusters showed 13.40% similarity with each other.

ISSR analysis

*Amplification and dendrogram observed by ISSR
primer A31*

Isolates of *Xanthomonas* species subjected to ISSR analysis showed thirty-two polymorphic bands by using primer A-31 (Plate 4). Six similar bands were observed in the case of *X. maltophilia* (V) and *X. nematophilus* (VII) at 250 bp, 350 bp, 450 bp, 600 bp, 700 bp and 1000 bp, showing homogeneity in their genetic profile.

Isolates of *X. axonopodis* exhibited intraspecific variation because one of the isolates *X. axonopodis* (I) showed amplification (distinct bands) at 250 bp

Table 1. List of isolates of *Xanthomonas* species used for molecular characterization.

FCBP Accession #	Strain	Substrate
1	<i>Xanthomonas axonopodis</i> (I)	Citrus fruit
186	<i>Xanthomonas axonopodis</i> (II)	Rhizospheric soil
8	<i>Xanthomonas campestris</i> (III)	<i>Mangifera indica</i>
118	<i>Xanthomonas maltophilia</i> (IV)	<i>Brassica campestris</i>
132	<i>Xanthomonas maltophilia</i> (V)	<i>Pisum sativum</i>
98	<i>Xanthomonas nematophilus</i> (VI)	Root nodules
115	<i>Xanthomonas nematophilus</i> (VII)	<i>Brassica campestris</i>

and 600 bp, whereas the other isolate, *X. axonopodis* (II), revealed bands at 200 bp, 500 bp and 900 bp. This result showed a marked discrimination in genetic profiles of pathogenic and nonpathogenic isolates. Four bands were observed in the case of *X. campestris* (III) at 250 bp, 350 bp, 450 bp and 600 bp. *X. maltophilia* (IV) displayed only two bands at 200 bp and 500 bp, whereas the amplification of 200 bp, 400 bp, 500 bp, 700 bp and 900 bp was observed in case of *X. nematophilus* (VI).

The dendrogram was comprised of two main clusters exhibiting 25.46% similarity with each other (Fig 3). Cluster 1 is further divided into two subclusters. *X. axonopodis* (II) and *X. nematophilus* (VI) fell under subcluster 1, showing 52.86% genetic homology with each other, whereas *X. maltophilia* (IV) was seen in the second subcluster showing 40% similarity with other members of cluster 1. *X. axonopodis* (I), *X. campestris* (III), *X. maltophilia* (V) and *X. nematophilus* (VII) formed cluster 2. Maximum genetic similarity (100%) was quite evident among the isolates of *X. maltophilia* (V) and *X. nematophilus* (VII). *X. axonopodis* (I) and *X. campestris* (III) exhibited 52.86% homology with each other. The least genomic similarity of 25.46% was found among two isolates of *X. axonopodis* from different substrates. i.e. citrus fruit and rhizospheric soil.

Amplification and dendrogram observed by ISSR primer D-3

The results obtained from primer D-3 showed that genetic variation exists among different isolates of

Xanthomonas (Plate 5). Similar amplifications at 300 bp, 500 bp, and 700 bp were observed among *X. maltophilia* (V) and *X. nematophilus* (VII). *X. axonopodis* (I) represented amplification with four bands at 300 bp, 350 bp, 500 bp and 700 bp, whereas three bands at 350 bp, 450 bp and 1000 bp were observed in the case of *X. axonopodis* (II). This showed that isolates of *X. axonopodis* from different substrates were genetically diverse from each other. *X. campestris* (III) exhibited amplifications at 300 bp, 350 bp, 450 bp and 600 bp. *X. maltophilia* (IV) revealed three bands at 350 bp, 450 bp and 500 bp; *X. nematophilus* (VI) showed two bands at 350 bp and 400 bp. Amplification patterns obtained through primer D-3 exhibited genetic diversity.

The dendrogram (Fig. 4) based on ISSR analysis using primer D-3 exhibited two main clusters showing 29.29% similarity with each other. *X. campestris* (III) and *X. nematophilus* (VI) exhibited unique genetic profiles. Cluster 1 consisted of two strains, i.e. *X. axonopodis* (II) and *X. maltophilia* (IV), indicating 42.26% genetic similarity among them. The second cluster was comprised of three strains of which *X. maltophilia* (V) and *X. nematophilus* (VII) showed maximum genomic homology (100%) with each other, whereas *X. axonopodis* (I) showed 59.18% similarity with both of them.

Amplification observed by ISSR primer A-16

The results obtained from ISSR primer A-16 showed amplification with only two isolates: *X. axonopodis* (I) and *X. maltophilia* (V). The strains exhibited no am-

Table 2: List of random primers and their sequences used for RAPD analysis.

S #	Primer	Sequence	Tm = 2(AT)+(GC)/2	nm
1	A-01	5'GGGTAACGCC3'	33.6	11.1
2	A-02	5'GTTGCGATCC3'	29.5	12.2
3	A-03	5'AGTCAGCCAC3'	29.5	11.2
4	A-04	5'AATCGGGCTG3'	29.5	11
5	A-05	5'AGGGGTCTTG3'	29.5	11.3
6	A-06	5'GGTCCCTGAC3'	33.6	12.4
7	A-07	5'GAAACGGGTG3'	29.5	10
8	A-08	5'GTGACGTAGG3'	29.5	10.6
9	A-11	5'CAATCGCCGT3'	29.5	11.9
10	A-12	5'TCGGCGATAG3'	29.5	11
11	A-13	5'CAGCACCCAC3'	33.6	11.8
12	A-14	5'TCTGTGCTGG3'	29.5	12.6
13	A-15	5'TCCGAACCC3'	21.5	12.4
14	A-16	5'AGCCAGCGAA3'	21.5	10.9
15	A-17	5'GACCGCTTGT3'	29.5	12.2
16	A-18	5'AGGTGACCGT3'	29.5	11
17	A-19	5'CAAACGTCCG3'	29.5	10.8
18	B-01	5'GTTTCGCTCC3'	13.7	29.5
19	B-04	5'GGACTGGAGT3'	10.6	29.5
20	B-05	5'TGCGCCCTTC3	33.6	13.9
21	B-11	5'GTAGACCCGT3'	11.4	29.5
22	B-12	5'CCTTGACGCA3'	11.9	29.5
23	B-13	5'TTCCCCCGCT3'	33.6	14.6
24	B-14	5'TCCGCTCTGG3'	33.6	13.3
25	B-16	5'TTGCCCCGGA3'	29.5	12.2
26	B-17	5'AGGGAACGAG3'	21.5	9.5
27	B-18	5'CCACAGCAGT3'	29.5	11.2
28	B-19	5'ACCCCGAAG3'	33.6	11.3
29	OPR-20	5'ACGGCAAGGA3'	22	77.7
30	OPR-02	5'CACAGCTGCC3'	26	164.8

plification at all, indicating that the primer sequence was incompatible with their genomes. *X. axonopodis* (I) represented three bands of size 350 bp, 500 bp and 700 bp, whereas *X. maltophilia* (V) displayed a single band at 700 bp (Plate 6).

Cluster dendrogram

The amplification data of all the primers (OPR-02, OPR-20, A-31 and D-3) were collectively used for the dendrogram construction as described in Fig. 5.

Table 3. List of primers and their sequences used in ISSR analysis.

S. #	Primer	Sequence	Tm = 2(AT)+4(GC)/2	nm
1	A-16	5'CACACACACACAR3'	29	55.8
2	A-31	5'AGCAGCAGCAGC3'	35	118.0
3	D-3	5'GACAGACAGACAGACA3'	43	80.7

Table 4. Genetic homology in different isolates of *Xanthomonas* species by RAPD and ISSR analysis using a cluster dendrogram.

Acc. #	Species	Substrate	Genomic similarity	Cluster
01	<i>Xanthomonas axonopodis</i> (I)	Citrus fruit	38.76%	C-2
186	<i>Xanthomonas axonopodis</i> (II)	Rhizospheric soil	50%	C-1a
008	<i>Xanthomonas campestris</i> (III)	<i>Mangifera indica</i>	29.29%	Unique isolate
118	<i>Xanthomonas maltophilia</i> (IV)	<i>Brassica campestris</i>	50%	C-1a
132	<i>Xanthomonas maltophilia</i> (V)	<i>Pisum sativum</i>	100%	C-2
98	<i>Xanthomonas nematophilus</i> (VI)	Root nodules	38.76%	C-1b
115	<i>Xanthomonas nematophilus</i> (VII)	<i>Brassica campestris</i>	100%	C-2

The cluster dendrogram comprised two main clusters representing 29.29% similarity with each other. Cluster 1 consisted of two subclusters, with *X. axonopodis* (II) and *X. maltophilia* (IV) in subcluster 1 with 50% similarity. The second subcluster comprised *X. nematophilus* (VI) and exhibited 38.76 % genomic similarity with subcluster 1. Cluster 2 displayed 100% similarity between *X. maltophilia* (V) and *X. nematophilus* (VII), whereas *X. axonopodis* (I) showed 38.76 similarity with other strains in the same cluster. *X. campestris* (III) remained unclustered in the cluster dendrogram. Genetic homology in the different isolates of *Xanthomonas* species by RAPD and ISSR analyses using cluster dendrogram is shown in Table. 4.

DISCUSSION

In this paper we describe the assessment of the genetic diversity among *Xanthomonas* isolates through Randomly Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR). In the case of RAPD analysis, amplifications in a size range of 200 bp-1000 bp were observed by using decamer OPR-20. Random primer OPR-02 only amplified four strains in the range of 350 bp-1000 bp. A high rate of amplification was observed by using ISSR primers. The amplification pattern in terms of size range when using primer A-31, was 200 bp-1000 bp. In the case of primer D-3, amplification was observed in the range of 300 bp -1000 bp.

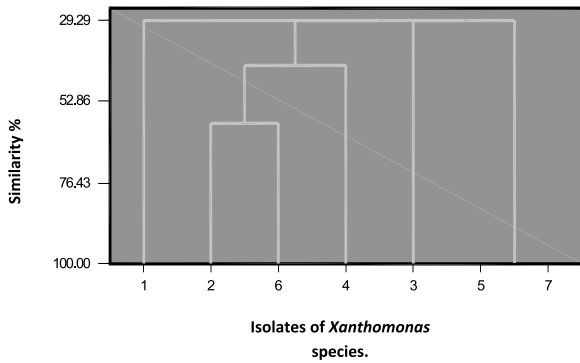


Fig. 1: Dendrogram obtained from amplification by primer OPR-02. 1: *X. axonopodis* (citrus fruit) 2: *X. axonopodis* (Rhizospheric soil) 3: *X. campestris* (*Mangifera indica*) 4: *X. maltophilia* (*Brassica campestris*) 5: *X. maltophilia* (*Pisum sativum*) 6: *X. nematophilus* (Root nodules) 7: *X. nematophilus*.

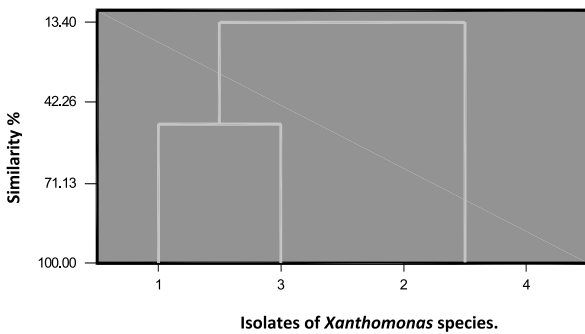


Fig. 2: Dendrogram obtained from amplification by primer OPR-20. 1: *X. axonopodis* (citrus fruit) 2: *X. axonopodis* (Rhizospheric soil) 3: *X. campestris* (*Mangifera indica*) 4: *X. maltophilia* (*Brassica campestris*).

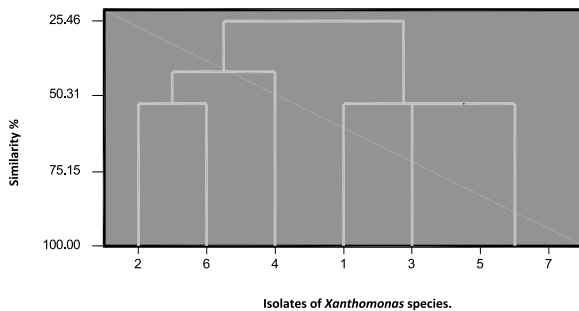


Fig. 3: Dendrogram obtained from amplification by primer A-31. 1: *X. axonopodis* (citrus fruit) 2: *X. axonopodis* (Rhizospheric soil) 3: *X. campestris* (*Mangifera indica*) 4: *X. maltophilia* (*Brassica campestris*) 5: *X. maltophilia* (*Pisum sativum*) 6: *X. nematophilus* (Root nodules), 7: *X. nematophilus* (*Brassica campestris*).

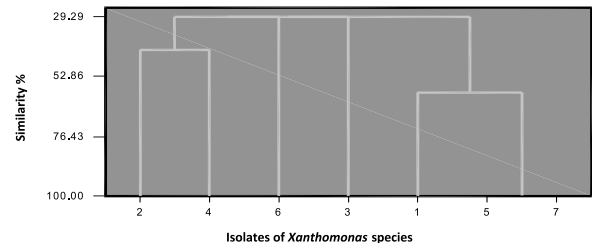


Fig. 4: Dendrogram obtained from amplification by primer D-3. 1: *X. axonopodis* (citrus fruit) 2: *X. axonopodis* (Rhizospheric soil), 3: *X. campestris* (*Mangifera indica*) 4: *X. maltophilia* (*Brassica campestris*) 5: *X. maltophilia* (*Pisum sativum*) 6: *X. nematophilus* (Root nodules) 7: *X. nematophilus* (*Brassica campestris*).

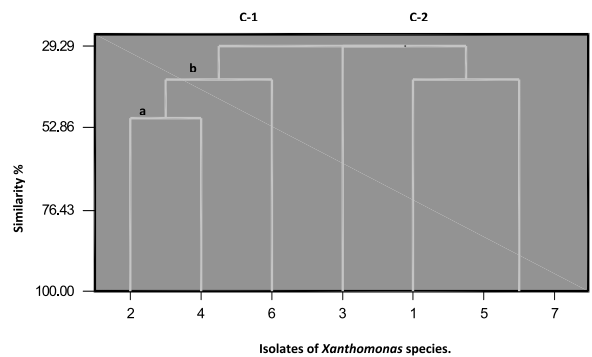


Fig. 5: Dendrogram obtained from amplification by RAPD & ISSR primers. 1: *X. axonopodis* (citrus fruit) 2: *X. axonopodis* (Rhizospheric soil), 3: *X. campestris* (*Mangifera indica*), 4: *X. maltophilia* (*Brassica campestris*), 5: *X. maltophilia* (*Pisum sativum*), 6: *X. nematophilus* (Root nodules), 7: *X. nematophilus* (*Brassica campestris*).

In this study, through combined analyses of RAPD and ISSR, it was found that genetic variability exists among seven *Xanthomonas* isolates in the range of 29.29-100% (in terms of similarity values). Maximum genomic homology was represented by two isolates, i.e. *X. maltophilia* (V) and *X. nematophilus* (VII) from different localities. This similarity might be because both the strains are pathogenic. Isolates belonging to different species may exhibit similar genomic similarity based on their pathogenic behavior. Similar to our findings, Pooler et al. (1996) used three PCR-based techniques that rely on different amplification priming strategies, i.e. RAPD, repetitive extragenic palindromic (REP), and enterobacterial repetitive intergenic consensus (ERIC) to study the genetic relationships among 25 isolates of *X. fragariae* from diverse geographic regions. The three methods gave

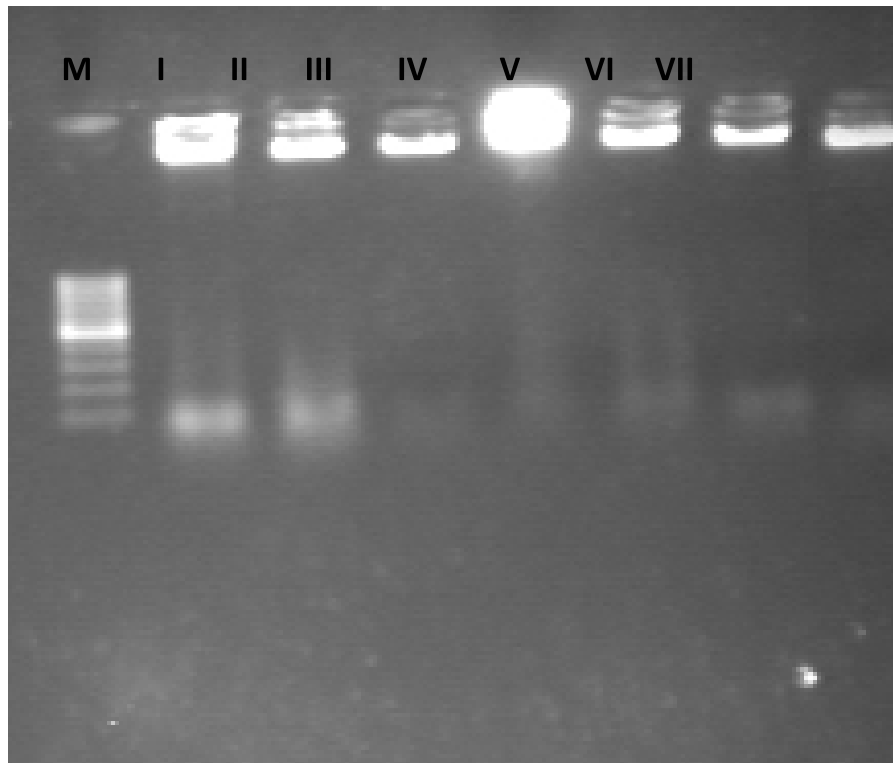


Fig. 6: Genomic DNA isolation from different isolates of *Xanthomonas* species. **M:** marker (100 bp) **I:** *X. axonopodis* (citrus fruit) **II:** *X. axonopodis* (Rhizospheric soil) **III:** *X. campestris* (*Mangifera indica*) **IV:** *X. maltophilia* (*Brassica campestris*), **V:** *X. maltophilia* (*Pisum sativum*) **VI:** *X. nematophilus* (Root nodules) **VII:** *X. nematophilus* (*Brassica campestris*).

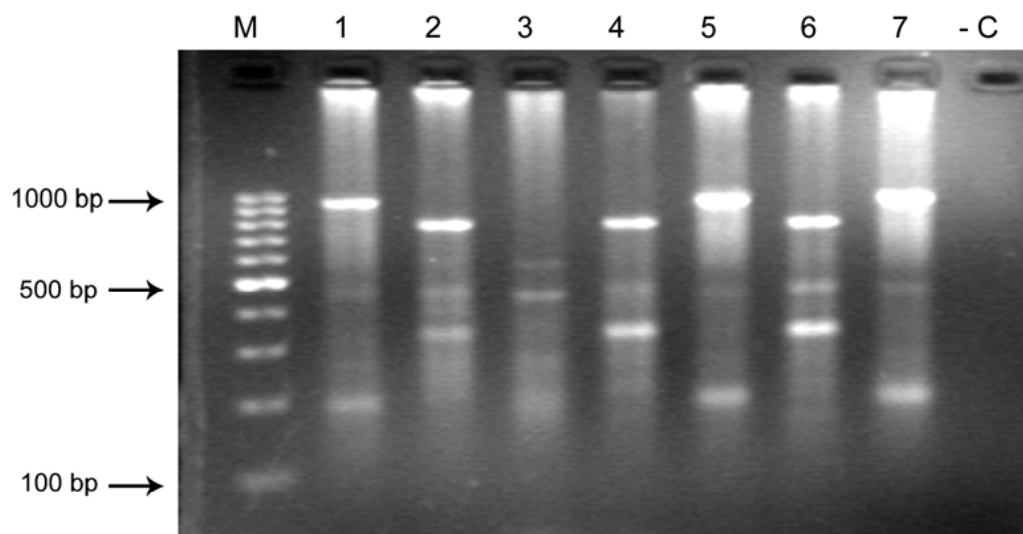


Fig. 7: RAPD analysis of different isolates of *Xanthomonas* by using primer OPR-02. **M:** DNA marker (100bp) **1:** *X. axonopodis* (citrus fruit) **2:** *X. axonopodis* (Rhizospheric soil) **3:** *X. campestris* (*Mangifera indica*) **4:** *X. maltophilia* (*Brassica campestris*) **5:** *X. maltophilia* (*Pisum sativum*) **6:** *X. nematophilus* (Root nodules) **7:** *X. nematophilus* (*Brassica campestris*) **-C:** Negative control.

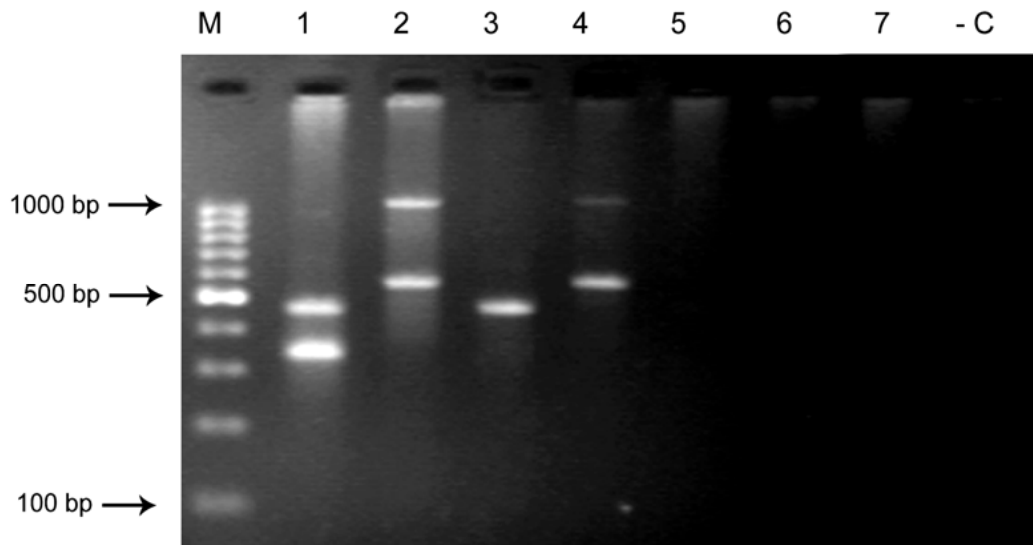


Fig. 8: RAPD analysis of different isolates of *Xanthomonas* by using primer OPR-20. **M:** DNA marker (100bp) **1:** *X. axonopodis* (citrus fruit) **2:** *X. axonopodis* (Rhizospheric soil) **3:** *X. campestris* (*Mangifera indica*) **4:** *X. maltophilia* (*Brassica campestris*) **5:** *X. maltophilia* (*Pisum sativum*) **6:** *X. nematophilus* (Root nodules) **7:** *X. nematophilus* (*Brassica campestris*) **-C:** Negative control.

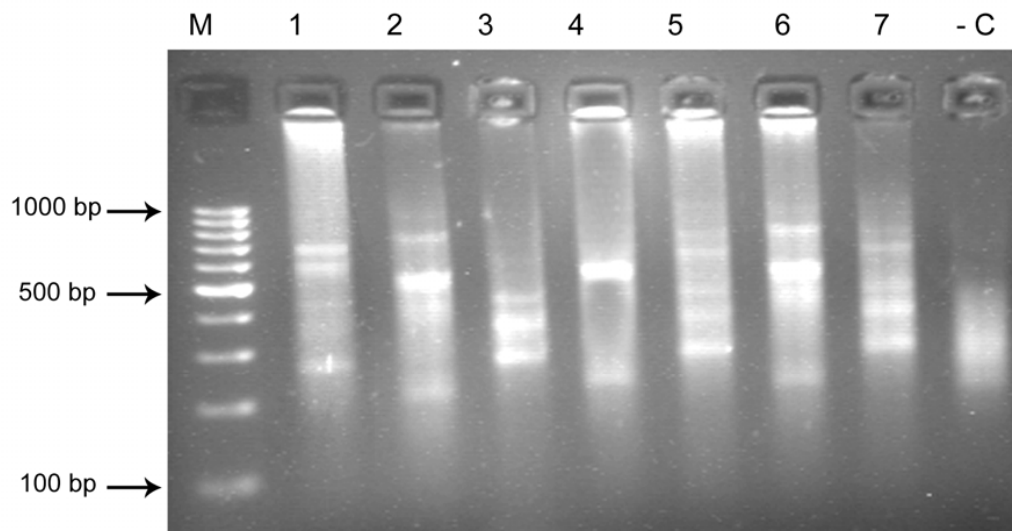


Fig. 9: ISSR analysis of different isolates of *Xanthomonas* by using primer A-31. **M:** DNA marker (100bp) **1:** *X. axonopodis* (citrus fruit) **2:** *X. axonopodis* (Rhizospheric soil) **3:** *X. campestris* (*Mangifera indica*) **4:** *X. maltophilia* (*Brassica campestris*) **5:** *X. maltophilia* (*Pisum sativum*) **6:** *X. nematophilus* (Root nodules) **7:** *X. nematophilus* (*Brassica campestris*) **-C:** Negative control.

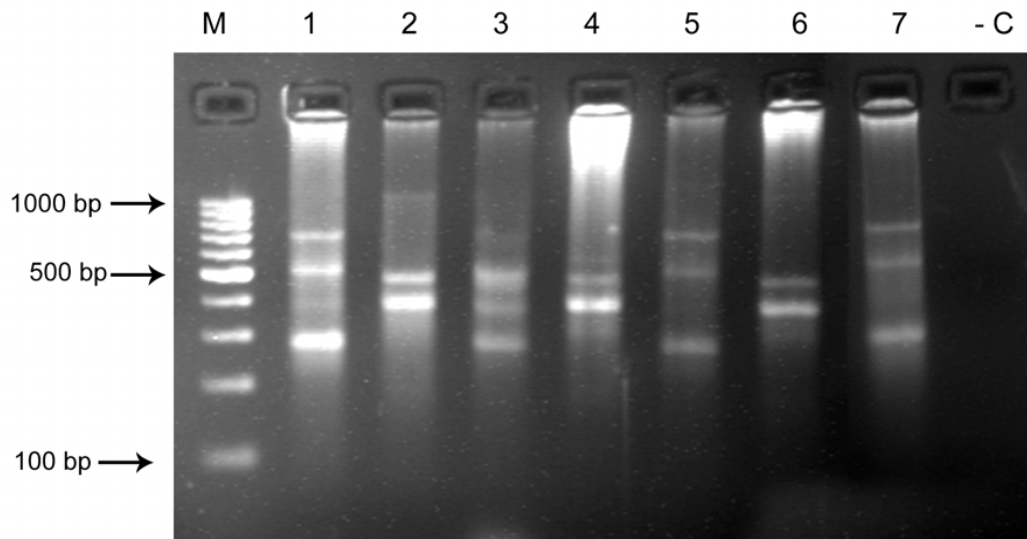


Fig. 10: SR analysis of different isolates of *Xanthomonas* by using primer D-3. **M:** DNA marker (100bp) **1:** *X. axonopodis* (citrus fruit) **2:** *X. axonopodis* (Rhizospheric soil) **3:** *X. campestris* (*Mangifera indica*) **4:** *X. maltophilia* (*Brassica campestris*) **5:** *X. maltophilia* (*Pisum sativum*) **6:** *X. nematophilus* (Root nodules) **7:** *X. nematophilus* (*Brassica campestris*) **-C:** Negative control.

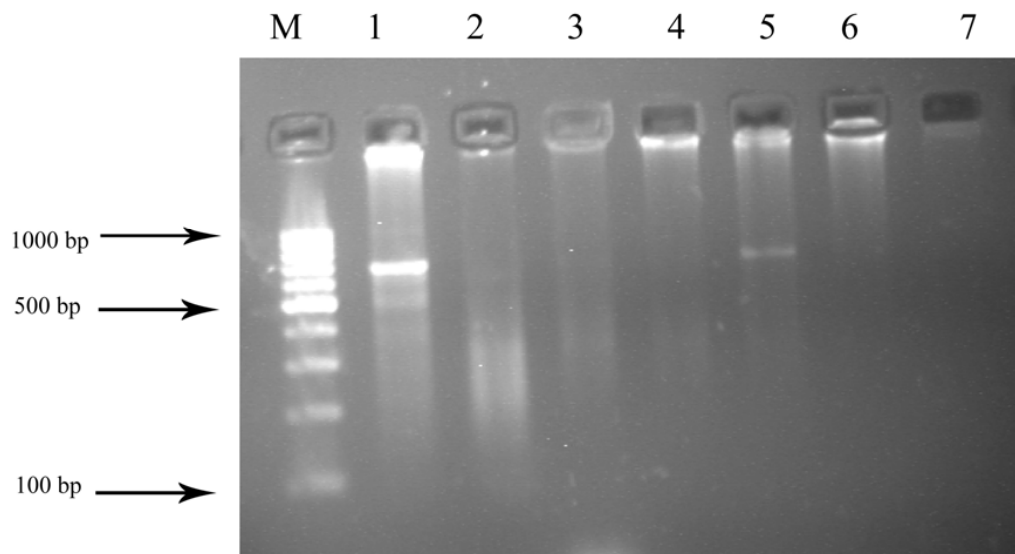


Fig. 11: ISSR analysis of different isolates of *Xanthomonas* by using primer A-16. **M:** DNA marker (100bp) **1:** *X. axonopodis* (citrus fruit) **2:** *X. axonopodis* (Rhizospheric soil) **3:** *X. campestris* (*Mangifera indica*) **4:** *X. maltophilia* (*Brassica campestris*) **5:** *X. maltophilia* (*Pisum sativum*) **6:** *X. nematophilus* (Root nodules) **7:** *X. nematophilus* (*Brassica campestris*).

consistent results, indicating that pathogenic strains are very closely related to each other.

Two isolates of *X. axonopodis* from different substrates, i.e. citrus fruit and rhizospheric soil, displayed high intraspecific variation (29.29% similarity value). In the cluster dendrogram, cluster 1 contained a nonpathogenic isolate whereas the pathogenic one was included in the second cluster. Similarly, Abdo-Hasan et al. (2008) detected genetic diversity among forty Syrian isolates of *X. axonopodis* pv. *malvacearum* (*Xam*), representing nine defined races, through RAPD and ISSR PCR-based techniques. Both techniques revealed high degrees of polymorphism among the studied races. Combined data showed that percent disagreement values (PDV) ranged between 0.13 and 0.37. The combined dendrogram based on UPGMA analysis contained two main clusters. The first cluster contained four isolates (races), three of which were the lowest in virulence, and the second cluster contained three highly virulent isolates (races). Thus, the strains used in our study showed a resemblance with Syrian isolates. It shows that isolates are mainly distinguished through pathogenicity criteria and not by geographical distribution. The variability of strains within the same species in our findings was further supported by Ogunjobi et al. (2007), who characterized the *X. axonopodis* population in Nigeria with Random Amplified Polymorphic DNA (RAPD). At a coefficient level of 90%, extensive genetic diversity was observed in the 74 strains of the bacteria studied by RAPD analysis, which made it difficult to categorize the strains. Ten clusters and five unclustered strains were identified at 75% similarity coefficient on the dendrogram. The diversities observed were not regionally influenced or agroecologically determined. Similar diversity has been described for *X. campestris* pv. *passiflorae* studied with the same molecular marker in southern Brazil (Goncalves and Rosato, 2000).

Similarly studies of Kishun and Gupta (2008) revealed that a *X. campestris* pv. *mangiferaeindicae* (*Xcmi*) population exhibited a significant level of genetic diversity as it formed 2 clusters in the phylogenetic tree. There was 7.66% polymorphism in the

individual isolates, which indicates significant polymorphism among the evaluated strains, with a mean difference of 0.33 (*Xcmi* 2 vs. *Xcmi* 8) and 0.29 (*Xcmi* 10 vs. *Xcmi*12). This result contrasts with the work of Assigbetse et al. (1998), who reported that RAPD analysis did not show a high level of polymorphism within the *Xanthomonas* strains used in their study. The results of Kaur et al. (2005) showed that RAPD markers have a high level of genetic diversity across the isolates of *X. axonopodis* pv. *cyamopsidis* in comparison with the other molecular markers employed.

Our findings contradict those of Odipio et al. (2009) who found very low genetic diversity among Ugandan isolates of *X. campestris* pv. *musacearum* (*Xcm*) by RAPD. Regardless of the source and geographical origin, similar fingerprints were generated from the tested isolates. Using a similarity coefficient of 58%, the unweighted pair group method with arithmetic averaging (UPGMA) analysis did not reveal any significant differences in clustering, with the exception of a single isolate (Wkk) that had unique fingerprints.

The cluster dendrogram based on the collective amplification results of all the primers indicated that isolates of *Xanthomonas* were divided into two main clusters. Cluster 1 subdivided into two groups (a and b) exhibiting approximately 50% genetic homology. Cluster 2 comprised three isolates of which *X. maltophilia* (V) and *X. nematophilus* (VII) showed 100% similarity with each other and 38.76% homology with *X. axonopodis* (I). *X. campestris* (III) remained unclustered in collective dendrogram analyses. It represented a unique genetic profile showing 29.29% genetic homology with other isolates used in the study. Both RAPD and ISSR techniques showed similar results. Support for this type of technique comes from Pooler and Hartung (1995), who showed that estimations of genetic distances within bacterial species based on RAPD analysis using carefully selected primers, is consistent with RFLP data.

In the absence of complete sequence information about the genome of the pathogen, RAPD is the ideal technique since it scans for sequence variation

throughout the whole genome. Recently, inter-simple sequence repeat (ISSR) markers have emerged as an alternative system with reliability and advantages of microsatellites (SSR). ISSR analyses are more specific than RAPD analyses, due to longer SSR-based primers with a higher primer annealing temperature, which enable higher stringency and greater band reproducibility (Wolfe et al., 1998). Coupled with the separation of amplification products on agarose gel, ISSR amplification can reveal a much larger number of fragments per primer than RAPD. The techniques are cost effective, sequence information of template DNA is not required and markers can be generated in the regions containing repetitive sequences.

To conclude, the employed biochemical and molecular techniques provide strong evidence for the existence of high levels of variability among indigenous *Xanthomonas* isolates.

REFERENCES

- Abdo - Hasan M., H. Khalil, B. Debis and N. Ali Mir (2008). Molecular characterization of Syrian races of *Xanthomonas axonopodis* pv. *Malvacearum*. *Journal of Plant Pathology*, **90** (3), 431-439.
- Assigbetse K., V. Verdier, K. Wydra, K. Rudolph and J.P. Geiger (1998). Genetic variation of the Cassava bacterial blight pathogen *Xanthomonas axonopodis* pv. *manihotis*, originating from different eco-regions in Africa In: Plant Pathogenic Bacteria, Proceedings of 9th International Conference, A. Mahadevan (ed.), Centre for Advance Study in Botany, University of Madras, Chennai, 223-229.
- Bornet B and M. Branchard (2001). Nonanchored Inter Simple Sequence Repeat (ISSR) Markers: Reproducible and specific tools for genome fingerprinting. *Plant Molecular Biology Reporter*, **19**, 209-215.
- Bradbury J.F (1984). Genus II. *Xanthomonas* Dowson. In *Bergey's Manual of Systematic Bacteriology*, Krieg NR. and Holt JG. eds. (Baltimore, Williams and Wilkins), 199-210.
- Gillings M.R., P.C. Fahy and J. Bradley (1998). Identification of *Xanthomonas fragariae*, the cause of an outbreak of angular leaf spot on strawberry in South Australia, and comparison with the cause of previous outbreaks in New South Wales and New Zealand. *Australasian Plant Pathology*, **27**, 97-103.
- Goncalves E.R. and Y.B. Rosato (2000). Genotypic characterization of *Xanthomonas* strains isolated from passion fruit plants (*Passiflora* spp.) and their relatedness to different *Xanthomonas* species. *International Journal of Systematic and Evolutionary Microbiology*, **50**, 811-821.
- Hayward A.C (1993). The hosts of *Xanthomonas*. In *Xanthomonas*, Swings JG. and Civerolo EL., eds. (London, United Kingdom, Chapman and Hall), 1-119.
- Herrick J.B., D.N. Miller, E.L. Madsen and W.C. Ghiorse (1996). Extraction, purification, and amplification of microbial DNA from sediments and soils. In: Burke J F, editor; Burke J F, editor. PCR: essential techniques. New York, N.Y: John Wiley & Sons. pp. 130-133.
- Kaur B., S. Purkayastha, N. Dilbaghi and A. Chaudhury (2005). Characterization of *Xanthomonas axonopodis* pv. *cyamopodis*, the Bacterial Blight Pathogen of Cluster Bean Using PCR-based Molecular Markers. *Journal of Phytopathology*, **153**, 470-479.
- Kishun R. and V.K. Gupta (2008). Detection of genetic diversity among Indian strains of *Xanthomonas campestris* pv. *mangiferaeindicae* using PCR-RAPD. *Nature Precedings* 2403.1
- Manulis S., L. Valinsky, A. Lichter and D.W. Gabriel (1994). Sensitive and specific detection of *Xanthomonas campestris* pv. *pelargonii* with DNA primers and probes identified by random amplified polymorphic DNA analysis. *Applied and Environmental Microbiology*, **60**, 4094-4099.
- Nagaoka T. and Y. Ogihara (1997). Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. *Theoretical and Applied Genetics*, **94**, 597-602.
- Odipto J., G. Tusiime, L. Tripathi and V. Aritua (2009). Genetic homogeneity among Ugandan isolates of *Xanthomonas campestris* pv. *musacearum* revealed by randomly amplified polymorphic DNA analysis. *African Journal of Biotechnology*, **8**, (21), 5652-5660.
- Ogunjobi A.A., A.G.O. Dixon and O.E. Fagade (2007). Molecular genetic study of cassava bacterial blight casual agent in Nigeria using Random Amplified Polymorphic DNA. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, **6** (9), 2364-2376.
- Permaul K., D. Pillay and B. Pillay (1996). Random-amplified polymorphic DNA (RAPD) analysis shows intraspecies differences among *Xanthomonas albilineans* strains. *Letters in Applied Microbiology*, **2**, 307-311.
- Pooler M.R. and J.S. Hartung (1995). Genetic relationships among strains of *Xylella fastidiosa* from RAPD-PCR data. *Current Microbiology*, **31**, 134-137.
- Pooler M.R., D.F. Ritchie and J.S. Hartung, (1996). Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive

extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. *Applied and Environmental Microbiology*, **62**, 3121-3127.

Roberts P.D., N.C. Hodge, H. Bouzar, J.B. Jones, R.E. Stall, R.D. Berger and A.R. Chase (1998). Relatedness of *Xanthomonas fragariae* strains by restriction fragment length polymorphism, DNA-DNA re-association, and fatty acid analyses. *Applied and Environmental Microbiology*, **64**, 3961-3965.

Saddler G.S. and J.F. Bradbury (2005). The Proteobacteria. In *Bergey's Manual of Systematic Bacteriology*. Edited by: Brenner DJ, Krieg NR, Staley JT, Garrity GM. New York: Springer.

Williams J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **8**, 6531-6535.

Wolfe A.D., Q.Y. Xiang and S.R. Kephart (1998). Diploid hybrid speciation in *Penstemon* (Scrophulariaceae). *Proceedings of the National Academy of Sciences of the USA*, **95**, 5112-5115.

Yao J.D., J.M. Conly and M. Krajden (1995). Molecular typing of *Stenotrophomonas* (*Xanthomonas*) maltophilia by DNA macrorestriction analysis and random amplified polymorphic DNA analysis, *Journal of Clinical Microbiology*, **33**, (8) 2195-2198.

