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Characterization of Newly Isolated *Xanthomonads* using 16s rDNA; ITS; rep PCR (ERIC, BOX)

Khalid Abdullah Ali AbdelRahim[1,2]*, El-Sayed Mohamed Soltan[2,3] and Klaus Rudolph[4]

[1]Botany and Microbiology Department, College of Science, King Saud University P.O. Box 2455, Riyadh 11451, Saudi Arabia.
[2]Botany Department, Faculty of Science, Sohag University, Sohag 82524, Egypt.
[3]Botany department, college of science, Al-Baha University, Saudi Arabia.
[4]Institute of Plant Pathology and Plant Protection, University of Göttingen, Grisebachstr. 6, D-37077 Göttingen, Germany.

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This study aimed to characterize newly isolated xanthomonads, using different genetics fingerprinting techniques. Rep-PCR fingerprinting (ERIC and BOX), 16S-23S Intergenic Transcribed Spacer-PCR (ITS), 16S rDNA amplification, were used for *Xanthomonads* strains characterization. By combining the ERIC and BOX PCR data using the UPGMA analysis, all strains from *Lobelia* and *Isotoma* were represented in one related group with a similarity coefficient of more than 93%. The strains from *Lobelia* and *Isotoma* could be identified as *Xanthomonas lobeliae*. The HV strains from cotton should be named *Xanthomonas axonopodis*pv. *malvacearum* race 20. The strains from *Catharanthus* should be named *Xanthomonas axonopodis* pv. *catharanthi.*

Key words: *Xanthomonas* species, 16S rDNA, rep PCR.

Xanthomonas campestris pv. *malvacearum* is a Gram negative bacterium, motile by a single polar flagellum, occurring in short rods, chemoorganotrophic and obligatory aerobic (Bradbury, 1986). The name of the species was changed to axonopodis by Vauterin *et al.* (1995). However, since this name is not yet generally accepted by the scientific community (Schaad *et al.*, 2000) the earlier species name *X. campestris* is being used here.

Many different genetic fingerprinting techniques are used for identification and characterization of the genetic diversity of phytopathogenic bacteria. AP-PCR, arbitrary primed PCR (Welsh *et al.*, 1990); RAPDs, randomly amplified polymorphic DNA (Williams *et al.*, 1990); rep-PCR, repetitive sequence-based PCR (Versalovic *et al.*, 1994) and AFLP, amplified fragment length polymorphism (Vos *et al.*, 1995).

Rep PCR is based on the specific conservative repeated sequences in different bacteria, for example REP (Repetitive Extragenic Palindromic sequences), ERIC (Enterobacterial Repetitive Intergenic Consensus sequences) and the BOX element.

Because of their sufficient conservation, the rRNA genetic locus is used in a universal organization of evolutionary relationships (Cedergen *et al.*, 1988). The utility of the rDNA sequence as a taxonomic tool has been amply demonstrated in bacteria, where 16S RNA sequence analyses have completely redefined phylogenetic relationships (Fox *et al.*, 1980; Lane *et al.*, 1985; Woese, 1987; Woese and Fox, 1977). In addition to highly conserved areas that have been used to study the relationships among distant taxa, the 16S sequence contains more variable regions that have been useful in the differentiation of genera

* To whom all correspondence should be addressed.
E-mail: Khalidfp7@gmail.com;
kabdelraheem@ksu.edu.sa

and species (Goebel *et al.,* 1987).

In prokaryotes, the rRNA genetic loci contain the genes for all three rRNA species, 16S, 23S, and 5S genes. These genes are separated by spacer regions which exhibit a large degree of sequence and length variation at the level of genus and species. Within a single genome there are frequently multiple rRNA genetic loci; spacer regions found within these loci also show a significant degree of variation in length and sequence. This diversity is due in part to variations in the number and type of tRNA sequences found within the spacer (Brosius *et al*., 1981; Loughney *et al.,* 1982). There is a different approach to identify the bacterial genera and species using this technique, the PCR product can be digested with a restriction enzyme, and the resulting fragments can be resolved electrophoretically.

If the PCR product contains the restriction endonuclease recognition sequence at unique locations, then the resultant fragment size pattern can be indicative of a particular species (Gardes *et al.,* 1991; Vilgalys and Hester, 1990).

Originally, each variant of the genus *Xanthomonas* showing a different host range or producing different disease symptoms was classified as a separate species, which can be described as the 'new host - new species method' (Starr, 1981). This led to a complex genus that finally contained more than 100 species.

Later on, more than 140 pathovars have been defined within the genus *Xanthomonas* (Bradbury, 1986; Hayward, 1993). The pathovars are defined by one single feature, i.e., pathogenicity, and thus have no place in a modern taxonomic environment (Vauterin*et al.*, 1990). This classification system may be useful if there were not three major practical problems with it. (i) In most cases, the host range of strains of a particular pathovar is not known, as no extensive host range studies, including numerous cross-inoculations, have ever been performed or at least published. (ii) In an early DNA hybridization study, Murata and Starr (1973) reported that there is significant heterogeneity within a number of pathovars, at that time nomenspecies, at the genomic level. (iii) Nonpathogenic xanthomonads, which are isolated from healthy as well as diseased plants cannot be classified in a pathovar system.

Several attempts have been made to classify pathovars and strains by using alternative features of the pathogen. Serological tests (Benedict, *et al.,* 1989; 1990), fatty acid profiling (Stead, 1992; Vauterin *et al.,* 1992), genomic and plasmid DNA analysis (Berthier *et al.,* 1993; Denny *et al.,* 1988; Hartung and Civerolo, 1987; Hildebrand *et al.*, 1990; King, 1989; Lazo, and Gabriel, 1987; Lazo *et al*., 1987; Leach, *et al.,* 1990; Pecknold and Grogan, 1973), and protein analysis (Van Zyl and Steyn 1990; Vauterin *et al*., 1991; Vauterin *et al*., 1990) have been used to classify pathovars and strains of different species. However, these techniques are often time-consuming, too expensive, or too insensitive for use in routine diagnosis. Therefore, new methods have been developed in recent years to rapidly identify and classify closely related pathogenic bacteria on the basis of genomic fingerprinting approaches.

The genome of diverse bacterial species includes a family of repetitive DNA sequences (Louws, *et al*., 1994). Three families, unrelated at the DNA sequence level, have been studied, namely the 35-to 40-bp repetitive extragenic palindromic (REP) sequences (Gilson *et al.,* 1984; Jigging *et al*., 1982), the 124- to 127-bp enterobacterial repetitive intergenic consensus (ERIC) sequence (Hulton *et al.,* 1991; Sharples and LIoyd, 1990), and the recently discovered 154-bp BOX element (Frank *et al.,* 1994). The organization of the bacterial genome is thought to be shaped by selection, and thus the dispersion of the REP, ERIC, and BOX sequences may be indicative of the structure and evolution of the bacterial genome (Gilson *et al*., 1987; Krawiec 1985; Krawiec and Riley, 1990; Lupski and Weinstock, 1992).

MATERIALS AND METHODS

Bacterial Strains

The tested strains obtained from the GSPB (Göttinger Sammlung (Collection) Phytopathogener Bakterien) bacterial collection (Table 1).

Extraction of genomic DNA.

Extraction of DNA was performed according to Koopmann, 1999, the quantification of DNA was done in a Gene Quant spectrophotometer (Pharmacia, Freiburg, Germany) at wavelengths of 260 and 280 nm for quantifying the amount of DNA (Ausubel *et al.,* 1995).

ERIC and BOX PCR fingerprinting

The primer sets used in this study for ERIC and BOX are listed in table 2 (Louws *et al.*, 1999).The reaction was done in 50 µl according to Louws *et al*., 1994, in 30 cycles as shown in table 3.

ITS amplification

DNA samples were diluted to a concentration of 20ng/µl prior to amplification. A 1.25-µl aliquot of bacterial genomic DNA was combined with 2.5 µl of reaction buffer (500 mMKCl, 100 mM Tris-HCl [pH 8.8 at 25 °C], 15 mM $MgCl_2$, 1% Triton X-100), 1 µl of a deoxynucleoside triphosphate (dNTP) mixture (concentration of each dNTP, 5 mM), 1.25 µl each of two 15-base oligonucleotide primers (primer G1 and L1[concentration, 50 ng/µl]), and 42 µl of deionized water (Jensen *et al*., 1993).

This mixture was heated to 94 °C for 5 min, and 1.3 U of a thermostable DNA polymerase were added. Twenty five amplification cycles were performed inT personal Biometrathermocycler according to the following format: 1 min at 94 °C; 2-min ramp to 55 °C; 7 min at 55 °C; 2 min ramp to 72 °C; and 2 min at 72 °C. The final cycle was followed by an additional 7 min at 72 °C to complete partial polymerization.

Primer Name	Sequence
G1	5‘-GAAGTCGTAACAAGG-3‘
L1	5‘-CAAGGCATCCACCGT-3‘

16 S rDNA amplification

The reaction had a final volume of 20 µl and contained 60 ng of each primerv(16S For, 16S Reverse), 1 unit *Taq* polymerase and 5-15 ng bacterial template DNA. The following PCR program was run: initial denaturation for 2 min at 95 °C and 29 cycles of 45 s at 95 °C, 1 min at 37 °C and 2 min at 62 °C(Maes, 1993).

Primer Name	Sequence
16S For	5‘-AAGGATCGGGTATTAAC-3‘
16S Reverse	5‘-AGAGTTTGATCITGGCTCAG-3‘

Visualization and documentation of PCR products

For visualization of ITS and 16S rRNA PCR products, DNA could be assayed by electrophoresis on a 1.5 % agarose gelin 0.5 x TBE buffer pH 8.0 (Agarose INEEO Ultra Quality gels, Roth, Kartsruhe). 100 bp Gene Ruler DNA ladder marker (Fermentas, St. Leon Rot) was run alongside the samples giving a ladder of fragments ranging from 100 bp to 1000 bp, allowing the molecular weight of the DNA to be estimated. For BOX and ERIC PCR products, 5- 10% gradient Poyacrylamide gel (PAGE) was used.

After DNA fragments were separated in the electric field, the gel was removed from its tank and carefully transported into a suitable dark staining tray containing 0.5 µg/ml ethidium bromide aqueous solution (Fluka, Neu Ulm) and incubated for 10 min. The gel was photographed with a Polaroid N4P4 camera provided with a red filter at 590 nm, using a black and white colourless film Polapan 667 (Polaroid, St. Albans, Hertfordshire, UK)

AFLP products were applied into ALFexpress II sequencer (serial nr. 56305130 P8 003404). The ALFexpress results were displayed as reconstructed gel image, electropherograms, or tabular data. ALFexpress results can be imported into the ALFexpress program for subsequent data analysis. This software identifies and measures bands ranging in size from 50 to 500 base pairs. The bands (alleles) were scored as present/absent, and a binary matrix was constructed. Four µl of ALFexpress (Alien Life Form sequencer) sizer 50-500 were loaded in the first and last lanes of the gel. Because the fragments were labeled with fluorescent dyes, they could be separated and quantified using the ALFexpressII software, Windows 98 version, which store data in tagged image file format and then process them with NTSYS-pc software (Rohlf, 1992). Cluster analysis was performed by the unweighted pair group method with average linkages (UPGMA). Banding patterns from AFLP analyses obtained after conversion of the peak patterns generated by ALFexpress gel electrophoresis.

Cluster analysis of rep-PCR (ERIC and BOX) and AFLP fingerprints

Each band was treated as a separate putative locus, and scored as present (1) or absent (0) in each accession. Estimates of similarities were based on Dice coefficient (Dice 1945). Cluster analysis was based on the unweighted paired-group method using arithmetic averages (UPGMA) of the NTSYS-pc software (Rohlf, 1992). The dendrogram was created with the TREE option of NTSYS and the goodness of fit of the clustering to

the data was calculated using the COPH and MXCOMP procedures (Rohlf, 1992). In order to identify major cluster groups, a principal coordinate analysis was conducted based on dissimilarity measures (1-S*ij*) using the DCENTER and EIGEN procedures in NTSYS (Rohlf, 1992).

RESULTS

16S rRNA genes and ITS (Intergenic Transcribed Spacer).

According to the amplified fragment patterns shown in fig. 1 (a and b), the newly isolated strains showed the same profiles as the reference

Table 1. Strains used in this study

GSPB Nr.	Origin anddate of isolation	Host plant	GSPB Nr.®	Origin anddate of isolation	Host plant	Pathovar
3085	Freising,	*Isotomaaxillaris*	1246	USA 1986	Cotton	*X. axonopodispv. malvacearum* (race 6)
3086	Germany, 2001		1583	Turkey		
3087			1584	1987		
3088			1585			
3089			1586			
3090			3005			
3091			1384	Nicaragua 1986	Cotton	*X.axonopodispv. malvacearum* (race18)
3092			1385			
3093			1386			
2940	Freising,	*Lobelia* spp.	1429			
3024	Germany 2000		1432			
3030			1435			
3034			1252	USA		
3036			3012	Sudan 1991		
3037			2217	Heidelberg, Germany 1994	*Brassicaceae*	*X.axonopodispv. Campestris*
3039			529	ATCC 29078	*Juglans*	*X.axonopodispv. juglandis*
2963	Stuttgart, Germany 2000					
2966	Bonn, Germany 2000					
2971	Netherlands 2000					
1828	Burkina Faso	Cotton	ATCC = American type culture collection® Reference strains			
1829	1984					
1830						
1831						
2921						
2922						
2923						
3006	Sudan					
2388	1994					
2801	India	*Catharanthuspusillus*				
2802	1997					
2803						

Xanthomonas strains. One band with a molecular weight of 480 bp in case of 16S rDNA amplification and one band of 600 bp in case of ITS amplification were amplified. Bacterial strains belonging to other genera than the xanthomonads were also analysed to find out whether these two bands are specific only to the genus *Xanthomonas*, but this strains showed different banding profiles than those of the *Xanthomonas* strains tested.

BOX-PCR fingerprinting-analysis of different xanthomonads

BOX fingerprint patterns of 10 HV strains are shown in fig. 2. Most of the banding profiles were very similar with the exception of strains 2388 (lane 6) and 3008 (lane 1).

As shown on fig. 3,BOX fingerprinting patterns of 9 *Xanthomonas* strains from *Isotoma* were identical, but differed clearly from 8 *Xcm* race 18 strains. Different DNA concentrations loaded per strain from *Isotoma* were the reason for the non-appearance of weak bands in some strains.

Fig. 4 shows the fingerprint patterns of 10 *Xanthomonas* strains from *Lobelia* which appear to be nearly identical to the strains from *Isotoma* shown in fig. 2, but again very different than the *Xanthomonas* pathovars *juglandis* and

Table 2. Primers used in ERIC and BOX fingerprinting

Technique	Primer name	Sequences (52 ————————32)	Target DNA
ERIC	ERIC IR	ATGTAAGCTCCTGGGGATTCAC	Repetitive sequences
	ERIC 2	AAGTAAGTGACTGGGGTGAGGG	
BOX	BOX A1R	CTACGGCAAGGCGAGGCTGACG	Repetitive sequences

Table 3. Amplification program of ERIC and BOX fingerprinting

Reaction	Initial Denaturation 1 cycle	Denaturation 30 cycles	Annealing	Elongation	Elongation 1 cycle
ERIC	95 °C 7 min	94 °C 1 min	52 °C 1 min	65 °C 8 min	65 °C 15 min
BOX	95 °C 7 min	94 °C 1 min	53 °C 1 min	65 °C 8 min	65 °C 15 min

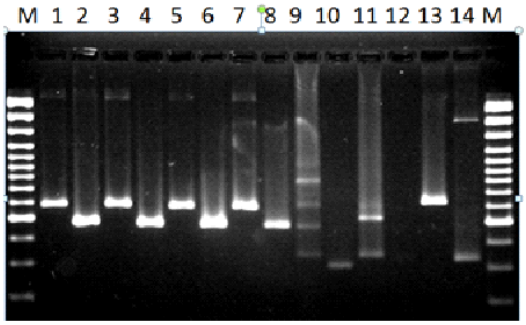


Fig. 1a: *Xa*pv. *malvacearum* race 6 (lane 1: ITS, lane 2: 16S rDNA),strain 2801 from*Catharanthus*(lane 3: ITS, lane 4: 16S RNA),pv. *campestris*(lane 5: ITS, lane 6: 16S rDNA),pv. *juglandis*(lane 7: ITS, 8: 16S rDNA), *Agrobacterium tumefaciens*(lane 9: ITS, lane 10: 16S rDNA) *Clavibactermichiganensis*(lane 11: ITS, lane 12: 16S rDNA), and *Pseudomonas syringae*pv.*tomato*(lane 13: ITS, lane 14: 16S rDNA). M: 100 bp Gene Ruler DNA ladder marker.

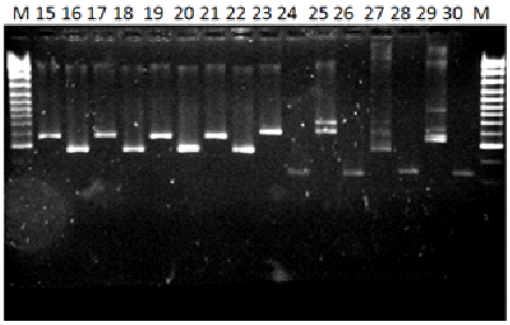


Fig. 1b:Strain 3090 from *Isotoma*(lane 15: ITS, lane 16: 16S rDNA), HV strain 3008(lane 17: ITS, lane 18: 16S rDNA), p*v. malvacearum*race18 (lane 19: ITS, lane 20: 16S rDNA), strain 3030 from*Lobelia*(lane 21: ITS, lane 22: 16S rDNA), *Pseudomonas syringae*pv. *tomato* (lane 23: ITS, lane 24: 16S rDNA), *Pseudomonas syringaepv. lachrymans*(lane 25: ITS, lane 26: 16S rDNA), *Azotobacterchroococcum* (lane 27: ITS, lane 28: 16S rDNA), and *Erwiniacarotovora ssp. atroseptica*(lane 29: ITS, lane 30: 16S rDNA).

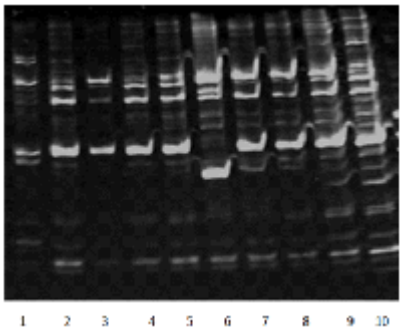
Fig. 1. Amplified fragments using 16S rDNA and ITS primers of representative strains of different standard or new xanthomonads and other bacterial genera.

campestris and *X*. strains from *Catharanthus*.

ERIC-PCR fingerprinting.

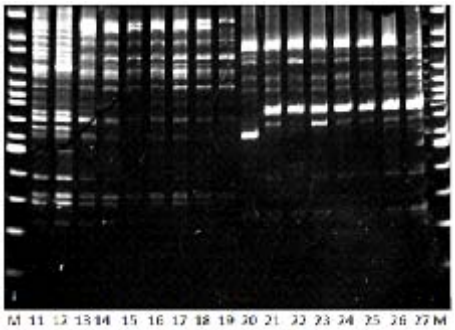
Fig. 5 shows the electrophoretic patterns of *Xanthomonas* strains from *Isotoma.* The strains appear to be identical in their profiles. Fig. 6 is a comparison of *Xcm*race 18 strains with *Xcm* HV strains. Both rather uniform groups differ slightly from each other. Exceptions from the general pattern are shown by race 18-strains 3012 (lane 17) and 1432 (lane 15), and by the HV strain 2923 (lane 25).

Fig. 7 shows that also by ERIC-PCR fingerprinting, the banding profile of the strains from *Lobelia* was very similar to that of the strains



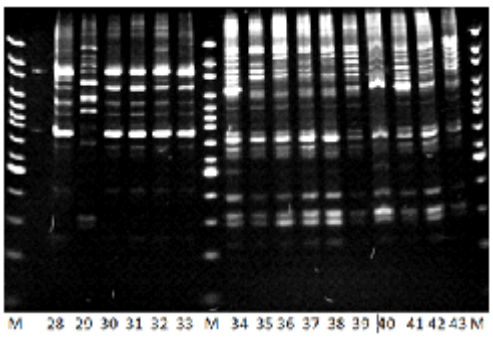
GSPB 3008 (lane 1), GSPB 3006 (lane 2), GSPB 2923 (lane 3), GSPB 2922 (lane 4), GSPB 2921 (lane 5), GSPB 2388 (lane 6), GSPB 1831 (lane 7), GSPB 1830 (lane 8), GSPB 1829 (lane 9), and GSPB 1828 (lane 10). M: 100 bp Gene Ruler DNA ladder marker.

Fig. 2. BOX-PCR fingerprinting patterns *X campestris* (HV strains)



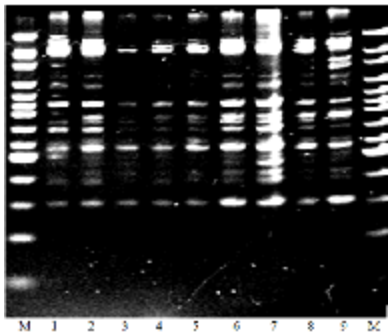
Strains from *Isotoma*:GSPB 3085 (lane 11), GSPB 3086 (lane 12), GSPB 3087 (lane 13), GSPB 3088 (lane 14), GSPB 3089 (lane 15), GSPB 3090 (lane 16), GSPB 3091 (lane 17), GSPB 3092 (lane 18), GSPB 3093 (lane 19). Strains from *X. c*.pv. *malvacearum* race 18: GSPB1252 (lane 20), GSPB 1384 (lane 21), GSPB 1385 (lane 22), GSPB1386 (lane 23), GSPB 1429 (lane 24), GSPB 1432 (lane 25), GSPB 1435 (lane 26), and GSPB 3012 (lane 27). M: 100 bp Gene Ruler DNA ladder marker.

Fig. 3. BOX-PCR fingerprinting patterns of strains from *Isotoma*and *X. c*.pv.*malvacearum(*race 18)



*Xanthomonasarboricola*pv.*juglandis* GSPB 529 (lane 28), *X.c*.pv. *campestris* GSPB 2217 (lane 29), *Xanthomonas* strains from *Catharanthus*:GSPB 2801 (lane 30), GSPB 2802 (lane 31), GSPB 2803 (lane 32 and 33), *Xanthomonas* strains from *Lobelia*:GSPB 3039 (lane 34), GSPB 3037 (lane 35), GSPB 3036 (lane 36), GSPB 3034 (lane 37), GSPB 3030 (lane 38), GSPB 3024 (lane 39), GSPB 2971 (lane 40), GSPB 2966 (lane 41), GSPB 2963 (lane 42), and GSPB 2940 (lane 43). M: 100 bp Gene Ruler DNA ladder marker.

Fig. 4. BOX-PCR fingerprinting patterns of *Xanthomonasar boricola* pv. *juglandis*, *X. c*. pv. *campestris* and *Xanthomonas* strains from *Catharanthus* and *Lobelia.*



GSPB 3093 (lane 1), GSPB 3092 (lane 2), GSPB 3091 (lane 3), GSPB 3090 (lane 4), GSPB 3089 (lane 5), GSPB 3088 (lane 6), GSPB 3087 (lane 7), GSPB 3086 (lane 8), and GSPB 3085 (lane 9). M: 100 bp Gene Ruler DNA ladder marker.

Fig. 5. ERIC-PCR fingerprinting patterns from genomic DNA of strains from *Isotoma*

from *Isotoma (*fig. 5), but different from the 3 rather homogeneous *Catharanthus* strains (lane 38-40) and *X. c*. pv. *campestris* as well as *X. arboricola*pv. *juglandis*.

Cluster analysis of rep-PCR (ERIC and BOX) fingerprints

A combination of the data from the two fingerprinting types (BOX and ERIC) was performed by using the UPGMA analysis. Diagram (fig. 8a,b) shows the dendrogram resulting from this application. The strains tested could be classified into three main clusters. The first group (I) includes all the strains from *Isotoma* and *Lobelia*. The similarity coefficient between these strains was more than 93%.

The second group (II) comprised only the strain of *Xanthomonas campestris* pv. *campestris.* This strain had a similarity coefficient of about 35% to the first group (strains from *Isotoma* and *Lobelia*).

The third group (III) includes all the *Xcm* strains of HVS and race 18, the strains from Catharanthus and one strain of *Xanthomonas arboricola* pv. *juglandis*. This group had a similarity coefficient of about 53% between all the strains represented in group III.

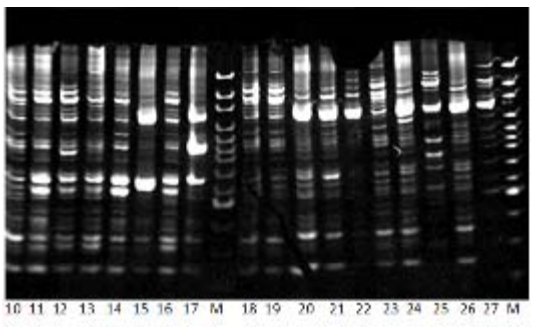
Within the group III two clusters were observed, IIIa which includes all the *Xcm* strains (HVS and race 18) with a similarity coefficient of about 75%, and cluster IIIb including *Xanthomonas arboricola* pv. *juglandis* and the new strains from *Catharanthus*.

The correlation coefficient between the newly isolated strains from *Isotoma* and *Lobelia* and the other strains used in this test was relatively low (about 22%).

The correlation of distance matrix and the dendrogram, that is the cophenetic of correlation was calculated as r = 0.9833. The cophenetic correlation coefficient measures the dendrogram and the data of the original similarity matrix.

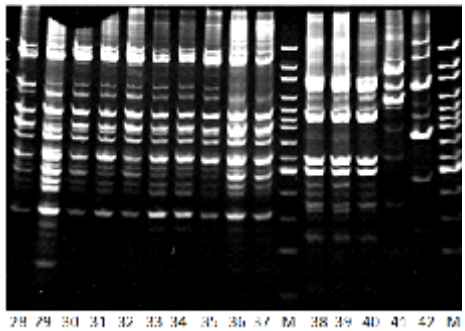
DISCUSSION

The development of the molecular genetic techniques allowed applying these methods to classify and/or reclassify related microorganisms parallel with evaluating phenotypic and physiological data. Our experiments aimed to classify *Xanthomonas* strains isolated from new host plants, i.e., *Lobelia*, *Isotoma* and *Catharanthus*, or of uncertain designation (HV strains from cotton). For further experiments, these results can also be used to understand the evolutionary relationship between the pathogen members of this genus, and by which mechanisms the bacterium may infect a new host plant.



Race 18: GSPB 1252 (lane 10), GSPB 1384 (lane 11), GSPB 1385 (lane 12), GSPB 1386 (lane 13), GSPB 1429 (lane 14), GSPB 1432 (lane 15), GSPB 1435 (lane 16) GSPB 3012 (lane 17). HV strains: GSPB 1828 (lane 18), GSPB 1829 (lane 19), GSPB 1830 (lane 20), GSPB 1831 (lane 21), GSPB 2388 (lane 22), GSPB 2921 (lane 23), GSPB 2922 (lane 24), GSPB 2923 (lane 25), GSPB 3006 (lane 26), and GSPB 3008 (lane 27). M: 100 bp Gene Ruler DNA ladder marker.

Fig. 6. ERIC-PCR fingerprinting patterns of 8 strains of *X. c.*pv. *malvacearum* race 18 and 10 HV strains.

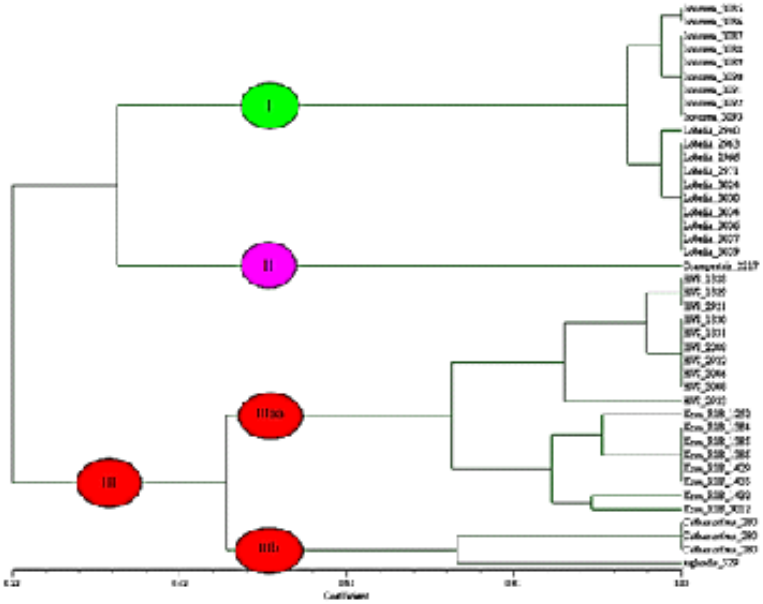


Strains from *Lobelia*:GSPB 2940 (lane 28), GSPB 2963 (lane 29), GSPB 2966 (lane 30), GSPB 2971 (lane 31), GSPB 3024 (lane 32), GSPB 3030 (lane 33), GSPB 3034 (lane 34), GSPB 3036 (lane 35), GSPB 3037 (lane 36), GSPB 3039 (lane 37). Strains from *Catharanthus*:GSPB 2801 (lane 38), GSPB 2802 (lane 39), GSPB 2803 (lane 40); *Xanthomonascampestris*pv. *campestris* GSPB 2217 (lane 41); and *Xanthomonasarboricola*pv. *juglandis*GSPB 529 (lane 42). M: 100 bp Gene Ruler DNA ladder marker.

Fig. 7. ERIC-PCR finger printing patterns of *Xanthomonas* strains from *Lobelia* and *Catharanthus*, *X. c.* pv. *campestris*, and *X. arboricola*pv.*juglandis*.

16S rDNA and ITS

According to our results of the 16s rDNA amplification, all of strains tested had the same fragment patterns which were unique to strains of the genus *Xanthomonas* and differed from other bacterial genera. These results agree with those of Maes (1993). She also concluded that in all *Xanthomonas* pathovars which she tested, a single 16S rDNA fragment of 480 bp was produced. In contrast, phytopathogenic bacteria from other genera showed different fragment patterns by agarose gel electrophoresis. The author used this method as a fast identification system for screening *Xanthomonas*-related organisms.



I- Strains from *Isotoma*and *Lobelia* II- *Xanthomonascampestris*pv. *campestris*. IIIa- Strains of *X. c.*pv. *malvacearum*: HVS and race 18. IIIb- *Xanthomonasarboricola*pv. *juglandis* and the new strains from *Catharanthus*.

Fig. 8a: Cluster analysis of ERIC and BOX-fingerprintings showing the correlation coefficient according to Dice (1945) using the UPGMA application.

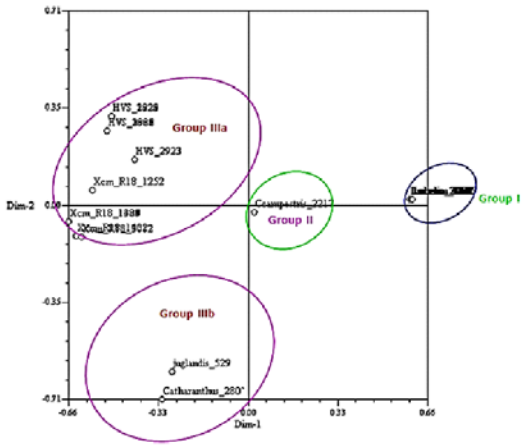


Fig 8b: Two dimensional principal coordinates plot based on ERIC and BOX fingerprints of 42 *Xanthomonas*strains showing the similarity coefficient according to Dice (1945).

Also, the results obtained by ITS amplification confirmed the earlier experiments. All the strains tested showed the same fragment (600 bp) which was unique to the genus *Xanthomonas* and differed clearly from fragments yielded by other bacterial genera (fig. 1a and 1b). It was concluded from our studies that the unique bands obtained by 16S rDNA or ITS amplification can be used as a genetic marker for preliminary identification of the xanthomonads. Toth *et al.* (2001) used the ITS-PCR for identification and differentiation of the soft rot erwinias. The authors reported that ITS-PCR generated unique patterns of all bacterial species tested, and in most cases these patterns were similar for strains within a species. They found three PCR groups within the soft rot erwinias. Slight differences were found

within group I (*E. carotovora* subsp. *atroseptica* and subsp. *beta vasculorum*) and II (*E. carotovora* subsp. *carotovora*, subsp. *oderifera*, subsp. *wasabiae* and *E. cacticida*). Group III comprised the strains of one species (*E. chrysanthemi*) and yielded six different but related patterns, which differed from those of group I and II. Also, one non-Erwinia strain gave similar patterns as group III.

BOX and ERIC PCR fingerprints

For *Xanthomonas* species, rep-PCRs have been used to assess variation among pathovars and revealed low levels of intra pathovar diversity (Louws *et al.,* 1994 and 1995; Opgenorth *et al.,* 1996; Pooler, 1996; Vauterin *et al.,* 2000).

In 1994, Louws *et al.* demonstrated the utility of the PCR technique with primers corresponding to ubiquitous repetitive DNA sequences (rep-PCR) to generate specific DNA fingerprints of *Xanthomonas* and *Pseudomonas* pathovars and strains. In addition, the authors showed the potential of rep-PCR fingerprinting as a diagnostic tool and in determining whether pathovars represent a single evolutionary line or are composed of several lines that have converged to a similar pathogenic phenotype.

Cubero and Graham (2002) demonstrated that under specific conditions, rep-PCR with BOX and ERIC primers are useful not only to separate pathotypes but also to differentiate strains of the same pathotype. By amplification of repetitive sequences (ERIC and BOX elements), the authors demonstrated that pathotypes of several *Xanthomonas* strains causing citrus bacterial canker could be differentiated. Subgroups specific for the pathotypes were identified, which were associated with certain geographic areas of the world.

The similarity between the rep-PCR (ERIC and BOX) patterns is calculated using the Pearson product-moment-correlation coefficient. This method may be more reliable than band-based calculations for comparing large numbers of patterns (Rademaker *et al.,* 2000). The authors concluded that the most highly significant and consistent results were obtained from combining the data obtained from BOX, ERIC and REP-PCR fingerprints. This is logical, since the total number of data points (bands/peaks) is greatly increased. Moreover, the genome is more extensively covered, since certain regions may have more (properly spaced) copies of a particular element than others (Lupski and Weinstock, 1992).

In figs 8 a, and b, and according to our results of combining data from ERIC and BOX PCR, three main clusters were observed. The first cluster (I) included all the strains from *Isotoma* and *Lobelia*. The strains of this group were very close to each other with a similarity coefficient of more than 92%. The relatedness of their host plants may explain this high degree of similarity as they infect plant host belonging to the same family (Campanulaceae). These genetic data are also supported by a report from Poschenrieder (2004) that cross-infection experiments with strains from *Lobelia* and *Isotoma* revealed no differences in host-specificity between these two groups. The reports of Poschenrieder *et al.* (1988; 2002) and Mavridis and Rudolph (2002) on the disease symptoms also indicate that these strains differ from many other xanthomonads.

Therefore, it can be concluded from our studies that the strains from *Isotoma* and *Lobelia* form a very distinct group different than all the other xanthomonads tested in this study and should be classified as distinct genomic group.

The third cluster (III) contained the HV strains, *Xcm* strains race 18, the strain of *X.arboricola*pv. *juglandis*and the strains from *Catharanthus.* Thus, the rep-PCR patterns did not differentiate between two different *Xanthomonas* species, i. e., *arboricola* and *axonopodis.* From these results we can conclude that the HV strains and strains from *Catharanthus* can be classified to belong to the species *axonopodis*. In figs 8a, the similarity coefficient between the HV strains and the reference strains of pv. *malvacearum* race 18 (cluster IIIa) was relatively high (about 75%). Therefore, the HV strains can be classified as a new race of pathovar *malvacearum*.

Our results confirmed those obtained by Huang (2000) and Huang *et al.* (2000). By using the same rep-PCR fingerprinting (REP, BOX and ERIC) techniques and 5 different HV strains in addition to most of the HV strains used in our studies, the authors concluded that the genetic fingerprints of the HV strains have a high semilarity degree to the fingerprints of the strains of *X. c.*pv. *malvacearum* race 18, but low similarity degree to the strains belonging to other pathovars of

Xanthomonas campestris. The authors concluded that the HV strains are belonging to *X. c*. pv. *malvacearum*.

Similar conclusions can be drown from the results of the principal coordination test (fig.8b). Thus, the strains from *Isotoma* and *Lobelia* represent one closely related group explaining that they should be classified as a distinct genomic group. According to these results, the HV strains can be considered as a new race belonging to the pathovar*malvacearum* and should be named race 20 as was suggested by Follin*et al.* (1988). The results shown in diagram 1 and 2 confirm that the strains from *Catharanthus*can be designated as *Xanthomonas axonopodis* pv. *catharanthi* - as suggested by Mavridis *et al.* (2000) - because these strains revealed a high similarity coefficient degree with the *Xcm* strains of race 6 and 18.

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