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Identification of Newly *Xanthomonads* using Amplified Fragment Length Polymorphism, AFLP.

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This study aimed to identify newly isolated xanthomonads, using Amplified Fragment Length Polymorphism (AFLP). The results show that, the strains from *Lobelia* and *Isotoma* with a similarity coefficient of 84% to each other, but a very far relation to all other *Xanthomonas* strains. The strains from *Lobelia* and *Isotoma* may named *Xanthomonas lobeliae* spec. nov. The highly virulence strains (HV) isolated from cotton plants can be classified as a new race of *Xanthomonas axonopodis* pv. *malvacearum* (race 20). The strains isolated from *Catharanthus* plants may classified as new pathovar (*Xanthomonas axonopodis* pv. *Catharanthi).*

Key words: Xanthomonads, Amplified Fragment length.

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.

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 Ønew host - new speciesØ method (Starr, 1981). This led to a complex genus that finally contained more than 100 species.

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

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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.

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).

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

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). This techniques named RFLP, and may not effective due to many fragments produced.

In 1999, Restrepo *et al.,* used AFLP as a novel PCR-based technique, to characterize the genetic diversity of Colombian *Xam* (*Xanthomonas axonopodis* pv. *manihotis*) isolates. The authors tested six *Xam* strains with 65 AFLP primer combinations to identify the best selective primers. Eight primer combinations were selected according to their reproducibility, number of polymorphic bands and polymorphism detected between *Xam* strains. Forty-seven *Xam* strains, originating from different Colombian ecozones, were analysed with the selected combinations. They demonstrated that results obtained with AFLP are consistent with those obtained with RFLP, using plasmid DNA as a probe. Some primer combinations differentiated *Xam* strains that were not distinguished by RFLP analyses, thus AFLP fingerprinting allowed a better definition of the genetic relationships between *Xam* strains.

MATERIALS AND METHODS

Bacterial Strains

The tested strains obtained from the GSPB (Göttinger Sammlung (Collection) Phytopathogener Bakterien) bacterial collection , and the references strains obtained from the LMG bacterial collection (Laboratorium voor Mikrobiologie, Gent, Belgium) are listed in 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).

Amplified fragment length polymorphism (AFLP)

The method used by Vos *et al.* (1995) was used. Primers for AFLP consist of a core region and a 32 - extension (E) of 0, 1, or 2 selective nucleotides. The *Eco*RI-primer is fluorescently-labelled. Five primer combinations were used as follows: (E_A/M_0); (E_C/M_C); (E_ACA/T_C); (E_A/M_C); (E_C/T_C). Table 5 shows the restriction enzymes and primer set used in this study.

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 ALFexpress II software, Windows 98 version, which store data in tagged image file format and then processe 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.

RESULTS

AFLP typing

The AFLP banding patterns of 20 representative new and 18 reference *Xanthomonas*-strains obtained by 2 enzyme sets and 5 different primer combinations are shown in figs. 1-5. The banding profiles varied in terms of the distribution of bands ranging from 100 to 500 bp in size. The banding profiles resulting from using the enzyme set *Eco*RI/*Taq*I with a C as a selective base (Rademaker *et al*., 2000) on both primers was not used in further experiments because too many bands with very small distances appeared so that evaluation was difficult.

Reproducibility of the AFLP profiles

The reproducibility of the AFLP profiles was defined as a > 95% similarity level between the duplicated samples. This was the mean similarity obtained between three independently obtained profiles of all 38 tested strains.

Cluster analysis of AFLP genomic fingerprints

To differentiate between the strains, the degree of similarity of banding patterns was calculated using the Pearson correlation coefficient and was expressed as percentage similarities. After cluster analysis by UPGMA, a dendrogram was constructed (Fig. 6 a, b). AFLP clusters were identified by banding patterns that grouped together and showed a linkage level with other clusters of less than 30%.

As shown in figs 6a, 6b, the strains could be classified into two main clusters (A and B) with a similarity coefficient of about 25%. Cluster (A) can be divided into 2 subclusters with a similarity coefficient of 29%. The first one (A1) includes all the strains of *Isotoma* and *Lobelia*. The *Lobelia* and *Isotoma* strains can be arranged into two groups with a very high similarity coefficient (84%). The second subcluster (S1) includes one strain each of *X. campestris* pv. *raphani* and *X. cucurbitae*.

The second main cluster (B) includes all HV strains, the strains from *Catharanthus* and all the references strains. Within cluster (B), three HV strains (1828, 1831 and 3008) came in subcluster (B1) with a similarity coefficient of about 54%. The strains from *Catharanthus* (2801, 2802, 2803) are grouped in the second subcluster (B2) with a similarity coefficient of about 62%. The third subcluster (B3) contains the HV strains 2388, 2921, 2922 and 2923 with a similarity coefficient of 52%. The strains from *Isotoma* and *Lobelia* represent a separate group with a very weak relation to the other strains. The HV strains and strains from *Catharanthus* represent a wide group which is relatively nearly related (66.6%) to the reference strains (subcluster S2) belonging to the species *axonopodis*, *vesicatoria, hortorum, pisi* and *arboricola*.

In fig. 6b, two main groups appeared, Group 1 can be divided into 2 highly related subgroups. Subgroup 1A includes two HV strains (1831 from Burkina Faso and 3008 from Sudan) together with three reference strains belonging to species *axonopodis* (*X. axonopodis* pv. *dieffenbachiae* LMG 695, *X. axonopodis* pv. *citri* LMG 862 and *X. axonopodis* pv. *phaseoli* LMG 7455) and *Xanthomonas arboricola* pv. *juglandis* LMG 747. The second subgroup 1B includes all the strains from *Catharanthus*, 5 HV strains and many reference strains, i.e. two strains of the species *axonopodis* (*X. axonopodis* pv. *alfalfae* LMG 497, *X. axonopodis* pv. *malvacearum* LMG 761), *X. vesicatoria* LMG 911 and *X. hortorum* pv. *pelargonii* LMG 7314.

The second group (2) is clearly separated from all the other strains and includes only the strains from *Isotoma* and *Lobelia*.

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

Table 1. Strains used in this study

GSPB Nr.	Origin and date of isolation	Host plant	GSPB Nr. ®	Origin and date of isolation	Host plant	Pathovar
3085	Freising, Germany, 2001	*Isotoma axillaris*	1246	USA 1986	Cotton	*X. axonopodis pv. malvacearum* (race 6)
3086			1583	Turkey 1987		
3087			1584			
3088			1585			
3089			1586			
3090			3005	Nicaragua 1986	Cotton	*X. axonopodis pv. malvacearum* (race 18)
3091			1384			
3092			1385			
3093	Freising, Germany 2000	*Lobelia* spp.	1386			
2940			1429			
3024			1432			
3030			1435			
3034			1252	USA		
3036			3012	Sudan 1991		
3037			2217	Heidelberg, Germany 1994	*Brassicaceas*	*X axonopodis pv. Campestris*
3039			529	ATCC 29078	*Juglans*	*X axonopodis pv. juglandis*
2963	Stuttgart, Germany 2000		**LMG**	**Strain ®**	**LMG**	**Strain ®**
2966	Bonn, Germany 2000		747	*X arboricola pv. juglandis*	844	*X axonopodis pv. phyllanthi*
2971	Netherlands 2000		837	*X axonopodis pv. phaseoli* var. *fuscans*	761	*X axonopodis pv. malvacearum*
1828	Burkina Faso 1984	Cotton	861	*X axonopodis pv. ricini*	7505	*X campestris pv. raphani*
1829			695	*X axonopodis pv. dieffenbachias*	690	*X cucurbitas*
1830			7303	*X axonopodis pv. begonias*	911	*X vesicatoria*
1831			862	*X axonopodis pv. Citri*	847	*Xanthomonas pisi*
2921			7455	*X axonopodis pv. phaseoli*	7314	*X hortorum pv. pelargonii*
2922			497	*X axonopodis pv. alfalfas*		
2923	Sudan 1994		538	*X axonopodis pv. xonopodis*		
3006			910	*X axonopodis pv. esicatoria*		
2388			7399	*X axonopodis pv. dieffenbachias*		
2801	India 1997	*Catharanthus pusillus*				
2802						
2803						

ATCC = American type culture collection

® Reference strains

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.

The determination of total genomic DNA-DNA homology values has persisted as a dominant component of taxonomic analysis. However, recent studies have shown that AFLP genomic fingerprinting analysis is an accurate approach for phylogenetic comparisons between bacteria (Huys *et al.*, 1996; Janssen *et al.*, 1997; Rademaker *et al.*, 2000).

Bacterial genomes are relatively small and, in general, one selective base for both primers yields scorable banding patterns (Janssen *et al.*, 1996). A complex and informative fingerprint can thus become useful by making small changes in the primer sequence. The use of a different set of restriction enzymes or of more or different primer

Xanthomonas Strains represented in Figs. 1-5

Lane no.	Reference strains		Strain no.
1	*Xanthomoans arboricola* pv. *juglandis*		LMG* 747
2	*Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans*		LMG 837
3	*Xanthomonas axonopodis* pv. *ricini*		LMG 861
4	*Xanthomonas axonopodis* pv. *dieffenbachiae*		LMG 695
5	*Xanthomonas axonopodis* pv. *begoniae*		LMG 7303
6	*Xanthomonas axonopodis* pv. *citri*		LMG 862
7	*Xanthomonas axonopodis* pv. *phaseoli*		LMG 7455
8	*Xanthomonas axonopodis* pv. *alfalfae*		LMG 497
9	*Xanthomonas axonopodis* pv. *axonopodis*		LMG 538
10	*Xanthomonas axonopodis* pv. *vesicatoria*		LMG 910
11	*Xanthomonas axonopodis* pv. *dieffenbachiae*		LMG 7399
12	*Xanthomonas axonopodis* pv. *phyllanthi*		LMG 844
13	*Xanthomonas axonopodis* pv. *malvacearum*		LMG 761
14	*Xanthomonas campestris* pv. *raphani*		LMG 7505
15	*Xanthomonas cucurbitae*		LMG 690
16	*Xanthomonas vesicatoria*		LMG 911
17	*Xanthomonas pisi*		LMG 847
18	*Xanthomonas hortorum* pv. *pelargonii*		LMG 7314
	New strains from	Origin and date of isolation	
19	*Isotoma*	Freising, Germany, 2001	GSPB 3086
20	*Isotoma*	Freising, Germany, 2001	GSPB 3087
21	*Isotoma*	Freising, Germany, 2001	GSPB 3089
22	*Isotoma*	Freising, Germany, 2001	GSPB 3093
23	*Lobelia*	Freising, Germany, 2001	GSPB 2940
24	*Lobelia*	Stuttgart, Germany, 2000	GSPB 2963
25	*Lobelia*	Bonn, Germany, 2000	GSPB 2966
26	*Lobelia*	Netherlands 2000	GSPB 2971
27	*Lobelia*	Freising, Germany, 2001	GSPB 3024
28	HVS	Burkina Faso, 1984	GSPB 2921
29	*Lobelia*	Freising, Germany, 2001	GSPB 3037
30	HVS	Burkina Faso	GSPB 1828
31	HVS	Burkina Faso	GSPB 1831
32	HVS	Sudan, 1994	GSPB 2388
33	HVS	Burkina Faso, 1984	GSPB 2922
34	HVS	Burkina Faso, 1984	GSPB 2923
35	HVS	Sudan, 1994	GSPB 3008
36	*Catharanthus*	India, 1997	GSPB 2801
37	*Catharanthus*	India, 1997	GSPB 2802
38	*Catharanthus*	India, 1997	GSPB 2803
S	ALFexpress Sizer 50-500 bp		

combinations can dramatically increase the number of polymorphisms detected.

For AFLP fingerprinting, we used 2 different enzymes sets. The first set was *Eco*RI/*Taq*I with C as a selective base (Rademaker *et al.*, 2000). However, the banding profiles resulting from this enzyme set contained too many bands which were difficult to evaluate (fig. 1). These results agreed with the observations of Restrepo *et al.* (1999) that banding patterns obtained by using restriction enzymes with G + C-rich recognition sequences, such as *Taq*I (T“!CGA) and *Pst*I (CTGCA“!G), contained more bands than fingerprints generated with restriction enzymes with A + T-rich sequences, such as *Mse*I (T“!TAA) and *Eco*RI (G“!AATTC), and the fingerprints were also more complex when G or C was the selective base on the primers.

The second enzyme set we used here was *Eco*RI/*Mse*I. The banding patterns were clear and easier to evaluate than the profile of *Eco*RI/*Taq*I. In 1996, Jansen *et al.*, demonstrated that the banding patterns produced by using the enzyme set *Eco*RI/*Mse*I and a primer combination with C as one selective base, correlated very well with results obtained on *Apa*I-*Taq*I templets which was chosen as one of the best enzyme sets for differentiation of Xanthomonas species and pathovars by AFLP fingerprints.

The cluster analysis of the AFLP banding patterns resulting from four primer combinations in our experiments is shown in table 2. The strains from *Isotoma* could be distinguished from the *Lobelia* strains although they were very close to

Table 2. Restriction enzymes and primer set used in this study

Enzyme (restriction site)	Primer core sequence	Adaptor
*Eco*RI (G/AATTC)	E-primer core sequence 52 - GACTGCGTACCAATTCE-32	52 -CTCGTAGACTGCGTACC-32 32 -CTGACGCATGGTTAA-52
*Mse*I (T/TAA)	M-primer core sequence52 - GATGAGTCCTGAGTAAE-32	52 -GACGATGAGTCCTGAG-32 32 -CTACTCAGGACTCAT-52
*Taq*I (T/CGA)	T-primer core sequence52 - CGATGAGTCCTGACCGAE-32	52 -GACGATGAGTCCTGAC-32 32 -TACTCAGGACTGGC-52
	Primer core used for preamplification: *Eco*4: 52 -GACTGCGTACCAATTC-32 *Mse13*: 52 -GATGAGTCCTGAGTAA32	

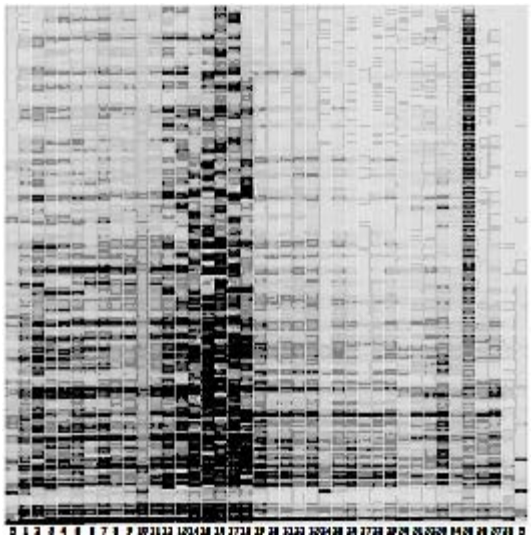


Fig 1. AFLP patterns of *Xanthomonas* strains using the E_C/T_C primer combination. DNA templates were digested with *Eco*RI/*Taq*I.

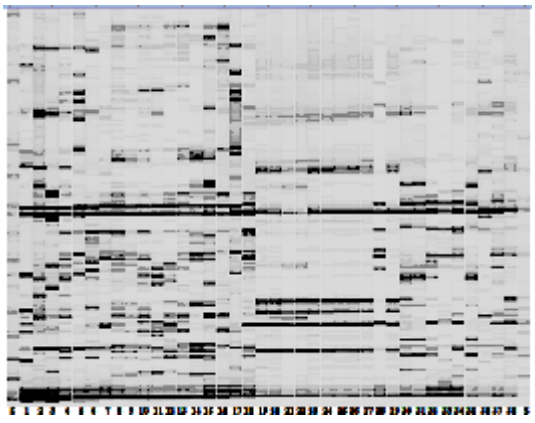


Fig 2. AFLP patterns of *Xanthomonas* strains using the E_C/M_C primer combination. DNA templates were digested with *Eco*RI/*Mse*I

each other with a similarity coefficient of more than 83%. These results reflect the power of AFLP in differentiating highly related strains belonging to the same pathovar. Also, Restrepo *et al.* (1999) could differentiate between strains of *Xanthomonas axonopodis* pv. *manihotis* by AFLP, although some strains fell into one group by using the RFLP analysis.

In order to decide to which species these bacteria should be integrated, a large group of reference *Xanthomonas* strains was included in the AFLP test. The reference strains were very carefully selected from all known xanthomonads, regarding disease symptoms provoked, and host plants which might in some way be related to those of the strains from *Lobelia* and *Isotoma*. The relatively highest similarity coefficient was observed between *Lobelia* and *Isotoma* strains and *X. campestris* pv. *raphani* LMG 7505 and *Xanthomonas cucurbitae* LMG 690 (28.3%). This similarity coefficient, however, is not high enough to conclude that the strains from *Isotoma* and *Lobelia* to belong to these species (*campestris* or *cucurbitae*).

Also, since cucumber and crucifers are botanically not very near to the host plants *Lobelia* and *Isotoma*, and since also the disease symptoms incited are different, it would not be reasonable to incorporate the strains from *Lobelia* and *Isotoma* into the species *X. cucurbitae* or *X. campestris*.

In 1997, Janssen *et al.*, concluded that, an AFLP analysis of *Acinetobacter* strains revealed that four of the tested strains convincingly grouped in a separate AFLP cluster, at (50.8 ± 2.2%) similarity to each other. The similarity of this group was relatively low to the other species (about 18%). The authors suggested that AFLP this cluster rep-resents a new genomic species in the genus *Acinetobacter.*

In conclusion from our results of AFLP fingerprints, the strains form *Isotoma* and *Lobelia* should be designated as a new species belonging

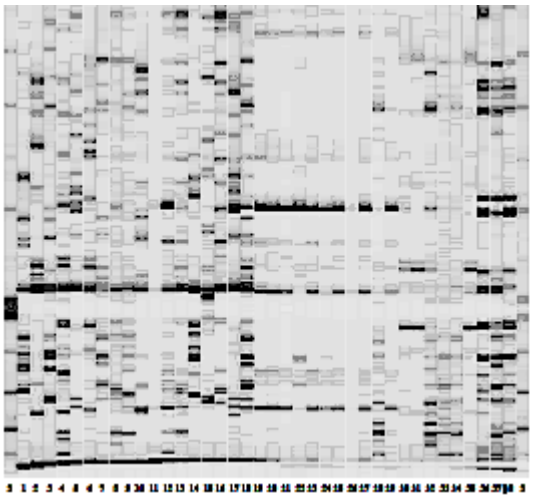


Fig 3. AFLP patterns of *Xanthomonas* strains using the E_A/M_0 primer combination. DNA templates were digested with *Eco*RI/*Mse*I .

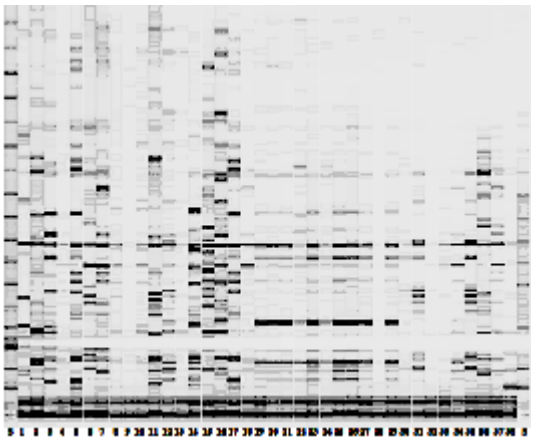


Fig 4. AFLP patterns of *Xanthomonas* strains using the E_C/M_0 primer combination. DNA templates were digested with *Eco*RI/*Mse*I .

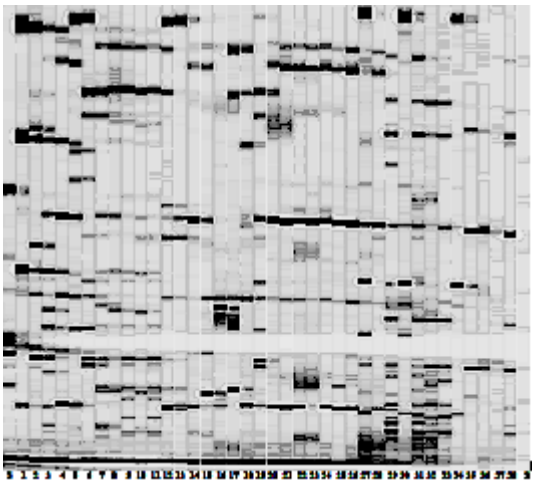
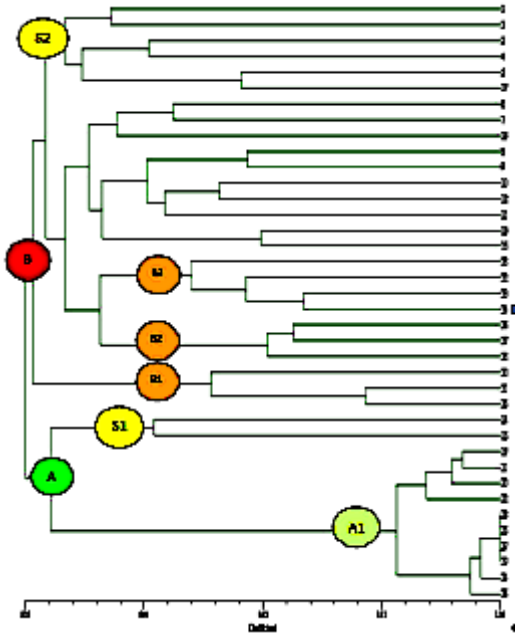


Fig 5. AFLP patterns of *Xanthomonas* strains using the E_A/M_C primer combination. DNA templates were digested with *Eco*RI/*Mse*I .



1- *X. arboricola* pv. *juglandis* LMG 747, 2- *X. axonopodis* pv. *phaseoli* var. *fuscans* LMG 837, 3- *X. axonopodis* pv. *ricini* LMG 861, 4- *X. axonopodis* pv. *dieffenbachiae* LMG 695, 5- *X. axonopodis* pv. *begoniae* LMG 7303, 6- *X. axonopodis* pv. *citri* LMG 7303, 7- *X. axonopodis* pv. *phaseoli* LMG 7455, 8- *X. axonopodis* pv. *alfalfae* LMG 497, 9- *X. axonopodis* pv. *axonopodis* LMG 538, 10- *X. axonopodis* pv. *vesicatoria* LMG 538, 11- *X. axonopodis* pv. *dieffenbachiae* LMG 7399, 12- *X. axonopodis* pv. *phyllanthi* LMG 844, 13- *X. axonopodis* pv. *malvacearum* LMG 761, 14- *X. campestris* pv. *raphani* LMG 7505, 15- *X. cucurbitae* LMG 690, 16- *X. vesicatoria* LMG 911, 17- *X. pisi* LMG 847, 18- *X. hortorum* pv. *pelargonii* LMG 7314, 19-22 strains from *Isotoma*, 23-27 +29 strains from *Lobelia*, 28 + 30-35 HV strains, 36-38 strains from *Catharanthus*.

Fig 6a. cluster analysis of AFLP fingerprints showing the similarity coefficient between the strains according to Dice (1945) using the UPGMA application by 4 primer combinations: *Eco*RI_C/*Mse*I_C, *Eco*RI_A/*Mse*I_0, *Eco*RI_A/*Mse*I_C and *Eco*RI_C/*Mse*I_0.

to the genus *Xanthomonas*, for which the name *Xanthomonas lobeliae* is proposed.

The HV strains and the strains from *Catharanthus* were closely related to the reference strains belonging to the species *axonopodis* (earlier *campestris*). Therefore, the HV strains can be classified and named as a new race of pv. *malvacearum* (*Xanthomonas axonopodis* pv. *malvacearum* race 20).

The newly isolated strains from *Catharanthus* showed to be a distinct group

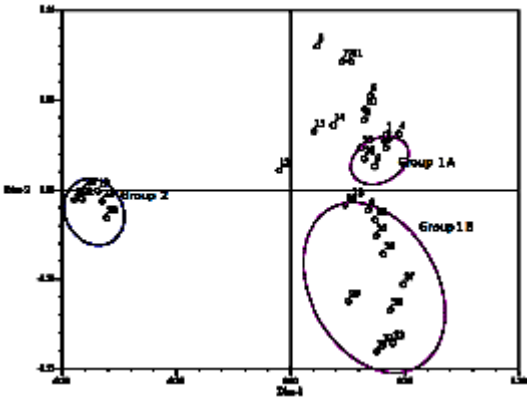


Fig 6b. Two dimensional principal coordinates plot based on AFLP fingerprints of 38 *Xanthomonas* strains showing the similarity coefficient between the tested strains by using 4 primer combinations, according to Dice (1945).

which is correlated to the strains of the pathovar *axonopodis* according to the results of AFLP fingerprinting showed in figs 6a, 6b. Therefore, the classification and name *Xanthomonas axonopodis* pv. *catharanthi* which was proposed by Mavridis *et al.* (2000) (although with the earlier species name *campestris*) can be confirmed.

Thus, Trébaol *et al.* (2000) identified a new bacterial species belonging to the genus *Xanthomonas* for which the name *Xanthomonas cynarae* was proposed by using polyphasic study including pathogenicity tests, DNA-DNA hybridization data, a numerical analysis of biochemical and physiological tests, G+C content and rRNA gene sequencing data.

In this context, AFLP should be seen as an ideal preliminary screening method for large numbers of isolates, with the ultimate confirmatory role reserved for DNA hybridization analysis, in which DNA-DNA pairing experi-ments to the other genomic species of Xanthomonads specially species of *campestris* can be more directed.

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