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Title: Detection of genetic diversity among Indian strains of *Xanthomonas campestris* pv. *mangiferaeindicae* using PCR-RAPD

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Short running title: Genetic diversity in *Xanthomonas campestris* pv. *Mangiferaeindicae*.

Key words: *Xanthomonas campestris* pv. *mangiferaeindicae*, variability, RAPD, MBCD Pathogen.

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Nature Precedings : hdl:10101/npre.2008.2403.1 : Posted 16 Oct 2008

Nature Precedings : hdl:10101/npre.2008.2403.1 : Posted 16 Oct 2008

Abstract

The randomly amplified polymorphic DNA (RAPD) technique was used to investigate the genetic diversity in 6 strains of *Xanthomonas campestris* pv. *mangiferaeindicae* (*Xcmi*), the causal pathogen of mango bacterial canker disease (MBCD). The RAPD analysis was also intended to identify molecular markers, specific to the species to develop PCR-based markers for detection of *Xcmi* in mango field and planting materials. Twenty RAPD primers (CP 1-CP 20) were evaluated to establish molecular characters and genetic variability in the genome of *Xcmi*. Among these, only 4 were found efficient for development of reproducible banding pattern. It has been observed that the largest and smallest amplified RAPD products were of 2.036 and 0.201 kbp. A total of 136 bands were scored against 6 strains of *Xcmi*. There was 7.66 per cent polymorphism in individual isolates which indicates significant polymorphism among the evaluated strains, with mean difference of 0.33 (*Xcmi* 2 *vs*. *Xcmi* 8) and 0.29 (*Xcmi* 10 *vs*. *Xcmi* 12). However, the single linkage euclidean distances were statistically significant ($P>0.05$), i.e., 0.58. The markers CP 5, 10, 16 and 19 were amplified in all the strains with polymorphic alleles, which indicates that these markers could be used for rapid detection of genetic variability in *Xcmi* strains.

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Introduction

Mango (*Mangifera indica* L.) suffers from a number of diseases caused by bacteria, fungi and other agents. The bacterial canker (black spot) incited by *Xanthomonas campestris* pv. *mangiferaeindicae* (*Xcmi*) is an endemic problem in the major mango-growing regions of the world, i.e., Asia, Southern and Eastern Africa, Western Oceania, and the Indian Ocean (CPC, 2005). Since last more than 2 decades the disease became important in India (Kishun, 1995). The pathogen affects all the above ground plant parts and results in substantial loss in fruit yield and quality (Shekhawat and Patel, 1975; Kishun, 1981). Lesions on leaves are angular, raised, black and necrotic, whereas on fruits these are star shaped and erumpent with an infectious gummy exudates. Occasionally, twig cankers can develop (Pruvost and Manicom, 1993). Bacterium survives mostly in lesions and on aerial parts of mango (Pruvost and Manicom, 1993; Pruvost and Luisetti, 1991) as well as epiphytes (Kishun and Chand, 1994). The pathogen is believed to spread between continents or countries through contaminated planting materials and on a smaller scale, by cultural practices and wind splashed rain (Manicom, 1986). Six *Xcmi* strains were selected from different geographical areas of mango genotype for detection of variability and classified as members of pathovar *mangiferaeindicae.* Intrapathovar diversity in *Xcmi* based on physiological and biochemical tests, antibiotics sensitivity and heavy metals, reaction towards bacteriophages, serological grouping, plasmid profiles and multilocus isozyme analysis are reported (Pruvost, 1989; Some and Samson, 1996; Kishun and Rajan, 2000). Though these techniques are useful for grouping strains for genus and species levels but limited in their utility to group strains at the pathovar level. Probably detection of variation based on genomic DNA may provide additional reliable tools to detect strain variability in *Xcmi*. One of the molecular techniques used for comparison of genome structure is restriction fragment length polymorphism

(RFLP) in *Xcmi* (Gagnevin, *et al.*, 1997), but it require several types of DNA probes for RFLP analysis. Genetic variations in isolates of different *Xanthomonas campestris* pathovars were also observed and measured using different molecular techniques such as RFLP and rep-PCR (Bragrad, *et al.*, 1995; Louws, *et al.*, 1995; Norman, *et al.*, 1999; Trindade, *et al.*, 2005). These techniques used either for detection of genetic variability in *Xcmi* and other *Xanthomonas campestris* pathovars have one or other limitations. Another technique, DNA-based markers is generally determined by the technology, used to reveal DNA-based polymorphism through PCR (Williams, *et al.,* 1990) with random primers can be used to amplify a set of randomly distributed loci in any genome and facilitated the applicability, i.e., the RAPD technique. Since this technique has not been used in case of *Xcmi* and may be reliable and efficient in differentiation of intrapathovar variation in Indian *Xcmi* strains, hence it has been attempted and results obtained are represented in this communication.

Materials and Methods

Collection of pathogen strains

MBCD infected plant samples were collected from different parts of India and isolations were made on nutrient agar medium. Cultures were purified by single colony transfer, proved pathogenic and designated as *Xcmi*. A total of six representive strains (*Xcmi* 2, 8, 10, 12, 20 & 22) from different mango cultivars/agroclimatic zones (Table A) were selected for molecular characterization.

DNA Extraction

An aliquot (1.5 ml) of bacterial suspension, grown for 48 hrs at 28 ± 1^{0}C, was washed with 550 µl TES buffer (50 mM Tris}HCl pH 7.8, 50 mM EDTA, 250 mM NaCl), centrifuged and resuspended in 400 µl of the same buffer. Fifty microlitres of SDS (20%, w/v) and 100 µg

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proteinase K were added and the mixture was incubated for 1 hr at 55 °C. Protein and cell debris were extracted with phenol and chloroform mixture (1:24) and the aqueous suspension dialysed against TE buffer (10 mM Tris HCl pH 7.8, 1 mM EDTA) for 48 hr. DNA concentration was estimated by comparison with known concentrations of Lamda DNA ladder in agarose gel electrophoresis (Sambrook, *et al*., 1989).

RAPD- Primers

Twenty oligodecamers CP 1- 5′ GGAGGGTGTT 3′ ; CP 2- 5′ ACAACGCCTC 3′ ; CP 3- 5′ GTAGACCCGT 3′ ; CP 4- 5′ GGACTGGAGT 3′ ; CP 5- 5′ GTTTCCCTCC 3′ ; CP 6- 5′ TCTGCTGAGC 3′ ; CP 7- 5′ GTGAGGCGTC 3′ ; CP 8 - 5′ AATCGGGCTG 3′ ; CP 9 - 5′ CCGCATCTAC 3′ ; CP 10- 5′ CATCCGTGCT 3′; CP 11- 5′ CAGGCCTTCA 3′; CP 12- 5′ TGCCGAGCTG 3′; CP 13- 5′ GGTCCCTGAC 3′; CP 14- 5′ GGGTAACCCC 3′; CP 15- 5′ AGGGGTCTTG 3′; CP 16- 5′ CTGGGCACGA 3′; CP 17- 5′ TTCCGCCACC 3′; CP 18- 5′ GGCGCAGTGT 3′; CP 19- 5′ GAGCGAGGCT 3′; CP 20- 5′ GATGACCGCC 3′ (Life Technologies, India) were used for RAPD marker studies.

Reactions and Conditions of RAPD-PCR

RAPD primer sets were used in Eppendrowf Master Cycler. RAPD-PCR was performed in 25 µl reaction volume containing 25 ng genomic DNA, 0.4 µl (5 pmole) primer, 1.5 µl dNTPs (25mM), 3µl of 10 X assay buffer with $MgCl_2$ (15mM), 0.5µl (3U/µl) of Taq DNA polymerase (Bangalore Genei Pvt.Ltd.). DNA was amplified by Eppendrowf Master Cycler programme to provide first denaturation for 5 min at 94^0C followed by 35 cycles of 1 min each at 94^0C and 35^0C followed by 2 min at 72^0C and final extension for 5 min at same temperature. PCR products were resolved by horizontal electrophoresis using agarose gel (1.2%) with TAE buffer (1%) containing ethidium bromide. Genetic variations like per cent polymorphism, similarity

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indices, phylogenetic distance etc. were calculated using statistical methods (Nei and Li, 1979). Comparative mean differences were also calculated by Newman-Keuls multiple comparison test using one way ANOVA.

Analysis of Molecular Data

Band positions for RAPD were visually determined and pair wise comparisons of degree of band sharing made. Similarity indices were calculated by the method (S.I. = 2Nab/Na+Nb) described earlier (Nei and Li, 1979). Dendogram was constructed by UPGMA (bootstrapped data using computer programme Phylip). The data was also subjected to principal component analysis (Manly, 1992).

Result and Discussion

Twenty RAPD primers (CP 1- CP 20) evaluated for detection of genetic variability in the genome of *X. campestris* pv. *mangiferaeindicae* (Fig. A-CP5, A-CP10, A-CP16 and A-CP19) and only four (CP 5, 10, 16 & 19) were able to produce reproducible banding pattern. The largest amplified RAPD products was 2.036 kbp, while the smallest was of 0.201 kbp. The primer CP 5 produced 39 bands with product size of 0.20 to 2.036 kbp and all showed a distinct profile but similar in at least one major band. Amplified fragments ranged from 0.396 to 2.036 kbp were obtained with CP 10 and having 23 bands. Similarly, other two primers CP 16 and CP 19 produced 32 and 42 bands with product size of 0.344 to 2.036 kbp and 0.201 to 2.036 kbp, respectively. These four primers produced a total of 136 bands, which were used to construct the dendogram based on a UPGMA algorithm (Fig.B). The numbers of scorable bands for corresponding primers ranged from 15 to 42 with an average of 34. Statistically significant ($P>0.05$) polymorphism (7.66 %) was also found in individual strains. Each representative *Xcmi*

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strain from the different agroclimatic zones/mango genotypes showed distinct pattern with similarity level of 50.18 per cent.

The average similarity indices based on 4 RAPD primers ranged from 0.5 to 1.0 and the distance matrices were 0.33 (*Xcmi* 2 *vs Xcmi* 8*)* and 0.29 (*Xcmi* 10 *vs. Xcmi* 12). The dendogram also revealed the similar difference among the six strains, but the single linkage euclidean distance (0.58) was statistically significant (P>0.05). It indicates that the evaluated *Xcmi* strains (6) are having less genetic difference and because of that falling in two different clads. First clad includes *Xcmi* 2 and 8, while second clad includes *Xcmi* 10, 12, 20 and 22 which further divided in 2 sub-clads showing intra cladal differences among the strains studied. The second clad having similar property comprises of strains almost of same groups (Fig.B) as obtained with principal component analysis where *Xcmi* 2 and 8 falls in one cluster, while *Xcmi* 10, 12, 20 and 22 in other (Fig.C). Thus, it can be concluded form the results that the 4 RAPD marker, i.e., CP 5, 10, 16 and 19 could be used as genetic variability markers for evaluated *Xcmi* strains.

Genetic analysis was an effort to understand the genomic structure of *Xcmi* population in India which will be helpful in selecting the efficient strain of the pathogen for screening mango gemplasm for resistance. The study showed that *Xcmi* population had a significant level of genetic diversity as it formed 2 clusters in phylogenetic tree or the PCA analysis. This result is contrary to work of Assigbetse *et al.* (1998), as they reported that RAPD analysis did not show high level of polymorphism within *Xanthomonas* strains used in their study. The RAPD technique have not been used in differentiation of *Xcmi* strains earlier. However, such work (PCR-RAPD) has been conducted in case of 55 *Xanthomonas campestris* pv. *passiflorae* strains (Goncalves and Rosato, 2000). The strains showed a high level of polymorphism with almost unique fingerprints. Fifteen clusters with a similarity of 70 per cent were identified, which

corroborates with present findings of 63 per cent similarity level among the strains of *Xanthomonas campestris* pv. *mangiferaeindicae.*

The RAPD pattern observed in this study is able to distinguish the minute difference differences within the same species of *Xcmi* strains. The quantitative estimate of genetic similarity obtained showed a significant level of genetic diversity. The *Xcmi* strains studied were not perfectly homogenous. Though RAPD has not been used in the evaluation of homogeneity among *Xcmi* strains but carbohydrate utilization profiles (Kishun, 1999) and *hrp*-RFLP study of Pruvost *et al*.(1998) showed almost similar trend. It emanates from present investigation that the molecular tools (PCR-RAPD) could be used for precise and reliable analysis of genetic variability in *Xcmi* strains. However, it is necessary to evaluate excising genetic biodiversity and detection of *Xcmi* strains present in India to determine the true genetic structure for formulating long term strategy so that the efficient strains could be used for resistant breeding programme against the pathogen.

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Acknowledgement

Authors are grateful to the Director, CISH, Lucknow for facilities, provided during the present investigations. The help of Shri P.K. Kulshrestha in the different experiments is thankfully acknowledged.

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Table A. Designation and source of *Xcmi* strains.

S. No.	Culture Designation	Source
1	*Xcmi* 1	Baig's Nursery, Sangareddy, A.P., India
2	*Xcmi* 2	Farmer's Field, Bazpur, U.P., India
3	*Xcmi* 3	IIHR, Bangalore, Karnataka, India
4	*Xcmi* 4	Nursery, CISH, Rahmankhera, Lucknow, U.P., India
5	*Xcmi* 5	Mango Field, Sitapur, U.P., India
6	*Xcmi* 6	Mango Garden, Chandigarh, India

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Fig A. Allelic pattern of *Xanthomonas campestris* pv. *mangiferaeindicae* strains using RAPD-PCR

M=1kb DNA Ladder (Gibco BRL)

L1-L6= *Xanthomonas camprestris* pv. *mangiferaeindicae* (*Xcmi*) strains

Fig B. Dendogram based on RAPD allelic pattern of *Xanthomonas campestris* pv. *mangiferaeindicae* strains

Fig C. Principal component analysis of *Xcmi* strains evaluated through RAPD.

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Fig. A-CP5

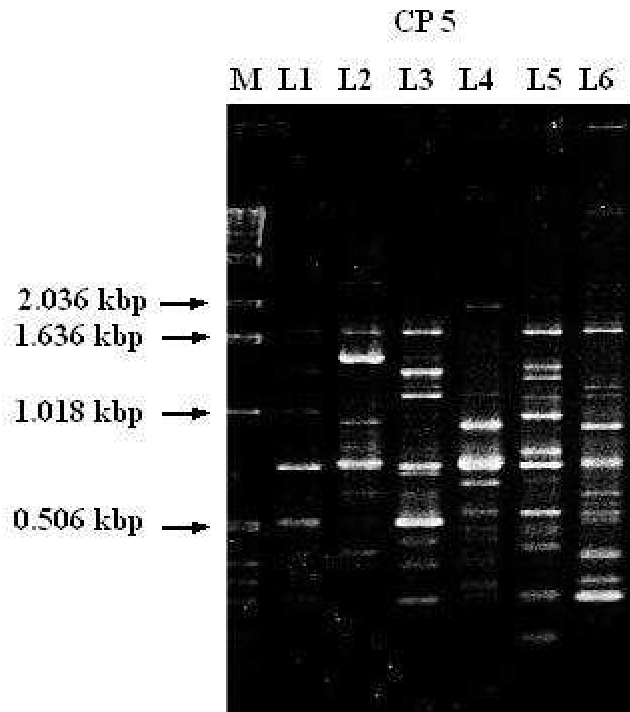

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Fig. A-CP10

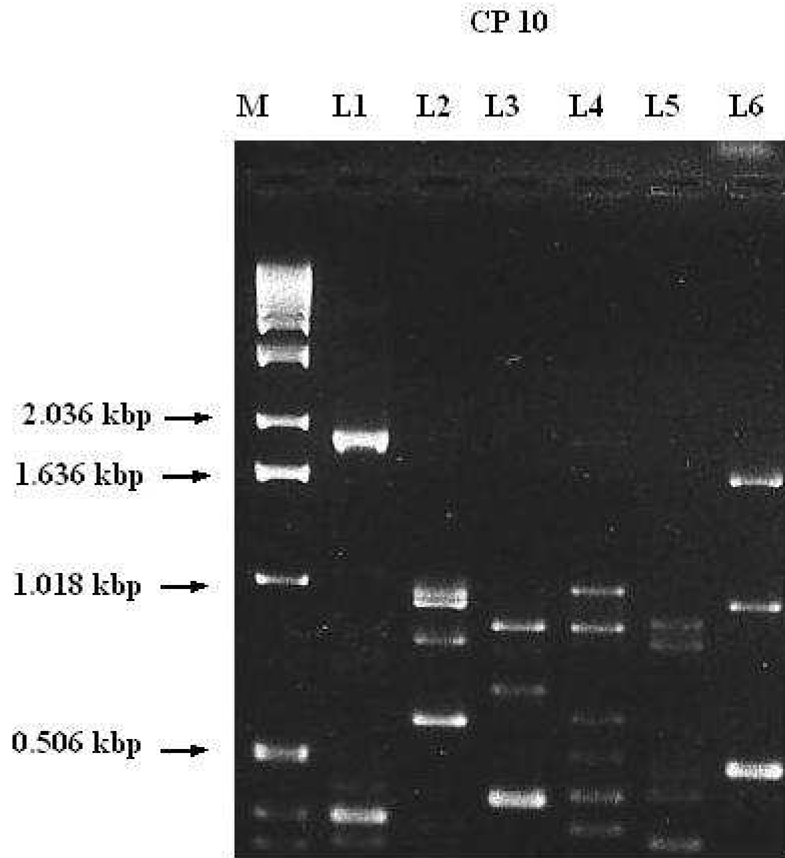

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Fig.A-CP16

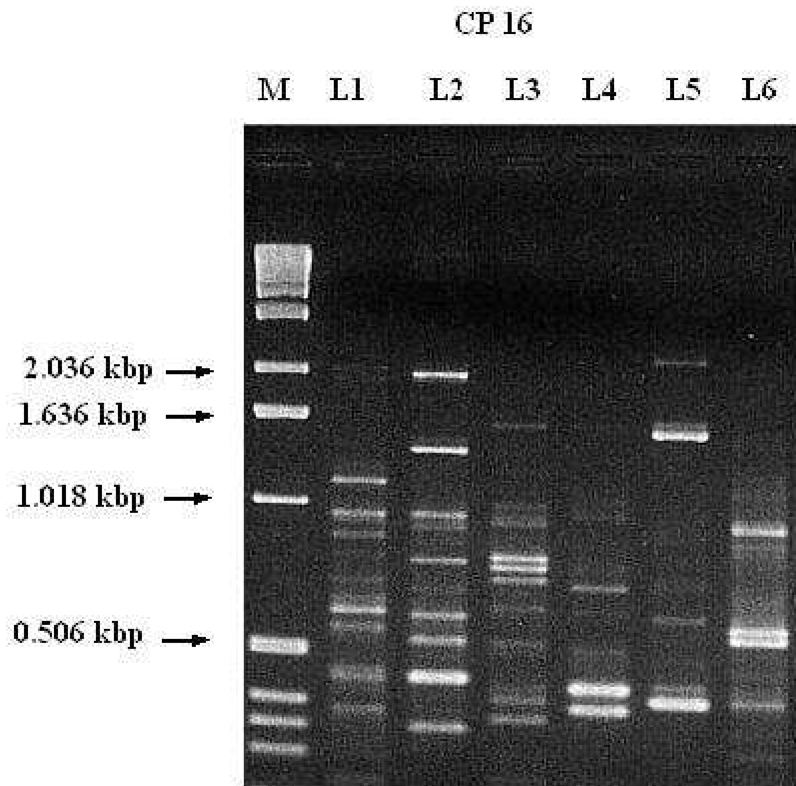

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Fig.A-CP19

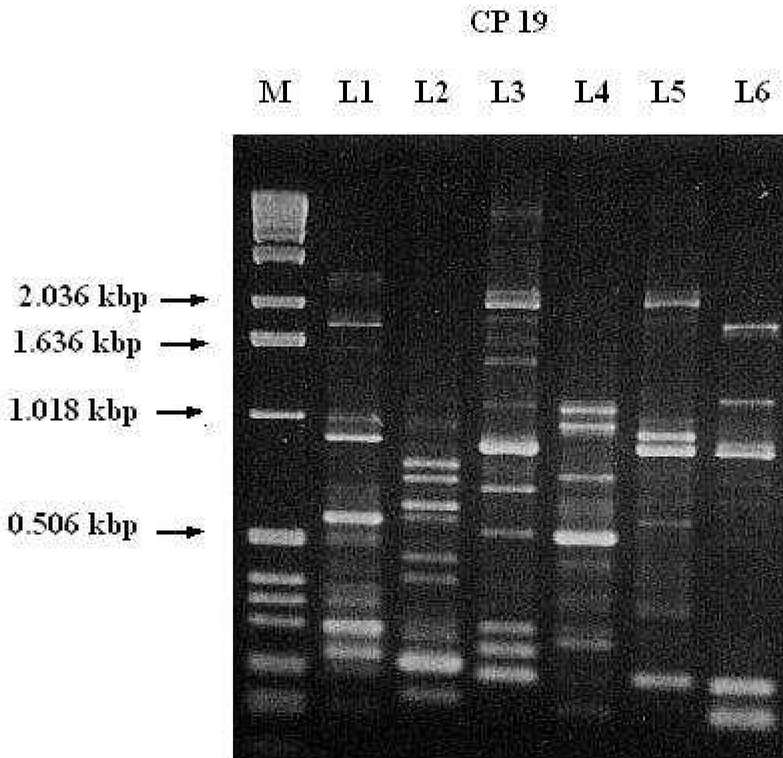

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Fig.B

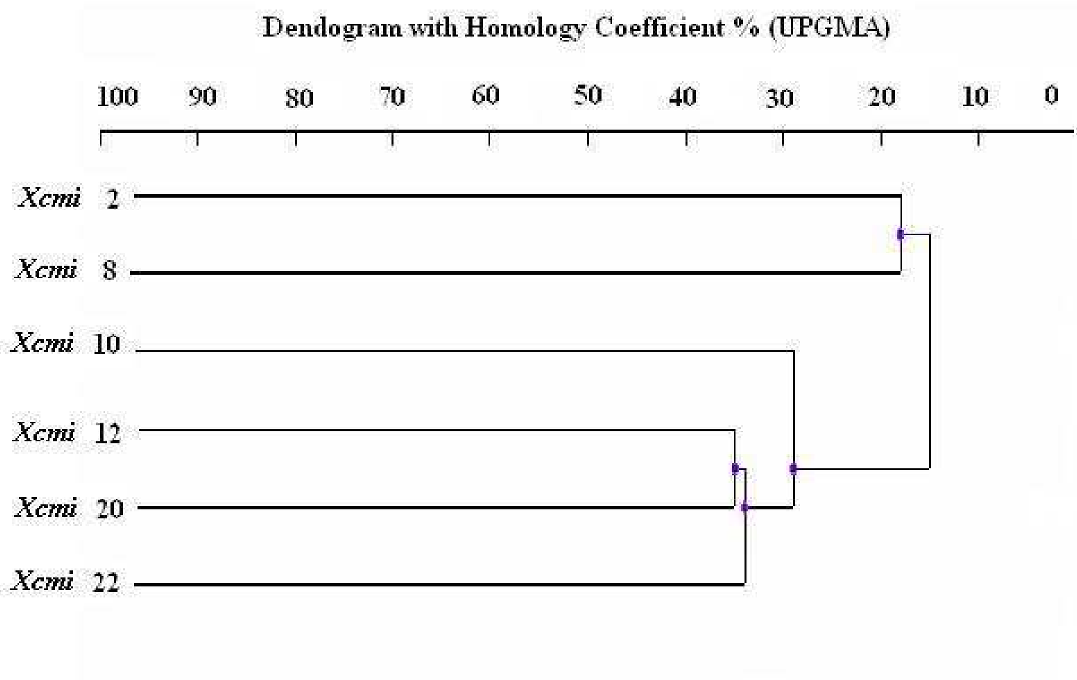

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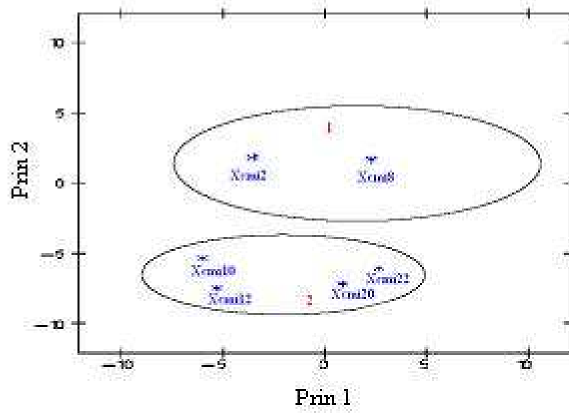
Fig. C



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