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Phylogeny Reconstruction of *Acetobacter* Species by RAPD (Random Amplified Polymorphic DNA) Markers

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Abstract

RAPD (Random amplified polymorphic DNA) analysis was performed to establish the phylogenetic relationship between *Acetobacter pasteurian* (2522), *Acetobacter xylinum* (NCIM 2526). Polymorphism was analyzed based on the dendrogram of RAPD patterns using UPGMA (Unweighed Pair GroupMethod with Arithmetic Mean). RAPD analysis in our study showed that there is a 80% similarity between these bacterial strains.

Key Words: RAPD, Phylogenetic relationship, *Acetobacter*, Polymorphism
Abbreviations: RAPD- Random amplified polymorphic DNA, PCR- polymerase chain Reaction, UPGMA - Unweighed Pair GroupMethod with Arithmetic Mean.

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Introduction

Acetobacter is Gram-negative, rod-shaped bacteria known for being a common plant pathogen. It is an obligatory aerobic, nitrogen-fixing bacterium that is known for producing acid as a result of metabolic processes [1](Flores *et al.,*1999) *Acetobacter sp.* are of particular importance commercially, because, they are used in the production of vinegar; they can destroy wine which it infects by producing excessive amounts of acetic acid or ethyl acetate, both of which can render the wine unpalatable; they are used to intentionally acidify beer during long maturation periods in the production of traditional Flemish Sour Ales; *Acetobacter xylinum* is the main source of microbial cellulose.

In the Acetobacteraceae family, *Acetobacter pasteurian* (2522) and *Acetobacter xylinum* (NCIM 2526) are very important. These two species, which are the subject of this study, differ significantly in morphology, physiology, behavior and genetics. It is important to understand the mechanisms that underlie these two phenotypes from the viewpoints of industrial vinegar production and basic microbiology. The molecular mechanism of ethanol oxidation has been extensively investigated in *Acetobacter* [2] (Adachi *et al.,* 1978). It has been demonstrated that RAPD-PCR may be useful for determination of taxonomic identity, establishment of systematic relationships and assessment of genetic differentiation of plants and animals including mammals [3] (Hadrys *et al., 1992*). Though reproducibility with RAPD markers is somewhat questionable, they are quite useful due to their simplicity, low cost and throughput capabilities [4] (Waugh and Powell , 1992)

Keeping the above background in mind, the present study is aimed at establishing the phylogenic relationship between *A. pasteurian* and *A. xylinum* through polymorphism analysis using RAPD markers and bioinformatics tools.

Materials and Methods

Culture and culture medium

Standard *Acetobacter pasteurian* (NCIM2522), *Acetobacter xylinum* (NCIM 2526) were obtained from the National Chemical Laboratory, Pune India. The culture was used as reference and subcultures were used as standard for the present study. Nutrient broth media was used for cultivation of *Acetobacter pasteurian* (NCIM2522), *Acetobacter xylinum* (NCIM 2526)The strains were routinely sub cultured and maintained in Nutrient agar media and were stored at 4°C in Nutrient agar slants as stock cultures. We also cultured the strains in different media to standardize its growth.

Isolation of bacterial DNA

1.5ml of the bacterial culture was taken in a sterile centrifuge tube and centrifuged at 10,000rpm for 10min at 4˚C. The supernatant was discarded, 1400µl of lysis buffer (50mM Tris-Hcl,25mM EDTA,250U of lysozyme/ml,pH-7.5) was added and vortexed to mix properly. The mixture was incubated at 69 ˚C for 70-80 minutes in water bath followed by incubation on ice for 5min. The mixture was centrifuged at 10000rpm for 10 min at 4˚C. The supernatant was transferred to fresh vials and double amount of chilled ethanol was added and mixed gently.This was inncubated overnight at -21˚C followed by centrifugation at 10000rpm for 10min at 4˚C. The supernatant was discarded and the remaining pellet was dried. 20µl of TE buffer (10 mM Tris-Hcl, 1mM EDTA, pH 8) was added to the dried pellet.

Random amplified polymorphic DNA (RAPD)

The procedure described by Williams *et al*., (1990) [5] with minor modification was done for carrying out PCR reaction to produce RAPD profiles. Amplification of DNA fragments was

carried out by the PCR using 10-mer arbitrary primers. The reaction mixture

consisted of 3 mM MgCl2, 100 µM each of dATP, dCTP, dGTP, dTTP, 0.2 µM primer, 15 ng of genomic DNA and 1 unit of Taq polymerase. to a total reaction volume of 25 µl performed in thermocycler with one cycle of initial denaturation 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, extension at 72°C for 2 min and with a final extension at 72°C for 5 min. Amplified products along with markers (Bangalore Genei) were resolved by gel electrophoresis on 1.2 % agarose gels in 1X TAE buffer containing 6 µl of 10 mg/mL ethidium bromide .

Data analysis

Comparison of each profile for each primer was done on the basis of the presence versus absence (1/0) of RAPD products of the same length. Bands of the same length were scored as identical. Analyses were based on the simple matching index,[6] (Sokal and Michener, 1958) which measures the proportion of common data (either 0 or 1) between the isolates. A dendrogram (Fig.1) was derived from the distance matrix by UPGMA (Unweighted Pair Group Method of Arithmetic means) [7] (Sneath and Sokal, 1973).

Results and Discussion

We screened seventeen RAPD primers (Table:1) to establish the phylogenetic relationship between *A. xylinum* and *A. pasteurian*. Since 16s rDNA cannot be used below strain level as it cannot resolve or establish the phylogenetic relationship between strains. RAPD is one of the powerful tools for studying genetic variations in living organisms as it amplifies segments of DNA which are essentially unknown. The limitations associated with pedigree data and morphological, physiological and cytological markers for assessing genetic diversity have largely been circumvented by the development of DNA markers such as restriction fragment length polymorphisms [8] (Botstein *et al.,* 1983), random amplified polymorphic DNAs [5] (Williams *et al.,* 1990). The RAPD technology is well suited to DNA fingerprinting [9](Thormann *et al.,* 1994) although it does suffer from a certain lack of reproducibility due to mismatch annealing [10] (Karp *et al.,* 1997). The exploration of RAPD (random amplified polymorphic DNA) as genetic markers has improved the effectiveness of r-DNA techniques. This method does not require DNA probes or prior sequences information. This method utilizes a single, arbitrarily primer to amplify a number of fragments for a given template of DNA to generate a discrete "fingerprints"when resolved by gel electrophoresis[11] (Danylchenko and Sorochinsky, 2005).

For the present study, we designed seventeen different primers based on the prior knowledge of polymorphism. We used seventeen 10mer primers whose sequences are known and we got amplifications only for seven primers whose names are genecity 11, genecity 12, genecity 13, genecity 14, genecity 15, genecity 16 and genecity 17. Based on amplification we generated a matrix (Table:1) which represents 1 for each loci and 0 for no loci. These loci were compared with the DNA marker, which ran along with RAPD-PCR products in the gel. The so generated matrix was used to generate the dendrograms (Fig 2 & Fig 3) by using MVSP (Multi Variate Statistical Package) software. Fig 2 shows the phylogenetic distance between *A. xylinum* and *A. pasteurian* based on polymorphism obtained in RAPD. The Jaccard's coefficient explains the polymorphism present between the microbes. Jaccard's coefficient (Fig.3) shows that *A. xylinum* is almost 25% identical for primer Genecity 11 & Genecity 14 and *A. pasteurian* is identical for Genecity 12 & Genecity 14 primers. Since these primers showed similarity for same organism it proved the similar type of polymorphism present in the organisms. Jaccard's coefficient also showed similarity between the two microbes for primer Genecity 15 and Genecity 16. It indicates that both strains have similar type of polymorphism and share a common evolutionary line. The polymorphism for various primers shows that these two strains belong to the same type of evolutionary line and represent 80%similarity.

Table 1: List of RAPD primers with name and sequences

Name of Primers	Sequence of Primers
Genecity 1	5'-TGT TGT CCA C-3'
Genecity 2	5'-CCT ACG GGG A-3'
Genecity 3	5'-AGG CTG TGC T-3'
Genecity 4	5'- GTG CCG TTC A-3'
Genecity 5	5'- GGG TGG GTA A-3'
Genecity 6	5'-CCG ACA AAC C-3'
Genecity 7	5'-TCA AGG GGA C-3'
Genecity 8	5'-TCC CCA TCA C-3'
Genecity 9	5'- CCA TGC GGA G-3'
Genecity 10	5'- CTG CTT CGA G-3'
Genecity 11	5' TGTGTATGGC 3'
Genecity 12	5' TGTACGGGGA 3'
Genecity 13	5' GGGCATTTGT 3'
Genecity 14	5' ATGTCTCTCA 3'
Genecity 15	5' ATGTGGGTTC 3'
Genecity 16	5' GCGTCAAACC 3'
Genecity 17	5' GGAAGGGGAT 3'

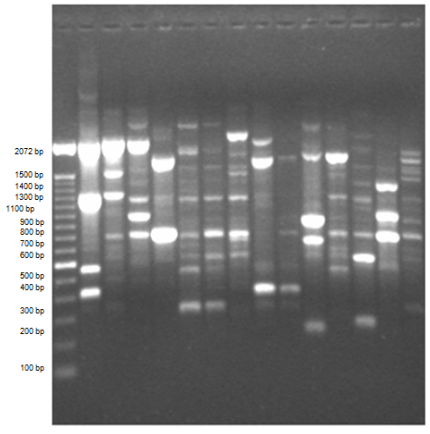

Figure 1 : RAPD-PCR product

Table 2: Matrix obtained from gel after RAPD-PCR by using ten different primers

Number of Loci	Number of samples													
1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
2	0	1	1	0	1	1	1	1	0	0	1	1	1	1
3	0	0	1	0	1	1	1	0	0	0	0	1	1	0
4	1	1	1	1	1	1	1	0	0	0	1	1	1	1
5	0	0	1	0	0	0	0	0	0	1	1	1	1	1
6	0	0	1	0	1	1	1	0	0	1	1	1	1	1
7	0	1	1	1	1	1	1	0	1	1	1	1	1	1
8	0	0	0	0	1	1	1	0	0	1	0	0	0	1
9	1	0	0	0	1	1	0	1	1	0	1	1	1	0
10	0	1	0	0	0	0	0	0	0	0	0	0	0	0
11	1	0	0	0	0	0	0	1	1	0	0	0	0	0
12	1	1	0	0	1	1	0	1	1	0	0	0	0	1
13	0	0	0	0	0	0	0	0	0	1	0	1	0	0

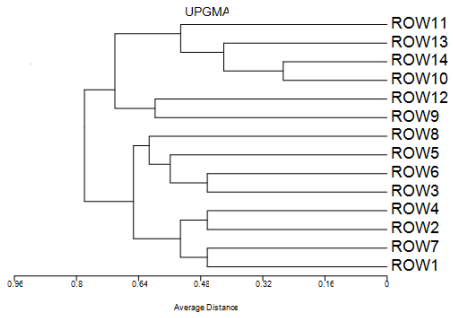

Figure 2 : Dendrogram obtained by MVSP software showing Average Distance

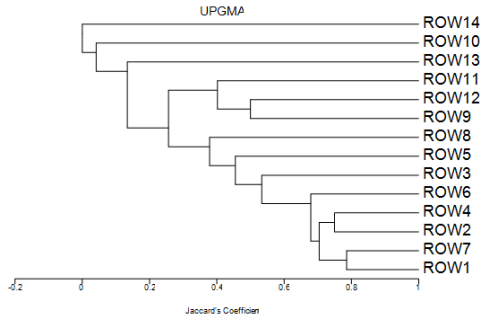

Figure 3 : Dendrogram obtained by MVSP software showing Jaccard's Coefficient

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