

MOLECULAR ANALYSIS OF *GANODERMA LUCIDUM* ISOLATES FROM LAHORE

GHAZALA NASIM*, MUHAMMAD ALI, AND NASIR MEHMOOD

Institute of Plant Pathology
University of the Punjab, Lahore-54590, Pakistan
Corresponding E-mail: ghazalanasim@hotmail.com

Abstract

Isolates of *Ganoderma lucidum* were collected randomly from the various regions of the Lahore and Changa Manga. The samples were brought to the Lab for their DNA isolation and quantification as well. Random amplified polymorphic DNA analysis was used to determine the % variability in the genomic profiles of randomly collected *Ganoderma lucidum* isolates from Lahore. For RAPD analysis three primers (5'ACCTGGACAC3', 5'GTATTGCCC3' and 5'GCTGTAGTGT3') were applied and designated as P1, P2 and P3 respectively. Only P1 was screened for the amplification of DNA fragments. Dendrogram of the RAPD analysis provided information of the genetic variability among the isolates.

Introduction

Ganoderma lucidum (Leyss. ex Fr.) Karst. (Ling Zhi) (Aphyllophorales, the family Polyporaceae) was first indexed in the Shen Nong's Materia Medica (206 BC–8 AD) as a longevity-promoting and tonic herb of the non-toxic superior class, and has been used in traditional Chinese medicine (TCM) for more than 2000 years to prevent and/or treat various human diseases such as hepatitis, chronic bronchitis, gastritis, tumor growth and immunological disorders. According to 'Fuzheng Guben', one of the major TCM therapeutic principles, *Ganoderma lucidum* (*Gl*) is capable of strengthening body resistance and improving constitutive homeostasis in patients (Lin, 2001). It was estimated that *G. lucidum* is used in TCM (Traditional Chinese medicine) for the treatment of cancers. In truth, the fungus must be one of the most convincing examples of ancient folk remedies being translated into new drug leads, and may represent an excellent paradigm for the general principal (Johnston, 2005). Molecular characteristics of *G. lucidum* are increasingly being used as additional taxonomic criteria in classification or to resolve controversies in taxonomic position of taxa. A wide range of molecular methods has been introduced, especially with the rapid development of polymerase chain reaction (PCR)-based techniques. Recently, a PCR-based method was developed for the identification of several decay fungi from the wood of broad leaved trees (Guglielmo *et al.*, 2007). Comparative analysis of coding and non-coding regions of ribosomal DNA has become a popular tool for construction of phylogenetic trees of many organisms including mushrooms. The internal transcribed spacers region is now perhaps the most widely sequenced DNA region in fungi. They are versatile genetic markers and have been used for phylogenetic analysis, evaluation of the evolutionary process, as well as for determination of taxonomic identities (Powers *et al.*, 1997). Karthikeyan *et al.*, (2009) stated that Molecular and immunological methods have been applied for detecting the *Ganoderma* disease of coconut. Polyclonal antibodies (PAb)s rose against basidiocarp protein of *Ganoderma* were used for detection. For polymerase chain reaction (PCR) test, the primer generated from the internal transcribed spacer region one (ITS 1) of ribosomal

DNA gene of *Ganoderma*, which produced a PCR product of 167 bp in size is used for early detection. The present study was focused to determine the percentage variability in the genomic profile of local *G. lucidum* isolates.

Material and Methods

Collection of *Ganoderma lucidum* isolates and DNA isolation:

Sample collection was done from within the premises of Punjab University campus as well as from places like Changa Manga and Allama Iqbal town (Fig. 1). The name of the host was also recorded at the time of sample collection. For this purpose mostly undisturbed areas were selected. Six samples of the *Ganoderma lucidum* were assigned code numbers as G-1, G-2, G-3, G-4, G-5 and G-6. The collection details are given in Table 1 & 2 and Fig. 2.

The total genomic DNA was extracted by cTAB method. Fungal tissues (100mg) were taken and grinded in pestle and mortar using liquid nitrogen. 2.5 ml of freshly prepared, prewarmed (65°C) extraction solution I (100 mM Tris HCl, 20 mM EDTA, 1.4 M NaCl, 2% cTAB and 0.2% β mercaptoethanol) was added to the powdered fungal mass. Samples were poured in 15ml culture tube and incubated at 65°C in water bath for 30 minutes. The samples were cooled on ice for 3-5 minutes; an equal volume of solution II (24 ml Chloroform, 1 ml Isoamylalcohol) was added. Vortex was done for 2 minutes to separate the phases and then it was dispensed into 4 eppendorf tubes (1.25ml in each). Centrifugation was done at 9000 rpm for 10 minutes at 4°C. Aqueous supernatant was transferred to new tubes and 2/3 volume of cold isopropanol was added to each and were incubated overnight at 4°C.

After 24 hours, the samples were again centrifuged at 9000 rpm for 10 min., and pellet was obtained. Pellet was washed with 70% ethanol, air dried and resuspended in 50 μ l of TE buffer and 1 μ l of RNase was added and incubated for one hour at 37°C to inactivate the RNA. After treatment with RNase the DNA was precipitated again with 100% ethanol and twice washed with 70% ethanol. After washing the extracted DNA sample was air dried at room temperature and resuspended in 25 μ l of T.E buffer.

Agarose gel electrophoresis: Agarose gel 0.7-1.2 % (w/v) was prepared by using 0.5 X TAE buffer (Tris HCl, EDTA). In the gel, 2 μ l Ethidium bromide (10mg/ml) was added to stain the gel as described by Heling *et al.*, (1997). The gel was placed in electrophoresis tank containing 500 ml of 0.5 X TAE buffer and DNA samples were loaded in gel wells after mixing with 0.2 volume of 6X DNA loading dye (Bromophenol blue, Xylene cyanol, Sucrose). The DNA fragments were separated on agarose gel by applying 100 volts for 1-2 hours. After running the samples on the gel, they were observed under UV transilluminator and results were documented by using gel documentation system.

Spectrophotometer quantification of DNA: DNA samples isolated from the fungal tissues were quantified by the means of spectrophotometric method (Table 3). 10-20 μ l of DNA of each sample was taken for analysis and DNA was quantified by means of absorbance at 260 nm by the measuring of optical density, following the procedure described by Hoinsington *et al.*, (1994).

RAPD analysis: In a preliminary test, selected random primers (5'ACCTGGACAC3'), (5'GTATTGCC3') and (5'GCTGTAGTGT3') were screened. The primers were ten bases long with 50% to 70% G + C content. From the test, the screened primers (5'ACCTGGACAC3') produced reproducible fragments. The primer was further checked to verify the reproducibility and consistency of RAPD banding patterns. Primer, which produced consistent patterns, was selected for RAPD analysis. After optimization of all the reagents, RAPD amplification was performed in a total volume of 25 µl reaction mixture which contained 2.5 mM MgCl₂, 1X PCR buffer [20 mM Tris-HCl (pH 8.4) and 50 mM KCl], 50 µM dNTP mix, 2.5 units *Taq* polymerase, 100 p mol of primer and 3 - 5 ng template DNA.

The RAPD amplification conditions were optimized by following method described by Williams *et al.*, (1990). Amplifications were performed in a TECHNE Thermalcycler (Model TC- 412) with the following general protocols: 4 min of initial denaturation at 94°C, 45 cycles of denaturation at 93°C for 30 seconds, annealing at variable T_m for 1 min and extension at 72°C for 1.5 min, and final extension at 72°C for 10 min., to ensure complete extension of the amplification products. Dendrogram was prepared by using SPSS software for the obtained bands of DNA which helped to check the similarity or variation among the collected samples of *Ganoderma lucidum*.

Results

The intact genomic DNA was isolated from six different isolates of *Ganoderma lucidum* by adopting cTAB DNA extraction method. The quantity of extracted genomic DNA was found satisfactory and determined by taking absorbance at 260 nm. The OD value was found to be in range of 120-170 µg/ µl.

The quality of extracted genomic DNA was determined by doing Agarose Gel Electrophoresis and the intact genomic DNA band at ~ 23 kb was observed (Fig. 3).

RAPD analysis: The RAPD amplifications were analyzed by doing 1 % Agarose Gel Electrophoresis. The amplification of different sizes by using primer P1 was observed on the Gel (Fig. 4). In all isolates except isolate "A" very prominent amplifications at 150 bp was observed. However, 225 bp, unique band was observed in isolate "A" only. In isolate "D" at 250 bp no amplification was observed as compare to the remaining five isolates. In isolates "B" and "C" sharp band at 325 bp were observed while in remaining all the isolates such band were not found. At 400 bp level.

Except isolate "A", in all the isolates amplification were observed. Similarly at 280 bp size in case of isolates "A" and "D" no amplification were observed while in remaining isolates prominent band at this level were quite clear.

At 500 bp level only isolates "B" and "C" showed amplification while in remaining isolates no such amplifications were observed. However, at size 590 bp and 700 bp the DNA band were only observed in isolates "A" while at these sizes no band were observed in rest of the isolates.

Isolates "B", "C" and "D" showed amplifications at 870 bp which were absent in isolates "A", "E" and "F". In isolate "A" and "E" 900 bp and 1100 bp amplifications were observed respectively while in remaining isolates no such amplifications were observed.

The dendrogram was constructed on the basis of pattern of amplifications in RAPD analysis (Fig. 5). In dendrogram analysis the highest similarity was found to be ~ 90 % in

case of isolates “B” and “C” while in case of isolates “A” and “E”, 68 % similarity was observed in the genome. However in case of isolates “F” and “D” the genomic diversity was quite evident in RAPD analysis and in dendrogram analysis due to unique pattern of amplifications in RAPD analysis.

Table 1. Collection details of *Ganoderma* isolates.

Sample code	Host tree species	Collection site	Date of collection
G-1	<i>Eucalyptus citriodora</i>	Quaid-e-Azam Campus, University of the Punjab, Lahore	15-03-2009
G-2	<i>Eucalyptus citriodora</i>	Quaid-e-Azam Campus, University of the Punjab, Lahore	15-03-2009
G-3	<i>Dalbergia sissoo</i>	Nishtar block, Allama Iqbal town, Lahore	27-03-2009
G-4	<i>Acacia arabica</i>	Changa Manga forest	07-04-2009
G-5	<i>Acacia arabica</i>	Changa Managa forest	07-04-2009
G-6	<i>Azadaracta indica</i>	Quaid-e-Azam Campus, University of the Punjab, Lahore	13-04-2009

Table 2. Summary of morpho-anatomical characters of *Ganoderma* isolates.

Sample code	Host tree species	Morpho-anatomical features of the basidiocarp					
		Size (cm)	Colour	Texture	growth rings	Growth pattern	Pore size (μm)
G-1	<i>Eucalyptus citriodora</i>	25-28	Brown	Leathery shiny	Fused	Single bracket	7-8
G-2	<i>Eucalyptus citriodora</i>	15-20	Light brown	Rough	Absent	Single bracket	5-6
G-3	<i>Dalbergia sissoo</i>	14-17	Yellowish brown	Shiny rough	Irregularly present	Storey form	5-7
G-4	<i>Acacia arabica</i>	15-18	Dull brown	Smooth dull	Absent	Storey form	8-9
G-5	<i>Acacia arabica</i>	20-25	Reddish brown	Rough	Absent	Storey form	6-7
G-6	<i>Azadaracta indica</i>	55-60	Yellowish to blackish brown	Rough	Fairly present	Single bracket	6-8

Table 3. Spectrophotometric quantification of DNA.

<i>G. lucidum</i> isolates	Absorbance at 260 nm	Quantity ($\mu\text{g}/\mu\text{l}$)
G-1	0.12	120
G-2	0.16	160
G-3	0.13	130
G-4	0.14	140
G-5	0.18	180
G-6	0.17	170

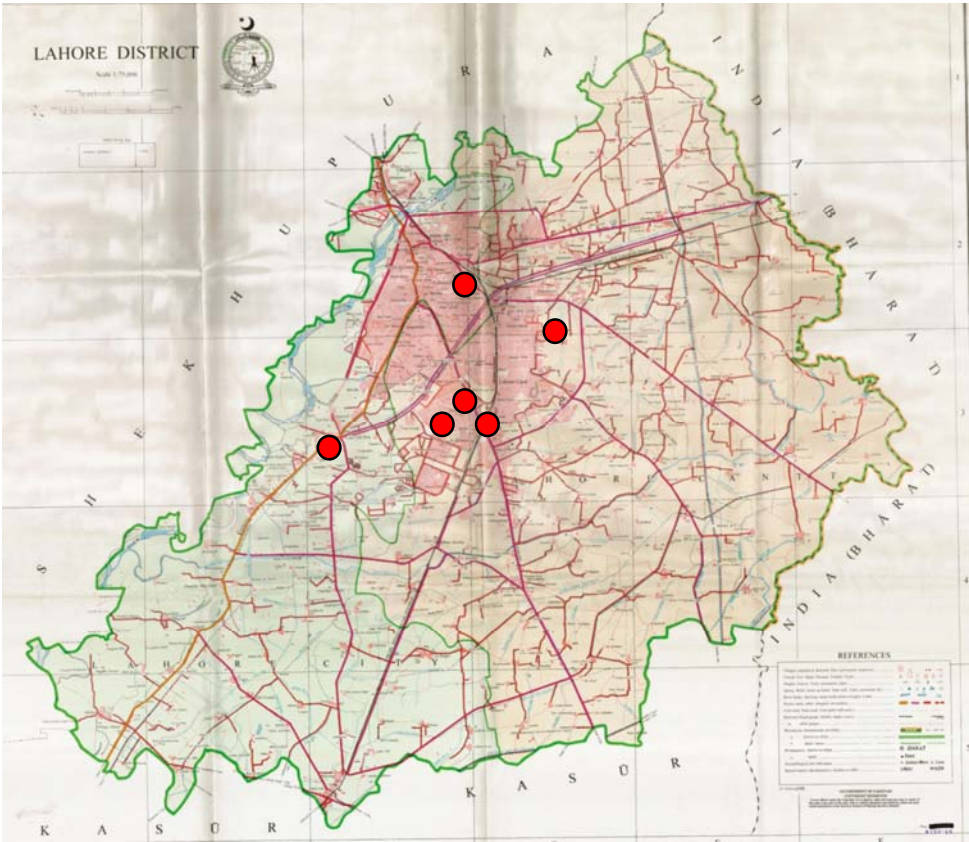


Fig. 1. Map of Lahore district showing the collection sites.

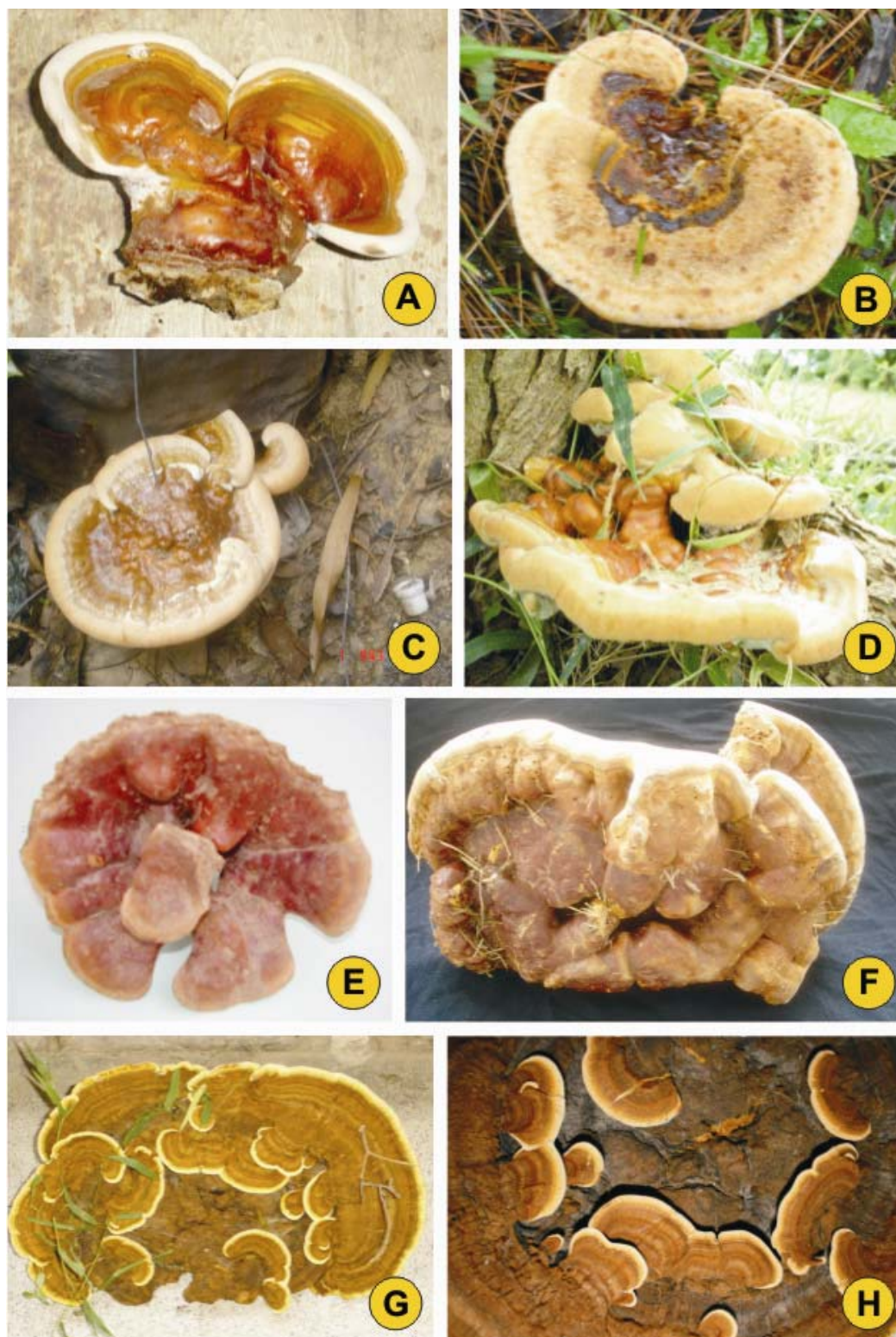


Fig. 2. **A: G-1** Growing on *Eucalyptus citriodora* collected from Quaid-e-Azam Campus, University of the Punjab, Lahore, Pakistan; **B: G-2** Growing on *Eucalyptus citriodora* collected from Quaid-e-Azam Campus, University of the Punjab, Lahore Pakistan; **C&D: G-3** Growing on *Dalbergia sissoo* collected from Nishtar Block, Allama Iqbal Town, Lahore, Pakistan; **E: Ganoderma** isolate **G-4** growing on *Acacia Arabica* collected from Changa Manga forest, Pakistan; **F: G-5** Growing on *Acacia arabica* collected from Changa Manga forest, Pakistan; **G & H: G-6** Growing on *Azadaracta indica* collected from Quaid-e-Azam Campus, University of the Punjab, Lahore, Pakistan.

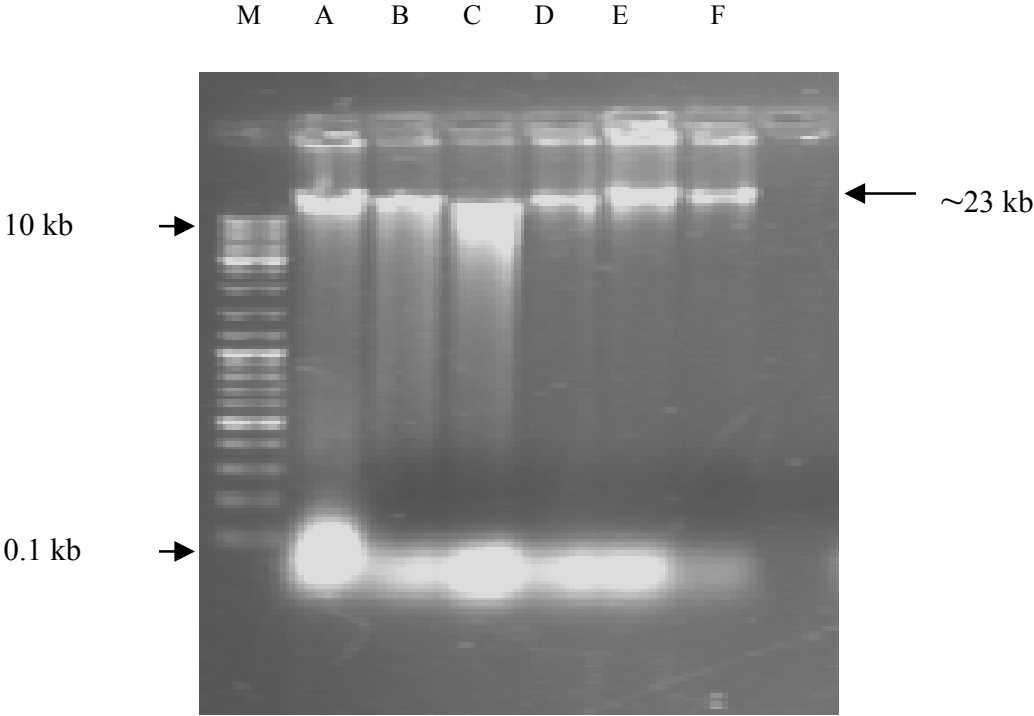


Fig. 3. DNA isolated from *G. lucidum* samples. M = Marker 1Kb; A = DNA isolated from G-1; B = DNA isolated from G-2; C = DNA isolated from G-3; D = DNA isolated from G-4; E = DNA isolated from G-5; F = DNA isolated from G-6

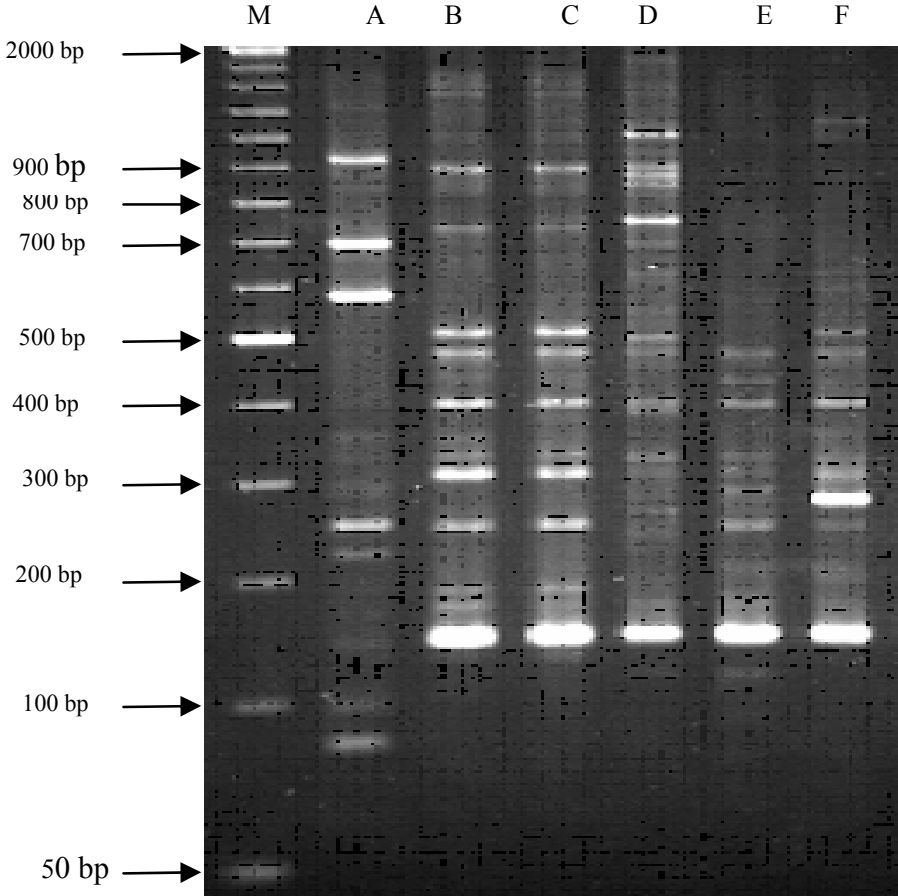


Fig. 4. Amplified DNA Fragments in RAPD Analysis.

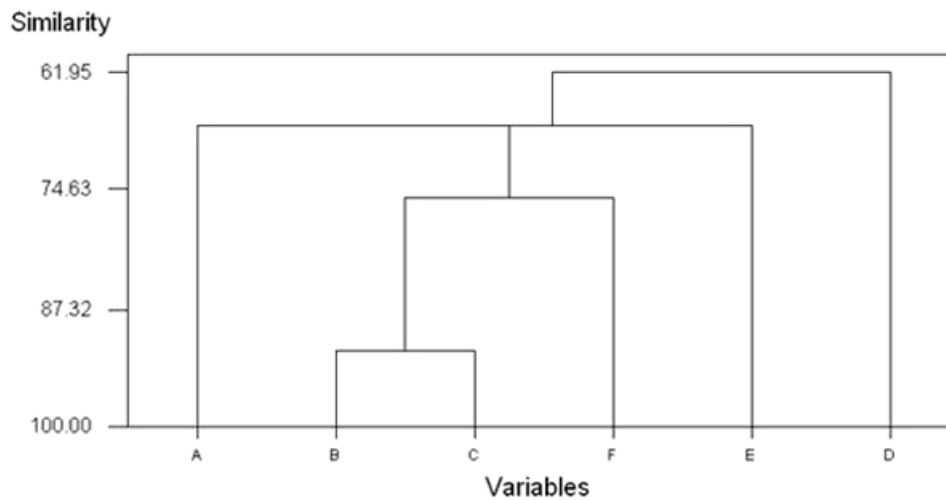


Fig. 5. Dendrogram showing the genomic % variations.

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