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RESEARCH ARTICLE

GENETIC IDENTIFICATION AND CHARACTERIZATION OF SOIL BACTERIA USING 16 S rDNA GENE SEQUENCE ANALYSIS

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Abstract

This work was undertaken with the aim to isolate, identify and characterize the bacteria from agricultural soil. The traditional method of bacterial species identification relies upon the phenotypic characteristics of biochemical testing and colonial morphology. Biochemical tests are simple to execute, require minimal equipment, and generally accurately differentiate between the more common species. However, they are time-consuming and present a delay to final identification due to long incubation times. Several genes common to all bacteria have been studied in sequence-based identification. The most well-known, the 16S rDNA gene, is considered a gold standard for identification for all bacteria and is widely recognized as a rapid and accurate method of identifying known and novel bacteria. Sequence identification can be completed as early as the next working day.

Fragment of 16S rDNA gene of the DNA of isolated bacteria was amplified by PCR . Forward and reverse DNA sequencing reaction of purified PCR amplicon was carried out with 16sF and 16sR primers. BLAST was performed and phylogenetic tree constructed using the sequences. The evolutionary distances were computed using the Kimura 2-parameter method and expressed in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA 4. 1447 nt contig region of sample D was homologous with sequence with Genbank accession no. AM396494.1, which was found to be *Acinetobacter sp. SS-2.*

Introduction

Traditional methods of bacterial identification have major drawbacks. First, they cannot be used for noncultivable organisms. Second, we are occasionally faced with organisms exhibiting biochemical characteristics that do not fit into patterns of any known genus and species. Third, identification of slow growing organisms would be extremely slow and difficult (Woo *et al.,* 2003).

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Several methods and approaches are now available to generate information on microorganisms that resides in soil, wherein molecular tools for the identification of microorganisms are now in common use, and 16S rDNA gene analysis is intensively used in phylogenetic investigations. Furthermore, 16S rDNA sequence typing approach now permits identification of the surviving and culturable bacterial species, based on employing highly conserved 16S rDNA oligonucleotide primers for the eubacteria with an intervening hypervariable gene sequence, which could be used as signature sequence to aid in species identification (Amann *et al*., 1995; Ward *et al*., 1990).

Bacteria classified as members of the genus *Acinetobacter* have suffered a long history of taxonomic changes. The genus *Acinetobacter* is now defined as including gram-negative coccobacilli, with a DNA G+C content of 39 to 47 mol%, that are strictly aerobic, nonmotile, catalase positive and oxidase negative (Bergogne-Bérézin and Towner 1996; Koneman *et al*., 1992)

Acinetobacter is a Gram-negative genus of bacteria belonging to the Gamma-proteobacteria (Vanbroekhoven *et al*., 2004). They are important soil organisms where they contribute to the mineralization of, for example, aromatic compounds (Hongjuan *et al*., 2011).Our knowledge on the ecology and epidemiology of many *Acinetobacter* species is limited, mostly due to the lack of accurate methods for routine identification of *Acinetobacter* isolates to the species level (Gundi *et al*., 2009). While phenotypic identification of *Acinetobacter* species has been found insufficient, (Chen *et al.,* 2007) several molecular methods have been shown to be adequate for this purpose.(Dijkshoorn *et al*., 2007)

The objective of this present study was to characterize the bacterial flora isolated from agricultural soil using molecular approach.

MATERIALS AND METHODS

Collection of soil sample, bacterial isolation and biochemical characterization

Field soil sample was collected in polythene bags from the surface layer (0-15cm) of the agricultural land of ICAR, Research Complex, Eastern Region, Palandu, Ranchi, as per the method of (Dutta *et al*., 2010). Bacteria was isolated from this by spread plate technique (Ahmed and Ahmed 2006), pure colony streaked out, subcultured on Nutrient Agar and labelled as sample "D". Morphological characteristics were studied and culture was gram stained. Different biochemical tests were performed by methodologies as described (Green and Bousfield, 1982) for further identification.

Molecular characterization of the isolated sample

DNA was isolated from the pure culture (marked as "D") as per the protocol of (Van *et al.,* 1997), which included mechanical lysis of cells, phenol and chloroform extractions, a potassium acetate precipitation. Its quality was evaluated on 1.2% Agarose gel (Peixoto *et al*., 2002) .The 16S rDNA gene was amplified as per the method of (Li *et al*, 2004) using universal primer and PCR product for the isolate was sequenced in both directions. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16sF and 16sR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer and chromatogram was plot using Chromas Lite 2.0 version Applied Biosystems and Amersham MegaBace automated sequencers. The 16S rDNA gene sequence was analysed using BLAST with nrdatabase of NCBI GenBank database to find closely related bacterial 16S rDNA sequences. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed according to Neighbour-joining method (Saitou and Nei, 1987) using MEGA 4.0 Molecular Evolutionary Genetics Analysis software version 4 (Tamura *et al*., 2007) for studying the evolutionary relationship. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

RESULTS AND DISCUSSION

Colony marked, "D" was circular, low convex, with entire margin, pale yellow to white in colour and slightly opaque (Table 1.). It showed negative reaction for gram stain , and was found to be related as *Acinetobacter sp*. as confirmed by the findings of (Constantiniu *et al*., 2004) and (Li *et al*., 2011). Similar results were obtained by (Nemec *et al*., 2003) for morphological characterization of *Acinetobacter* colonies.

Further biochemical characteristics were studied and it was observed that bacterial isolate was identified as *Acinetobacter* as shown in (Table 2.). In biochemical characterization, *Acinetobacter* showed negative result for oxidase test and VP test (Table 2.) which is favoured by similar observations of (Constantiniu *et al*., 2004) who stated that *Acinetobacter* does not produces oxidase but produces catalase and thus was

catalase positive as in our case also. This is also in agreement with the reports of (Li *et al*., 2011) who carried out several biochemical tests on *Acinetobacter sp*. and found same results except for the nitrate reduction test, which is in contrary to our findings; which may be explained due to difference at species level.

These morphological and biochemical properties of strain were consistent with bacteria of the genus *Acinetobacter*.

On quality evaluation of all isolated DNA, a single band of high molecular weight DNA was observed on 1.2% Agarose Gel . A single discrete PCR amplicon band of 1500bp of 16S rDNA was observed when resolved on agarose Gel (Kimura, 1980) as in (fig. 1).Similar results were obtained by (Orengo *et al*., 2010),on amplification of 16S rDNA using universal primer an amplicon band of 1.5kb was obtained on electrophoresis. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 1437 positions in the final dataset of sample "D". Table 3. and Table 4. describes the significant alignments produced and distance matrix respectively. BLAST (fig. 3) was carried out with the nrdatabase of NCBI GenBank database. These were homologous to sequences with GenBank Accession No:- AM396494.1. Chromatogram (fig 2.) gives the detailed gene sequence of the sample obtained. Based on nucleotide homology and phylogenetic analysis, (Gurtner *et al*., 2001) sample marked as "D" was found to be *Acinetobacter sp. SS-2*.

A phylogenetic tree or evolutionary tree is a branching diagram or tree showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics. Fig. 4. shows the phylogenetic tree of sample "D" with the ten most similar sequences.

Similar results were obtained by (Ibrahim *et al*., 1997) while carrying out identification of *Acinetobacter* by 16 S rDNA technique. The taxa joined together in the tree are implied to have descended from a common ancestor. Unrooted trees illustrate the relatedness of the leaf nodes without making assumptions about ancestry at all. In the case of unrooted trees, branching relationships between taxa are specified by the way they are connected to each other, but the position of the common ancestor is not (Maher, 2002).

Table 1. Morphological details of the isolated bacterial sample labelled as "D"

Sample Labeled as	Isolated organism	Shape of colony	Elevation	Edge/ Margin	Colour	Grams stains	Cell type
D	*Acinetobacter sp. SS-2*	Circular, smooth	Low convex	entire	Pale yellow to white	Negative	Small rods

Table 2. Biochemical characteristics of the bacterial isolate marked "D"

Isolated organisms	Voges Proskauer	Glucose fermentation	Nitrate reduction	Catalase	Oxidase	Motility
Acinetobacter sp. SS-2	-	+	-	+	-	-

Table 3 : Sequence Producing Significant Alignments (Sample "D")

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM184280.1	Acinetobacter sp. WAB1941	2660	2660	100%	0.0	99%
AM184273.1	Acinetobacter sp. WAB1934	2660	2660	100%	0.0	99%
EU337121.1	Acinetobacter johnsonii strain 3B2	2658	2658	100%	0.0	99%
GU570643.1	Acinetobacter sp. enrichment culture clone B1-5	2652	2652	100%	0.0	99%
AM396494.1	Acinetobacter sp. SS-2	2647	2647	100%	0.0	99%
FJ193151.1	Uncultured Acinetobacter sp. clone GI6-10b-C07	2645	2645	100%	0.0	99%
FJ193024.1	Uncultured Acinetobacter sp. clone GI5-002-G04	2645	2645	100%	0.0	99%
FJ192815.1	Uncultured Acinetobacter sp. clone GI5-007-G03	2645	2645	100%	0.0	99%
FJ192797.1	Uncultured Acinetobacter sp. clone GI5-007-E05	2645	2645	100%	0.0	99%
FJ192775.1	Uncultured Acinetobacter sp. clone GI5-007-B09	2645	2645	100%	0.0	99%

Table 4 : Distance Matrix (Sample "D")

Sample-D	1		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
AM184280.1	2	0.001		0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
AM184273.1	3	0.001	0.000		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
EU337121.1	4	0.001	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001	0.001
GU570643.1	5	0.001	0.001	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001
AM396494.1	6	0.002	0.003	0.003	0.003	0.003		0.002	0.002	0.002	0.002	0.001
FJ193151.1	7	0.001	0.001	0.001	0.002	0.002	0.003		0.001	0.001	0.001	0.001
FJ193024.1	8	0.001	0.001	0.001	0.002	0.002	0.003	0.001		0.001	0.001	0.001
FJ192815.1	9	0.001	0.001	0.001	0.002	0.002	0.003	0.001	0.001		0.001	0.001
FJ192797.1	10	0.001	0.002	0.002	0.002	0.002	0.003	0.003	0.003	0.003		0.001
FJ192775.1	11	0.001	0.000	0.000	0.001	0.001	0.003	0.001	0.001	0.001	0.002	

Fig. 1 : Gel Image of 16SrDNA amplicon of Sample "D"

Lane 1: 16S rDNA amplicon band

Lane 2: DNA marker

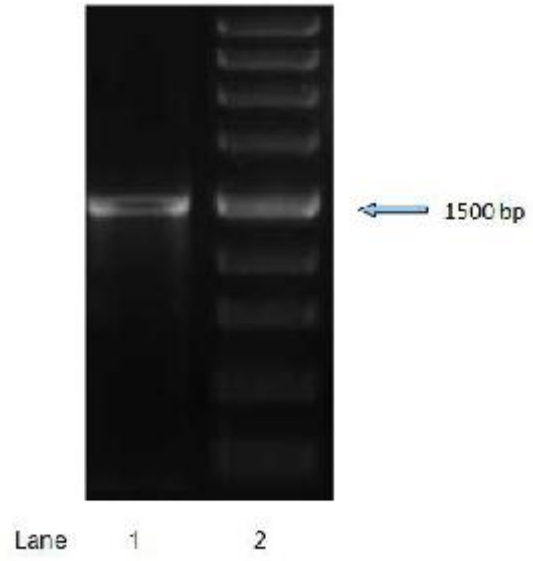


Fig. 2 : Chromatogram plot of *Acinetobacter sp. SS-2*

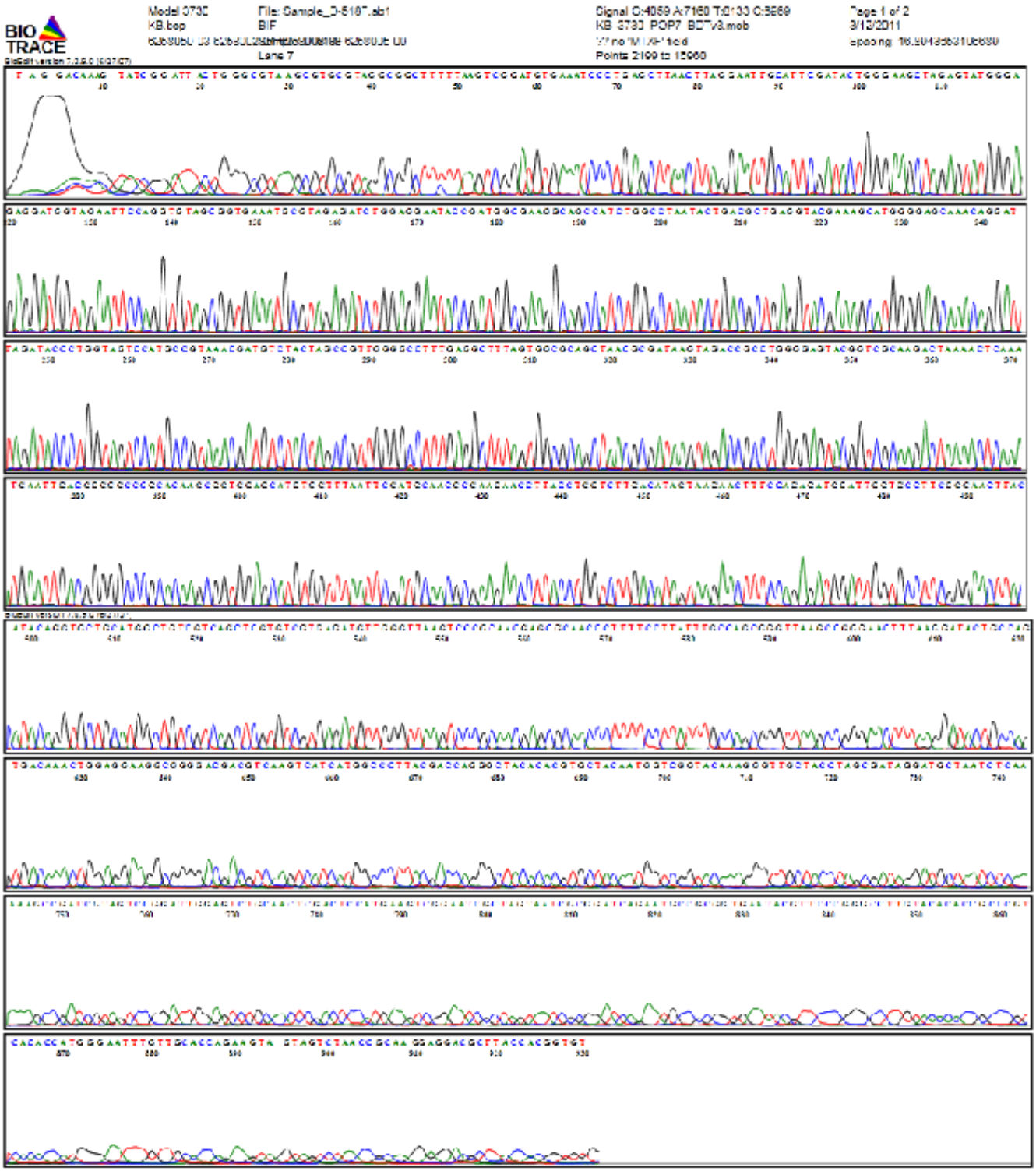


Fig. 3 : BLAST data for sample "D" : (Alignment view using combination of NCBI GenBank)

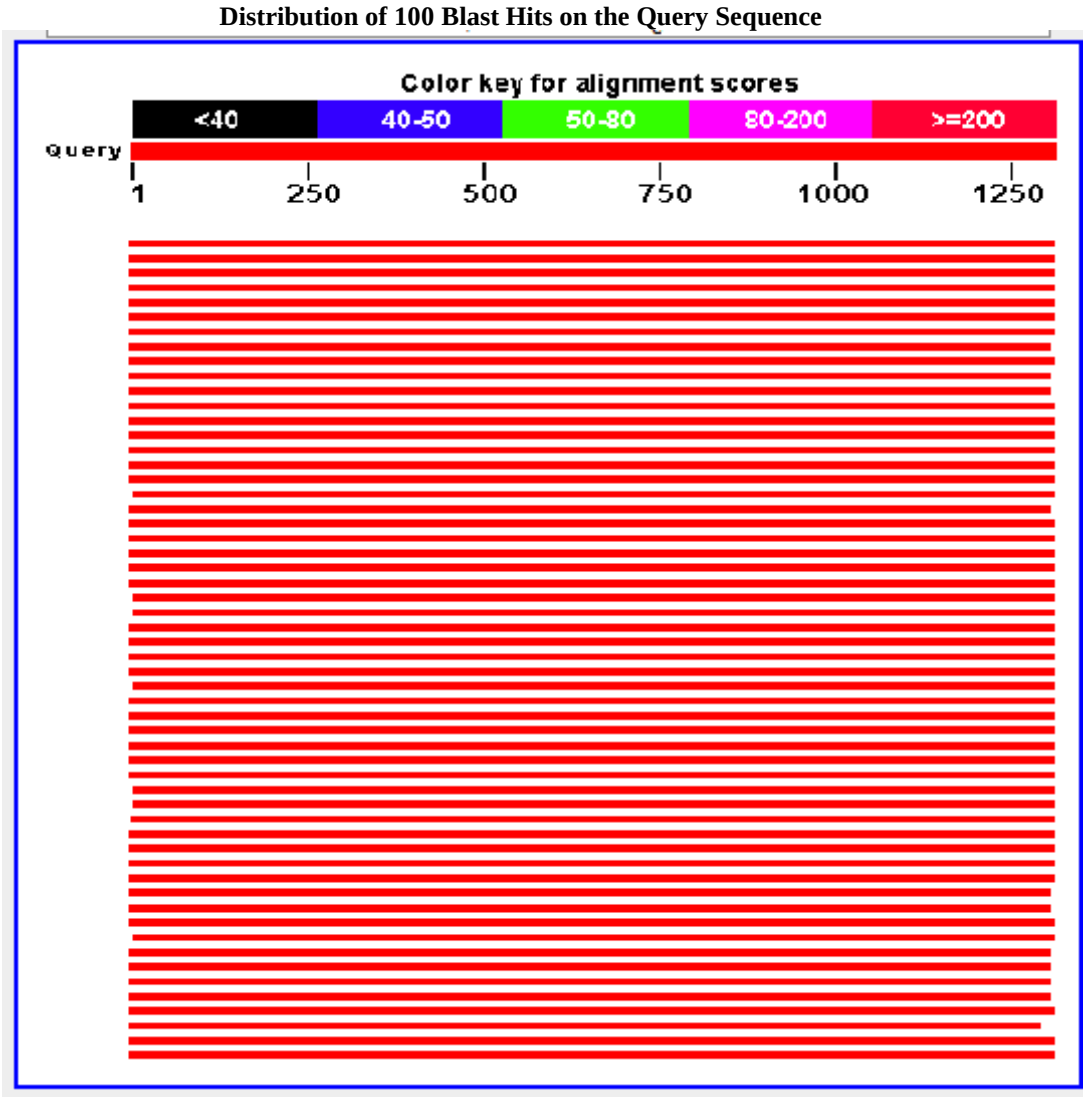
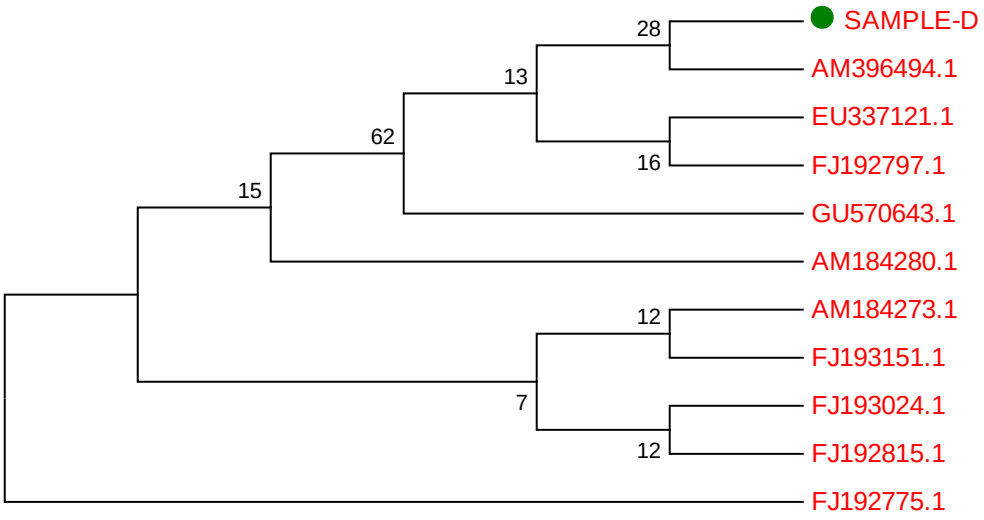


Fig. 4 : Phylogenetic Tree : Evolutionary relationships of 11 taxa including sample "D"



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