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Phylogenetic Analysis of Endophytic Bacteria from Nakshtra Trees

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A B S T R A C T

Endophytic bacteria were inhabitant microflora of plant part. This association was presumably long term and they were mostly commensalisms. The microorganisms which were living within the plant cell without causing any harmful effect on the plant tissue. These bacteria were secrets the plant growth promoting hormones like indole acetic acid which helps for growth of plant. In the present study the endophytic bacteria was isolated from medicinal plants of nakshtra udyan and screened out for the different characteristics of these microorganisms. The isolates were identified by 16s r DNA technique and the phylogenetic analysis was done for the 96 isolates from Nakshtra Udyan plant. The isolates were grouped into 23 different types of group of species and subspecies of microorganisms in which *Bacillus species* are more predominant than *Pseudomonas sp, Enterobacter sp, Alcaligene spp, Arthrobacter sp, Micrococcus sp, Klebsiella pneumoniae and kocuria sp.* These bacteria were important for different properties like antimicrobial peptides, plant growth promoting hormones and used as biocontrol agent.

Introduction

A niche environment previously untapped has been explored for study of bacterial population within leaf portions of huge medicinal trees. This study has yielded into isolation of a few peculiarly characterized as endophytic bacteria hosting an unique culture collection at VSBT. This culture collection of endophytic bacteria has yielded into phylogenetic patterns.

They are known to secrete different types of secondary metabolites like antimicrobial peptides, growth promoting hormones and bioactive compounds which are used as biocontrol agent, in pharmacology used as antimicrobial drugs preparation, in food industry and agriculture (Dhanya *et al.*, 2013 Susheel kumar *et al.*, 2013). These endophytic bacteria play an important role for improvement in growth and health of plant and it has several mechanisms for it (Taghavi *et al.*, 2009). Endophytes may directly produce chemical defense in plants through the production of secondary compounds which inhibit insects and pathogenic organisms. The *in vitro* secretion of substances by endophytes that limit the growth of other microbial species, including pathogens (Vania Specian *et al.*, 2012). The many of endophytes re poorly investigated group of microorganisms that produces secondary metabolites which is used in modern medicine and about 40% of the prescription of drugs are based on them

(Shukla *et al.,* 2014). The endophytic bacteria produces Indole Acetic acid (IAA) (Pedraza *et al.,* 2004), cytokinin (Ergun *et al.,* 2002) and Gibberellic acid (GA) (Kharwar *et al.,* 2008) which are required for the plant growth. In the 70's, endophytes were initially considered neutral, neither causing benefits nor showing detrimental influence on plants, but from the results of more recent studies it has been possible to show that in many cases, they have an important role in host protection against pathogens. Several studies have now shown that the interaction between plants and some endophytic bacteria is associated with beneficial effects such as plant growth promotion and biocontrol potential against plant pathogens (Lalande *et al*., 1989; Bashan *et al*., 1990; Chen *et al*., 1995; Hallmann *et al*., 1997).

Materials and Methods

Selection of medicinal plants and Explants collection

For the isolation of bacteria the explants were collected from different parts of medicinal plants from Nakshtra Udyan which located at Vidya Pratishthan's school of biotechnology. The cultivation of these plants was strictly maintained in organic package and the age of plants was fourteen years.

Pretreatment and surface sterilization of explants

The explants used for the isolation were leaves, stem and root of the plants. The collected explants was brought to the laboratory and washed under running tap water. After this these were thoroughly washed with distilled water. Surface sterilization protocol was standardized which contained surface sterilizing agents like 1% phenolic compounds containing solution for 5 min followed by 0.1% sodium hypochlorite treatment for 5 min. Afterwards the explants was washed with sterile distilled water.

Isolation of endophytic bacteria

The samples were aseptically ground in a motor and pestle in potassium dihydrogen phosphate buffer (pH 6.8) and inoculated into sterile nutrient broth medium with negative and positive control i.e. sterilized medium without inoculation of explants and uncrushed surface sterilized explants. The broth was incubated for 24 hrs at 30^0C on rotary shaker incubator at 120 rpm. The grown culture was plated onto sterile nutrient agar plates. The plates were incubated at 37C for 24 hrs. The isolated bacteria were plated onto selective medium after their morphological, biochemical and molecular identification and preserve it by lyophilization.

Biochemical characterization of isolates

The standard tests for the characterization were done according to the Beregey's Mannual of Determinative Bacteriology. The isolates were characterized for colony characterization which includes size, shape, color, consistency, opacity, Gram's nature, Capsule staining and presence of endospores. The biochemical characterization was done for IMViC test, starch hydrolysis, gelatin liquefaction, and different sources of carbon utilization, Oxidase and catalase tests by standard methods.

Molecular Characterization of isolates

The isolated strains were identified by using 16 s r DNA techniques. The genomic DNA of endophytic bacteria was isolated by using

CTAB method. The amplification of template DNA was done by using universal primes, R1 forward (5'AGTTTGAT CCTGGCTCAG 3') and R2 reverse (5' GGACTACCAGGGTATCTAAT3'). The 50 ul PCR reaction contains $Mgcl_2$ (0.45mM), dNTPs (0.2mM), forward primer (10 pmol), reverse primer (10pmol), Taq polymerase (0.5U) having 10X assay buffer (1X), genomic DNA(1 ug/ul) and sterile MilliQ water is used. Amplification of DNA was done in automated thermocycle machine provided by applied biosystem and product was checked on 1% agarose gel. The gel was eluted by using SIGMA gel elution kit.

The sequencing reaction was carried out in 3130 genetic analyzer at VSBT. The BLAST of the sequences was done for sequences of bacteria to NCBI GeneBank.

Phylogenetic tree analysis

The phylogenetic tree was constructing by using MEGA software. The neighbor-joining method was used to construct the phylogenetic tree. The bootstrap resampling test with 100 replications was also applied.

Results and Discussion

These observation form one of the most significant and comparable account of endophytic bacteria in tropical tree species (Table.1). This high through put isolation followed by classification and identification of endophytic bacteria using biochemical and 16 s rDNA techniques has culminated into reliable unique culture collection center at our center. Biochemical preferences of these bacteria has shown acetate as preferred carbon source only to put forth a heavy duty of acetate scavenging function in tree species in longest longitude. The phylogeny study specifies an almost ubiquitous presence of *Bacillus sp*. Among 96 endophytic bacterial isolates 34 were from the same group i.e. *Bacillus sp.* (Table.2).

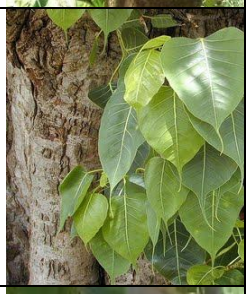
Table.1 Plants used for isolation of endophytic bacteria.

Sr. No.	Name of the plant	Botanical Name	Plant family	Image of plant
1	Adulsa	*Adhatoda vasica*	Acanthaceae	
2	Arjun	*Terminalia arjuna*	Combretaceae	

3	Awla	*Emblica officinalis*	Euphorbiaceae	
4	Bakul	*Mimusops elengi*	Sapotaceae	
5	Bel	*Aegie marmelos*	Rutaceae	
6	Chandan	*Santalum album*	Santalaceae	
7	Jai	*Jasminum auriculatum*	Oleaceae	
8	Jamun	*Eugenia jambolina*	Myrtaceae	

9	Kadamb	*Anthocephalus kadamba*	Rubiaceae	
10	Khair	*Acacia catechu*	Mimosaceae	
11	Kuchala	*Strychnos nuxvomica*	Loganiaceae	
12	Mango	*Mangifera indica*	Anacardiaceae	
13	Moha	*Madhuca indica*	Sapotaceae	

14	Nagkeshar	*Messua ferrea*	Clusiaceae	
15	Nagchapha	*Michelia Champaka*	Mangoleaceae	
16	Palas	*Butea frondosa*	Fabaceae	
17	Payar	*Ficus infectoria*	Aricaceae	

18	Phanas	*Artocarpus iniegrifolia*	Moraceae	
19	Pimpal	*Ficus religiosa*	Articaceae	
20	Raal	*Veteria indica*	Dipterocarpaceae	
21	Rui	*Calotropis gigantea*	Asclepiadiaceae	
22	Savar	*Salmalia malabarica*	Malvaceae	

23	Shami	*Prosopis spicigera*	Mimoceae	
24	Umbar	*Ficus racemosa*	Articaceae	
25	Vad	*Ficus bengahalensis*	Articaceae	
26	Velu	*Bambusa arundinacea*	Garminae	
27	Vet	*Calamus roteng*	Palmae	

Table.2 Classification of endophytic bacteria on the basis of identification:

Name of isolated bacterial species	Total number of new strains	Code of isolate	Endo Code	Name of plant and plant part used	Sequence length
Bacillus subtilis	02	VCC.16.SB	Endo_25	*Messua ferrea* Stem	776
		VCC.5.S	Endo_1	*Acacia catechu* Stem	776
Bacillus megaterium	03	VCC.4.RA,	Endo_7	*Eugenia jambolina* Stem,	789
		VCC.22.LD	Endo_35	*Anthocephalus kadamba* leaf	799
		VCC.4.SB	Endo_21	*Eugenia jambolina* Stem	793
Bacillus lichniformis	02	VCC.8.SA	Endo_12	*Ficus religiosa* Stem	701
		VCC.15.LE	Endo_82	*Terminalia arjuna* leaf	754
Bacillus axarequiensis	01	VCC.22.LC	Endo_81	*Anthocephalus kadamba* leaf	773
Bacillus safensis	02	VCC.7.LC	Endo_71	*Bambusa arundinacea* leaf	751
		VCC.21.L	Endo_83	*Prosopis spicigera* leaf	766
Bacillus firmus	01	VCC.26.LB	Endo_86	*Calamus roteng* leaf	768
Bacillus pumilus	01	VCC.22.LG	Endo_50	*Anthocephalus kadamba* leaf	700
Bacillus aryabhattai	01	VCC.16.SC	Endo_9	*Messua ferrea* Stem	780
Bacillus niacin	01	VCC.16.SA	Endo_20	*Messua ferrea* Stem	786
Bacillus cereus	20	VCC.8.SB	Endo_19	*Ficus religiosa* Stem	778
		VCC.22.LF	Endo_45	*Anthocephalus kadamba* leaf	736
		VCC.15.LG	Endo_41	*Terminalia arjuna* leaf	780
		VCC.25.LF	Endo_80	*Madhuca indica* leaf	774
		VCC.25.LE	Endo_75	*Madhuca indica*	762

				leaf	
		VCC.15.LD	Endo_77	*Terminalia arjuna* leaf	777
		VCC.27.LD	Endo_74	*Arthrocarpus iniegrifolia* leaf	775
		VCC.27.LC	Endo_69	*Arthrocarpus iniegrifolia* leaf	771
		VCC.27.LB	Endo_73	*Arthrocarpus iniegrifolia* leaf	769
		VCC.14.LC	Endo_76	*Aegle marmelos* leaf	775
		VCC.26.LD	Endo_88	*Calmus roteng* leaf	769
		VCC.26.LC	Endo_87	*Calmus roteng* leaf	769
		VCC.26.LE	Endo_89	*Calmus roteng* leaf	688
		VCC.18.L	Endo_84	*Salmalia malabarica* leaf	776
		VCC.2.LB	Endo_51	*Emblica officinalis* leaf	791
		VCC.24.LD	Endo_54	*Aegle marmelos* leaf	786
		VCC.24.LE	Endo_59	*Azadirachta indica* leaf	780
		VCC.23.LD	Endo_60	*Mangifera indica* leaf	770
		VCC.7.LB	Endo_66	*Bambusa arundinacea* leaf	703
		VCC.1a.LB	Endo_72	*Adhatoda vasica* leaf	783
Klebsiella pneumonia	01	VCC.18.RT	Endo_16	*Salmalia malabarica* root	648
Alcaligens species	04	VCC.15.LB	Endo_93	*Terminalia arjuna* leaf	773
		VCC.25.LB	Endo_94	*Madhuca indica* leaf	763
		VCC.24.LA	Endo_96	*Azadirachta indica* leaf	660
		VCC.20.LC	Endo_67	*Calotropi gigantean* leaf	778

Enterobacter spp	02	VCC.19.S,	Endo_2	*Veteria indica* Stem,	766
		VCC.8.RA	Endo_30	*Ficus religiosa* Stem	677
Arthrobacter globiformis and Arthrobacter protophormiae	02	VCC.15.SA	Endo_23	*Terminalia arjuna* Stem	758
		VCC.24.LC	Endo_449	*Azadirachta indica* leaf	751
Kocuria sediminis and Kocuria rosea	02	VCC.4.SD	Endo_91	*Eugenia jambolina* Stem,	746
		VCC.4.RB	Endo_21	*Eugenia jambolina* Root	749
Micrococcus luteus Micrococcus lulae and Micrococcus sp	03	VCC.11.SC	Endo_14	*Butea frondosa* Root,	745
		VCC.13.SA	Endo_15	*Jasminum auriculatum* Stem,	712
		VCC.13.RB	Endo_31	*Jasminum auriculatum* Stem,	724
Pantoea dispersa	01	VCC.15.SB,	Endo_10	*Terminalia arjuna* Stem,	766
Psychrobacter spp	02	VCC.2.LC,	Endo_56	*Emblica officinalis* leaf	746
		VCC.14.LB	Endo_52	*Aegle marmelos* leaf	768
Ochrobacterium spp	04	VCC.2.LA	Endo_46	*Emblica officinalis* leaf	720
		VCC.23.LB	Endo_57	*Mangifera indica* leaf	872
		VCC.22.LB	Endo_58	*Anthocephalus kadamba* leaf	704
		VCC.27.LE	Endo_79	*Artocarpus iniegrifolia* leaf	711
Pseudomnas spp	01	VCC.3.RC	Endo_22	*Ficus racemosa* Stem	757
Staphylococcus hemolyticus	01	VCC.CLIED	Endo_92	*Strychnos muxvomica*	769
No significant sequence matching	17	VCC.5.R,	Endo_3	*Acacia catechu* root,	
		VCC.15.SC,	Endo_6	*Terminalia arjuna* Stem,	

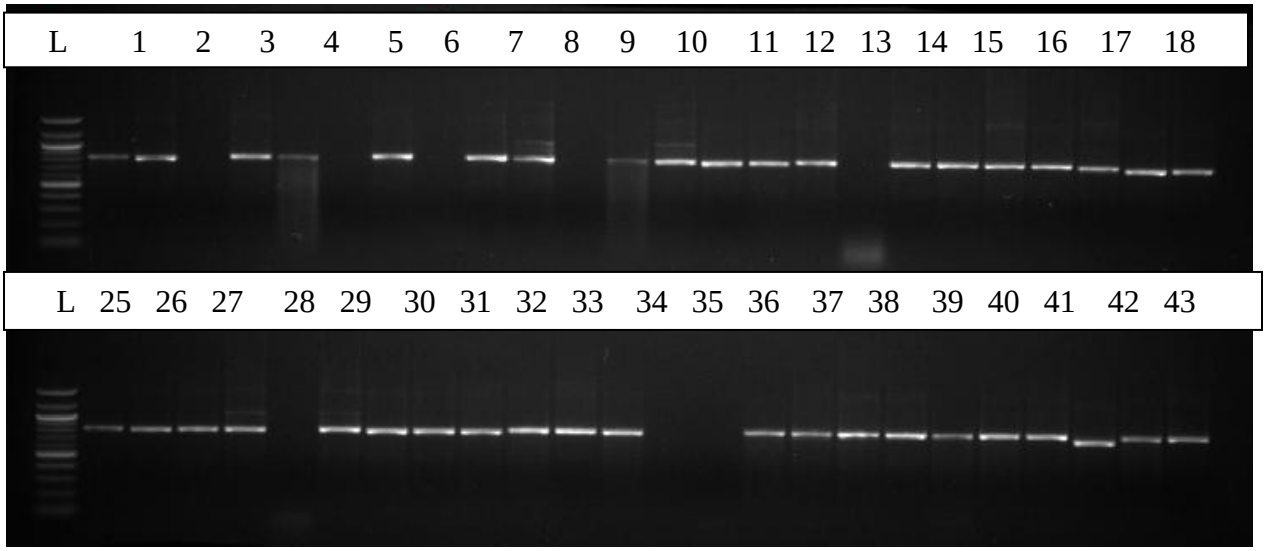
		VCC.16.R,	Endo_8	*Messua ferrea* root,	
		VCC.13.RA,	Endo_11	*Jasminum auriculatum* Root,	
		VCC.3.RB,	Endo_18	*Ficus racemosa* root,	
		VCC.3.RA,	Endo_29	*Ficus racemosa* root,	
		VCC.11.SB, ,	Endo_33	*Butea frondosa* stem,	
		VCC.1a.LA	Endo_38	*Adhatoda vasica* leaf,	
		VCC.25.LI,	Endo_44	*Madhuca indica* leaf,	
		VCC.23.LC,	Endo_55	*Mangifera indica* leaf,	
		VCC.25.L, ,	Endo_63	*Madhuca indica* leaf,	
		VCC.25.LC,	Endo_65	*Madhuca indica* leaf,	
		VCC.27.LA	Endo_68	*Artocarpus iniegrifolia* root,	
		VCC.20.L,	Endo_78	*Calotropis gigantean* leaf,	
		VCC.13.SB,	Endo_90	*Jasminum auriculatum* stem,	
		VCC.4.SD,	Endo_91	*Eugenia jambolina* Stem,	
		VCC.24.LB	Endo_95	*Azadirachta indica* leaf	
Unculturable bacteria	02	VCC.4.SC,	Endo_17	*Eugenia jambolina* Stem,	733
		VCC.14.L	Endo_42	*Aegle marmelos* leaf	764
Terribacillus saccharophilus	01	VCC.4.SA	Endo_7	*Eugenia jambolina* Stem	726

Table.3 No significant sequence matching

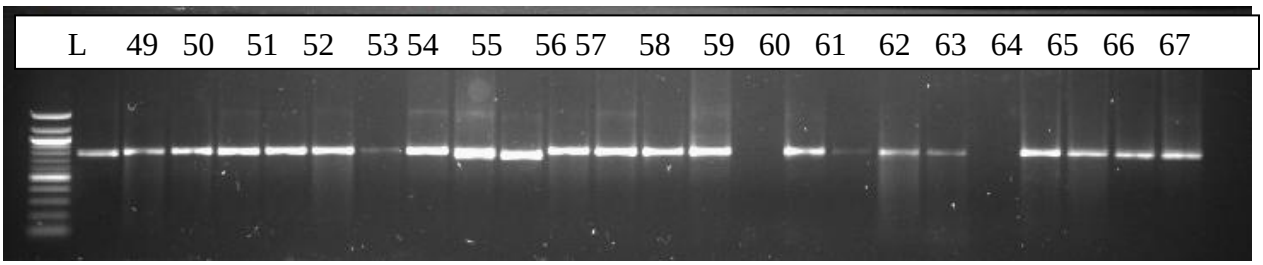
Code of endophytic bacteria	Name of the plant and plant part used	Gram's Nature	Capsule	Endospore
VCC.5.R	*Acacia catechu* root,	Gram positive cocci	+	_
VCC.15.SC	*Terminalia arjuna* Stem,	Gram positive cocci	_	_
VCC.16.R	*Messua ferrea* root,	Gram negative cocci	+	_
VCC.13.RA	*Jasminum auriculatum* Root,	Gram positive cocci	+	_
VCC.3.RB	*Ficus racemosa* root,	Gram positive cocci	_	_
VCC.3.RA	*Ficus racemosa* root,	Gram positive cocci	+	_
VCC.11.SB	*Butea frondosa* stem,	Gram negative cocci	+	_
VCC.1a.LA	*Adhatoda vasica* leaf,	Gram negative rod	+	_
VCC.25.LI	*Madhuca indica* leaf,	Gram positive rod	+	_
VCC.23.LC	*Mangifera indica* leaf,	Gram positive rod	+	+
VCC.25.L	*Madhuca indica* leaf,	Gram negative rod	+	_
VCC.25.LC	*Madhuca indica* leaf,	Gram positive rod	+	+
VCC.27.LA	*Artocarpus iniegrifolia* root,	Gram negative rod	+	+
VCC.20.L	*Calotropis gigantean* leaf,	Gram negative rod	+	+
VCC.13.SB	*Jasminum auriculatum* stem,	Gram positive cocci	+	+
VCC.4.SD	*Eugenia jambolina* Stem,	Gram positive cocci	+	+
VCC.24.LB	*Azadirachta indica* leaf	Gram positive rod	+	+

(Note: +=Present, _= Absent)

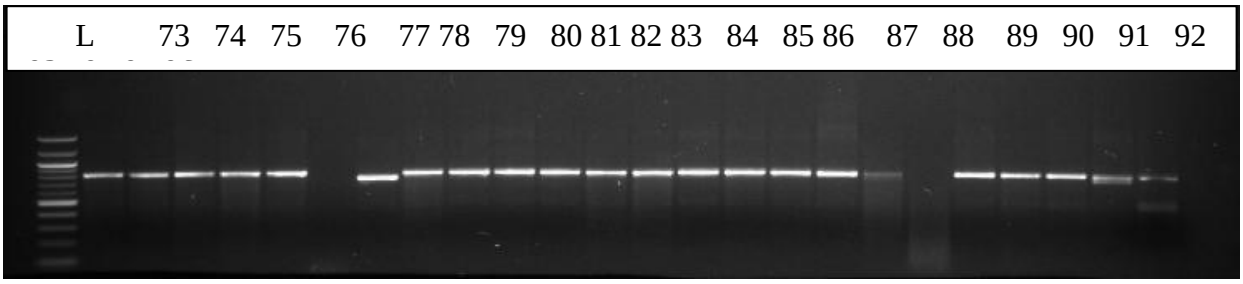
Fig.1 PCR amplification for 16 s rDNA



LaneLMarker,lane1VCC.5.S.,lane2:VCC.19.S.,Lane3:VCC.5.R,Lane4:VCC.4.RA,Lane5:11.SA, Lane6:VCC.15.SC,Lane7:VCC.4.SA,Lane8:VCC.16.R,Lane9:VCC.16.SC,Lane10:VCC.15.SB, Lane11:VCC.13.RA,Lane12:VCC.8.SA,Lane13:VCC.3.SA,Lane14:VCC.11SC,Lane15:VCC.13 .SA,Lane16:VCC.18.RT,Lane17:VCC.4.SC,Lane18:VCC.3.RB,Lane19:VCC.8.SB,Lane20:VCC .16.SA,Lane21:VCC.4.SB,Lane22:VCC.3.RC,Lane23:VCC.15.SA,Lane24:VCC.4.SD,Lane25:V CC.9.SB,Lane26:VCC.27.S,Lane27:VCC.27.R,Lane28:VCC.3.SB,Lane29:VCC.3.RA,Lane30:V CC.8.RA,Lane31:VCC.13.RB,Lane32:VCC.4.RB,Lane33:VCC.11.SA,Lane34:VCC.25.LG,Lan e35:VCC.22.LD,Lane36:VCC.15.LF,Lane37:VCC.26.L,Lane38:VCC.1a.LA,Lane39:VCC.25.L H,Lane40:VCC.22.LE,Lane41:VCC.15.LG,Lane42:VCC.14.L,Lane43:VCC.15.L,Lane44:VCC. 25.LI,Lane45:VCC.22.LF,Lane46:VCC.2.LA,Lane47:VCC.23.LA,Lane48:VCC.15.LC.

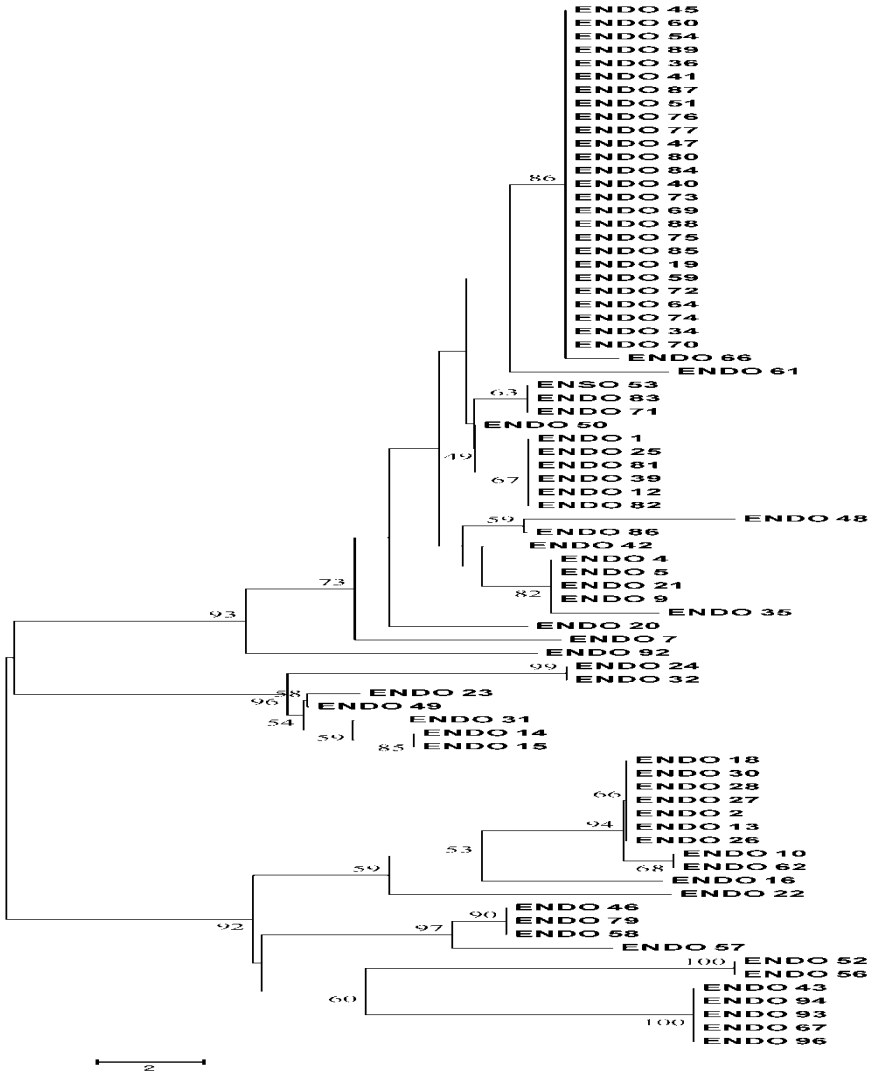


L:Marker,Lane49:VCC.24.LC,Lane50:VCC.22.LG,Lane51:VCC.2.LB,Lane52:VCC.1.4.LB,Lan e53:VCC.22.LA,Lane54:VCC.24.LD,Lane55:VCC.23.LC,Lane56:VCC.2.LC,Lane57:VCC.23.L B,Lane58:VCC.22.LB,Lane59:VCC.24.LE,Lane60:VCC.23.LD,Lane61:VCC.7.LA,Lane62:VC C.23.LB,Lane63:VCC.25.L,Lane64:VCC.12.L,Lane65:VCC.25.LC,Lane66:VCC.7.LB,Lane67: VCC.20.L,Lane68:VCC.27.LC,Lane69:VCC.27.LC,Lane70:VCC.25.LD,Lane71:VCC.7.LC,Lan e72:VCC.1a.LB



L:Marker,Lane73:VCC.27.LB,Lane74:VCC.27.LD,Lane75:VCC.25.LE,Lane76:VCC.14.LC,Lane77:VCC.15.LD,Lane78:VCC.20.L,Lane79:VCC.27.LE,Lane80:VCC.25.LF,Lane81:VCC.22.LC,Lane82:VCC.15.LE,Lane83:VCC.21.L,Lane84:VCC.18.L,Lane85:VCC.21.LB,Lane86:VCC.26.LB,Lane87:VCC.26.LC,Lane88:VCC.26.LD,Lane89:VCC.26.LE,Lane90:VCC.13.SB,Lane91:VCC.4.SD,Lane92:VCC,Lane93:VCC.15.LB,Lane94:VCC.25.LB,Lane95:VCC.24.LB,Lane96:VCC.24.LA

Fig.2 Phylogenetic tree analysis



Other genera found to contribute to these humongous tree genomes are *Klebsiella pneumoniae, Alcaligens species, Enterobacter spp Arthrobacter globiformis and Arthrobacter protophormiae, Kocuria sediminis and Kocuria rosea, Micrococcus luteus, Micrococcus lulae and Micrococcus sp, Pantoea dispersa, Psychrobacter spp, Ochrobacterium spp, Pseudomnas spp, Staphylococcus hemolyticus,* Unculturable bacteria, *Terribacillus saccharophilus* in tree *Salmalia malabarica* root, *Terminalia arjuna* leaf, *Madhuca indica* leaf, *Azadirachta indica* leaf, *Calotropi gigantean* leaf, *Veteria indica* Stem, *Ficus religiosa* Stem, *Terminalia arjuna* Stem, *Azadirachta indica* leaf, *Eugenia jambolina* Stem, *Eugenia jambolina* Root, *Butea frondosa* Root, *Jasminum auriculatum* Stem, *Jasminum auriculatum* Stem, *Terminalia arjuna* Stem, *Emblica officinalis* leaf, *Aegle marmelos* leaf, *Emblica officinalis* leaf, *Mangifera indica* leaf, *Anthocephalus kadamba* leaf, *Artocarpus iniegrifolia* leaf, *Ficus racemosa* Stem, *Strychnos muxvomica*, *Eugenia jambolina* Stem, *Aegle marmelos* leaf, *Eugenia jambolina* Stem. An interesting finding of these endophytic bacterial isolation is as many as 17 isolates are amongst those which could not have significant matching with any of the known sequences on NCBI. Further biochemical investigation to characterize them shall provide a novel appendage to the present culture collection. Bioprospecting through these endophytic bacteria producing useful metabolites would make a valid value addition to the whole process of culture collection.

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