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Phylogenetic analysis of a gut bacteria of pine caterpillar: *Kunugia latipennis*.Walker

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ABSTRACT

Insect gut microflora contribute towards the utilization of complex food resources in the gut of the host. The interaction of the host and the micro organisms can be symbiotic or transient. In this study The *Kocuria rhizophila* strain (NCBI gene accession number NR_026452.1) was isolated from the gut of pine caterpillar (*Kunugia latipennis*.Walker) in and around North Eastern hill University campus, Meghalaya, India.16s rRNA was targeted for isolation and amplification. The isolated sequence was compared to representatives strains of *Kocuria rhizophila sp*. Phylogenetic analysis revealed that the isolated strain belongs to the genus *Kocuria* and is most similar to *Kocuria varians strain* G33 (Accession number NR_029297.1 similarity % = 90) and *Kocuria marina* strain KMM 3905 (Accession number NR_025723.1 similarity % = 99). This appears to be the first report of this strain from terrestrial insect larval gut. Importance of this strain in the context of the host food quality is discussed.

KEYWORDS

16s rDNA;
Insect gut;
Blast;
Bacteria;
Phylogenetic analysis.

INTRODUCTION

Insect gut has been extensively studied because insects are one of the most diverse groups of living organisms on earth[7] . Due to their diverse behaviour and feeding habits, almost no terrestrial food source can escape consumption by one or more insect species. Despite the diversity, the highly inter dependent and well-regulated symbiotic interactions with micro-organisms seem to be an important common property for different insect species. Protozoa and bacteria play a key role in the host physiology and gut ecology. They supplement the host with nitrogen through nitrogen fixation[2,13], assist in cellulose degradation[3] and also provide energy, for example, through acetogenic reduction of CO_2 [12,17]. Termites and cockroaches are dependent upon facultative mutualistic bacteria in their hindgut, which take part in digestion[5]. In a few other studies, mutualistic gut bacteria were shown to be advantageous to their host by providing nutrients or by degrading toxic compounds[6]. Many of the microorganisms involved in such associations belong to the family *Enterobacteriaceae*. In addition to these facultative symbionts, transient microorganisms can be found in the gut. Transients are

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ingested during food intake, but are removed with the faeces without colonizing the gut and hence they are not transmitted to the next generation.

Molecular techniques independent of culturing live bacteria is an important addition to the culture dependent approach and provide the opportunity to describe microbial diversity[16]. The most common molecular approach for identification of bacteria is to target the 16S rRNA gene (16S rDNA), which is approximately 1300 to 1500 bp long, and codes for the small sub-unit of ribosomal RNA of prokaryotes. The 16S gene contains highly conserved and variable regions useful for identification of bacteria by their 16S sequences. The 16S sequences of the total bacterial community is obtained through extraction of the bacterial communities' total DNA, PCR amplification of 16S rRNA genes using bacteria specific primers[10], and finally by generating the sequence and using them for the bacterial identification through BLAST, and phylogenetic analysis using MEGA-5. In this study we combined both molecular and bioinfomatics techniques to identify the bacteria isolated from the gut of the larva (caterpillar).

MATERIALS AND METHODS

The Pine Caterpillars (*Kunugia latipennis*.Walker) were collected from in and around North Eastern Hill University (NEHU), Mawlai Umshing, Shillong. They were raised in insect boxes till the fourth instar. The caterpillars were starved for 24 hours prior to being killed either by exposure to chloroform, or by keeping them in deep freeze refrigerator for 15 – 20 minutes. Individuals were then dissected under aseptic conditions, and the guts were isolated for further processing.

Scanning electron microscopy (SEM) study

Cold immobilized larvae were surface sterilized through sequential washes with 70% ethanol (2 minutes), 5.25% sodium hypochlorite (2 minutes) and several washes with sterile distilled water prior to being fixed by injecting 3% Glutaraldehyde directly into the gut of whole specimens. After 24h. the larvae were dissected and the whole gut was aseptically removed and stored submerged in specimen tubes containing 3% Glutaraldehyde. The guts were opened up and carefully washed in 3% Glutaraldehyde to remove the gut contents, and mounted and processed for viewing of the inner gut lining under SEM (JSM-6360.JEOL).

Microbial culture of gut contents

External surface sterilization of the insect was done through sequential washes with 70% ethanol (2 minutes), 5.25% sodium hypochlorite (2 minutes) and several washes with sterile distilled water. The gut was removed aseptically using sterile forceps and scissors and were macerated and inoculated along with its contents in 100ml L-B agar petri plates and incubated at 32 ± 2^0C for 32- 48 hours. All steps were done under a laminar air flow to avoid contamination. From the colonies developed after 48 hrs., pure cultures were obtained by spread plate method. The pure cultures were subjected to conventional gram staining and primary identification was done using Bergy's Manual[1] All reagents used were of analytical reagent grade.

Extraction of 16S RNA

The Bacterial genomic DNA was extracted by standard Cetyltrimethyl ammonium bromide (CTAB) method and the quality of the DNA was evaluated on 1.2% Agarose Gel. A single band of high-molecular weight DNA was observed and amplification of the 16s RNA gene was carried out by using standard Polymerase Chain Reaction (PCR) protocol and specific forward and reverse primers were used (B27F AGAGTTTGATCCCTCAG and U1492R - GGTTACCTTGTTACGACTT) to amplify the 16sRNA region, and the sequences were generated using sequencer (ABI3730xl, Applied Biosystem). Similarity search of the sequence was done under Basic Local Alignment Search Tool (BLAST) followed by a phylogenetic analysis using Molecular Evolutionary Genetic Analysis tool (Mega-5 software)

Phylogenetic analysis

The phylogenetic analysis was done based on the first 10 maximum identity score followed by the 10 mid identity score, and the least 10 identity score. These were aligned using Mega – 5 tool and the tree was generated using methods like Neighbor joining, Minimum evolution, and Maximum Likelihood.

RESULTS

The SEM of the gut lumen clearly indicated the pres-

ence of the microbes (Figure 1).

Preliminary tests on the isolated strain by Gram staining method revealed the microbe to be gram positive, cocci, non spore former (Figure 2).

Further, a positive result for catalase test[1] confirmed that the microbe belongs to the Group V1 i.e. the family of *Micrococcus, Planococcus* or *Staphylococcus*. A 1290 bp rDNA gene was generated from forward and reverse primers and the sequence was also generated (Figure 3 & 4).

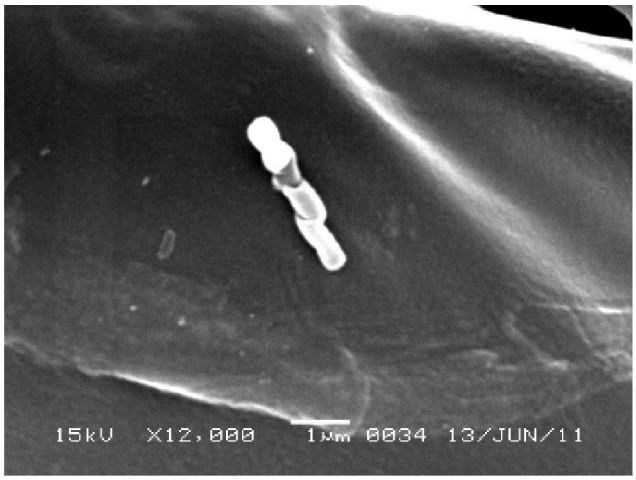
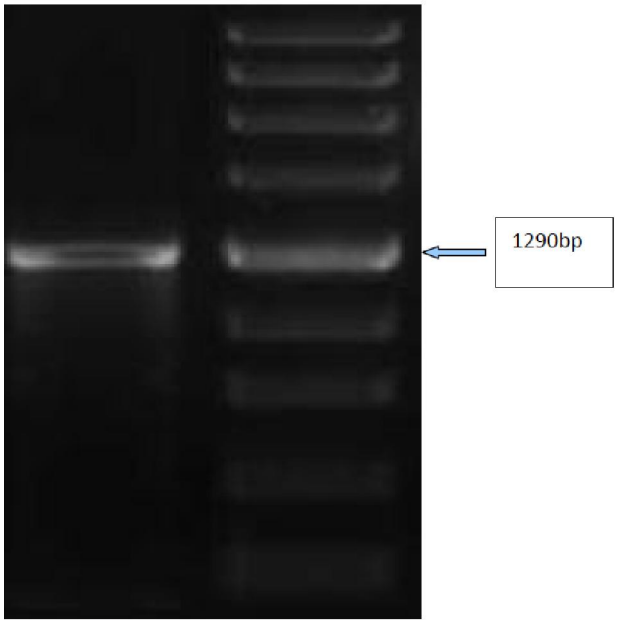


Figure 1 : SEM image of a microbe inside the caterpillar gut



Lane 1: 16S rDNA amplicon band, Lane 2: DNA marker
Figure 3 : Gel image of 16SrDNA amplicon

When similarity search of the sequence of the isolated strain was performed using BLAST, it was it revealed 97% similarity with the family of *Micrococcus luteus* strain DSM (Acc no NR_03711.31), while with *Kocuria rhizophila* strain TA68 (Acc no NR_026452.1) the similarity was 99%. This was further confirmed from the different phylogenetic trees

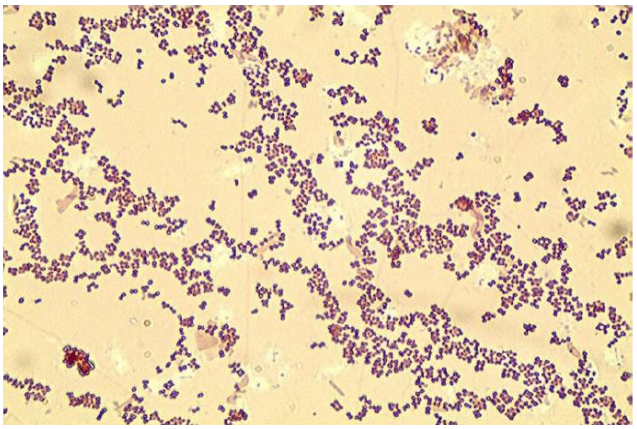


Figure 2 : Cocci gram positive bacteria under 100x microscope

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CAACCTTTGTGCCTGGCCTGGGTGGATGAGTGGCGAACGGGTGAGTAATACGTGAGTAACCTGCCCTTGACTCTGGG
ATAAGCCTGGGAAACTGGGTCTAATACTGGATACGACATGTCACCGCATGGTGGTGTGTGGAAAGGGTTTTACTGGT
TTTGGATGGGCTCACGGCCTATCAGCTTGTTGGTGGGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAG
AGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCACGGAAGAAG
CGAAAGTGACGGTACGTGCATAAGAATCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTACGGCGCAAGCG
TTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGCCCGGGGCTTAACCCCG
GGTGTGCAGTGGGTACGGGCAGACTTGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGA
TATCAGGAGGAACACCGATGGCAAAAGCAGGTCTCTGGGCTGTTACTGACGCTGAGGAGCGAAAGCATGGGGAGCGA
ACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGAACATTCCACGTTTTCCGCGC
CGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAACCGGG
CCAGAGATGGTCTTTCCCCCTTGTGGGGCTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTGATGGTGGGGACTCATAGGAGACTGC
CGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACA
ATGGCCAGTACAATGGGTTGCGATGCCGCGAGGTGGAGCTAATCCCAAAAAGCTGGTCTCAGTTCGGATCGTGGTCT
GCAACTCGACCACGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATGCTGCAATC
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Figure 4 : 16s rDNA sequences generated

constructed (Figure 5 & 6).

Phylogenetic analysis

The isolated 16s rRNA sequence was aligned (Figure 7) and the phylogenetic trees were constructed. The tree using Neighbour joining method (Figure 8) shows similarity of the isolated sequence with the genus that fallsunder the *Kocuria* family.

The isolated strain (Sequence 1 in the Figure) shows highest similarity (90 %) with *Kocuria varians* strain G33 (Acc no – NR_029297.1) The trees constructed by Minimum Evolution (Figure 9) and maximum likelihood (Figure 10) shows almost consistent scores with each other and sequence 1 is distantly related to *Amycolatoptopsis niigatensis* strain LC11 with very low bootstrap value.

The tree has 6 clusters viz. *Kocuria, Anthrobacter, Nesterenkonia,* Curtobacterium, *Rhodococcus* and

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Amycolatopsis. From the similarity search and using different tree building algorithms we confirmed that the isolated strain from the gut of the pine caterpillar is the *Kocuria rizophila* strain. The isolated sequence was also analysed for the percentage of GC content using on line tool GENSCAN.

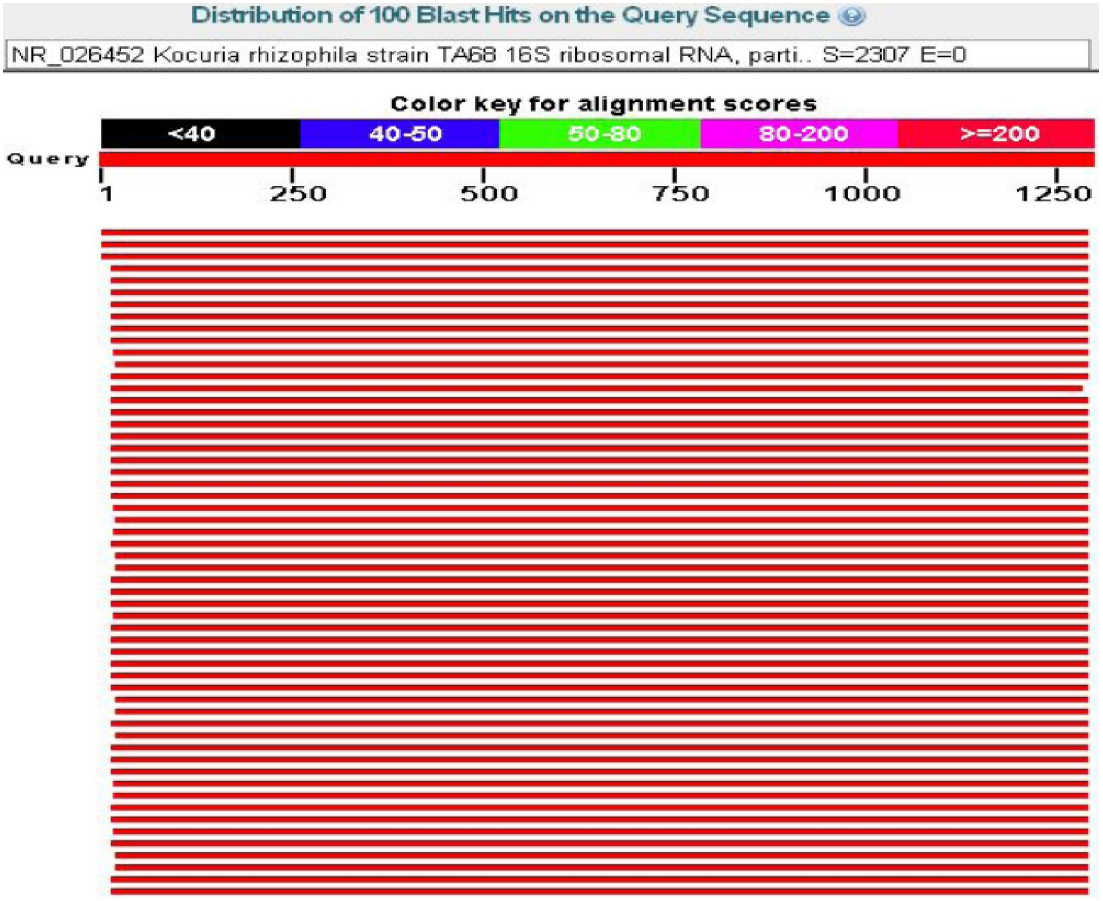

Figure 5 : Blast data (Alignment view using combination of NCBI GenBank)

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
NR_026452.1	Kocuria rhizophila strain TA68 16S ribosomal RNA, partial sequence	2307	2307	99%	0.0	99%	
NR_029297.1	Kocuria varians strain G33 16S ribosomal RNA, partial sequence	2207	2207	99%	0.0	98%	
NR_025723.1	Kocuria marina strain KMM 3905 16S ribosomal RNA, partial sequenc	2170	2170	99%	0.0	97%	
NR_027193.1	Kocuria carniphila strain CCM 132 16S ribosomal RNA, partial sequer	2167	2167	98%	0.0	97%	
NR_026451.1	Kocuria palustris strain TAGA27 16S ribosomal RNA, partial sequenc	2100	2100	98%	0.0	97%	
NR_044871.1	Kocuria rosea 16S ribosomal RNA, partial sequence	2098	2098	98%	0.0	97%	
NR_028924.1	Kocuria polaris strain CMS 76or 16S ribosomal RNA, partial sequenc	2087	2087	98%	0.0	96%	
NR_044308.1	Kocuria flava strain HO-9041 16S ribosomal RNA, partial sequence	2056	2056	98%	0.0	96%	
NR_043511.1	Kocuria aegyptia strain YIM 70003 16S ribosomal RNA, partial seque	2056	2056	98%	0.0	96%	
NR_043899.1	Kocuria turfanensis strain HO-9042 16S ribosomal RNA, partial sequ	2050	2050	98%	0.0	96%	
NR_044025.1	Kocuria halotolerans strain YIM 90716 16S ribosomal RNA, partial se	2039	2039	98%	0.0	96%	
NR_026189.1	Arthrobacter crystallopoietes strain DSM 20117 16S ribosomal RNA,	2025	2025	97%	0.0	96%	
NR_026199.1	Kocuria kristinae strain DSM 20032 16S ribosomal RNA, partial seque	2023	2023	98%	0.0	96%	
NR_043323.1	Kocuria himachalensis strain K07-05 16S ribosomal RNA, partial seq	2012	2012	97%	0.0	96%	
NR_025424.1	Arthrobacter nasiphocae strain M597/99/10 16S ribosomal RNA, par	1997	1997	98%	0.0	95%	
NR_025310.1	Rothia nasimurium strain CCUG 35957 16S ribosomal RNA, partial se	1967	1967	98%	0.0	95%	
NR_043968.1	Rothia terrae strain L-143 16S ribosomal RNA, partial sequence	1951	1951	98%	0.0	94%	
NR_028928.1	Nesterenkonia lacusekhoensis strain EL-30 16S ribosomal RNA, part	1949	1949	98%	0.0	94%	
NR_043765.1	Zhihengliuella halotolerans strain YIM 70185 16S ribosomal RNA, par	1947	1947	98%	0.0	94%	
NR_044894.1	Arthrobacter woluwensis strain 1551 16S ribosomal RNA, complete :	1947	1947	98%	0.0	94%	
NR_028925.1	Arthrobacter roseus strain CMS90 16S ribosomal RNA, partial seque	1945	1945	98%	0.0	94%	
NR_042250.1	Arthrobacter pigmenti strain : LMG 22284 16S ribosomal RNA, partia	1940	1940	98%	0.0	94%	
NR_025083.1	Arthrobacter methylotrophus strain TGA 16S ribosomal RNA, partial	1936	1936	98%	0.0	94%	
NR_043279.1	Nesterenkonia jeotgali strain JG-241 16S ribosomal RNA, partial seq	1932	1932	98%	0.0	94%	
NR_025362.1	Arthrobacter luteolus strain CF-25 16S ribosomal RNA, partial seque	1930	1930	97%	0.0	94%	
NR_044873.1	Rothia mucilaginosa 16S ribosomal RNA, partial sequence	1930	1930	98%	0.0	94%	
NR_041545.1	Arthrobacter oryzae strain KV-651 16S ribosomal RNA, partial seque	1927	1927	98%	0.0	94%	
NR_029119.1	Nesterenkonia sandarakina strain YIM 70009 16S ribosomal RNA, pa	1927	1927	97%	0.0	94%	
NR_026187.1	Arthrobacter globiformis strain DSM 20124 16S ribosomal RNA, parti	1927	1927	97%	0.0	94%	

Figure 6 : BLAST Sequences producing significant alignment showing scores, query coverage and max identity

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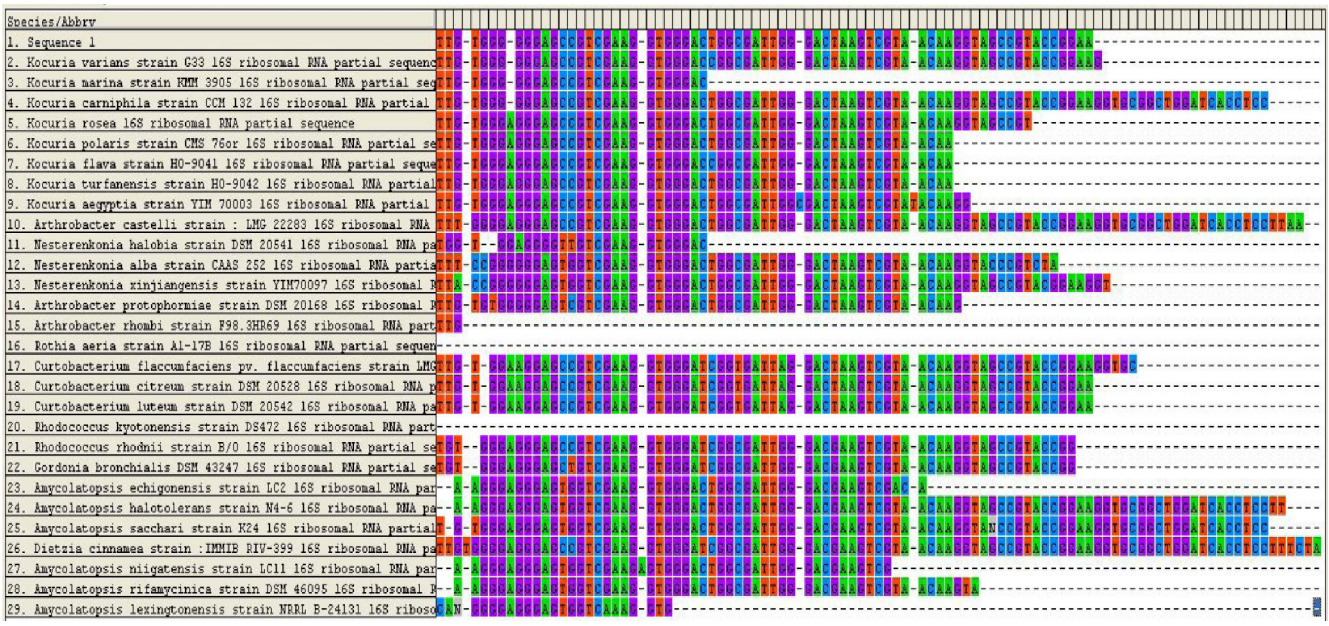

Figure 7 : Mega 5 (Alignment explorer) aligning the sequence for phylogenetic analysis

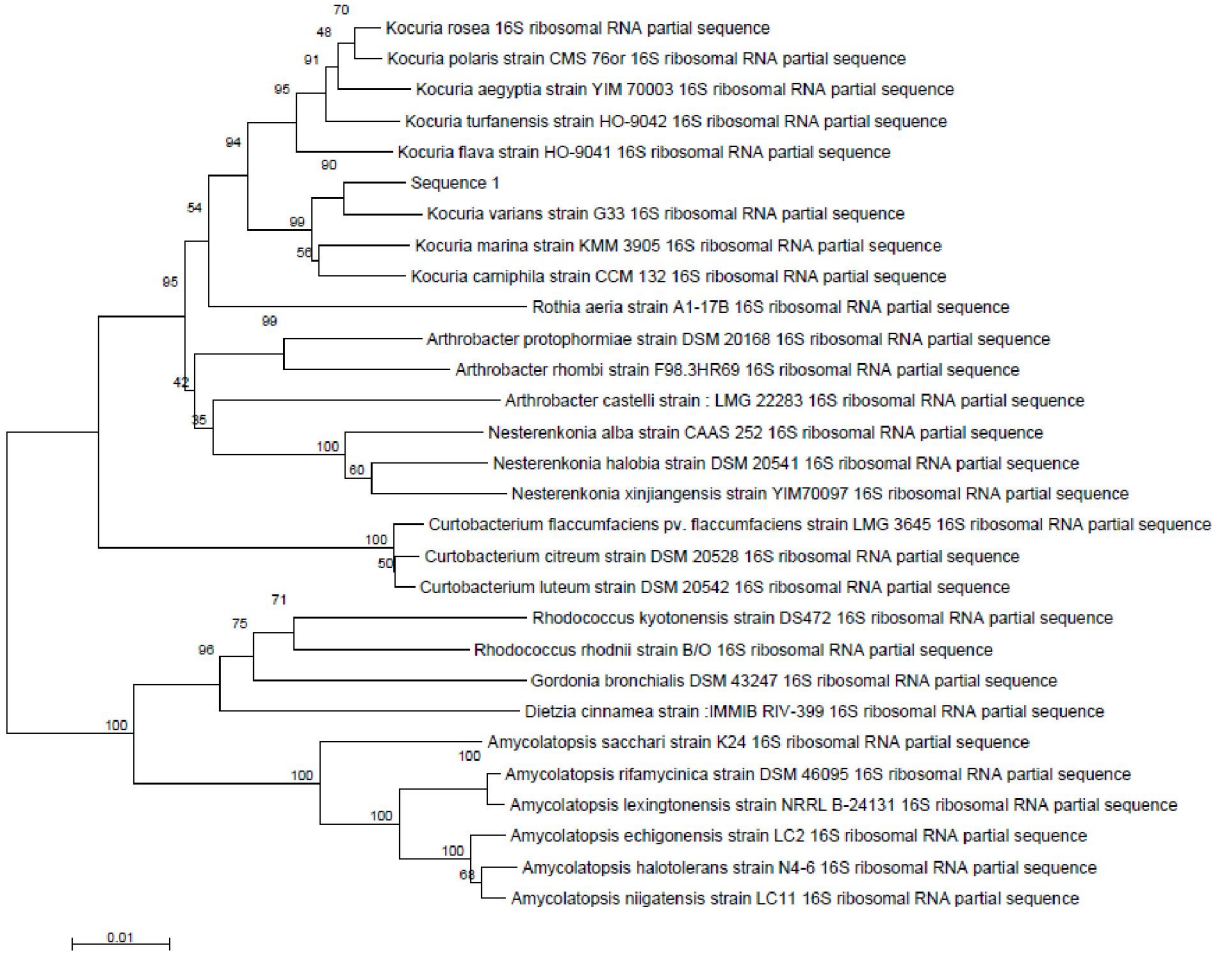

Figure 8 : Neighbor joining phylogenetic tree based on 16s r RNA gene sequences showing the distance of isolated strain (sequence 1) within the genus *Kocuria*. *Amycolatolatopsis nigatenesis* strain LC11 was used as an outgroup. Bootstrap values are show at the nodes of the tree. Bootstrap values greater than 90% are shown by the genus *Kocuria*. Bar shows 0.01 substitutions per nucleotide positions.

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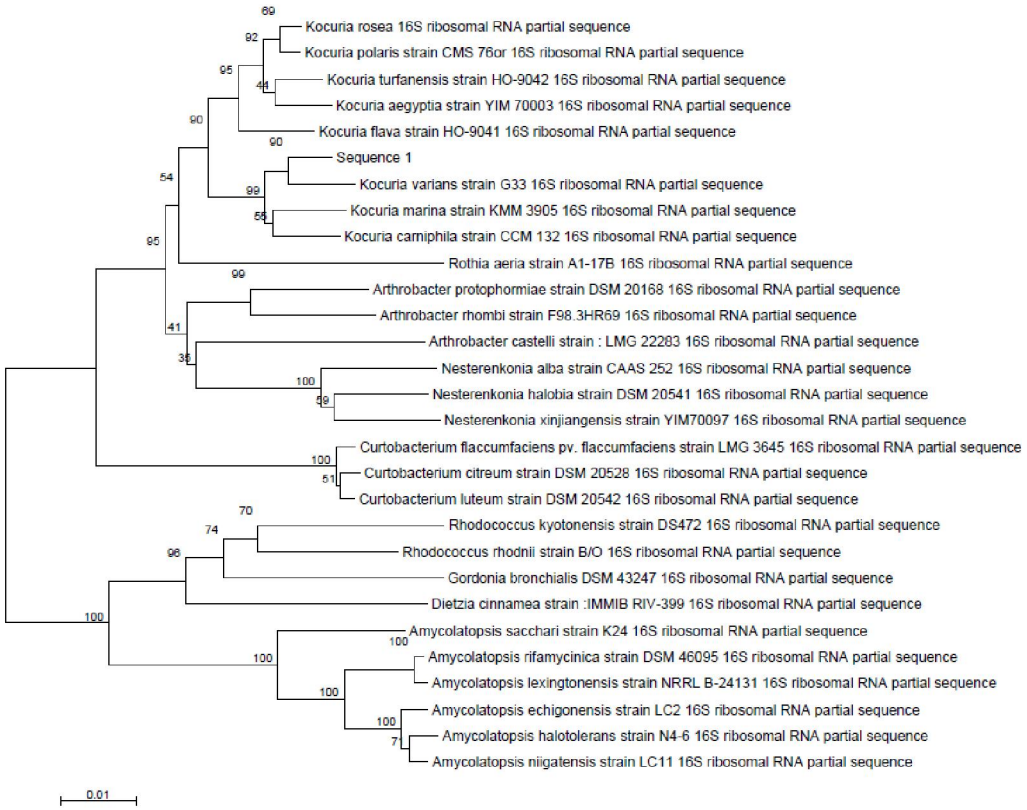

Figure 9 : Minimum evolution bootstrap phylogeny of *Kocuria* family inferred from 16s rRNA sequences. Bootstrap probabilities are shown at the nodes

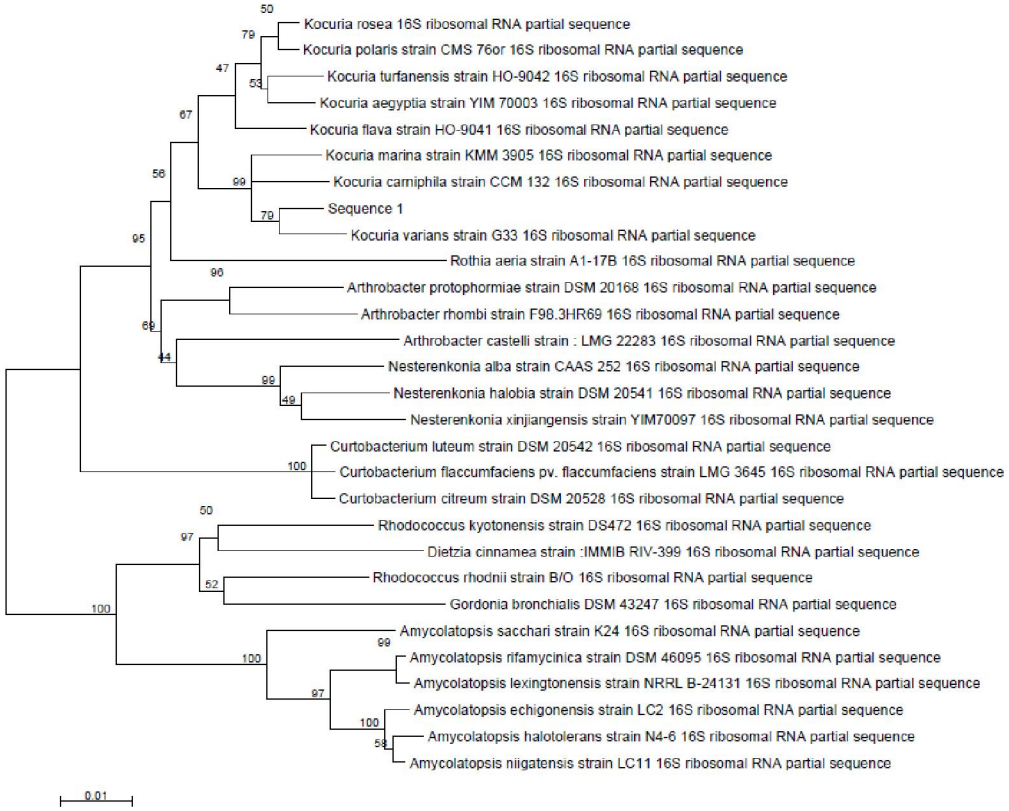

Figure 10 : The topology shown here is an unrooted tree and branches are found by maximum likelihood (branches significantly positive at P < 0.01)

DISCUSSION

Phylogenetic tree building is the most common method of classifying organisms, and by basing it on 16s rRNA makes it even more reliable. In this study the crucial part is the amplification of the 16s rRna and the alignment of the sequence. The functional role of *Kocuria rhizophila* appears to be the degradation of phenolic compounds[14] and it also is halotolerant (10% NaCl). These bacteria have been reported in fermented foods, clinical specimens, and fresh water and marine sediments[8], and also in the rhizosphere of narrow leaf cattail[9] suggesting that each *Kocuria* species is highly adapted to respective ecological niche[8]. The presence of probable metabolic pathways for the transformation of phenolic compounds generated from the decomposition of plant materials, and the presence of a large number of genes associated with membrane transport, particularly amino acid transporter and drug efflux pumps, may contribute to the organisms utilization of root exudates as well as the tolerance to various organic compounds[14]. In the present instance, the host food (pine needles) is a resource containing lignins and polyphenols[15] which appears to be the cue for host to harbor *Kocuria rhizophila* for the utilization of such lignocellulosic resources as food.

The GC content of the sequence was found to be 56.58% by using GENSCAN. The GC content of the species is indicative of reasonable stability of its genome, and is in the intermediate range reported for a wide variety of bacterial genome[11].

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REFERENCES

[1] Alfred E.Brown; Microbiological application, 9th Edition, Mc Graw Hill: NY, 269 - 273 **(2005)**.

[2] J.A.Breznak, W.J.Brill, J.W.Mertins, H.C.Coppel; Nitrogen fixation in termites. Nature, **244**, 577- 580 **(1973)**.

[3] J.A.Breznak, A.Brune; Role of microorganisms in the digestion of lignocellulose by termites. Ann.Rev.Entomol, **39**, 453-487 **(1994)**.

[4] J.A.Breznak, J.M.Switzer; Acetate synthesis from H2 plus CO2 by termite gut microbes. Appl.Environ.Microbiol, **52**, 623-630 **(1986)**.

[5] J.A.Breznak; Intestinal microbiota of termites and other xylophagous insects. Annua Rev.Microbiol., **36**, 323-343 **(1982)**.

[6] B.C.Campbell; On the role of microbial symbiotes in herbivorous insects. E.A.Bernays (Ed); Insect-plant interactions. CRC Press, Boca Raton, F. 1-45 **(1990)**.

[7] M.Dana, E.Cook, H.Crescenzo, U.Rima, J.B.D.Peterson; Isolation of polymer-degrading bacteria and characterization of the hindgut bacterial community from the detritus-feeding larvae of *Tipula abdominalis*. Applied Environmental Microbiology., 5683-5686 **(2007)**.

[8] S.B.Kim, O.I.Nedashkovskaya, V.V.Mikhailov, S.K.Han, K.O.Kim, M.S.Rhee, K.S.Bae; *Kocuria marina* sp. nov., A novel actinobacterium isolated from marine sediment. Int.J.Syst.Evol. Microbiol., **54**, 1617 -1620 **(2004)**.

[9] G.Kovacs, J.Burghardt, S.Pradella, P.Schumann, E.Stackebrandt; *Kocuria palustris* sp.nov. and *Kocuria rhizophila* sp.nov., Isolated from the rhizoplane of the narrow-leaved cattail *Typha angustifolia*. Int.J.Syst.Bacteriol., **49**, 167-173 **(1999)**.

[10] W.T.Liu, T.L.Marsh, H.Cheng, L.J.Forney; Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Applied and Environmental Microbiology, 4516-4522 **(1997)**.

[11] A.Muto, S.Osawa; The quinine and cytosine content of genomic DNA and bacterial evolution. Proc.Natl.Acad.Sci.USA., **84**, 166-169 **(1987)**.

[12] D.A.Odelson, J.A.Breznak; Volatile fatty acid production by the hindgut microbiota of xylophagous insects. Appl.Environ.Microbiol, **45**, 1602-1613 **(1983)**.

[13] M.Ohkuma, S.Noda, R.Usami, K.Horikoshi, T.Kudo; Diversity of nitrogen fixation genes in the symbiotic intestinal microflora of the termite reticulitermes speratus. Appl.Environ.Microbiol, **62**, 2747-2752 **(1996)**.

[14] H.Takarada, M.Sekine, H.Kosugi, Y.Matsuo, T.Fujisawa, S.Omata, E.Kishi, A.Shimizu, N.Tsukatani, S.Tanikawa, N.Fujita, S,Harayama; Complete genome sequence of the soil actinomycete *Kocuria rhizophila*. J.Bacteriol., **190(12)**, 4139-4146 **(2008)**.

FULL PAPER

[15] B.K.Tiwari, R.R.Mishra; Dry weight loss and changes in chemical of pine (*Pinus keiya* Royle) needles and teak (*Tectona grandis* L) leaves during processing in a fresh water lake. Hydrobiologia, **98**, 249-256 **(1983)**.

[16] H.K.Urakawa; Kita-Tsukamoto, K.Ohwada; Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. Microbiol., **145**, 3305-3315 **(1999)**.

[17] D.Waller; Nitrogen fixation by termite symbionts. In: Nitrogen fixation: A model system for analysis of a biological process. E.W.Triplett (Ed); Horizon Scientific Press, Wymondham, UK., **(2000)**.