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Molecular Identification of Newly Isolated *Bacillus paramycoides* SEQ179_SEZ 0001 – from Tasar Silkworm

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A B S T R A C T

The present study was carried out to isolate and identify the bacteria from the body of tasar silkworm cadaver using 16SrRNA gene sequence analysis. The outdoor rearing of silkworm was carried out during 2016 to 2018.The phylogenetic tree constructed on the basis of 16SrRNA gene sequence revealed that bacteria strain isolated as clustered was closest member of *Bacillus sps* and is identified as *Bacillus paramycoides* with 100% similarity and length of 1404 bp.

***Key words*:** Tasar silkworm, 16SrRNA, *Bacillus paramycoides*

Mulberry and non-mulberry silks are produced by silkworms which feed on different food plants. Among the non-mulberry, tropical tasar silkworm, *Antheraea mylitta* is an economically important silk from products of Saturniidae silkworm called Tusah, Tussore, Tusser or Tussur, these uneven filaments are being reared outdoor on tall host plants like *Terminalia tomentosa*, *Terminalia arjuna*, *Shorea robusta* etc. The filament that is reeled out of the cocoons are coarser, stronger and shorter than the normal domesticated silk with less damage when exposed to high temperature. Thousands of tribal families are engaged in tasar rearing which helps the vulnerable section of people for income generation with the locally available food plants in the wild sericulture areas which is sprawling over Central and Asian plateaus extending over the states of Bihar, Madhya Pradesh, Orissa, Maharashtra, Andhra Pradesh, Telangana, Karnataka, Uttar Pradesh, Meghalaya, Manipur, Assam and Arunachal Pradesh, Nagaland [1-2]. Tasar is copperish, unique, durable, rich, textured which gives feel and dignity of its own, thus creating employment avenues to aboriginal families of India with high returns from low investments for past few decades. It is a great boon with financial independency to the tribals especially women for enhancing their economic status not only within the household but also in the society [3-4]. Tasar culture helps in providing year-round employment, protection of our bio resources, provides regular economic returns, generates employment opportunities and check environmental degradation hereby forms a subsidiary occupation of forest fringe dwellers whose livelihood is linked with tasar culturing and also helps in gender empowerment as all activities are undertaken by women folk [5].

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Tasar silk worms are affected by different bacteria due to various biological, chemical, nutritional, physical and environmental causes. Tasar silkworms are reared outdoor on food plants cultivated in 17 Indian states in the form of 44 eco races which are exposed to adverse environmental conditions, attacked by pests and predators and also suffers from various diseases which account for a loss of 40-50% during early instars [6-7]. Non mulberry silkworms are also affected by different bacteria like gram – positive bacillus and gram – negative micrococcus that causes sealing of anal lips, another species of bacteria causes chain type excreta while the third bacteria cause rectal protrusions through the anal opening. Quality of foliage, density of worms, handling of tasar larvae, usage of improved and control rearing techniques, exposure to different climatic conditions not only adversely affects the physiological functioning of the silkworm but also determines its robustness subsequently the crop yield which has strong impact on quantity and quality of silk [8].

The four silkworm diseases common in India are viz., grasserie (viral), flacherie (bacterial), muscardine (fungal) and pebrine (protozoan), of these diseases, bacterial flacherie in tasar silkworm accounts for a loss of 15-18%, the factors responsible for outbreak of flacherie disease is mainly due to change in climatic conditions. Flacherie is caused by different types of bacteria that prevail throughout the rearing period in tasar culture regions [9-10]. Based on the data it was found that the incidence of bacterial diseases was 15.01% followed by grasserie (12.08%), pebrine (10.58%) and others account for 7.07% at silkworm larval, moth and cocoon levels. Bacterial diseases are classified as bacterial septicemia, bacterial toxicosis and bacterial gastro-enteric diseases according to the type of bacteria which is more pronounced in larval period throughout the year in tasar culturing. The bacteria that induced the flacherie include bacillus sps., streptococcus sps., staphylococcus sps., proteus sps., pseudomonas sps., micrococcus sps etc.

The three different types of features found in the larvae suffering with bacteria.

Sealing of anal lips: A soil colored, sticky, semisolid fluid seals the anal lips. The larva shrinks length wise, becomes immobile, excretes brown sticky fluid that seals the anal lips.

Chain type excreta: The faecal matter in form of beads is excreted out with a jelly like substances in the form of chain and hangs with presence of infectious microorganisms. The larvae become thin, long and soft, stops responding to stimuli.

Rectal protrusion: The rectum protrudes out of the anal opening in the forms of transparent bag filled with green haemolymph [6]. The anal lips dilate and the body contracts lengthwise.

The other symptoms of flacherie include darkness of the body, loss of appetite, sluggishness of worms with slow growth, shrinkage, thorax swelling, brown specks on the skin, oral and anal discharges, liquefaction of inner organs, rupturing of skin and foul-smelling brown liquid oozing out of the body [11-12].

MATERIALS AND METHODS

Rearing of tasar silkworms was carried out in the sericulture field, Kakatiya University in the months of September-October 2016-2018. The tasar silkworms of third instar larvae that showed the symptoms of bacterial infection were collected during outdoor rearing for further studies.

Isolation of bacteria from tasar silkworms by serial dilution, agar plating method

The whole body of silkworm showing bacterial infection were surface sterilized with 0.1% mercuric chloride solution and wash thrice with distilled water, the larvae were triturated in sterile water using a glass rods, with the help of inoculation needles the suspension was streaked on nutrient agar media and incubated at 37ºC for 24 hours for pathogen growth[13] followed by the isolation of single colony of bacteria using serial dilution agar plating method.

The bacteria were cultured in the liquid broth and send for molecular identification

16srRNA gene sequence analysis for identification bacteria

16srRNA gene sequencing can be used for identification, classification and quantitation of bacteria within complex mixture and gut samples. This gene is highly conserved component, is suitable as a target gene for sequencing DNA in a sample containing different types of bacteria from a single sample. The sequencing files were edited using chromaslite (version 1.5) and further analyzed by Blast with closet culture sequence retrieved from NCBI. Database base were in the programme that compares nucleotide or protein sequence to sequence data bases.

The 16srRNA sequence of strain SEQ179_SEZOO 01_NC220319 showed closest relation to *Bacillus paramycoides* (100%), followed by (100%) homology with *Bacillus albus* strain with NR_157729. Our test strain which was isolated from the tasar silkworm cadaver showed closest homology with *Bacillus paramycoides* with an objective to identify the bacterial strain using 16srRNA PCR amplification and sequence analysis.

16srRNA Amplification

Chromosomal DNA was extracted by using spin column kit (Himedia, India). Bacterial 16srRNA gene (1500bp) was amplified using polymerase chain reaction in a thermal cycler and were purified using Exonuclease-I-Shrimp Alkaline Phosphatase [EXO-SAP] [14].

The purified amplicons were sequenced by Sanger method in ABI 3500xL genetic analyzer (Life Technologies, USA) and sequencing of the files (.ab1) was done with CHROMASLITE (version 1.5) and further analyzed by Basic Local Alignment Search tool (BLAST) with closest culture sequence retrieved from the National Centre of Biotechnology Information (NCBI) database that finds regions of local similarity between sequences [15].

The phylogenetic relationship of this bacterial strain isolated from tasar silkworm larvae were analyzed with other closely related bacterial species present in the GenBank, the sequence generated to table of closely similar bacterial organisms with the test bacterial strain, the sequence was obtained in FASTA format. Each isolate is reported for the first five-ten hits observed in the said database. The sequence that was collected from the BLAST were checked for multiple sequence alignment (MSA) T-Coffee tool from EBI (EMBL) and the data that was saved was analyzed using CLC sequence viewer and then converted into Nexus format finally tree was developed.

RESULTS AND DISCUSSION

Isolation, molecular identification of the bacteria

Molecular techniques are carried out for distinct classification and identification of bacteria by isolating the genomic RNA. The use of 16srRNA gene sequence for studying bacterial phylogeny has been most common genetic marker used for number of reasons. On basis of multiple sequence alignments and phylogenetic analysis of 16srRNA sequence by Chromaslite (version 1.5) and the strain SEQ 179_SEZOO 01_NC 220319 exhibited high level (100%) of similarity with the known sequence in the public database NCBI and Blast results and identified as *Bacillus paramycoides.*

Sequencing

Partial 16sgene of (1404bp) was obtained after sequencing and shown below FASTA format.

>530F_Seq 179_SEZOO 01
AGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGA
GATATGGAGGAACACCAGTGGCGAAGGCGACTTT
CTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTT
TCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCA
CTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACT
CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
TTACCAGGTCTTGACATCCTCTGACAACCCTAGAG
ATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTG
GTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATC
TTAGTTGCCATCATTTAGTTGGGCACTCTAAGGTG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG
ACGTCAAATCATCATGCCCCTTATGACCTGGGCTA
CACACGTGCTACAATGGACGGTACAAAGAGCTGC
AAGACCGCGAGGTGGAGCTAATCTCATAAAACCG
TTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTAC
ATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCA
TGCCGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCGCCCGTCACACCACG

Fig 1

Sequence analysis

The total 1404bp partial 16srRNA was retrived in FASTA format and subjected for BLAST search in GenBank, which showed that the bacterial strain from tasar silkworm was similar to *Bacillus paramycoides* with 100% similarity and E value of 0.0.

Query ID: SEQ 179_SEZOO 01_NC 220319.
Description: Bacterial strain from tasar silkworm cadaver.
Molecular type: Nucleic acid
Query Length: 1404
Sequences producing significant alignment:

Sequencing results in tabular form

Strain	Aim	Primer	NCBI BLAST (Type Strain)	Remarks
SEQ179_SEZ OO 01_ NC220319	Identification of 16S rRNA gene ++ Strain received from Prof. Dr. Edla Sujatha.Kakatiya University	530F_907 R(1404bp)	NR_157734 *Bacillus paramycoides* Identities: 1404/1404(100%) Int. J. Syst. Evol. Microbiol. 67 (8). 2499-2508 (2017) ++ NR_157729 *Bacillus albus* Identities: 1403/1404(100%) Int. J. Syst. Evol. Microbial. 67 (8). 2499-2508 (2017)	Strain showed closest homology with *Bacillus*sp. (Closer to *paramycoides*)

Fig 2 BLAST score, query coverage and E-value for the query sequence

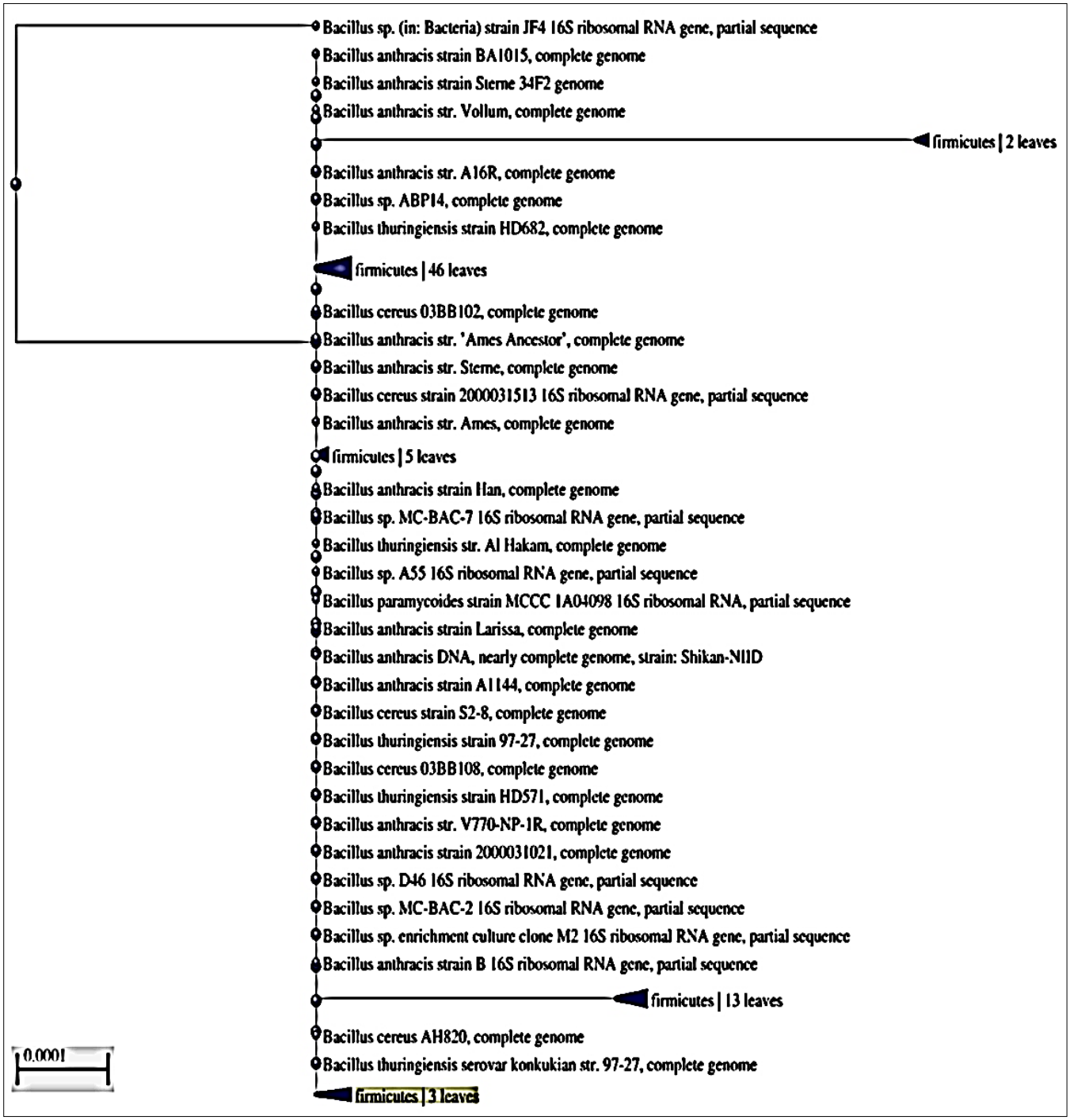

Fig 3 Similarity of the tested organism based on16srRNA sequence

Phylogenetic relationship of the bacterial strain from tasar silkworm cadaver was analyzed with 16srRNA, sequence of similar bacterial strains. Finally, it was found that the sequences of closely related bacillus species when matched under multiple sequence alignment and phylogenetic analysis showed that the tasar bacterial strain was under the group of Bacillus sps and the strain showed closest monology with *Bacillus paramycoides.*

On the basis of molecular identification and phylogenetic analysis revealed that the bacterial strain comes under Bacillus group and is closely related to *Bacillus paramycoides*. It forms white opaque colony is that are characteristically hairy in appearance repeated spiral pattern, growing in aerobic and anaerobic inhabitable. Gram Positive, rod shaped that form circular colony with a cell length of 1.8 to 2.2µm. Colony size 2 to 3mm, colony shape circular, temperature range 15 to 39ºC, and pH 5 to 9.

CONCLUSION

In conclusion species specific 16s rRNA gene sequencing is an important tool for rapid identification and distinguishes closely related species. It also helps in resolving phylogenetic relationships at genus level. The phylogenetic tree constructed on sequence bade revealed that the newly isolated Bacillus paramycoides was closely member of Bacillus species with 100% similarity and length.

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