Assessment of gut microbiota associated with oak tasar silkworm, Antheraea proylei J. (Lepidoptera: Saturniidae)

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July 31, 2020

Abstract

Antheraea proylei J, is an economically important silkworm of North Eastern region of India reared for the production of the tasar silk. The silkworm is often exposed to various microbial diseases caused by bacteria and viruses. The disease causes significant damage to larvae and elicit pupal mortality, thus posing a serious threat to the linked economic activities. The gut microbiome of silkworms play an important role, in nutrient acquisition and immunity. In this study, we have reported molecular characterization and histopathological assessment of gut associated bacteria of healthy and diseased tasar silkworms. As compared to healthy silkworms, diseased infected silk glands shows loss of turbidity, secretory layer not distinguishable to tunica propria and lumen distorted. Both secretory and absorptive cells were found to be hypertrophied. Body fat becomes vacuolated and soft when compared to the healthy silkworms. Bacterial profile of healthy and diseased silkworm respectively was identified by 16S rRNA gene sequencing and analysis. Bacillus toyonensis and Bacillus thuringiensis were commonly found in healthy larvae whereas Bacillus aryabhattai and Bacillus megaterium were found in diseased larvae. The family Bacillus of phylum Firmicutes was dominant in both healthy and diseased silkworms. To the best of our knowledge, this is the first attempt to study A. proylei midgut microbiota from a biodiversity hotspot in North Eastern India. The present study might be helpful in disease prognosis and further comprehensive analysis on midgut microflora may lead towards the development of effective strategies for management of these economic silkworms.

INTRODUCTION

The North eastern region of India is home to a number of wild sericigenous insects and is centre of wild silk culture including muga, eri, oak tasar, and mulberry silk (Lokeshwari & Shantibala, 2010). Tasar silk industry has lot of socio-cultural and traditional linkages in India since immemorial and plays a vital role on rural economy and hence indicates its impact on the county's economy simultaneously with agriculture(Reddy, 2010).The oak tasar silkworm, *Antheraea proylei* J, is an economically important silkworm of Manipur reared for the production of tasar silk. It is interbred between the male Indian species of *A. proylei* with the female of Chinese species of*A. pernyii* G(Jolly, 1969).The yield and quality of tasar silk cocoons are depended on the climatic conditions, silkworm health and nutrient absorption. Physiology and pathology of the silkworm's digestion, absorption, nutrient utilization and diseases emergence of the silkworm was closely related to microbiota of the larvae's midgut. Till date, there are no silkworm races at present, which are deemed as totally resistant to diseases or pests. At the end of the larval stage, silk productions are aided by silk glands whose infection affects silk production, resulting in substantial economic losses to the

farmers and industries(Brancalhão, Torquato, & Fernandez, 2009).As disease infected silkworms fail to spin cocoons, analysis of cytological damages in the silk gland is essential. Some histopathological studies showed the susceptibility of silk gland cells to BmNPV (Brancalhão et al., 2009; Khurad, Mahulikar, Rathod, & Rai, 2005; Rahman & Gopinathan, 2004). Hence knowledge of the microfloral changes of the gut in diseased conditions will help in understanding the health and nutrition of the silkworm(Yuan, Lan, Yang, Xiao, & Zhou, 2006)and give us ideas of management to improve the diseased condition during infection as such.

The intestinal tract microflora plays an important role in the health of the host by maintaining a normal ecological balance, regulating absorption, digestion, and assimilation (Jeon et al., 2011). Gut microflora are required in pheromone production, pesticide degradation and survivability, vitamin synthesis, and immunity against pathogens(Reeson, Jankovic, Kasper, Rogers, & Austin, 2003).In addition, these bacteria also resist and compete with the invading microbes and their propagation and strengthen the immune system (Tokuda, Watanabe, Matsumoto, & Noda, 1997). Microbial pathogens infect all animal species leading to disease and death. But their immune defence system helps in protection and survival(Miyashita, Kizaki, Kawasaki, Sekimizu, & Kaito, 2014). Insects especially their larval forms are more susceptible to pathogenic bacterial diseases and their virulence factors than vertebrates which further leads to alteration in host defense mechanism (Waterfield, Daborn, & Ffrench-Constant, 2004).Culture studies in laboratory conditions of insect gut bacterial communities do not reflect the entire microbial types and strains (Gilliam, 1997). Culture dependent methods screen only a few predominant bacteria genera and are unable to detect bacterial genera with low abundance. The 16S rRNA gene is often used as a marker for identifying the diversity of bacterial species in insect gut microbiota (Reeson et al., 2003). It is of notable fact that gut microbiota is involved in regulating the growth, development and environmental adaptation in the host.

However, information on the gut bacterial communities of many insects, including *A. proylei* is limited. The molecular information regarding this economically important silk moth remains severely limited till date and only a handful of DNA sequences are available (Shantibala, Devi, Lokeshwari, Anju, & Luikham, 2018; Yang et al., 2019). Additionally, very little is known about the effects of pathogens, its nutrient utilization and disease emergence of silkworm in its gut microbiota. Therefore research of the gut microbiota is of great importance .Little is known about the bacteria inhabiting the gut of this silk moth and even basic information on the insect's microbial symbionts is lacking. To date, there is no report on profiling of gut microflora in silkworm. Hence, in this present study, we aimed to compare the intestinal microflora of *A. proylei* of normal and Tiger band disease infected fifth instar larva reared under the same conditions using 16S rRNA-based sequencing method. Furthermore, we also assess the histopathological changes in the midgut and silk glands after infection to assess the tissue damages. Therefore, our study might provide insights for improvement and management of disease as a step towards conservation of wild seri biodiversity for ecological balance and for sustainable economic viability.

MATERIALS AND METHODS

Sample Collection

Healthy and infected fifth instar larvae of *A. proylei* were collected from Regional Sericulture Research Station, Mantripukhri, Manipur during summer season of 2019. The details of the location are furnished in Table 1. Healthy tasar silkworms and disease silkworms showing the symptoms of Tiger band disease were collected from the above mentioned area (Fig. 1 a & b). The infected larvae suffering from Tiger band disease were recognized from their black band pattern similar to tiger stripes appearing on the larva (Shantibala, Fraser, et al., 2018). Proper sterilized equipment such as forceps, scissors and hand gloves were used while collecting the insect sample. The collected samples were stored in an ice cold storage box. Samples were brought from the field and surface sterilization was done with 70% ethanol by submersion and rinsing three times using sterile distilled water prior to dissection. The gut tissue of larvae and midgut was dissected out in a sterile environment using dissection scissors. The collected intestinal contents will be immediately stored at 50% glycerol at -800C for future use (Broderick, Raffa, Goodman, & Handelsman, 2004). Three larvae from each location were taken for gut dissection. Individual larvae were used for the isolation of gut bacteria. After isolation of gut bacteria the larvae were transferred to laboratory conditions maintained at

26 ±1º C and 70 % RH at Animal Resources Division Laboratory, Institute of Bioresources and Sustainable Development, Takyelpat, Manipur, India.

Isolation and culture of the intestinal bacteria

Guts of healthy and diseased *A. proylei* larvae were homogenized in 0.86% NaCl solution (Broderick et al., 2004).The stock solution was prepared by taking 1 ml of the suspension and was mixed with 9.0 ml saline. Using the serial dilution method seven dilutions were prepared. 1ml of each dilution was added to a separate plate in triplicates. Then 15 ml of nutrient agar was added to each medium and incubated for 24 hours at 37°C. The dominant colonies were identified, purified three times by successive inoculation and culture on the corresponding agar plates, and further transferred to agar slants (Huang & Xin, 1999).

Histopathological Investigation

For histopathological investigation healthy and diseases infected fourth instar larvae were collected from Regional Tasar Research Institute, Manipur, India. Different organs like silk glands and gut were removed from normal and nucleopolyhedrovirus (NPV) infected silkworms. The removed tissues were preserved in 10% formaldehyde solution. The tissues were again fixed in Bouin's fluid. The water molecules were removed with the help of alcohol and embedded in paraffin wax for sectioning. 5-7µm thick tissues were stained with haematoxylin and eosin. Structural examination and histopathological changes were identified by visualization through Leica DM 3000 LED and photographed with Leica DFC450 C.

DNA extraction, amplification, purification and sequencing

Total genomic DNA was extracted from the intestinal contents using Gsure Bacterial Genomic DNA isolation kit. The quality of the extracted DNA was assessed by electrophoresis in 1% (w/v) agarose gel. The concentration of the extracted DNAs was determined using a Nanodrop (Thermo fisher Scientific) and then normalized to 200 ng/µl. Universal 16S rRNA genes were amplified by PCR in a volume of 25µl containing 200 ng DNA, 5X Phusion HF buffer, 10mM each dNTP, 2.5 U of Phusion DNA Polymerase, 0.5µM of forward and reverse primers as listed before (Weisburg, Barns, Pelletier, & Lane, 1991).

The PCR amplification was carried out with the following process: 94^0C for 5 min; 35 cycles at 94 ^{0}C for 1 min, 50 ^{0}C for 30 sec, 72 ^{0}C for 2 min and a final extension at 72 ^{0}C for 10 min. The amplified PCR products were analysed by electrophoresis on a 1% agarose gel and visualized under Gel Documentation System (Bio-Rad). The PCR product was further purified using Gene JET purification column and sequenced at sequencing was done using BigDye® terminator kit following manufacturer's instructions (Applied Biosystems Inc. ABI, Foster City, CA) and products analysed on an ABI 3500xL Genetic Analyser platform (at Eurofins Pvt Ltd, Bangalore, India). Amplicons were sequenced from both directions using forward and reverse primers.

The purified PCR products were sequenced and aligned with the corresponding sequences in the GenBank database by using Blast search algorithm (Altschul et al., 1997).These sequences were deposited in the GenBank.

Sequence analysis

Online similarity searches were performed using the BLAST algorithm of GenBank. Sequence alignment was carried out with the CLUSTALW program by MEGA7 software(S. Kumar, Stecher, & Tamura, 2016). The phylogenetic analyses based on the 16S rDNA gene for proteolytic bacteria were further used to establish relationships among them. The partial 16S rDNA sequences obtained for were utilized in the search of reference nucleotide sequence available in NCBI GenBank database using BlastN algorithm to retrieve the similar sequences(Altschul et al., 1997). Phylogenetic tree and evolutionary relationships of bacterial isolates was constructed by Neighbor-Joining method (Saitou & Nei, 1987) with Kimura 2-parameter corrections (Kimura, 1980). To calculate the support for each clade, bootstrap analysis were performed with 1000 replications (Felsenstein, 1985).

RESULTS

Histopathological Observations

Silk glands

The three layers of silk glands such astunica propria, glandular layer and tunica intima were visible in normal silkworm. The secretory cells have rich secretory granules with a wider layer compared to tunica propria. Tunica propria is narrow and the lumen consists of silk mass. Glandular zone has a large number of haemocytes, whose nuclei are branched. (Fig. 2 a). In disease infected worm, the silk gland shows loss of tunica propria integrity and secretory layer is not easily distinguishable from tunica propria. Lumen is seen distorted with vacuolation in the silk mass. The silk mass loss compactness and becomes less dense (Fig. 2b).

Midgut

Mid gut in the larvae is mainly involved in digestion and absorption. Histologically, a stratum of enteric epithelium, the outer ends of whose cells rest upon a basement membrane lines the midgut. The latter is followed by an inner layer of circular muscles and an outer layer of longitudinal muscles. The midgut cell are of two types, columnar cells and secretory or goblet cells. In addition regenerative cells replacing the destroyed epithelial cells during moulting are also present. As healthy larvae mid gut are rich in digestive activity, so the secretory cells are seen normal and the absorptive cells are normal indicative of good transport of absorbed material. Regenerative cells and their nuclei are prominent. The basement membrane and musculature are normal (Fig. 3a).

However in the diseased silkworm, the epithelial layer of the midgut lacked continuity. After infection, the hypertrophy was much more pronounced and large vacuoles were formed in epithelial cells of*A. proylei* midgut. Both secretory and absorptive cells were hypertrophied. Absorptive cells are fully loaded indicating loss of its absorption. The inter-cellular spaces widened to a great extent and epithelial tissues detached from the basement membrane. Also bacterial populations were seen with dark masses inside lumen indicative of its infections corroding the epithelial layer (Fig. 3b).

Phylogenetic Affiliation of 16S rRNA

We analysed the 16S rRNA sequences for the phylogenetic affiliation of both unidentified and identified strains by neighbour-joining analysis with the optimal criteria set for distance in MEGA 6. 16S rRNA sequences were compared with sequence data deposited in GenBank using the BLAST search program. Comparative analysis with GenBank sequences revealed that most of the intestinal bacteria were showing 99% similarity and a few were showing 98% similarity to their closest relatives retrieved from the Gene bank database. The 16SrRNA sequences of gut bacterial isolates generated from our study were submitted into GenBank vide accession nos.MT416410.1 to MT416415.1 and the details are presented in Table 2.

Bacterial isolates obtained from the midguts of *A. proylei* were screened based on their colony characteristics and identified on the basis of 16S rRNA gene sequences. Based on 16S rRNA gene sequence analysis, tasar silkworm, *A. proylei* showed Firmicutes as the dominant group forming a major clade with Bacillus as the dominant genus with ten different species.

Bacillaceae was found to be abundantly represented in the gut of both healthy and diseased A. *proylei* (Table 3). Bacillus was found to be the predominant genera in our study from cultivable gut bacterial isolates of *A. proylei* of North Eastern India. The predominant Bacillus genus was represented by species such as *Bacillus toyonensis, Bacillus thuringiensis, Bacillus pacificus, Bacillus mobilis* and *Bacillus mycoides* in healthy worms. The diseased group on the other hand are represented by species such as*Bacillus megaterium, Bacillus aryabhattai, Bacillus zanthoxyli, Bacillus flexus* and *Bacillus simplex.*

Phylogenetic analysis revealed that gut microflora diversity of diseased and normal tasar silkworms is almost similar with only 6 % differences between the groups. The evolutionary distances were computed using the Maximum Composite Likelihood method(Tamura, Nei, & Kumar, 2004). A phylogenetic tree was constructed

(Fig. 4) using the 16S rRNA sequences by neighbour-joining method 1000 bootstraps, to better understand the evolutionary relationship of the intestinal bacteria.

In the diseased silkworms, *B. aryabhattai* seems to be the most abundant genera found in all the diseased worms. In healthy silkworms, microflora is affiliated with the species *B. toyonensis* and*B. thuringiensis* , showing that these species share high sequence similarity. This showed more than 99% identity with the sequences deposited in the GenBank database.

Generally, *Bacillus* species are widely used as biopesticides and biofertilizers in agricultural practice (Pérez-García, Romero, & De Vicente, 2011), as potential probiotics for both animals and humans (Cutting, 2011; Elshaghabee, Rokana, Gulhane, Sharma, & Panwar, 2017). Hence our results also might help in potential application of*Bacillus* species against a variety of pathogenic gut microflora.

DISCUSSION

Many Lepidopteran insects including silkworms are described as beneficial insects in the past two millennia. Gut microflora of most of the insects comprises a wide variety ranging from obligate endosymbionts to facultative bacteria and others (Dillon & Dillon, 2004). Comprehensive analysis of bacterial diversity within the gut of insects from different geographical regions will enable us to better understand their ecological roles and their interactions with the insect host. Very little information is available on the gut bacterial diversity of tasar silkworm, *A. proylei* which is exclusively present in the world in North Eastern regions of India. Culture-dependent 16s rRNA gene sequence based approaches in this study was used to assess the cultivable gut bacterial communities associated with these tasar silkworm populations in Manipur, North Eastern state of India.

Our analysis shows that the tasar silkworm harboured unique bacterial flora in its gut with Firmicutes group as the major one and Bacillus being the predominant genera constituting about 100 per cent of total gut bacterial isolates from *A. proylei.* Earlier studies have also documented the presence of Pseudomonas and Bacillus species as the dominant bacterial communities in the gut of gypsy moth, *L. dispar* (Lepidoptera)(Broderick et al., 2004). These results were in agreement with that of *Bombyx mandarina* and *Bombyx mori*gut bacterial diversity where phylum Firmicutes was found to be the predominant bacteria and Enterococcus to the predominant bacterial genus through 16S rRNA gene sequencing(D. Kumar et al., 2019). Bacillus was also the most dominant bacterial genus in an earlier study, accounting for 18% of the total gut community in 21 different insect species (Yun et al., 2014).

Gut morphology was early being described as a potential phylogenetically informative character in a study on Passalids (Bess beetles)(Reyes-Castillo, 1970). A limited diversity was revealed in 16S rRNA gene analysis of gut bacteria of *P. xylostella* larvae (Indiragandhi et al., 2007). The phylogenetic analysis of gut bacterial isolates from tasar silkworm indicates that tasar silkworm has limited gut bacterial diversity.

We also investigated the histopathological importance of healthy and diseased silkworm larvae to understand in-depth pathogenic effects a disease imposes on the cells, tissues and organs. As infected silkworms fail to spin cocoons analysis of cytological damages in the silk gland is very essential. The digestive system in silkworm larvae needs much attention as oral entry of pathogenic microbes are quite common. Apart from dual function of digestive and absorptive, the midgut region also provides a barrier to invading parasites too. Here we report for the first time the histopathological effects in *A. proylei* silkworm. Our results show that silk glands that contains tissue of infected larvae were ruptured and deformed along with the formation of lump cells compared with healthy larvae. In disease infected worm, the silk mass shows a notable destruction and vacuolation of the fat body cells and the fat tissues became soft and compactness as compared to those of the healthy insect. Also the IV instar larvae with disease stop feeding and colour change appears in the integument. The abnormal swelling, cell hypertrophy and other cytotoxic effects are the result of loss of silkworm ability to maintain homeostasis.

Histopathological changes in midgut by bacterial or viral infections have been documented in other silkworms (Choudhury et al., 2004; Jurat-Fuentes & Jackson, 2012; Mohanta et al., 2015; Ponnuvel et al., 2003).

The midgut portion of nuclear poly hydrosis infected silkworm larvae showed hyper trophism of nuclei. Hypertrophic nuclei were swollen compared to normal nuclei. We observed notable hypertrophy, hyperplasia and multi-layered epithelial cells of the midgut.

Also, phylogenetic tree analysis of the gut bacterial 16S rRNA gene sequences in our study (Fig. 4) showed Firmicutes as the dominant family forming a major clade with Bacillus as the dominant genus with ten different species.

It appears that the histopathological effects with respect to the Tiger band disease are mainly localized in the silk glands, midgut, and muscles surrounding the alimentary canal and body fat. The most common pathological changes observed include hypertrophy, vacuolation ultimately leading to insect death. Molecular phylogenetic analysis further reveals that Firmicutes and Bacillus being the predominant clade and genera in both healthy and diseased silkworms. Detailed characterization and further investigation from different geographical locations in the North eastern region of India would advance our understanding of diversity and composition of gut bacteria of oak tasar silkworms and its disease management.

CONCLUSION

To the best of our knowledge, this is the first attempt towards a genetic and histopathological characterization of the midgut microbiota of *A. proylei* found in Manipur, North East India. A more comprehensive knowledge about midgut bacteria may lead to better understanding the direct or indirect involvement of microbiota in the immune response, nutrition and reproduction of these important silkworm. This present study not only offers insight into the process of gradual degradation *A. proylei* larvae midgut, but also elucidates the extent of damage that occurs therein, after the infection which also affects the silk glands. The probiotic candidature of role of the potential gut microflora will also be a challenge ahead. Further our findings might advance our understanding in the field of disease resistance traits and silk worm *A. proylei* management.

ACKNOWLEDGEMENTS

The authors are gratefully acknowledged the help and support rendered by Director, Institute of Bioresources and Sustainable Development (IBSD), Manipur, Dept. of Pathology, RIMS and Regional Sericultural Research Station, Imphal for the conduct of this study. While the first author acknowledges Institute of Bioresources and Sustainable Development, Manipur India for financial assistance through IBSD-Ph.D. programme.

Conflict of interest: The authors have no conflict of interest to declare.

AUTHORS CONTRIBUTION

Conceived and designed the experiments: YRD, DSL, TS, YR. Sample collection and performed the experiments: YRD, SS. Analysed results and data: DSL YRD. Wrote Manuscript DSL, YRD, YR. YR supervised the study.

DATA AVAILABILITY STATEMENT

DNA sequences used in study have been released in NCBI with GenBank Accession numbers MT416410.1 – MT416415.1.

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Figure Legends

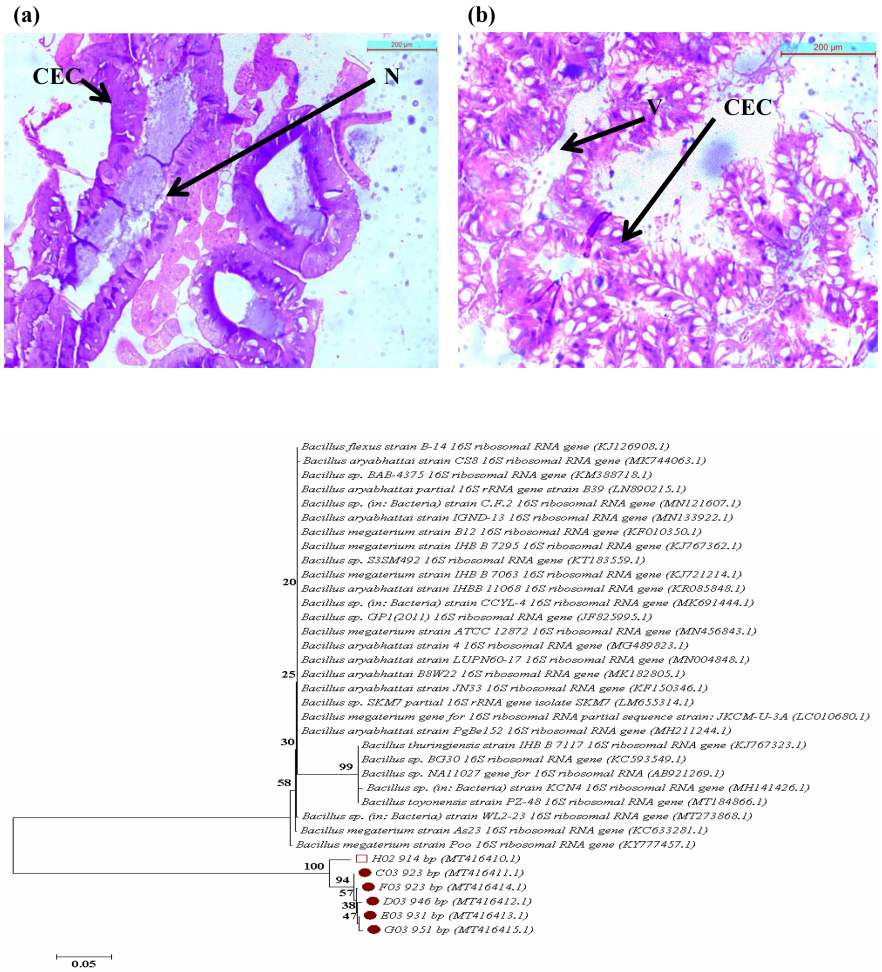
Figure 1: Oak tasar silkworm larvae, *Antheraea proylei* J (a) healthy and (b) disease larvae suffering from Tiger band disease.

Figure 2 : Microscopic examination of silk gland of normal silkworm and diseased at 10x magnification. (a) Silk gland of normal silkworm showing intact tunica propria (TP) and rich nuclei showing horseshoe shaped nuclei (N) (Bar=100μm) (b)The diseased silk gland showing loss of tunica propria (TP) integrity. At advance stage of infection, larvae shows small spherical nuclei (N) with appearance of vacuoles (V) (Bar=100μm).

Figure 3: (a) Cross section of midgut of healthy fourth instar larvae of *A. proylei* showing columnar epithelial cells (CEC)and compact darkly stained nucleus (N) (Bar= 200μm) (b) Cross section of midgut of TBD fourth instar larvae of *A. proylei* showing hyper tropheid columnar epithelial cells (CEC) and vacuoles (V) in cytoplasm (Bar= 200μm).

Figure 4: Phylogenetic tree of gut bacteria isolated from gut of*Antheraea proylei*

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.66091549 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. The tree is 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 Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7. Diseased silkworm (red circles) and healthy silkworms (square) are represented in analysis.



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