IJIE Int. J. Indust. Entomol. 47(1) 1-11 (2023)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2023.47.1.1

ISSN 1598-3579 (Print)
ISSN 2586-4785 (Online)
[Review]

Molecular methods for diagnosis of microbial pathogens in muga silkworm, *Antheraea assamensis* Helfer (Lepidoptera: Saturniidae)

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Abstract

The Indian golden muga silkworm, *Antheraea assamensis* Helfer is an economically important wild silkworm endemic to Northeastern part of India. In recent years, climate change has posed a threat to muga silk production due to the requirement that larvae be reared outdoors. Since the muga silkworm larvae are exposed to the vagaries of nature, the changing climate has increased the incidence of microbial diseases in the rearing fields. Accurate diagnosis of the disease causing pathogens and its associated epidemiology are prerequisites to manage the diseases in the rearing field. Although conventional microbial culturing methods are widely used to identify pathogenic bacteria, they would not provide meaningful information on a wide variety of silkworm pathogens. The information on use of molecular diagnostic tools in detection of microbial pathogens of wild silk moths is very limited. A wide range of molecular and immunodiagnostic techniques including denaturing gradient gel electrophoresis (DGGE), random amplified polymorphism (RAPD), 16S rRNA/ITSA gene sequencing, multiplex polymerase chain reaction (M-PCR), fluorescence in situ hybridization (FISH), immunofluorescence, and repetitive-element PCR (Rep-PCR), have been used for detecting and characterizing the pathogens of insects with economic significance. Nevertheless, the application of these molecular tools for detecting and typing entomopathogens in surveillance studies of muga silkworm rearing is very limited. Here, we discuss the possible application of these molecular techniques, their advantages and major limitations. These methods show promise in better management of diseases in muga ecosystem.

Int. J. Indust. Entomol. 47(1), 1-11 (2023)

Received : 16 Jun 2023
Revised : 12 Jul 2023
Accepted : 24 Jul 2023

Keywords:
Muga silkworm,
Antheraea assamensis Helfer,
Pathogen diagnosis,
Molecular techniques,
Metagenomics

Introduction

North East India has the unique distinction of producing all the four major varieties of commercially exploited silks *viz*. Eri, Muga, Tasar and Mulberry. Muga silkworm, *Antheraea assamensis* Helfer (Lepidoptera: Saturniidae) is an economic

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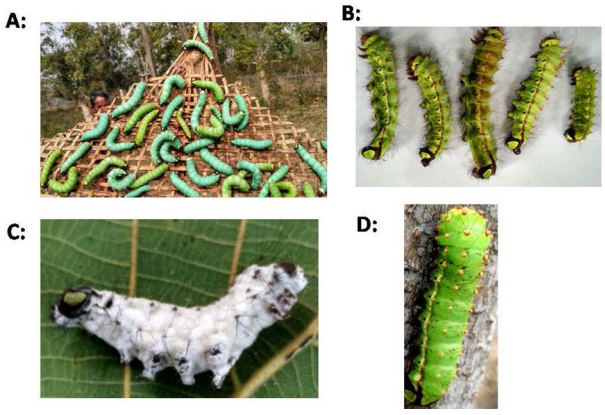

Fig. 1. A: Healthy muga silkworms (V instar, both blue and green morphotypes); B: Bacterial infected flacherie diseased dead cadavers of muga silkworm (V instar); C: White muscardine infected muga silkworm (V instar); D: Pebrinized muga silkworm (V instar)

insect, prerogative and Geographical Indicator (GI) to the North Eastern region of India (Tikader *et al*., 2013; Unni *et al*., 2011). Unlike other domesticated silkworms (*Bombyx mori* L. and *Samia ricini*), muga silkworms are wild and reared under outdoor conditions. Muga silkworm feeds primarily on "Som" (*Persea bombycina*) and "Soalu" (*Litsea monopetala*). The other important secondary food plants include "Digloti" (*Litsea salicifolia*) and "Mejankori" (*Litsea citrata*) (Tikader *et al*., 2013). Muga culture plays a significant role in sustainable rural livelihood generation and poverty alleviation. The production of muga raw silk in India during 2021-2022 was estimated to be 255 MT (http://texmin.nic.in/). The golden muga silk is considered to be one of the costliest silks in the world and price for a kilogram of muga raw silk yarn is approximately 390 US dollars. Due to the outdoor nature of rearing of muga silkworms, outbreaks of various diseases *viz*., flacherie, muscardine and pebrine (Fig. 1) are the major constraints encountered in muga industry (Chakravorty *et al*., 2007). Virosis, flacherie, and pebrine, which are caused by viruses, bacteria, and fungi, respectively, usually occur during every season and results in the death of considerable number of larvae, which ultimately affects the cocoon production (Subrahmanyam *et al*., 2019).

The golden muga silkworm is polyphagous, multivoltine (6 crops per year) and semi-domesticated in nature (Subrahmanyam *et al*., 2018). On the basis of environmental condition and cocoon quality, entire muga silkworm rearing seasons are divided into three distinct crops *i.e*. commercial (April-May; October-November), seed (August-September; February-March) and pre-seed crop (June-July; December-January) and all the crops play a key role in muga silk industry (Fig. 2). To

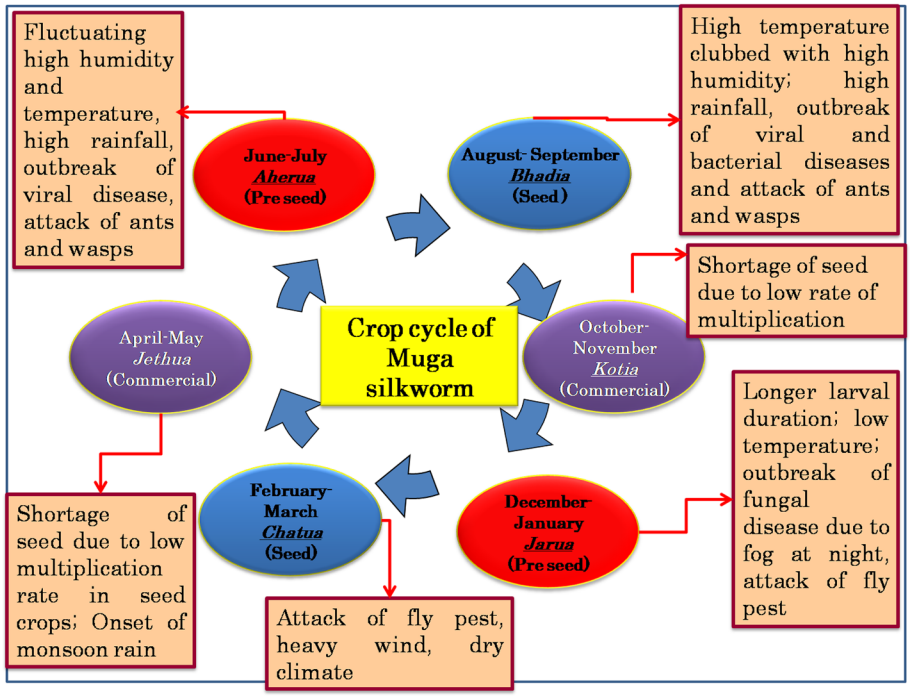

Fig. 2. Crop schedule of muga silkworm rearing in a year and their corresponding challenges. Local Assamese names for muga seasonal crops are marked with italics and underlines

raise commercial crops (Spring: April-May; Autumn: October-November), two seed and two pre-seed crops have to be raised in two separate rearing cycles. However, the climate during seed and pre-seed crops are very harsh, challenging and is responsible for incidence of various microbial diseases (Fig. 2). Even after providing quality foliage and suitable environmental conditions, in every rearing around 40% (average) crop loss normally occurs due to incidence of diseases (Subrahmanyam *et al*., 2018; Singh *et al*., 2014). In one study, it has been estimated that severe bacterial infection led to crop loss up to 80-90% in summer and rainy seasons (Das *et al*., 2014).

The incidence of diseases at the time of silkworm rearing severely affects the production of silk. Among the various diseases identified, bacterial flacherie is very common and has been reported to be caused by *Lysinibacillus* sp., *Serratia marcescens* and *Pseudomonas aeruginosa* (Subrahmanyam *et al*., 2023; Sharma *et al*., 2005; Choudhury *et al*., 2002). Nevertheless, these pathogenic bacteria were not obtained and a set of new bacterial pathogens (*Bacillus thuringiensis* and *Streptococcus bombycis*) which are common pathogens of *Bombyx mori* were reported in muga rearing field. Predisposing factors for flacherie are likely to be fluctuation in temperature, high humidity and poor quality of leaves (Chakravorty *et al*., 2007). When silkworms are physiologically weak, bacterial pathogens can attack them eliciting a heavy toll on sericulture. Hence, it is important to study the unknown pathogenic bacteria for developing effective control measures.

Entomopathogenic bacteria are classified within Eubacteria. This group contains three major divisions based on the presence or structure of the cell walls: bacteria with a Gram-negative type cell wall (Gracilicutes), Gram-positive type cell wall (Firmicutes), and Eubacteria lacking a cell wall (Tenericutes) (Vega and Kaya, 2012). However, their distribution and dynamics in muga silkworm host pathology is poorly understood (Subrahmanyam *et al*., 2023). Despite the fact that traditional microbial culturing techniques are frequently employed to identify pathogenic bacteria, they fail to provide useful information on a wide range of silkworm pathogens. Particularly, opportunistic pathogens and uncultivable bacterial/fungal members including microsporidia could not be identified through traditional microbiological practices. Furthermore, laboratory culture methods could underestimate the distribution, diversity and community dynamics of insect pathogens (both species evenness and richness).

Over the past decade, many molecular tools and techniques have been developed for understanding aetiology of bacterial pathogens (Gurtler and Subrahmanyam, 2021). These unprecedented advancements in modern molecular biology, particularly in those of DNA marker technology, have created a wealth of information in molecular microbial ecology in insects (Gurtler and Subrahmanyam, 2021). These molecular tools can be positively implemented and utilized in muga culture for improving production and productivity of silk. Therefore, the present article provides an overview of few important molecular techniques that can be helpful in understanding the pathobiology of muga silkworms.

Molecular Techniques

The immense phenotypic and genetic diversity found in insect microbial communities makes it one of the most difficult communities to investigate (Sangannavar *et al*., 2021; Gurtler and Subrahmanyam, 2021). It has been suggested that at least 99% of bacteria observed under a microscope are not cultured by common laboratory techniques. Given the high genetic and physiological diversity of bacteria, it is impossible to investigate all of them by using one single method (Sangannavar *et al*., 2021). However, different types of techniques can be applied to explore microbial diversity. Problems with studying microbial communities are caused not just by methodological issues, but also by a lack of taxonomic expertise.

A number of technological advancements have been made recently, resulting in the development of novel and more sophisticated methods for resolving the microbial community structure of insects and analysing community dynamics in relation to biotic and abiotic factors (Sangannavar *et al*., 2021). Methods for measuring microbial diversity in insect pathology can be divided into two categories: biochemical-based techniques and molecular-based techniques, each with its own set of advantages and disadvantages (Gurtler and Subrahmanyam, 2021). Following are some of the important molecular tools for exploring microbiota of insects including bacterial pathogens.

Polymerase chain reaction (PCR) based technologies

16S rRNA gene/Internal transcribed spacer (ITS) sequencing

A universal method for detection and identification of given bacterial species is PCR based 16S rRNA gene sequencing (Barghouthi, 2011). In this method, genomic DNA will be extracted,

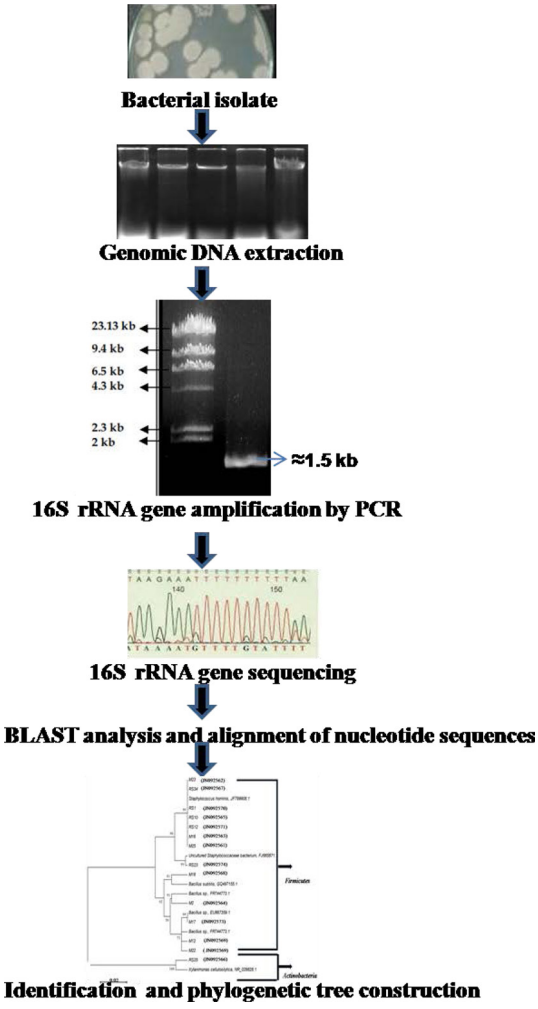

Fig. 3. Schematic diagram of molecular identification of bacterial isolate by 16S rRNA gene sequencing analysis

amplified with eubacterial 16S rRNA gene primers and sequenced (Fig. 3). The nucleotide sequence is then compared with a database library (for example, https://www.ncbi.nlm.nih.gov/; https://www.ezbiocloud.net/) to identify the bacterium. This method is simple and rapid to decipher phylogenetic information of the bacteria under investigation. Bacterial strains, *Bacillus cereus* SW7-1 and *Klebsiella granulomatis* (Li *et al., 2015;* Mohanta *et al*., 2015) pathogenic to domesticated mulberry silkworm *Bombyx mori* are identified by the aforementioned method. Rahul *et al*. (2019) identified *Staphylococcus argenteus* and *Bacillus carboniphilus* by 16S rRNA gene sequencing and the isolates were found to be pathogenic to *Bombyx mori* causing mortality upto fifty percent. Haloi *et al.* (2016) characterized the gut microflora of healthy and diseased muga silkworm by using 16S rRNA gene sequencing analysis. Results indicated that *Pseudomonas aeruginosa* (DRK1), *Ornithinibacillus bavariensis* (DRK2), *Achromobacter xylosoxidans* (KH3) and *Staphylococcus aureus* (FLG1) strains were commonly found in healthy as well as diseased larvae whereas, *Bacillus thuringiensis* (MK1) was found only in diseased larvae. Further, *Bacillus mycoides and Pseudomonas* sp., causing flacherie disease in muga silkworm were identified based on 16S rRNA gene sequencing analysis (Subrahmanyam *et al*., 2023). 16S rRNA gene sequencing will be helpful in understanding the disease prognosis and colonization of bacteria causing the disease in muga silkworm.

The 18S rRNA gene and internal transcribed spacer (ITS) region of the rRNA operon are widely considered as DNA markers/barcodes for investigating taxonomic identification of fungal species (Banos *et al*., 2018). ITS sequencing will be more descriptive to resolve species level classification of fungal pathogens (Ceballos-Escalera *et al*., 2022; Badotti *et al*., 2017). The causative fungal species for white muscardine disease in muga silkworm was identified as *Beauveria bassiana* through 18S rRNA gene sequencing (Subrahmanyam *et al*., 2018). Different strains of *Beauveria bassiana* were isolated from infected mulberry silkworms and identified by 18S rRNA gene sequencing analysis (Zhang *et al*., 2022; Wang *et al*., 2013). Further, ITS sequencing analysis revealed the identity of entomopathogenic fungi associated with lepidopteran insects which include *Aspergillus flavus*, *Penicillium* sp., *Fusarium chlamydosporum, Fusarium fujikuroi, Fusarium oxysporum*, *Fusarium solani* and *Alternaria* sp. (Gielen *et al*., 2022). Similarly, numerous studies also reported taxonomic identification of microsporidian pathogens (*Nosema* sp.) of silkworms and other lepidopteran insects through ITS/RNA sequencing (Bojko *et al*., 2022; Subrahmanyam *et al*., 2019). Nevertheless, the main limitations of this technique include methodological difficulties of DNA extraction, non-specific gene amplifications and sequence errors.

Despite the fact that ITS region works effectively as a fungal barcoding marker, it has been subject to debate in the recent past. The ITS based identification was not found to be efficient in some highly speciose taxa (Mongkolsamrit *et al*., 2020; Raja *et al*., 2017). Numerous species within a given genus cannot be separated using this non-coding region of DNA and moreover it isn't an essential gene that is going through evolutionary pressure (Raja *et al*., 2017). Thines *et al*. (2018) detailed a multitude of reasons why a sequence-based nomenclature will not be relevant for fungus anytime soon. Many researchers believe that the identification of fungi at the species level may necessitate

polyphasic taxonomic approaches, primarily combining morphological profiles (phenotypic data) as well as sequencing data (molecular data) from different genes (Raja *et al*., 2021).

Multiplex polymerase chain reaction (M-PCR)

The second group of PCR techniques encompasses assays where several non-homologous target sequences are amplified simultaneously in the same reaction tube (Kalle *et al*., 2014). Each target sequence is amplified with its own primer set. This type of PCR is referred to as multiplex PCR and is widely used in diagnostics (Kalle *et al*., 2014). M-PCR can potentially detect two or more pathogens simultaneously in a single PCR reaction system. M-PCR for individual and simultaneous detection of three major insect pathogens such as microsporidians, nucleopolyhedrovirus (NPV) and densovirus (DNV) infecting the silkworm, *Bombyx mori* was developed by Ravikumar *et al*. (2011). In their study, the authors designed three primer pairs, two from the conserved 16S small subunit ribosomal RNA gene of microsporidians and polyhedrin gene of NPVs, and a third from the internal sequences of BmDNV. Products of multiplex PCR differ in size and can be fractionated. An agarose gel is usually used for separation of amplicons as each type of amplicon can be visualized as a distinct band. This technique was successfully applied to detect six different insect microbial pathogens such as *Serratia marcescens, Pseudomonas aeruginosa, Bacillus thuringiensis, Metarhizium anisopliae, Beauveria bassiana* and *Oryctes rhinoceros nudivirus* (Kwak *et al*., 2015).

The technique will have potential implications in muga silkworm rearing to diagnose different pathogens *viz*., *Nosema* sp., bacterial and viral pathogens, simultaneously. This will facilitate timely control measures to be taken to save the crops. Simultaneous detection of distinct pathogens enables to understand the prevalence of latent/co-infection in muga rearing fields. The disadvantages of M-PCR include the need of higher initial concentration of the template sequences, low detection limit, and the competition between primers for the target sequences and for the reagents.

Loop-mediated isothermal amplification (LAMP-PCR)

LAMP is an advanced modification of PCR technique which will be a preferred choice as it is easy, fast, reliable, relatively cheap and sensitive (10 to 100 times) than conventional PCR methodology. Japanese scholar Notomi established the loop-mediated isothermal amplification (LAMP) in 2000 (Notomi *et al*., 2000). This technology has been continuously improved and widely used for the detection of pathogenic microorganisms (Parida *et al*., 2008). Recently, LAMP-PCR was developed to detect *N. bombycis* in mulberry silkworm using primers (LSU296) designed based on the sequence of the LSU rRNA. The minimum detection concentration was ten spores/mL (Yan *et al*., 2014). Furthermore, the LAMP method established in their study could detect *N. bombycis* infection in silkworm 24 h earlier than microscopy. Rapid detection and differentiation of *N. apis* and *N. ceranae* in honeybees was developed based on LAMP assay (Ptaszyńska *et al*., 2014). In their study, LAMP assays were performed at a constant temperature of 60 °C using species-specific primers, recognising eight distinct fragments of 16S rDNA gene. Recently, a field friendly LAMP technology was developed for rapid diagnosis of *N. bombycis* infecting *B. mori* silkworms (Sivaprasad *et al*., 2021a). The detection limit of the developed LAMP assay was found to be approximately 10^{1} dilutions of *N. bombycis* spores (Sivaprasad *et al*., 2021a). The technique is considered to be 100 times more sensitive than conventional PCR. This method can be readily utilized in muga silkworm grainages/seed production centres and rearing fields for testing large numbers of samples towards pebrine detection. The main disadvantage of LAMP-PCR includes limitations for multiplex system and involves complex primer designing protocol which can limit target site selection and specificity.

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA involves the PCR amplification of conserved 16S rRNA gene/ITS region followed by restriction digestion with tetracutter endonucleases enzymes such as HaeIII and AluI (Panigrahi *et al*., 2019). The digested fragments are further resolved on polyacrylamide or agarose gels. This technique is virtually an extension of PCR-RFLP technique. Universal eubacterial 16S rRNA gene primers such as 27F (Sequence 5' GAG AGT TTG ATC CTG GCT CAG) and 1107R (5' GCT CGT TGC GGG ACT TAA CC) are suitable for the amplification of 16S rRNA gene (Subrahmanyam *et al*., 2012). ARDRA pattern of each bacterial species will be utilised to generate dendrogram by binary scoring system (1 for the presence of the band and 0 for absence of band) with an input matrix using UPGMA (unweighted pair group method with arithmetic mean) clustering (Subrahmanyam *et al*., 2012). Each bacterial pathogen has a unique ARDRA profile and comparing the ARDRA banding pattern, one can readily diagnose the type

of the pathogen. ARDRA may be useful for rapid monitoring of various microbial pathogens in muga silkworm ecosystem. One can compare the diversity among microbial pathogens under contrasting environmental conditions through ARDRA.

Repetitive sequence based polymerase chain reaction (rep-PCR)

Repetitive sequence based polymerase chain reaction (rep-PCR) technique has been originally devised for characterization of bacteria and is widely employed to distinguish species, strains and serotypes. Eukaryotic or prokaryotic genome contains endogenous repetitive DNA elements that are distributed in multiple locations throughout the genome. The rep-PCR technique involves use of three specific primers, designated as BOXA (A sub unit of BOX element), ERIC (enterobacterial repetitive intergenic consensus) and REP (repetitive extragenic palindromic sequence) which have been designed to match the conserved sequences distributed in diverse bacterial/fungal genomes. These primers amplify regions located between repetitive sequences of genome. The resultant multimeric-PCR products with varied sizes are separated by agarose gel electrophoresis and a species/strain specific pattern will be generated (Rademaker *et al*., 2008).

Occasionally, rep-PCR patterns differentiate bacteria at subspecies level. Isolates with a similar fingerprinting will tend to cluster with each other. Full identification can be ensured with nucleotide sequencing of a single bacterial/fungal isolate from each group within the cluster. Rep-PCR has been widely used in diversified studies that account for accurate differentiation of bacterial, fungal and plant species (Rampadarath *et al*., 2015; Abdollahzadeh and Zolfaghari, 2014). Further, the technique was utilized for differentiation of rhizobacterial diversity (Lisek *et al*., 2011). This technique will be useful for muga silkworm pathobiology by allowing species-level identification of bacteria, fungi, and microsporidia.

Ribotyping through denaturing gradient gel electrophoresis (DGGE)

Genetic fingerprinting tools provide a pattern of the genetic diversity in a microbial community. DGGE is one of the routinely used molecular fingerprinting tools for studying microbial diversity including functional gene diversity in a range of environments. Muyzer, 1999 first time applied DGGE technique to study microbial communities in soil microbial ecology. For this technique, community DNA is extracted from soil samples and amplified using PCR with universal primers targeting part of the 16S rRNA (for bacteria) or 18S rRNA (for fungi) gene sequences. A 40 base pair GC clamp is attached to the 5'-end of the forward primer. The general principle is "On denaturation, DNA melts in domains, which are sequence specific and will migrate differentially through the polyacrylamide gel with a gradient of the denaturant (in general 40 to 60%)". In other words, separation of DNA fragments depends on the decreased electrophoretic

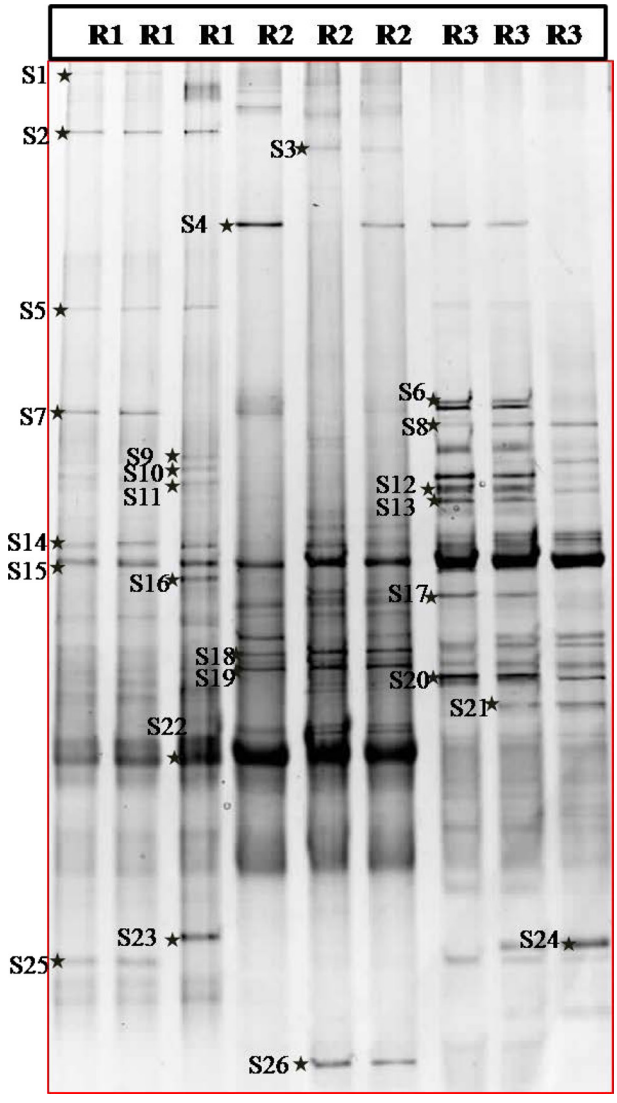

Fig. 4. Typical DGGE profiling of bacterial community structure. Each numbered band (shown with star mark) on the gel indicates one specific phylotype of the bacterial genera (Adapted from Subrahmanyam *et al*., 2016). Sequencing of the bands will identify the spatiotemporal dynamics of the bacterial genera

mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of urea and formamide. Theoretically, DGGE can separate DNA with one base-pair difference (Muyzer, 1999). DGGE has the advantages of being reliable, reproducible, rapid and somewhat inexpensive. Most importantly, multiple samples can be analysed simultaneously, making it suitable to detect changes in microbial populations. Typical representative DGGE bacterial profiling is shown in Fig. 4 (Subrahmanyam *et al*., 2016). Nevertheless, it has few limitations such as PCR biases and variable DNA extraction efficiency.

The technique has been successfully utilized to decipher gut microbial diversity of lepidopteran insects such as *Spodoptera littoralis* (Chen *et al*., 2016) and *Mythimna separata* (He *et al*., 2013). Further, the technique was utilized to address the gut microbial dynamics of Coleopteran insect, *Dastarcus helophoroides* (Zhang *et al*., 2014) and *Diabrotica virgifera* (Dematheis *et al*., 2012). Similarly, the technique can be utilized in the muga sericulture to understand the silkworm gut microbial profiling, dominant microbial species, diversity and dynamics of bacterial and fungal pathogens. Information on microbial species richness and evenness including the information on the opportunistic pathogens can be deciphered by this technique.

Antigen and antibody based methods

Immunofluorescence assay (IF)

Immunofluorescence assay relies on the binding interactions between antigen (Ag) and fluorophore labelled antibody (Ab). This method is widely used in immunohistochemistry based on the use of fluorochromes to visualize the location of the Abs through fluorescent microscope. There are four different types of IF assays among which direct immunofluorescence is very rapid and one-step procedure to identify antibodies bound to specific antigens of pathogens. This method allows researchers to visualise pathogen colonisation in different tissues and analyse insect pathogen interactions at the cellular level. This technique is widely employed to understand the *Nosema bombycis* colonization, life cycle and expression of distinct virulence genes in *B. mori* tissues (Dong *et al*., 2021; Wang *et al*., 2020; Chen *et al*., 2017). Expression of various microsporidia spore wall proteins (SWP 7, SWP9, SWP 26, EOB13250), polar tube proteins (NbPTP1, NbPTP2, NbPTP6) and their functions have been elucidated with IF assay (Lv *et al*., 2020; Wang *et al*., 2020; Yang *et al*., 2017; Yang *et al*., 2015; Li *et al*., 2009). The possible application of this fluorescence immune localization technique in muga culture would be to address the microsporidial pathogen recognition patterns, pathogen virulence mechanisms and concurrent host defence strategies to counter it. Details on identification of silkworm pathogens by immunodiagnosis were detailed by Sivaprasad *et al*. (2021b).

The main limitations of this technique include photobleaching, autofluorescence and non-specific binding of target proteins, which could result in reduced sensitivity and specificity of fluorescence signal. The technique is relatively expensive and demands sophisticated instrumentation and expert work force to get optimal fluorescence signal.

Fluorescence in situ hybridization (FISH)

FISH is a common name assigned to a variety of techniques employed for visualizing gene transcripts especially in eukaryotic cells. This technique is now further modified to visualize spatial localization of viruses and bacteria during the infection process. FISH targets RNA or DNA molecules in the cell and is a relatively easy and fast method for studying spatiotemporal localization of insect pathogens for diagnostic purposes. This method can be reliable and reasonably simple to implement when the procedures employ short hybridizing, commercially purchased probes. The method involves simple preparation, fixation followed by hybridization of entire insect mounts and dissected organs with 20 base pairs (preferably) short DNA probes conjugated to fluorescent dyes on their 5' or 3' ends. This approach has been effectively applied to a variety of insect and plant tissues, and it can be used to examine expression of mRNAs or other RNA or DNA species in the cell (Kliot *et al*., 2016; Kliot *et al*., 2014).

FISH probing is very popular and has several advantages over conventional molecular techniques as it offers a non-invasive cultivation independent approach for identification, quantification and localization of cellular targets including nucleic acids, proteins, cell membranes, vesicles etc. 16S DNA FISH probes have enabled three-dimensional detection of bacteriomes in aphids and in eggs, larvae, pupa and male and female adults of whiteflies and other important pests (Chung *et al*., 2014; Aharon *et al*., 2013). In a previous study, sericin gene-1and chymotrypsin inhibitor-13 have been successfully localized in *Bombyx mori* by using FISH (Song *et al*., 2008). However, getting success in a FISH experiment depends on specific probe design, labelling

and identification of target including optimal hybridization conditions.

Metagenomics of insect gut microbiota

Over the past decade, metagenomics research has gained a lot of momentum to elucidate the genomes of both culturable as well as non-culturable microorganisms with an aim of understanding microbial dynamics of a wide variety of environments including insect gut microbiota (Dee Tan and Bautista, 2022; Chen *et al*., 2020; Shi *et al*., 2010). The metagenome analysis combined with the next generation sequencing approach will provide enormous genetic information allowing in-depth microbial diversity analysis (Chen *et al*., 2018a; Chen *et al*., 2018b). In this approach, insect gut metagenome will be extracted by suitable protocols (Subrahmanyam *et al*., 2016). The extracted metagenome will be assessed with gene specific PCR, or molecular fingerprinting methods or FISH or metagenomic sequencing (by next generation sequencing methods) followed by downstream data analysis with various bioinformatics mining tools.

Metagenomics revealed that gut microbiota plays a vital role in insect growth, development and reproduction. The composition of gut microbiome influences the absorption and utilization of nutrients (Dee Tan and Bautista, 2022). Very recently, the next generation based metagenomic analysis was carried out to compare the differences in gut microbial community structure as related to feeding habits of silkworm (Chen *et al*., 2018a). Metagenomics of *Bombyx mori* L. gut microbiome revealed the dominant microbiota such as *Enterobacter, Acinetobacter* and *Enterococcus* (Chen *et al*., 2018b). Further, *B. mori* gut microbiome analysis highlights the correlation between bacterial diversity, larval developmental stage and the age of mulberry leaves. Microbial diversity and species evenness were significantly increased in matured *B. mori* larvae (Dee Tan and Bautista, 2022). 16S rRNA gene based high throughput sequencing analysis deciphered the major bacterial genera (*Acinetobacter*, *Methylobacterium, Delftia, Sphingomonas* and *Pseudomonas* sp.) of *B. mori* L. gut samples (Dee Tan and Bautista, 2022). Muga silkworm pathogens and their spatiotemporal dynamics under contrasting environmental factors can be understood through high-throughput metagenomic sequencing analysis. Sample processing and efficient metagenome extraction processes are two critical elements to consider while performing insect gut microbiome research using metagenomic studies.

Conclusion

Timely and accurate detection of microbial pathogens are considered as one of the prime aspects of muga silkworm pathology for developing crop protection measures. In general, bacterial species will be identified by a polyphasic (genotypic, chemotaxonomic and phenotypic) approach. Although polyphasic approach is a gold standard in deciphering the identity of silkworm pathogens, it is very cumbersome and time consuming. On the other hand, frequent identification of microbial pathogens in large collections is not always affordable and possible in sericulture. Distinguishing bacterial isolates on the basis of physiological, biochemical and biological tests is not always successful and hardly one can draw any accurate information. Thus, accurate, reproducible and less time consuming molecular diagnostic techniques are need of the hour. With the advent of recent developed molecular techniques discussed in this review, one can deduce the etiological information of the microbial pathogens in muga silkworm rearing.

Acknowledgements

The authors are thankful to Central Silk Board, Ministry of Textiles, Govt. of India for the financial support in projects ARP5878 and ARP08007MI.

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