

Behavioral genomics: a way to study insect behavior in the post-genomics era

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Abstract: Thanks to recent developments in biology in the last 20 years, the classical genetics mode of studying insect behavior is now being actively replaced by new biotechnologies including massively parallel nucleotide sequencing and genome engineering. These two technologies have changed the way the researchers study insect behavior even in non-model organisms. Owing to the lack of research resources it was once considered a difficult task to study behavior in non-model insects, especially in serious agricultural pests, which is now a possibility due to cost effective sequencing and ease of gene disruption in these species. Several studies are now underway to understand the molecular genetic basis of behavior in several insects. This would provide a large body of information on peculiar behavior of many economically important insects, which would certainly help to address several longstanding questions in entomology.

Key words: Insect genomics, CRISPR/Cas9, phototropism, insect behavior.

The study of the genetic control of behavior is fundamental to an understanding of its evolution (Ewing and Manning, 1967) and we can now make a fairly consistent interpretation of behavioral evolution by comparative genomics approaches. The insects have proved to be an excellent material for the understanding of how genes influence behavior for three main reasons. First, the short generation time and high fecundity of many insect species make them well suited to a variety of quantitative genetic breeding designs. Secondly, many behaviors in male and female insects are highly stereotyped. Finally, insects have relatively simple genomes and many have been sequenced increasing the ease and effectiveness of genomic studies investigating the specific genes that regulate behavior (Hunt et al., 2018).

Previously, the techniques of classical genetics were used to study insect behavior and such studies were complicated by the limitations of classical genetics and the difficulty of detecting segregating units of behavior. Also, classical genetics techniques were mostly useful for comparing insect behaviors that differ at one locus only but became complicated when they differ at more than one locus. Today the scenario has changed. With the tools and techniques available currently, the behavioral genetics can be studied easily through the genomics route. Insect behavior controlled by QTLs can also be identified now and functionally characterized. In this article, I discuss why the current scenario is best suited to study insect behavior through the genomics approach and what are the biotechnologies that come in handy in achieving this? I have given a few examples

of how insect behavior can be studied through the application of new technologies.

Several studies have begun to identify the genetic toolbox that controls behavior in insects. Studying insect behavior is one of the very important aspects of insect biology as it is central to developing better management strategies for controlling agriculture pests, medically important insects as well as beneficial insects. Understanding the molecular genetic basis of why some insects behave the way they do will help to develop better insecticides for pest species and aid in improving the productivity of beneficial insects. The new biology heralded by technological advances in cost effective massively parallel sequencing of genetic materials and subsequent development in the field of genome engineering that allows for precise editing of the genome of an organism is leading to unprecedented progress in studies on the genetic effect of insect behavior. Subsequent studies can use methods for complex trait analysis in economically important insects to map behavioral genes, map out gene expression patterns and study gene -gene and gene - environment interactions.

The behavior observed in different animals is the result of the interaction between the genome and the environment. Here the broad view of 'behavior' as the 'response of an individual to a particular stimulus' is considered. Given that the macro-environment will be more or less the same for all the individuals of a species inhabiting a particular location, the differences in the behavior of individuals may be attributed mainly to the genomic makeup of the organism. Understanding the

genome would help us in linking the genetic loci to a behavior. Once linked the underlying information will be useful in practical applications. The newly developed DNA sequencing technologies have made the job relatively easy.

NGS and third generation sequencing techniques and their application in insects

Regular advances in sequencing technologies have spurred the rapid accumulation of insect genome sequences, setting the stage for a new era of insect science. The last two decades of decreasing DNA sequencing costs, simplified data analysis and proliferating sequencing services in core labs and companies have brought the de-novo genome sequencing and assembly of insect species within reach for many entomologists. However, sequence production alone is not enough to generate a high-quality reference genome, and in many cases, poor planning can lead to extremely fragmented genome assemblies preventing high quality gene annotation and other desired analyses. Owing to constant improvements in sequencing technologies, numerous insect genomes have been sequenced. More than a thousand insect genome sequencing projects have been registered with the National Center for Biotechnology Information, including 401 that have genome assemblies and 155 with an official gene set of annotated protein coding genes (Li et al., 2019).

Now that a large amount of DNA sequence data is available, the challenge remains to make its sense. Several labs are now working on utilizing this data in various ways. Thanks to inexpensive sequencing technology, currently the researchers are

getting representational sequences from whole genome from individuals of mapping population and analyzing to identify the genetic loci responsible for complex traits. This has shortened the research period from many years to less than a year. Global gene expression profiles can be prepared from tissues obtained from different treatments and one can identify the genes linked to specific behavior of the insect.

Differential gene expression studies

Genes influence behavior through the presence or absence of allelic gene variants in the genome or through differential gene expression. The best way to study the effect of genes on behavior is to link the dynamic expression patterns to behavior using different molecular biological studies such as mRNA quantification through massively parallel sequencing of mRNA transcripts. This has been made possible through technological advances that allowed the researchers to simultaneously examine the expression of thousands of genes via RNA-Seq. This kind of global transcriptomic analysis allows for an unbiased view of genes potentially underlying a behavior (Wong and Hoffman, 2010).

Differential gene expression studies help in identifying the genes linked to behavior of insect under study. A research was conducted in Honey bees to study

associations between grooming behavior and the expression of selected immune, neural, detoxification, developmental and health-related genes. It was found that the level of grooming behavior may be related to the expression pattern of vital honey bee genes (Hamiduzzaman et al., 2017). These genes may be further studied to find their suitability as bio-marker for behavioral traits and also as genetic markers in marker assisted selection to breed better honey bee strains.

Genome wide association studies

Association studies are presently conducted across the genome usually with Genome wide association studies (GWAS). GWAS searches for genetic associations using many thousands of markers spanning the entire genome. The markers, mainly Single Nucleotide Polymorphisms (SNPs) are single base-pair changes occurring at a higher frequency in a genome. GWAS utilizes many of the same methodologies that were used as classic linkage based QTL studies and they have become highly successful in identifying loci linked to quantitative traits of interest in many insects. Once the linked loci are identified the functional genomics approaches can be used to generate information about possible gene functions and study the genetic interactions between gene complexes and the environment (Hunt et al., 2018).

Using GWAS a few studies have been carried out to understand the behavior and adaptation of insects. The genetic landscape of insect behavior was assessed using GWAS in *Drosophila* (York, 2018). The results of the study provided evidence that different types of behavior are associated with variable genetic bases and suggested that, across animal evolution, the genetic landscape of behavior



is more rugged, yet predictable, than previously thought. However, the findings revealed several important caveats and prospects for future behavior genetic studies. The QTL mapping methods possess inherent limitations in detecting the complete genetic architecture of certain traits. Therefore, future studies of the genetic architecture of behavior will thus benefit from integrating QTL methods with results from genome-wide sequencing and genetic interrogations directed by genome editing (York, 2018).

CRISPR/Cas9 based Genome engineering in insects

With the development and application of genome editing, both the scope and depth of research on economically important insects were improved. Custom-designed nuclease

Fig. 1. Negative phototropism exhibited by the Indian Golden silkworm larvae. The late instar larvae tend to move towards ground when there is bright sunlight and less leaves on the trees

technologies such as the clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) system provide attractive genome editing tools for insect functional genetics. The targeted gene mutagenesis mediated by the CRISPR/Cas9 system has been achieved in several insect orders including Diptera, Lepidoptera and Coleoptera. Initially, the genome editing was mainly used to confirm some known functions or to verify candidate genes discovered by positional cloning. With the increased

applications of genome editing, functional genomics researches in insects have evolved, in which large scale analysis of genes in an entire pathway was conducted. Compared with RNAi technology, which regulates gene expression on the transcription level, the CRISPR/Cas9 system works on the genome level and is more penetrating and heritable, and therefore useful for functional gene analysis.

The CRISPR/ Cas9 technology has captured the imagination of insect scientists. It has been optimized and explored in more insect species beyond *Drosophila*. Nevertheless, the successful editing of insect genomes using this technology will require careful attention to the design and

assessment of the gRNA, the delivery of Cas9 and the gRNA, and the screening for and detection of insects carrying the mutation of interest. Finally, the reassessment of how genetically altered organisms are regulated, based on the latest science will definitely play an important role in the possibility of the future release of CRISPR-edited insects into the environment (Taning et al., 2017).

Studies are now going on that combine the genomics information and gene disruption techniques in economically important insects to address the questions linked to their behavior (eg., Yan et al., 2017).

Negative phototropism in Indian Golden silk moth, *Antheraea assamensis*

The Indian Golden Silkworm, *A. assamensis*, locally known as muga silkworm, is reared outdoors and it completes the larval stage on its host plants. The peculiar behavior exhibited by muga silkworms is negative phototropism towards the end of their larval stage. The larvae mainly in the fifth instar climb down the tree when they sense bright sunlight. This happens at around 10AM-12PM when there is bright sunlight (Fig. 1). This has been observed to happen when there are fewer leaves in the tree, which exposes the larvae to sunlight. This induces the movement of larvae towards the ground. Once they climb down they search for nearby host plants and climb them. This kind of behavior the farmers believe that the larvae sense the dearth of leaves for feeding and then they decide to leave the tree, which in reality is not the case.

When the larvae mature and ready to spin the cocoons, where they metamorphose to emerge as moths, they again climb down the tree and find a specific place to spin the cocoon. But this time they will start moving toward the ground at the time of sunset. This phenomenon is called as positive geotropism. These two behaviors are not observed in closely related Saturniid silkworm species *Antheraea mylitta* and *Antheraea proylei*. These two species of silkworms stay on trees the whole time of their larval duration and also prepare cocoons on the trees.

This peculiar behavior of muga silkworms is not yet studied in detail owing to lack of genomic information and gene editing technologies in this insect species. The ongoing efforts of Central Silk Board, India to prepare a high-quality genome sequence will aid in studying the genetic and molecular basis of negative phototropism and positive geotropism exhibited by muga silkworm larvae. Also, other closely related silkworm species are also being sequenced by different laboratories, the comparative genomics approach would identify the linked genetic factors and thereby help in disrupting them through gene editing.

To study the genetic basis of negative phototropism in muga silkworms, the transcriptomes from tissues of the larvae that climb down the tree maybe compared with those of the ones that do not show this behavior and continue the feeding on the tree. This will provide the information on the expression of the linked genes and such genes may be functionally annotated through transient knockdown via RNAi. The genes identified as linked to the behavior can be disrupted using CRISPR/Cas9

technology and look for loss of behavior. Thus, using the behavioral genomics datasets the causal relationship between gene expression and behavior, and their associations with time can be addressed.

Nocturnal behavior of *Spodoptera litura*

Insect pests cause significant damage to crops and affect agricultural productivity significantly. *Spodoptera litura* is an important polyphagous pest in India, China and Japan. It is a serious pest of various economically important crops such as cotton, groundnut, chilly, tobacco, castor, brinjal, pulses, etc. It is a noctuid moth nicknamed ‘night thief’ in Japan because of the voracious night feeding of the late instar larvae which steal crops and vegetables when farmers are asleep while cryptically hiding in the soil during the day.

A very interesting aspect of the *Spodoptera* study is that *S. litura* is extremely polyphagous and attacks over a hundred important crops, while another moth species well studied the domesticated silkworm, *Bombyx mori*, is strictly monophagous. A comparison between these two species will lead to a deeper understanding of insect-host plant interaction and also the nocturnal and polyphagous nature of *S. litura*. The genus *Spodoptera* constitutes a good model to explore host-plant specialization within phytophagous insect groups and to address exciting questions in phylogeny and ecological adaptation (monocots *versus* dicots or monophagy *versus* polyphagy). *Spodoptera picta*, which is closest to *S. litura*, is found in Japanese southern islands and feeds only on monocots of Liliaceae, whereas *S. litura* feeds mainly dicots. The comparison between *S. litura* and *S. picta*

could be very informative to study ecological adaptation. In addition, ‘‘Polyphagy’’ presents an interesting subject to be studied. Since each plant family has its own defense system against herbivores, polyphagous insects such as *S. litura* may have a ‘‘master system’’ to evade a wide-range of defense systems in host plants. The understanding of the molecular basis of behavior and adaptation mechanisms of *S. litura* could provide the required knowledge for devising pest control strategies.

Towards this end, the genome of *S. litura* has been sequenced and a physical map is made available. The genome sequencing project provided strong evidence on how this polyphagous insect has evolved to become a deleterious and powerful global pest through adaptive changes and subsequent selection of gene expansions (Cheng et al., 2017). Additionally, the CRISPR/Cas9 system induced efficient gene mutagenesis has also been standardized for *S. litura* (Bi et al., 2016). Utilizing these two main developments, many outstanding questions in this notorious pest can be studied.

As nocturnal insects like *S. litura* are active and also feed at night but sleep or rest during the day. The dichotomy of the two phases, namely feeding and hiding, possibly by circadian control, may have contributed to make them the most efficient herbivore in the field. Existence of such a mechanism can also be studied using the tools currently available to study this insect.

Foraging behavior in insects and its link to domestication of the silkworm *B. mori*

Population density varies over time and space, and plays a significant role in the evolution of characteristics within

populations. Population density affects a number of processes, for example, predator-prey and parasite-host interactions, the spread of disease, competition, population regulation, and territoriality. Population density was one of the main factors that affected the domestication of silkworm *B. mori* more than 5000 years ago. In nature, maintaining lower density will increase the chances of survival of organisms as it leads to the increased availability of food.

wild silkworm species closely related to the domesticated silkworm, do not show crowding behavior indicating that crowding behavior was selected for during the domestication process. Studying the molecular basis of this behavior would help in the domestication of wild silkworm species such as Tasar (*A. mylitta*) and muga. Muga silkworms exhibit non-crowding behavior; an adaptation for its survival in limited availability of food. The larvae



Fig. 2. Population density was one of the main factors that affected the domestication of silkworm *B. mori*(A). Similarly, the domesticated Saturniid silkworm Eri, *Samia ricini* (C) also can be grown indoors in higher population density. The same is not true for *B. huttoni* (B), a close relative of *B. mori*, and also for the saturniid silkworm *A. assamensis* (D).

However, for commercial sericulture, we need a higher density of silkworm larvae per unit area, which is a contrasting requirement. The continuous selection has made possible to rear a very high number of silkworms in the small area thereby increasing productivity. *Bombyx mandarina* and *Bombyx huttoni*, two of the bombycid

though feed in groups in the initial larval stage, move away from each other in search of food in late instars.

The foraging behavior has been studied well in the fruit fly *Drosophila melanogaster*. Normal individual differences in the *foraging(for)* gene of *Drosophila*

result in two behavioral types called rover and sitter. Larval rovers show a greater behavioral response to changes in their food environment than sitters. The *for* gene makes an enzyme called PKG, which is found in the head of the fly, as well as in most other organisms, including humans (Kent et al., 2009). As larvae, rovers move more and feed less in the presence of food than sitters, but do not differ in locomotion in the absence of food.

In the case of silkworms *B. mori* and *Samia ricini* larvae stay in groups while feeding and therefore are grown indoors, where the rearing environment can be controlled to get the desired yield (Fig. 2). However, *A. mylitta* and *A. assamensis* larvae tend to move away from each other. Identification of genetic factors linked to such behavior in silk moths through application of transcriptomics, GWAS and genome engineering would help in breeding new strains of tasar and muga silkworms that can be reared indoors in higher density.

Conclusions and future prospects

Almost all behaviors are associated with some form of heritable genetic variation. The interplay between genetic and environmental forces that shape behavior is complex and disentangling it requires an array of research endeavors spanning several disciplines. Previously, owing to limits in data and availability of research methods, the extents to which genetic architectures that vary across a full spectrum of behaviors and animal taxa remained largely unexplored (York, 2018). Now that the tools and data becoming available, behavioral biology in insects may begin to produce a more nuanced and predictive understanding

of the interplay of genetic forces governing the evolution of behavior, especially in the economically important insects.

Genome editing has not only accelerated fundamental research but has also shown promising applications in agricultural breeding and therapy. The recent publication of genome sequences even in non-model insects along with the development of CRISPR/Cas9 based gene disruption technique in non-model insects has provided required tricks and tools for behavioral genomics studies in insects including the non-model species. This ease of doing disruption of candidate genes as identified through genomics and transcriptomics approaches will pave new ways of studying insect behavior. Several ongoing studies to analyze the enormous sequence data in many economically important insects will provide deeper insights into genetic manifestations of the peculiar behavior of insects in their habitat. Such studies will be fascinating for basic biology studies in ecology and evolutionary biology as well as in addressing the long standing problems in agriculture and allied fields.

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