Review article

Molecular genetics and insect pest management

Genética molecular y control de plagas de insectos

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Abstract

The use of molecular approaches has dramatically changed the entire field of genetics, and this in turn has greatly affected applications of genetic technology including the area of pest management. The shift to molecular based techniques has brought many advantages, including the broader applicability to a wide range of species. In particular, this shift has done much to move the field away from the frustrations often associated with attempts to transfer genetic tools developed in *Drosophila* directly to pest species. Current applications of molecular genetic technologies in pest control include cutting-edge systems for genome editing and the use of RNA interference for selectively knocking out the expression of individual genes. Finally, as the field of genetics has shifted its focus from the analysis of individual genes to that of entire genomes, the application of genetic technology for management of insect pests has moved along a parallel track and brought even greater opportunities for enhancing the success of control programs.

Keywords: Sterile insect technique; RNA interference; Y chromosome; Insect pest management; Transposable elements; Genome editing.

Resumen

El uso de técnicas a nivel molecular ha cambiado por completo el campo de la genética y esto a su vez tuvo su efecto en las aplicaciones en la ingeniería genética como ser el área del control de plagas. Dicho cambio de las técnicas a nivel molecular ha traído numerosas ventajas, entre ellas la aplicación de las mismas sobre un mayor número de especies. En especial, este cambio hizo mucho para superar las frustraciones relacionadas generalmente con los intentos de transferir herramientas genéticas desarrolladas en *Drosophila* directamente a especies de plagas. Las aplicaciones actuales de la ingeniería genética molecular en el control de plagas incluyen sistemas de última generación para la manipulación del genoma y para la utilización de RNA interferente que permite suprimir selectivamente la expresión de un gen. Para finalizar, como el campo de la genética ha cambiado su perspectiva desde el estudio de genes individuales hacia el estudio del genoma en su conjunto, la aplicación de la ingeniería genética en el control de plagas de insectos ha incorporado nuevas ideas y brindado mejores oportunidades para optimizar el éxito de los programas de control de plagas.

Palabras clave: Técnica del insecto estéril; ARN de interferencia; Cromosoma Y; Manejo de plagas de insectos; Elementos transponibles (transposón); Manipulación del genoma.

Introduction

Historically, the need for improved agricultural practices has developed in parallel with the growth of human populations. As agricultural practices changed, insect pests would increasingly take advantage of the newly created ecological niches, and the need to manage them rose in direct proportion to the damage they inflicted. In a similar manner, the methods used to manage the pests had to change as well. Methods based on genetics have long played a key role in changing management practices, and the use of these methods has evolved as the field itself has developed and changed over time. This includes the progression from classical methods involving simple collections of mutations and selective breeding to improve strains used for control purposes to the use of sophisticated methods that are at the forefront of molecular biology.

Early on, the field of genetics was focused primarily on investigating heritable changes in or-

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ganisms without even knowing the true physical nature of genes. Much effort was placed on the collection and characterization of interesting mutations that affected everything from eye color to wing structure and, in some cases, behavior. The field of genetics was also among the first to recognize the importance of chromosomes studies in relation to heredity, and later genetics led the way to merge with the biochemical discipline of molecular biology to enhance the fundamental understanding of the nature of genes and inheritance. The field of genetics has now become focused almost entirely on analyses done at the level of the *genome*, or at least parts of it, as opposed to individual genes (Dale *et al.*, 2012).

The application of genetic methods in pest management began with methods using relatively slow and labor intensive techniques based on breeding or selection strategies to achieve heritable genetic changes or to understand basic biological processes such as mechanisms of sex determination. Much of this effort was directed towards the development of new strains for the sterile insect technique (SIT). The more contemporary methods for genetic manipulation use techniques of molecular biology that can produce virtually instant modifications of individuals and genomes through applications of genetic engineering technology. These changes have been applied with new technologies that allow researchers to work with entire genomes of many insect pest species instead of just focusing on one gene at a time. Finally, even as regulatory agencies continue to debate the cost *vs* benefits of issues such as allowing field releases of genetically modified flies, the latest advances at the forefront of molecular genetic manipulation using techniques of genome editing may soon render much of this debate irrelevant.

In short, information and ideas from both classical and contemporary molecular genetics have been applied to the management of insect pests. Each has played valuable roles in the development and applications of new methods for pest control and will continue to do so for the future. This review will focus on the more contemporary molecular genetic applications in the management of insect pests of agricultural importance. Many parallels can be found in efforts to use molecular genetic methods to control other insect pests such as mosquitoes (James, 2000), ticks and a variety of other species of medical importance (reviewed in Robinson, 2002 and Boetel *et al.*, 2015).

Early genetic research on insect pests

Many of the early efforts incorporating genetic methods into pest management relied heavily on conceptual thinking but were somewhat passive in nature, and much of this work was intended to parallel some of the massive body of information from basic research already in place for the genetics of the vinegar fly, *Drosophila melanogaster* (*e.g.* Lindsley and Grell, 1968). For example, in the 1980s, considerable effort went in to collecting interesting mutations and developing gene linkage relationships for some insects of agricultural importance such as the Mediterranean fruit fly, *Ceratitis capitata* (reviewed in Stratikopoulos *et al.*, 2008) and the apple-maggot fly *Rhagoletis pomonella* (reviewed in Roethele *et al.*, 2001). Around the same time, some very talented and dedicated cytologists began focusing on the development of maps of polytene chromosomes found in many pest species including the sheep blowfly (Childress, 1969; Foster *et al.*, 1980) and Tephritid fruit flies (Stratikopoulos *et al.*, 2008; García-Martínez *et al.*, 2009; Zepeda-Cisneros *et al.*, 2014). To some extent this was again done to parallel the highly developed maps available for *D. melanogaster* (Ashburner, 1989).

Genetics merges with molecular biology

As this work was progressing, the field of genetics itself inevitably began to shift toward incorporation of more molecular based approaches. These initially took several forms (Beverly and Wilson, 1984), but ultimately, for a number of different reasons, methods based on the use of DNA came to the forefront (Dale *et al.*, 2012). One of the major advantages of using the molecular methods was that a wide variety of specific types of DNA sequences could be directly isolated from the genomes of other insect species without the need for extensive homology or sequence similarities to *Drosophila* genes (Hoy, 2013).

Of the different types of DNA sequences that could be directly isolated from new insect genomes using these techniques, two of the most common were repetitive DNA sequences known as microsatellites and minisatellites (Haymer, 1994; Bonizzoni *et al.*, 2000; Stratikopoulos *et al.*, 2008; Lanzavecchia *et al.*, 2014). The isolation of these sequences took advantage of the vast reservoir of genetic variation present in portions

of the genome such as the repetitive DNA within centromeres and telomeres of chromosomes (Haymer, 1994). Consequently, the number of species of agriculture importance that became amenable to the development of basic genetic tools such as chromosome linkage maps incorporating these types of DNA based markers expanded dramatically to include other flies (Drosopoulou *et al.*, 2010) as well as beetles (Hawthorne, 2001) and bees (Hunt and Page, 1995) among others, including parasitoid species under consideration as agents for biological control programs of pest species (Homchan *et al.*, 2014).

Although not directly employed in the development of linkage maps and genetic control methods, the availability of these repetitive sequences can be invaluable for the characterization of the genetic structure of pest populations as well as other population parameters (Lanzavecchia *et al.*, 2014). Similar to studies of mitochondrial sequences, this information can play a vital role in related management applications such as area wide eradication and/or suppression efforts directed toward pest species (Alberti *et al.*, 2008).

Genetics and applied research in pest management

In terms of more direct applications for pest management, one of the areas where genetics played a clear role was in the development of new strains for improvement of the sterile insect technique (SIT). The basic concept of SIT, which had been first described more than 50 years previously (reviewed in Klassen and Curtis, 2005), is that large numbers of male flies of a pest species are reared, sterilized and then released to mate with wild females. When this method works, wild females are effectively eliminated from the breeding population, and over time the target pests should simply disappear through a lack of reproductive success. The use of this strategy has steadily grown and continues to be employed on a global basis to control a wide range of pest species (Klassen, 2005). Although SIT itself is not strictly a genetic method, several different forms of genetic manipulation played key roles in the development of new strains for use in SIT. The following section will review this material.

Sterile Insect Technique

Relatively soon after being conceived, SIT had already been effectively employed for control program targeting certain species in various localities around the world. However, it was also recognized that in some cases there was a clear need for new strains that could improve the efficiency of this method (Condona *et al.*, 2007; Cladera *et al.*, 2014; Meza *et al.,* 2014). The new strains were often euphemistically referred to as "genetic sexing" strains, and to develop these it was clear that there was a need for basic molecular genetic information on species where this approach could be applied (reviewed in Robinson and Hendrichs, 2005).

The concept of genetic sexing referred to the fact that although the goal of the SIT method was to have large numbers of sterilized males released to mate with wild females, during the rearing of the strain chosen for SIT, large numbers of females were needed only to build the populations up to the millions of flies per week typically required for releases. In fact in the early days of SIT, strains were selected for rearing primarily based on female fecundity and egg productivity in the laboratory (Knipling, 1955). At the final stage of rearing, females were collected and released along with the sterile males mostly because there was no efficient way to selectively remove them. Even though sterile, the released females would inflict extensive damage in the form of fruit stings when they attempted to lay eggs. This could serve as entry points for mold and bacterial infections, etc. and was largely considered to be unacceptable. Besides, the released females would draw the sterile males into mating with them instead of the wild females that were the intended targets. Clearly, if the females could be selectively eliminated just prior to release, the efficiency of the whole method could be dramatically improved, but how to accomplish this?

Chromosome translocations and SIT

One early genetic approach to achieving the desired separation was to use translocations to link visible or selectable markers to sex determination mechanisms, in particular to the Y chromosome (Condona *et al.*, 2007). This was feasible in many of the Tephritid species considered to be amenable to SIT because the presence of at least part of the Y

chromosome was sufficient for male sex determination (Lifschitz and Cladera, 1988; Anleitner and Haymer, 1992; Wilhoeft and Franz, 1996; Douglas *et al.*, 2004; Meza *et al.*, 2014). This system was functionally similar to that of mammals, and was in sharp contrast to the *Drosophila* system that depended on the ratio of sex chromosomes to autosomes for sex determination (Ashburner, 1989).

Fortunately also, a number of the visible mutations that had been more or less passively collected in the early genetic studies of different species were potentially usable for this purpose. For example in the medfly, which normally produce pupae with brownish color, a strain carrying a recessive mutation producing white colored pupae had been established. A chromosome translocation was generated linking the wild type allele of this gene to the Y chromosome (Franz and Robinson, 2011). This allowed production of males with the normal, brown colored pupae that were easily distinguishable from the females with white pupae. In mass rearing, large numbers of pupae could be produced and machines using photoelectric sensors could be used to sort the different colored pupae. With this technology, 99% or better separation of the sexes could be achieved at the pupal stage of development. An additional benefit here was that the female pupae could be recycled back into the rearing system while the male pupae were packaged, sterilized and released (McInnis *et al.*, 2004) for the control program.

Temperature sensitive lethals and other conditional mutations

A further improvement on this type of system became possible when mutations were identified that were expressed in a conditional manner. Conditional mutations are those where expression is dictated by, for example, environmental conditions such temperature. Especially for mass rearing situations, conditional mutations exhibiting sensitivity to temperature were in fact the most highly desired (Alphey and Andreasen, 2002; Schetelig *et al.*, 2009).

A series of mutations exhibiting such temperature sensitivity were identified in the medfly. For example, a temperature sensitive mutation was identified where the exposure of larvae to temperatures equal or exceeding 33 ^{o}C was lethal. In a manner similar to that described for the visible white pupal color mutation, a translocation was used link to the wild type allele of this temperature sensitive lethal (tsl) gene to the Y chromosome. This provided a system where females could be selectively eliminated at any time during the rearing phase simply by exposing the larvae to an elevated temperature. Male larvae would survive this temporary exposure to high temperature, and the pupae that emerged would be packaged for irradiation and shipment to the release point (Franz, 2005).

However, the strains utilizing either pupal color phenotype or temperature sensitivity both rely on translocations to achieve the sex separation. Especially in mass rearing situations, all strains carrying translocations have some tradeoffs in terms of reduced fertility. Also, over time, the chromosome rearrangements tend to break down and revert to a state close to wild type (Robinson and Hendrichs, 2005). These facts were part of the motivation for development of system where individual genes could be introduced at will into strains to achieve the desired goal.

Transposable elements and SIT

One way this could be achieved involved the use of mobile or transposable elements. Using these elements, genes can potentially be moved directly into strains without reliance on major chromosome rearrangements. However, the use of such elements still needed to be tied to some type of system where expression of a gene or construct was controllable and/or limited to only one sex. This need served as a primary motivation for identifying and characterizing genes involved in sex determination in these species with the hope that this would lead to the identification of gene specific promoters or other regulatory systems where expression was controllable or limited to only one sex.

As the interest in using alternative approaches involving transposable elements and genes involved in sex determination for management of pest species was developing, the breadth and scope of sophisticated genetic tools available to researchers utilizing *D. melanogaster* continued to expand dramatically (Ashburner, 1989) in both of these areas. First, some newly discovered mobile or transposable elements were discovered that could be used to introduce individual genes into strains as a new and more direct form of genetic manipulation (Handler, 2000) and potentially circumvent the

need for the use chromosome rearrangements in constructing new strains for SIT. Second, a series of genes involved in sex determination pathways, including several that were limited in expression to one sex, were discovered and characterized in a systematic fashion from *Drosophila*. The possibility that the genes and/or elements from *Drosophila* in both of these areas might be directly transferrable to insects of economic importance held great promise for researchers attempting to bring such sophisticated genetic tools to bear on the species that they were interested in, including many pest species (Robinson *et al.*, 2004). The following sections will look at both of these areas.

P and other transposable elements

One specific transposable element known as the P element was rapidly developed into a powerful transformation tool in *Drosophila*. Plasmids genetically engineered to carry P elements and other genes of interest were injected directly into developing embryos and transformation frequencies as high as 5-10% were routinely achieved in certain strains of *D. melanogaster* (Ashburner, 1989).

Given these successes, beginning in the late 1980s, much effort was expended in using this technology to introduce genes of interest directly into the genomes of Tephritid pest species, including the medfly *Ceratitis capitata* (Ashburner, 1995). In these cases, in addition to the P elements, genes for resistance to the antibiotic resistance neomycin were also included on the plasmids (McInnis *et al.*, 1990). Because the antibiotic resistance was in effect dominant and neomorphic in expression, it was potentially much more broadly applicable than markers used previously that depended on complementation of existing mutations to detect successful transformation events. In terms of novel strategies for pest control, this approach could be to introduce genes that could control and/or disrupt sex determination mechanisms or to introduce genes whose expression could be precisely controlled in a conditional manner (Alphey and Andreasen, 2002; Handler, 2004).

Despite considerable effort, with one or two rare exceptions, the use of P elements for genetic transformation of species other than *D. melanogaster* was never achieved (Ashburner, 1995; Handler 2000). In retrospect, it should not have been surprising that this approach failed to be applicable to other species. Extensive evidence showed that the P system of transposable elements, based on the concept of hybrid dysgenesis, was not universal. Even within *D. melanogaster*, the mobilization of the P elements only occurred when a male from a "P" strain was crossed with a female from an "M" strain. Despite much searching, the occurrence of true M strains appeared to be essentially unique to *D. melanogaster*, and this technology could not be readily transferred even to closely related sibling species such as *D. simulans* and/or *D. mauritiana,* let alone the more distantly related pest species such as many of the Tephritidae (Beverly and Wilson, 1984).

Efficient transgenesis in non-Drosopholidae species

However, the interest in the use of transposable elements for transformation systems continued to grow. New elements, such as the Minos (Pavlopoulos *et al.*, 2007), Mariner (Lampe *et al.*, 2000), Hermes and other elements (Atkinson and O'Brochta, 2000) were identified in different species, and some of these were the first shown to be capable of achieving transformation at a reasonable frequency in species outside of *D. melanogaster* (Ashburner, 1995; Atkinson, 2002; Sagri *et al.*, 2014).

Further down the road, what may be the closest thing to a universal system for insect transformation was developed based on the use of another transposable element known as *"piggybac"* based on a gene originally isolated from the cabbage looper *Trichoplusia ni* (Fraser, 2000). Handler (2002) adapted it into a vector that could be injected into insect embryos. Since its introduction in early 2000, this system has been used to genetically transform a wide variety of insect species, and is widely considered to be the key development that would force the USDA to adopt rules and regulations regarding the use and release of genetically modified insects for control programs (Hoy, 2000).

Sex specific patterns of gene expression

In terms of genes that might exhibit a sex specific pattern of expression, a number of genes involved in the sex determination pathway of *Drosophila* had been identified, and complete DNA sequences were available for many of them. These included genes such as *doublesex* (dsx) and *trans-*

former (tra) that exhibited some type of sex specific pattern of expression during development (Saccone *et al.*, 2011). However because of fundamental differences in the mechanisms of sex determination and the extensive evolutionary divergence between these insects (Beverly and Wilson, 1984), it was not clear that the same sex specific type of expression would be seen in pest species such as the Tephritids. Nonetheless, the extensive DNA sequence information available for these genes made them clear targets for isolation and characterization in other insect species based on the idea that there would be some homology with the *Drosophila* genes.

Unfortunately, as had been seen in the efforts to directly utilize *Drosophila* based transformation systems (such as the P element) in other insect species, the hope that this type of cross species gene isolation would be routine was quickly dashed. The theoretical basis for this approach is based on the idea that there will be some degree of DNA sequence similarity for each gene in the different species. In reality, once again the information and tools from the *Drosophila* based systems turned out to be unusual and not broadly applicable. One major reason for the inability to rely on cross species homology was the fact that significant differences in codon usage patterns at the DNA level were apparent even for highly conserved genes such as actin in these comparisons (He and Haymer, 1995). This meant even for genes that were functionally and structurally similar at the amino acid level, the use of DNA sequences from *Drosophila* to identify similar sequences in another insect species was not going to be simple or straightforward.

Perseverance did pay off in some cases, however, and a few homologs of *Drosophila* genes apparently involved in sex determination pathways were successfully isolated in Tephritids. These included homologs of *tra* and *dsx* that exhibited some sex specific difference in expression in a range of species (Shearman and Frommer, 1998; Kuhn *et al.*, 2000; Scali *et al.*, 2005; Saccone *et al.*, 2011). Also, Y chromosome sequences were isolated *de novo* directly from species such as the medfly, etc. (Anleitner and Haymer, 1992; Wilhoeft and Franz, 1996; Zhou *et al.*, 2000). This approach potentially represented a more direct way to isolate and characterize genes needed to at least initiate sex determination in these species.

Separate from these efforts, the advent of broadly applicable transformation systems based on vectors incorporating the *piggybac* element (Handler, 2002) did provide a way for researchers to propose alternative means for achieving the type of genetic sexing considered to be necessary for improvement of the sterile insect method. They proposed a system known as RIDL, an acronym for release of a dominant lethal (Alphey and Andreasen, 2002). This approach still depended on the availability of gene promoters that exhibited sex specific patterns of expression, but it took advantage of the conditional expression of *tet* promoter (RIDL).

Regulatory challenges for GMOs

Regardless of the approach used, the ability to produce transgenic arthropods, either for new strain development for improvements to the sterile insect technique or for the use an RIDL type approach, approval from the USDA and/or other appropriate governmental agencies will still be required. And although some detailed procedures for initiating the process of obtaining this regulatory approval have been described in Young *et al.* (2000), many of the issues described some years ago in detail by Hoy (2000) still remain. One of the great ironies here may be how quickly, as described below in the section on genome editing, the technological developments continue to outpace the regulatory process.

Genome level approaches

One feature common to all of the previously mentioned cases is the fact that they represent manipulations of single genes or single sites in the genome. However, as described in the beginning of this review, the field of genetics itself has moved from the level of single genes to one focused on collections of genomes, including that of whole genomes.

To a great extent this is possible because the genome of each organism can be analyzed *de novo*. In other words, as described for other molecular approaches, these methods can be carried out newly for each genome and with no dependence on gene homology or the transfer of technology from *Drosophila* (Schmitt-Engel, 2014). Comparisons are still often made to *Drosophila* to facilitate the annotation of genome level data derived from these studies, but this is not strictly a requirement. For example, to identify genes involved in insecticide

resistance in the oriental fruit fly, *Bactrocera dorsalis* (Hsu *et al.*, 2012) used a *de novo* assembly of the transcriptome (the transcribed sequences of the genome) of this species to identify several scores of genes actually or potentially involved in chemical resistance. Some *Drosophila* genes were used for comparison during the annotation process, but the raw data were generated and could be analyzed without the reliance on direct homology from *Drosophila* genes.

Another example of a genome level approach relevant to insect pest management, and one that was also developed independently of *Drosophila* centric work, involves the use of RNA interference (RNAi) technology. This approach was used to carry out functional studies of several thousand genes identified in genome of the flour beetle *Tribolium castaneum*. The RNAi method is designed to either completely eliminate, or at least knockdown, the expression of genes to a point where the functional properties of the gene can be clearly identified. In this method, small segments of RNA are used to inhibit expression by interfering with the translation of RNA transcripts from individual genes. Using this approach, Schmitt-Engel (2014) developed an extensive database of individual genes important in the early development of *Tribolium*. Many of the genes they identified have great potential for control applications, and many would likely have escaped identification using traditional candidate gene approaches based on cross species homology.

Genome editing

As the field of genetics continues to evolve, a host of new, recently developed techniques for genetic manipulation of insects (and other species) have been developed under the heading of "genome editing". This term is broadly defined exactly as the name implies. Specifically, here the genome of an organism is modified by editing or changing native DNA sequences rather than by introducing foreign or non-native genetic material as done when creating "traditional" genetically modified organisms.

A major advantage of this approach is the ability to target specific regions of the genome for modification. Prior to this, techniques for genome modification were literally "shotgun" approaches where the user had little or no control over the sites of integration and the subsequent fate of the genetic material introduced. Genome editing techniques, by contrast, are designed to target highly specific regions of the genome for modification (Haimovich *et al.*, 2015). Consistent with many previous advances in genetics, this technology has already been used extensively in *D. melanogaster*. Although a number of mechanisms have been employed to achieve this type of genetic modification, system based on the use of CRISPR-Cas9 technology appears to be the most widely applicable to any number of different insect species (Hsu *et al.*, 2014).

In this system, the CRISPR acronym stands for "clustered regularly interspersed short palindromic repeats", and the Cas9 appendage describing this system refers to an enzyme with endonuclease activity. This method was originally identified in bacteria where it is employed as a defense mechanism to mitigate damage that might otherwise be caused by invading viruses and/or foreign plasmids. Together, these two components form a complex that is capable of targeting and altering the DNA making up a specific region of a genome in almost any organism (reviewed in Sander and Joung, 2014). Versions of this system are already commercially available that include customized molecules designed based on the needs of the individual genome and/or system where the modifications are to be introduced, either through some type of embryo injection system or other cell based transformation system.

The CRISPR component of this system includes a single strand "guide" RNA (sgRNA). This guide RNA can be modified to carry a specific sequence, usually about 20 bases in length, which will be complementary to a specific region of the genomic DNA. This is somewhat analogous to the ability of single strand DNA primers used in the polymerase chain reaction to align with specific regions of the genome for targeted amplification. As described by Hsu *et al.* (2014), this targeting system can also be thought of as similar to the search function of contemporary word processors that can identify a specific string of letters in a lengthy word document.

The pairing of the complementary sequence in the sgRNA also serves to recruit the Cas9 protein to the specific site, and the exonuclease activity of this protein allows changes to be introduced to the genomic sequence. These changes include double strand breaks and repairs, modification of terminal sequences and other editing type functions (re-

viewed in Bassett and Liu, 2014). In short, this is a system that can modify the DNA of a genome in vivo, without the introduction of exogenous segments as is currently done in transgenic systems (Hsu *et al.*, 2014).

In *Drosophila*, two versions of this system have been most widely used for genome editing. One is based on the injection of plasmids into early embryos, either together or separately, containing the two parts of this system (the CRISPR and the gene encoding the Cas9 enzyme). A second approach uses strains that have been engineered separately to contain each of the two different components. This system is activated when the strains are crossed and the appropriate offspring are generated containing both elements (Bassett and Liu, 2014).

Conclusion

In conclusion, the use of genetic tools in pest management is likely to increase dramatically in the future, especially in the realm of biologically based control methods. The advent and increasing use of genome level tools holds great prospects for novel approaches to achieve this and for moving away from the need to transfer *Drosophila* based technologies to pest species. *Drosophila* will continue to serve as a model organism in many realms of biology, and will no doubt continue to contribute to the genetic understanding of pest species. However, given the fact that insects are among the most diverse organisms found on the planet, for the future it is clear that it will be to everyone's advantage to use technologies that consider each species independently and without the need to impose constraints for understanding the biology of each species.

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