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Evolve Genomix

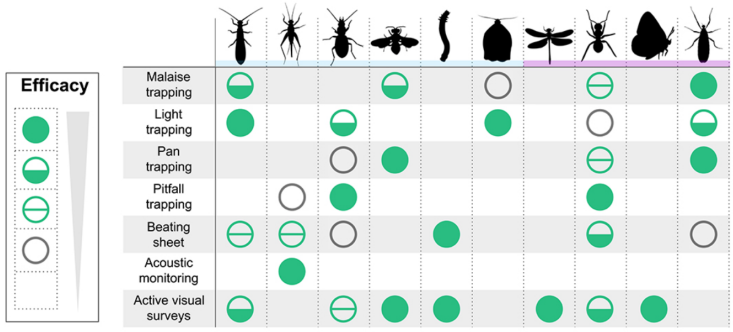
APPLICATION NOTE

Evolve Pest-Seq™: DNA Sequencing-based Pest Monitoring Test

Introduction

The economic impact of agricultural pests in the US is significant, running into billions of dollars annually. According to the USDA APHIS, the damage to crops caused by invasive insects and plant diseases in the US is about 40 billion USD (1) whereas globally this is about 290 billion USD, according to the UN Food and Agriculture Organization (2). The pesticide expenditures vary by crop type, but with high-value fruits and vegetables like tomatoes and strawberries, this exceeds USD 800 and USD 1600 per acre, respectively (3).

The pest monitoring market in the US is growing due to the increasing awareness of pest damage and the need for more sustainable pest management practices. The market size was estimated at around USD 1.7 billion in 2021 and is projected to reach USD 2.5 billion by 2026, with a CAGR of 6.6%. Increased adoption of precision agriculture, growing demand for organic and eco-friendly pest control methods and need for early pest detection to minimize yield losses and reduce pesticide use are some of the factors driving the growth in this market.



Commonly monitored insect-guilds and the efficiency associated with different traditional benchmarking methods (from G.A. Montogomery et al., 2021) (5).

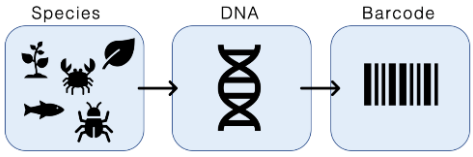
The traditional methods for pest monitoring and surveillance such as visual field inspection and microscopic examination of insects trapped in different types of traps for morphological differences are time-consuming, laborious and can be expensive (4). They also require specially trained personnel and the results can sometimes be subjective and not reproducible between different examiners as illustrated in the figure (5). Morphology can also be different according to the stage of the life of the insect, which can be challenging for the taxonomist to identify to species-level resolution. While modern technologies such as remote sensing using drones, imaging using high-resolution cameras and sensors, AI/ML based approaches, etc. are starting to improve upon some of these challenges, the taxonomical sensitivity, specificity and reproducibility are still lingering concerns. DNA-based

pest monitoring is a revolutionary approach gaining traction in the agricultural sector. This method involves analyzing insect traps or environmental samples like soil, water, or plant parts for the presence of specific pest DNA (6). While there are a few commercial methods based on PCR that are available to detect the presence of specific pests or plant pathogens, they are limited in identifying unknown or new pests.

Evolve Genomix has developed a next generation sequencing (NGS) based assay workflow called **Evolve Pest-Seq™** to detect the presence and provide the taxonomic identity of all insect pests and any vector-borne bacterial and fungal pathogens in sticky insect traps or other lure traps, as two independent modules (one for insects and another for microbes). The Microbe module of the assay can also be performed on plant tissue such as stem/bark, leaves, fruits or roots.

Technology Overview

NGS-based assays such as **Evolve Pest-Seq™** have several advantages over other molecular methods such as PCR, qPCR or ELISA that are used for pathogen diagnostics in humans, animals and plants. Unlike the 'Yes' or 'No' binary answer for a specific target that one gets in these tests, the NGS test can provide the sequence information of the target which confers them great specificity and is also very useful in elucidating the taxonomic information such as genera and species. Also, the NGS tests can be designed for "Hypothesis-free" testing, where the user does not have to assume or know what to look for in a sample and can gather information about all the different species present in a particular sample.



Evolve Pest-Seq™ sequencing assay targets multiple internationally accepted universal meta barcode regions for insects, bacterial and fungi in the genomic DNA extracted from the sticky insect traps. This test is potentially far more superior than the traditional morphology-based identification as it can detect all forms of the insect pests (fully grown insects, nymphs, male/female, larvae, eggs, etc.) that are stuck on the insect traps. Also, this DNA-based molecular identification is less subjective and far more reproducible and repeatable between the operators unlike the traditional morphology-based approach. This method is also a lot more scalable compared to the morphology-based detection since many samples can be processed together in large batches and unlike the traditional method, the cost of testing scales inversely proportional to the number of samples tested. Because of this, the turn-around time for this assay for even a batch size up to 96 samples can be limited to 5-7 business days, compared to weeks or months with some of the traditional methods.

Asian Citrus Psyllid (ACP), the vector for Citrus Greening disease

This method can monitor insect pests, including but not limited to the most dangerous Asian Citrus Psyllid (ACP) and Fruit flies, in farms, vineyards, orchards, ranches, residential areas and food storage warehouses. Farmers, regulatory and environmental agencies can also use this test to monitor the nature and abundance of the pests periodically to get a temporal picture of the pests in specific geographic areas and correlate that to the use of pesticides, climate changes or agricultural activity.

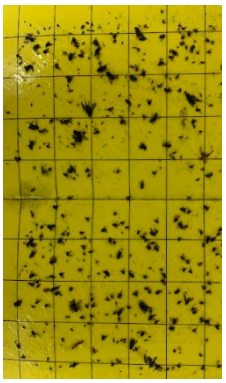
Along with identifying the insect pests, this test can also identify the taxa of the vector-borne bacterial and fungal pathogens present in the guts of the insect pests. Since this test can directly detect the presence of bacterial and fungal DNA in the insect guts, there is no need for isolating the pathogens and culturing them. This is a significant advantage as many of these vector-borne pathogens are not culturable in labs and even if they are, they require special culture media and growth conditions and take several weeks to grow. This aspect of detecting the insect pests and associated pathogens simultaneously can be very useful in understanding the pathogen-host interactions for new invasive pests and transmission routes for vector-borne pathogens. Besides, this test provides the farmers the mechanism for early detection of pests, needed to preemptively address any dangerous vector-borne pathogens like *Candidatus Liberibacter asiaticus* infecting their farms.

Case Study

In this application note, we showcase a pilot study of the **Evolve Pest-Seq™** test performed in our lab by our trained lab personnel. The scope of the pilot study is limited only to the detection & identification of the insect pests through **Evolve Pest-Seq™** and the identification of the pathogens in the pests was performed in a separate study.

The samples for the tests were Yellow Sticky Traps (insect) collected from 14 different citrus orchards that were spread across an area of 2500 sq. miles in the central valley in California. These insect traps were then shipped at room temperature and processed in our laboratory in Pleasanton, CA. Two different specimens of Asian Citrus Psyllid (ACP, Taxonomic name *Diaphorina citri*) were also processed alongside these insect trap samples as positive controls (7).

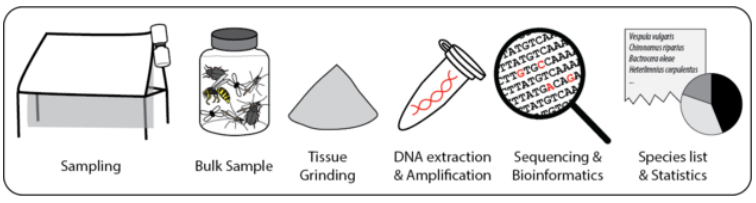
I. Experimental Methods



Yellow sticky insect trap with insects

Each of the insect traps was first treated and washed with roughly 5 ml of 70% ethanol to collect all the insect material collected on the traps into a trough. Then, the insect matter was homogenized thoroughly into a paste and about 1g was sub-sampled for cell lysis and DNA extraction. The same process was followed for the pure ACP specimens as well. The samples were then subjected to cell lysis by incubating at 56C overnight in Lysing matrix A (from MP Biomedicals). Then DNA was extracted and purified from the respective samples using a modified version of the NucleoSpin Tissue kit (from Machery Nagel). The elution volume was limited to produce a concentrated amount of purified extracted DNA, which was quantified using Qubit and subsequently used for sequencing library preparation. Each sample was processed as two replicates to ensure that the reported taxa is reproducible.

Universal metabarcode markers were selectively amplified from the genomic DNA of each of the samples using a proprietary optimized multiplex primer mix that contained multiple internationally



An example of Metabarcoding workflow that is optimized in Evolve Pest-Seq

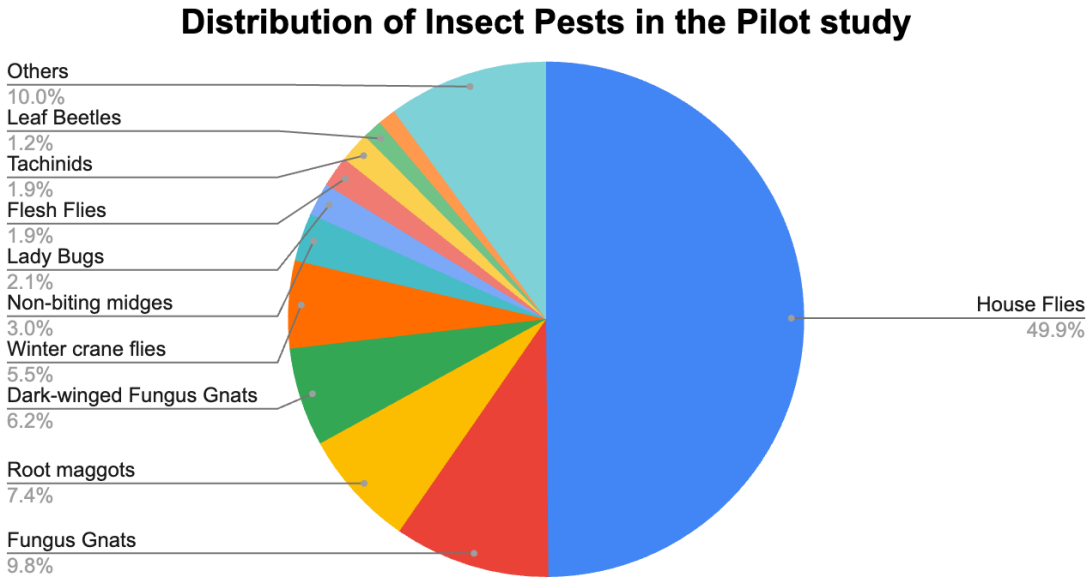
approved genetic markers common for all species in the animal kingdom. The amplicons from each of the samples were cleaned up using a bead-based purification method, attached to unique dual sample-level barcodes, followed by Illumina sequencing adapters and subsequently sequenced on MiSeq sequencer using the 2x250bp v2 nano kit for approximately 27 hours. Multiple run-level controls were included in this sequencing run as well. The FASTQ data from the sequencer was then analyzed using our proprietary bioinformatic analysis software and a specially-curated database to provide sample-level identification results. The results were then manually verified to create a sample-level taxonomical ID report.

II. Results & Discussion

- Out of the 14 insect trap samples, the **Evolve Pest-Seq™** test was able to
    - Detect & Identify 53 unique genera of insects
    - Detect & Identify 33 unique species
    - Resolve 55% of samples taxonomically to species-level resolution

  The actual number of genera and species that can be identified is significantly higher as the **Evolve Pest-Seq™** assay targets the Cytochrome oxidase subunit I (COI) region, which is well characterized and has a very large reference database (8).

- A high-level distribution of the different insect families identified in this study is shown in the chart below. While almost 50% of the insects captured in the insect traps used in this pilot study belonged to the family Muscidae (House flies), the assay also identified a lot of interesting insects belonging to very different families. Some of these orchards are having some fungal rot diseases and discoloration of fruits and interestingly, nearly 25% of the insects (Fungus gnats, Black-winged fungus gnats and Root maggots) could cause this. Potentially the abundance of these fungal vectors could be even higher since one of the most abundant genera identified in the House flies family is Tiger flies, which are known predators of fungus gnats. The category “Others” in the figure actually is a long list of clearly resolved families of very small abundance percentages.



- Individual sample-level reports provide the taxonomic distribution of different species present in the sample and the % of DNA of each species in the sample. An example of such a sample-level report is shown in the table below. The % of DNA present in the trap does not necessarily mean the % of abundance of specific insects, as many factors such as ploidy of the specific insect's genome, no. of copies of the universal metabarcode markers in the genome and to some extent, the refractory nature of certain insect tissue to the lysis method used can impact such a determination.

Sample ID	Taxonomical ID of Pests found	% of DNA in Insect trap
Orchard 1	Delia platura	44.9
Orchard 1	Muscina levida	31.3
Orchard 1	Scatopsciara atomaria	7.5
Orchard 1	Helicobia rapax	5.1
Orchard 1	Coenosia Sp.	4.2
Orchard 1	Chrysoperla Sp.	2.1
Orchard 1	Apis Sp.	1.7
Orchard 1	Phasia Sp.	1.1
Orchard 1	Ceratagallia omani	1.1
Orchard 1	Pegomya betae	0.3
Orchard 1	Macrosteles Sp.	0.2
Orchard 1	Lasioglossum Sp.	0.2
Orchard 1	Musca domestica	0.1
Orchard 1	Lonchaea cristula	0.1

- The two positive controls (ACP) ran alongside the insect trap samples were identified correctly as *Diaphorina citri*. The only other species present in one of the positive controls is the fungus *Penicillium citrinum*, which is a common fungal species present sometimes in moldy citrus fruits. It is quite possible that the control ACP insect specimen might have contacted this mold and it was present on the body of the insect.

Advantages and Limitations

Evolve Pest-Seq™ leverages the power of highly accurate targeted short-read sequencing to differentiate the amplicon sequence variations of a few universal barcodes present in the mitochondrial DNA to detect a very broad range of insect pests, concurrently in a large number of samples. This method is highly advantageous compared to other conventional methods that rely on the differences in the physical structure & shape of insects and hence are subjective, not very scalable and may not have the species-level resolution. Also, as demonstrated in the results of the pilot study (table above), this DNA based test has a high sensitivity to detect fragments of the insects from their different metamorphic stages. This test can have a number of practical applications to the agriculture industry:

- Routine monitoring of farms for early detection of insect pests and vector-borne pathogens can prevent crop losses, improve yields and reduce the expenditure in pesticides

- Species-level resolution of pests present in a farm can enable precision pest control methods that are crop-friendly and planet-positive.
- A detailed listing of all the pests present can be a great tool for diagnosing and root cause identification of crop diseases, in conjunction with other plant pathology data.
- An agnostic approach of detecting all vector-borne pathogens, including the ones that are not culture-able in labs or those that require special media and special incubation conditions is a huge advantage for properly identifying the root-cause for many plant diseaes
- The unprecedented faster turn-around time of 5-7 business days for both pest and vector-borne pathogens for large sample sizes enables rapid corrective action to protect the crops and the farm at large.
- Chronological data from a Pest Surveillance program can provide deeper insights into the effect of seasonal changes, weather patterns, global warming, deforestation and population movements on the type and abundance of pests emerging in a monitored area and how to mitigate them
- Data on relative abundance of pests can be used to understand predator-prey dynamics and can be harnessed to develop more natural ways to control insect pests

Sequencing-based tests are often considered as expensive, time consuming and requiring specific expertise, compared to other molecular tests. But, with the advent of many sequencing platforms and kits, the costs have significantly reduced over the years and with adequate batching of samples in a run, the price per sample for a targeted test like **Evolve Pest-Seq™** is very much comparable to other molecular tests. Besides, the wealth of information we can gain from a sequencing-based proactive pest surveillance program can more than offset the costs & time incurred in losses, damage control and reclamation once a farm or a warehouse has been ravaged by pests and vector-borne pathogens.

Conclusion

The growing menace of agricultural pests and the increasing pressure to ensure food security for a rising global population demand innovative solutions. Traditional methods for pest monitoring often fall short, lacking the sensitivity, resolution, and scalability needed for effective management. **Evolve Pest-Seq™**, our NGS-based assay, represents a leap forward in this arena. It unlocks a comprehensive understanding of the insect pest landscape in farms, vineyards, orchards, and beyond. The ability to detect a wide range of pests at all stages, coupled with the identification of associated vector-borne pathogens, empowers farmers with unprecedented knowledge.

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