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Rapid and cost-effective screening of genetic markers associated with pyrethroid resistance in Musca domestica using RNAse H2 PCR (rhPCR)

Alden Estep

alden.estep@usda.gov

US Department of Agriculture Agricultural Research Service

Neil Sanscrainte

US Department of Agriculture Agricultural Research Service

Alexandra Pagac

US Department of Agriculture Agricultural Research Service

Christopher Geden

US Department of Agriculture Agricultural Research Service

Edwin Burgess IV

University of Florida

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Abstract

Pyrethroid resistance, particularly knockdown resistance (*kdr*), is widespread in insect pest populations, but rarely has *kdr* been associated with field-level pest control failure. The prevailing understanding is that *kdr* contributes to a resistant phenotype, but this knowledge has remained largely an academic pursuit and has not translated to tools and strategies needed by agricultural producers to make rapid decisions for effective resistance management. As a first step in providing these operational tools, we developed robust assays using the high specificity of rhPCR to reduce *kdr* assessment time by approximately 80% and costs ~ 75% from the traditional Sanger based method used for *Musca domestica*. An important consideration for the use of an operational tool is the ability to get an accurate result on the first attempt, so we used Nanopore sequencing to confirm genotypes in a subset of samples and found the first pass genotyping accuracy of rhPCR method to be 75.0%, versus 41.2% with the traditional Sanger method. To demonstrate the broad applicability and comparability of screening for *kdr* SNPs using rhPCR, we conducted the largest assessment of *kdr* genotypes of *M. domestica* in United States dairy operations and found similar *kdr* patterns to other recent studies using traditional methods.

Introduction

Pesticide resistance affects the control of numerous arthropod pests worldwide, including those relevant to crops[1,2], animal production[3–5], public health[6], and the urban environment[7]. Consequences of pesticide resistance include the loss of crops[8], reduced animal performance (reviewed in Brewer et al.[9]), risk of outbreaks of vector-borne diseases (reviewed in Rivero et al.[10]), and litigious infestations of urban pests[11], to name a few. Pest surveillance, which includes pesticide resistance surveillance, is an important facet of an effective Integrated Pest Management program [12].

Numerous resistance mechanisms have been described across arthropod species, including metabolic detoxification, reduced cuticular penetration, and modifications of the pesticide target site (i.e., target site mutations). Of these, target site mutations are often implicated in strains with strong resistance phenotypes[13,14]. Target site mutations are single nucleotide polymorphisms (SNPs) resulting in changes in amino acid codons of the target ion channels or proteins (e.g., acetylcholinesterase), which, when translated into the protein, alters various properties of the target, including its susceptibility to pesticides[15]. Often target site SNPs act in ensembles with additive properties to create higher levels of phenotypic resistance. Common target site mutations include “resistance to dieldrin”, or “*rdl*,” which is a mutation in the *rdl* subunit of arthropod GABA/glutamate-gated chloride channels, conferring resistance to dieldrin[16], fipronil, abamectin[17,18], but not fluralaner[19]. Another common set of mutations is found in the gene for acetylcholinesterase, an enzyme that hydrolyzes the neurotransmitter acetylcholine into choline and acetic acid[20]. These mutations alter affinity for organophosphates and carbamates. One of the most implicated target site mutations due the widespread use of pyrethroids in countless pest control systems all over the world is “knockdown resistance,” or “*kdr*,” which was first described in house flies by Busvine[21] (1951) shortly after DDT was brought to market. All three of these target site

mutations have been described in bed bugs[13] and importantly, in house flies[14], as this offers an exemplary model for surveillance tool development for broad application.

Commonly used methods for the detection of target site mutations include Sanger sequencing[4], multiplex PCR[22], and allele-specific PCR[23] (which includes melt curve analysis (MCA)). However, as with all methods, each of these has benefits and limitations. While Sanger sequencing is the traditional "gold standard" for target site mutation detection[4,14], it is usually reliant on a remote facility (which may not be locally available) and cannot be multiplexed, thus making it challenging to use for large sample sizes. There is also a level of subjectivity in play when determining if two in-phase peaks can be "called" a heterozygote during inspection of electropherograms, and the threshold of the minimum signal needed to make a call is rarely identified. Multiplex PCR, as another method, is considered inexpensive and fast but can result in false positives if methods are not optimized properly[24,25]. Allele-specific PCR is also inexpensive and fast but relies on visual inspection of gels where faint bands pose a challenge [26]. The MCA approach with variously tailed SNP-specific primers, resulting in distinct melting temperatures, has become the predominant method used for *kdr* assessment in mosquitoes due to cost effectiveness, the rapidity of producing results for thousands of samples and the ease of implementation with existing laboratory infrastructure often used for mosquito-borne pathogen screening[27–29]. However, MCA is difficult when mutations of interest are tri-allelic due to side-by-side SNPs, such as the substitution of leucine for phenylalanine or serine at amino acid position 1014 (L1014 F/S) for the mosquito *Culex quinquefasciatus* and valine for glycine or isoleucine at position 1016 (V1016 G/I) in *Aedes aegypti*[30,31]. Notably, the 1014 mutation in house flies is also due to adjacent SNPs resulting in the wildtype leucine becoming a histidine or phenylalanine (L1014 H/F)[4].

An alternative form of allele-specific PCR that has not yet been explored for use in detecting SNPs implicated in insecticide resistance target site mutations is RNase H-dependent PCR (rhPCR)[32]. This method utilizes blocked primers, with an RNA base in the primer located complementary to the SNP of interest on the template DNA, with several DNA bases 3' of the RNA base and a final blocking group that, when present, inhibits amplification by DNA polymerase. When the RNA base is correctly matched to the complimentary DNA base on the template, RNAse H2, derived from *Pyrococcus abyssi* (Thermococcales: Thermococcaceae), cleaves the primer at the 5' side of the RNA:DNA heteroduplex. This cleavage relieves the steric hindrance caused by the blocked 3' end of the primer allowing the DNA polymerase to amplify as in standard PCR. This SNP-specific limitation acts at each cycle to reduce spurious amplification. This allows for highly specific amplifications of targeted SNPs and is quickly being adopted for detection of critical SNPs in biomedical research[33–35]. The advantages of rhPCR are that it is relatively inexpensive, can be conducted in high throughput on common qPCR equipment and produces results quickly. The specificity of the RNAse H2 cleavage reaction enables discrimination among triallelic mutations and produces very low error rates when properly controlled and optimized. The products of rhPCR can also be visualized with simple gel electrophoresis and transillumination and even sent for Sanger sequencing with no additional steps required.

To demonstrate the utility of rhPCR to rapidly and efficiently detect insecticide resistance target site mutations, we developed rhPCR assays to detect *kdr* SNPs in house fly collections from dairies located in eight states (Table 1). As a significant agricultural and public health pest, house flies have become a model organism for pesticide resistance research (reviewed in Geden et al.[36]) due to their wide geographic distribution and pest status. House fly populations have long been challenging to control due to their rapid development of insecticide resistance[37–39]. Of the insecticides registered for use against house flies, pyrethroids are one of the most widely used and often fail due to resistance[4,40–42]. This resistance is due in part to well-characterized and numerous *kdr* mutations, including the triallelic L1014 F/H mutation, a methionine to threonine mutation at 918 (M918T), a threonine to isoleucine mutation at 929 (T929I), and a rare aspartic acid to asparagine mutation at 600 (D600N)[4]. Traditionally, the 1014F mutation is considered to be a gatekeeper that must be present before the 918, 929 or 600 mutations are found[4,14,40,43–45]. As the ability to get an accurate answer from the first test attempt is an important consideration for the use of an operational, rather than academic, research tool, we used Nanopore sequencing to confirm genotypes in a subset of samples.

The rhPCR method adds a new tool to resistance monitoring programs and can extend to virtually all pests or arthropod disease vectors and any resistance mutation that is conferred by a SNP. Additionally, rhPCR can be scaled to a wide range of instrumentation, from simple PCR and gel electrophoresis to quantitative PCR and next-generation sequencing. The rhPCR method makes resistance genotyping accessible to a wider range of lab types, especially those in low and middle-income countries.

Results

Development of RNAse H-dependent PCR methods to assess *knockdown resistance* mutations in *Musca domestica*

We developed a rhPCR based method to assess the well-described markers of pyrethroid insecticide resistance found in the voltage-gated sodium channel (VGSC) of the house fly *Musca domestica*, resulting in a tool that could improve operational fly management in dairy or livestock production operations. Relying upon the reported single base specificity of rhPCR and the design guide provided by the manufacturer (IDTDNA, Coralville, IA), we designed primers to detect the presence of the SNPs for the 1014 (Fig. 1A), 918 (Fig. 1B), 929 (Fig. 1C) and 600 (Fig. 1D) VGSC mutations based on previously reported sequences[43,44] (Table 1). For the 1014 SNPs, three reactions assess the presence of two possible SNPs in consecutive base pair positions (Fig. 1A). The primers for the wildtype 1014 lysine and the *kdr* mutant 1014 histidine interrogate for the presence of the codons CTT (L) or CAT (H) (position 4371952 of NW026712250.1). The primer for the *kdr* mutant 1014 phenylalanine assesses the presence of the codon TTT (F) (position 4371953 of NW026712250.1). We observed that the specificity of the rhPCR primers prevented misamplification at the two adjacent SNPs at the site of the 1014 *kdr* mutations.

Primers for the 918, 929 and 600 *kdr* mutations assess the presence of the following SNPs: 918 – wildtype methionine (ATG) to *kdr* mutant threonine (ACG) (Fig. 1B, position 4376601 of NW026712250.1); 929 – wildtype threonine (ACA) to *kdr* mutant isoleucine (ATA) (Fig. 1C, position 4376568 of NW026712250.1); and 600 – wildtype aspartic acid (GAC) to *kdr* mutant asparagine (AAC) (Fig. 1D, position 4392528 of NW026712250.1).

Comparison of samples with a specific *kdr* SNP to those without the SNP were discernable with a difference in cycle threshold (C_t) of at least 4 cycles. This allows for the genotyping of unknowns for specific *kdr* alleles by comparison to known controls. While we used the amplification curves produced by the higher capacity of standard qPCR equipment to call genotypes, it was also possible to make presence or absence calls using lower throughput visualization on agarose gels as commonly done with AS-PCR (Fig. 2), although the very short amplicon (~ 60bp) for the 600D or N was difficult to visualize.

Comparison of genotyping methods

We compared rhPCR genotyping and the traditional Sanger based method of genotyping to genotypes generated by Nanopore-based next generation sequencing. We evaluated the ability of each method to determine the complete genotype by evaluating success after only a single attempt, what we call the “first pass” accuracy of each method. First pass accuracy is critical for cost-effectively genotyping large sample numbers as in large MCA based mosquito insecticide resistance (IR) studies[28,29,31]. For this comparison, we selected 68 samples from the 1,209 tested by rhPCR. These included genotypes obtained by the rhPCR assays that were unexpected based on previous literature, including those with 918 or 929 mutations without the presence of the 1014F gatekeeper mutation as well as those with more common genotypes (Supplementary Table 1) and we did not consider the 600 *kdr* locus in our calculations because its presence in the samples tested was so rare[5]. To be considered a success, the 7 individual rhPCR reactions (3 for 1014, 2 for 918, 2 for 929) or the 2 individual Sanger reactions (one for 1014, 1 for 918/929) needed to match the result from nanopore sequencing.

Three of the 68 samples resulted in a different genotype by each of the three methods (Table 2). The rhPCR assays resulted in successful genotyping for 75.0% of samples while individual assay success rate for each allele was greater than or equal to 89.7%. We obtained a complete and accurate genotype result from commercial Sanger sequencing (2 reactions) on the first pass for only 41.2% of the samples.

We also assessed the time needed to generate genotypes by the three different methods based on a group of 96 samples (Fig. 3). Including sample preparation and homogenization time (Fig. 3A), which was similar regardless of method, rhPCR was able to produce results in 6.5 hours (Fig. 3B). Both Sanger based genotyping (Fig. 3C) and nanopore based sequencing (Fig. 3D), which shared much of the same procedure, both required more than 30 hours to generate results. We note that our timeline for Sanger includes only 24 hours for overnight shipment, purification and sequencing by a commercial provider which could certainly be longer depending on individual laboratory facilities. Based on this comparison, rhPCR reduced the time needed to produce results by 80%.

We also considered the costs associated with each method to develop a complete genotype (Supplementary Table 2). Based on the same 96 sample group on one plate, rhPCR was able to produce results for $4.00 USD per sample or less than $400 USD for the plate. Sanger costs resulted in a per sample cost of $17.50 or $1,680 USD for the plate. Most of this cost was the purification and Sanger sequencing expense charged by the provider. We also calculated the costs for genotyping by nanopore and it was comparable to Sanger at $14.72 USD per sample with the major expenses being the sequencing flow cell and library prep reagents.

Assessment of *kdr* mutations in wild populations of *Musca domestica* from dairy production facilities by rhPCR

Using the rhPCR assays described above, we assessed the presence of *kdr* mutations in populations of *M. domestica* from eight states, which totaled 1,209 individual flies with nearly 77% (926/1,209) producing a result from each of the 9 individual genotyping reactions (File S1). Genotype calls were made for each wild individual by comparison to the results from laboratory strains with known *kdr* genotypes included in each assay. Negative controls, containing no DNA, were also included. Raw genotyping data is included in the data repository at DOI: 10.15482/USDA.ADC/25044329. We observed 27 different *kdr* genotypes (abbreviated by summarizing the amino acids resulting at 1014, 918, 929 and 600) among all the locations (Supplementary Table 1). The three most common genotypes (described by a shortened genotype notation for 1014, 918, 929, and 600 respectively, LLMMTTDD, HHMMTTDD, FFMMTTDD) were homozygotes at position 1014 without any other *kdr* mutations (Fig. 4A). These together accounted for nearly 50% of the successfully genotyped *M. domestica*. Heterozygotes at 1014 with no other *kdr* mutations accounted for 3 of the 4 (HFMMTTDD, LHMMTTDD, LFMMTTDD) next most common genotypes. Notably, the 1014F homozygote paired with heterozygous 929 (genotype FFMMTIDD) was found at similar frequency as the 1014-only heterozygotes. Together, these 7 genotypes represented more than 70% of the total genotypes detected. We did detect low frequencies of unexpected genotypes where *kdr* mutations were found without the 1014F, which is considered a gatekeeper for the other *kdr* mutations[4]. Notably, the 600N mutation was rare and only detected in a single sample from Minnesota.

We observed considerable variation in allele frequencies between states (Fig. 4B). The wildtype 1014L allele ranged from absent in the NC samples to nearly 0.7 in WI although the average frequency was 0.35. The frequency of the 1014H mutation averaged 0.328 (range: 0.082–0.726) and the 1014F frequency was similar at 0.326 (range: 0.095–0.609). Both the 918T and 929I mutations were identified in all states although not in all locations within a state (Figs. 4B & 5). The 918T SNP frequency averaged 0.077 (range: 0.012–0.191) and was highest in MN and KS and less than 0.02 in the southeastern states of FL, GA, and NC. In contrast, the 929I mutation frequency, which averaged 0.102 (range: 0.016–0.316) across all states, was higher in FL, GA, and NC and low (< 0.061) in all other states including TX. As noted above, the 600N mutation was only identified in a single sample from MN. For multiple samples within a state, we observed a similar presence of genotypes although the relative frequencies of each genotype varied (Fig. 5).

Assessment of genotype frequency variation by NMDS

To formalize our observation that genotype frequencies varied by location, we conducted non-metric multidimensional scaling (NMDS) analysis using our genotype data. The original dissimilarities among sites, with regard to genotype composition, were well-preserved in reduced dimensions (non-metric R^2 = 0.993, linear R^2 = 0.946). Model stress was 0.085. Permutational ANOVA revealed a statistically significant difference among the states (Fig. 6A; F = 2.34, df = 7, 14, P = 0.001). There was also a statistically significant difference among the regions (Fig. 6B; F = 2.43, df = 2, 19, P = 0.002). Among regions, only the pairwise comparison of the South and Midwest regions was significant (F = 3.14, df = 1, P = 0.006; R^2 = 0.156).

Discussion

This study is the first to describe a method using rhPCR for resistance genotyping in insects. This robust tool will enable more rapid field-level sampling and could positively impact operational decision-making by allowing inclusion of IR information into pest control programs in livestock production operations. We introduced a series of rhPCR assays to detect key genotypes from large numbers of flies with a relatively high rate of success at the first testing event. Notably, the rhPCR-based genotyping we conducted in this study encompassed over 1,200 organisms from 22 locations across 8 states, making it one of the largest *M. domestica* IR genotyping studies conducted to date [5,45–48] . The rhPCR assays can be completed quickly and cost effectively; in this study only 12 days were needed to conduct testing at a consumable cost of less than $4,900 (in 2024 USD); this is significantly cheaper than obtaining the same data through Sanger sequencing which would have cost around $21,000, a savings of slightly more than 75%. Additionally, processing time for these rhPCR assays (qPCR performed on homogenized samples) was only about a quarter of the time required for a Sanger sequencing workflow, which requires multiple procedures for each sample (DNA purification, PCR assays, PCR cleanup, sequencing onsite or through a 3rd party).

Traditionally, the Sanger based *kdr* assessment in flies has been applied in a manner to screen first for the mutations at 1014. If 1014F was detected, further reactions and Sanger were conducted to assess the presence of the 918, 929 and 600 SNPs. This reduced the overall costs by not running additional Sanger reactions for SNPs that were not expected to be present without the gatekeeper 1014F mutation. The rhPCR method described here was able to successfully identify several unexpected genotypes where the 918 and 929 mutations were not paired with 1014F. These initial genotyping results were also frequently obtained by both Sanger and nanopore-based methods, thus confirming that rhPCR correctly identified these unexpected genotypes.

The rhPCR method described here has a few distinct advantages for expanding the operational utility of IR assessment in flies and other insects. At a minimum, we demonstrate that rhPCR can easily screen for SNPs and is highly specific even when dealing with adjacent variation (as in the 1014L, H, or F SNPs in *M. domestica*). This method could conceivably be a useful improvement for genotyping other tri-allelic

mutations such as those that exist in *Ae. aegypti* and *Cx. quinquefasciatus*. The same rhPCR method we applied to the *kdr* gene here could test for acetylcholinesterase or *rdl* mutations, which are indicative of IR to other modes of action in various insect taxa, including flies. A second advantage of the rhPCR method is the ability to screen ecologically relevant numbers of organisms at relatively low cost (1/5 the cost) and in a short time period compared to traditional methods. Screening larger sample sizes will allow for a better understanding of which genotypes survive sprays conducted in agricultural and urban settings, where both survivors and dead are screened for genotype and compared statistically[28,49]. The speed of rhPCR would allow for multiple assessments being conducted during a treatment to understand the dynamics of *kdr* spread in a population under selection. The rhPCR method could also be applied to understand the distribution of *kdr* genotypes over geographic space. Our NMDS analysis showed significant differences in genotype between geographically dispersed collection sites, but we did not have adequate sample density in any one area to use methods that can quantify the spatial distribution of resistance mutations, which would guide sampling strategies and operational interventions in adjacent areas not yet tested[29].

While there are clear advantages to the use of rhPCR for IR assessment, it has disadvantages that are common to surrogate methods. The rhPCR method provides a screening tool for rapid assessment only of known mutations; it is not a tool for discovery of novel mutations and so a foreknowledge of the SNP or SNPs of interest is required. Along with this, rhPCR, like other surrogate methods, requires that the SNP being assessed is a high value marker in order for the resulting phenotype to be meaningful. It is clear from work in mosquitoes that many mutations exist in the VGSC but that not every mutation results in much resistance; some act as part of an ensemble of SNPs and are found in both susceptible and resistant organisms. Third, proper use of rhPCR requires that controls for the alleles being tested must be included in every assay conducted, optimally including heterozygotes to ensure the assay is indicating adequate sensitivity. This study benefitted from the kindness of other researchers in the field who provided strains with the specific *kdr* mutations we required, but these strains may not be as accessible to other workers, especially those in developing countries. Though controls in the same format as the collections (i.e. legs, whole bodies, purified DNA, etc.) may not always be possible to source, this does not relieve the experimenter from including proper controls. Improperly controlled surrogate assays should never be used for operational decision making or in academic publishing. Long synthetic DNA oligos can be ordered and should be used as allele-specific controls when proper control strains are not available.

In summary, we demonstrate an rhPCR based assay system that can move IR assessment from the academic laboratory to an operationally useful tool. This easy method can be used to better define important ecological parameters of IR in the field, by increasing sample sizes and increasing the number of locations that can be assessed for a given quantity of resources. This may allow more effective use of IR information in integrated pest management programs. Monitoring IR informs decision-making about pesticide usage by reducing use and expense when a formulation is likely to be ineffective. Because house flies in the US are widely resistant to all the products currently available for their management, the

ability to monitor resistance as new products enter the market will be critical for their effective management. Improved IR monitoring will also be an emerging tool in parts of the world where resistance to current products is not as severe as in the US, and alternating insecticidal modes of action remains a viable option.

Methods

Musca domestica strains and samples

Musca domestica strains with known *kdr* genotypes were kindly provided by Dr. Jeffrey Scott. The abyss (no 1014, 918, 929 or 600 mutations), ALkdr (1014F mutation), NChis (1014H), Type N (1014F, 918T, 600N), kdr1B (1014F, 929I) and JPSkdr (1014F, 918T) strains were used for initial assay development and as specific allele containing controls for rhPCR assays. The CAR21 strain (no 1014, 918, 929 or 600 mutations) was also used as a *kdr* negative control. This strain, which was originally obtained from Carolina Biological Supply, has been maintained at the USDA Center for Medical, Agricultural and Veterinary Entomology and is recognized as being insecticide-susceptible[50]. Maintenance and rearing of these strains was conducted as previously described[51].

Samples of field strains were collected from eight states by collaborators in Hatch Project S-1076. Samples were collected from livestock facilities using sweep nets. Collected samples were knocked down by freezing and placed into 50 mL tubes (Thermo Fisher, Waltham, MA) or 1 pint paper containers (WestRock, Atlanta, Georgia, USA) for overnight shipping on ice blocks and were maintained at -80 ^{o}C after arrival. Samples from CA and TX were delayed in shipping and spent more than 24 hours at room temperature causing significant degradation but were tested anyway.

Sample preparation and homogenization

Samples provided by collaborators were stored at -80°C until sorting and identification on ice. Flies were visually identified as *M. domestica* using morphological characters, and forceps were submerged in 100% ethanol between the handling of each fly. *Musca domestica* individuals had a mid and hind leg removed if present (if not, any two legs were chosen) with forceps where the femur meets the trochanter. The legs were then placed in a 96-deep well homogenization plate (Omni International, Kennesaw, GA) and the rest of the sample placed in a corresponding 96-well plate in the same well position. The homogenization plate containing the legs was prepared by adding 2.0 mm cubic zirconium beads (BioSpec Products, Bartlesville, OK) to each well using a LabTie bead dispenser (BioSpec Products, Bartlesville, OK), followed by the addition of 200 µL of deionized water. All plates were labeled with the relevant collection information to keep the leg samples for testing linked with the remainder of the sample. Once all samples from a collection event were processed, the 96-well plate containing the remainder of the sample was covered with a foil plate seal (USA Scientific, Ocala, FL) and stored at -80°C in case further testing was required. The homogenization plate containing the legs was then covered

with a silicone sealing mat (Omni International, Kennesaw, GA) and stored at -20°C until homogenization. Sample preparation was conducted separately from sample testing.

On the day of assay, homogenization plates containing legs, beads, and deionized water were defrosted in a 22 ^{O}C water bath and then homogenized for 2 minutes on a Beadruptor 96 (Omni International, Kennesaw, GA). After homogenization, 96-well plates were spun for 2 min at 805 x g then placed on ice until assay setup.

rhPCR assays

Master mixes for rhPCR were prepared for each allele using the primers (IDTDNA, Coralville, IA) and reaction quantities listed in Tables 2 & 3. The effective concentration of H2 RNAse (IDTDNA, Coralville, IA) was optimized for each allele-specific reaction as recommended by the manufacturer using laboratory strains with known genotypes[43,44]. Reactions were assembled in 384-well plates using an EpMotion 5750 liquid handling system (Eppendorf, Hamburg, Germany) in a final reaction volume of 10 µL. Eight microliters of each master mix was added by the liquid handling system followed by 2 µL of spun homogenate. Each 96-well plate of homogenates required three 384 well plates for all the assays needed: 1st – 1014L, 1014H, and 1014F; 2nd – 9198M, 918T, 929T, and 929I; and 3rd – 600D and 600N.

Plates were sealed with clear optical covers (Thermo Fisher, Waltham, MA), mixed gently on a plate vortex (IKA Works, Inc., Wilmington, NC), spun to remove bubbles and then cycled on a QuantStudio 6 Flex system (Thermo Fisher, Waltham, MA) running QuantStudio Real-Time PCR Software V1.3 using standard FAST conditions of 95 ^{O}C for 20 sec, then 40 cycles of 95 ^{O}C for 3 sec and 60 ^{O}C for 20 sec. This was followed by a melt curve phase of 95 ^{O}C for 15 sec, 60 ^{O}C for 1min, and a ramp step from 60 ^{O}C to 95 ^{O}C at 0.05 ^{O}C /sec with continuous fluorescence recording to assess amplicon melting temperature as an extra check to ensure the specificity of the reaction.

The presence of an allele in an organism was made using two metrics. First, the Ct of a curve had to be within 3 cycles of the known positive controls for the allele in question and at least two cycles above the Ct of the known negative controls. As a second factor, the melting temperature (Tm) of the resulting amplicon had to be ± 0.4 ^{O}C of the Tm of the known positive control. A sample that did not meet both metrics above was considered negative for the specific allele. A detailed version of the protocol for this assay has been deposited in the data repository.

PCR amplification and Sanger sequencing of sodium channel mutations

Reactions for PCR amplification of regions containing *kdr* mutations used a combination of previously published and novel primers (Table 2). All reactions were conducted in 25 µL volumes using 1 µL of fly homogenate or DNA with final concentrations of 1.5 mM $MgCl_2$, 0.2 µM dNTPs, 0.4 µM forward and reverse primers, and 0.04 U/µl of Platinum Taq (Thermo Fisher, Waltham, MA). Reactions to amplify the 1014 containing region were conducted on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with the following conditions: 94 ^{O}C for 2 min 40 cycles of 94 ^{O}C for 30 sec, 60 ^{O}C for 25 sec and 72 ^{O}C

for 30 sec; and 72 ^{o}C for 5 min. Reactions for the 918/929 and 600 *kdr* containing regions were amplified on the same equipment but with the following conditions: 94 ^{o}C for 2 min; 40 cycles of 94 ^{o}C for 30 sec, 55 ^{o}C for 30 sec and 72 ^{o}C for 1 min; and then 72 ^{o}C for 5 min. Select amplicons were verified on 2% agarose gels. ExoSAP purification and Sanger sequencing were conducted by PsomagenUSA (Rockville, MD). Visualization of rhPCR amplicons for Fig. 2 used 10 µl of PCR reaction with 1.1 µL of 10X Blue Juice. A 100 base pair ladder (Thermo Fisher, Waltham, MA) was included and the samples were electrophoresed at 102 V for 1 hr and then visualized on an iBright imaging system (Thermo Fisher, Waltham, MA).

Nanopore sequencing of *kdr* amplicons and sodium channel coding region

Nanopore sequencing was conducted according to the manufacturer provided protocol using R10 chemistry and Native Barcoding Kit V14 (SQK-NBD114.96; Oxford Nanopore Technologies, Oxford, England). The protocol version used was: NBA_9170_v114_revL_15Sep2022 updated 02/10/2023 and a marked up version is included in the data repository. Samples for sequencing *kdr* amplicons were prepared from unpurified PCR products by combining 0.34 µL of the 1014 amplicon PCR, 0.27 µL of the 918/929 amplicon PCR, and 0.27 µL of the 600 amplicon PCR with 11.37 µL of nuclease free water. Samples for VGSC sequencing used 12.25 µL of the unpurified VGSC PCR and were placed into a 96-well plate and end prepped using NEBNext Ultra II End Repair/dA-Tailing Module (New England BioLabs Inc, Ipswich, MA) per the protocol. Each sample was then affixed with a unique barcode and all barcoded samples were then combined, cleaned with 80% ethanol and then final sequencing adapters were ligated per the ONT protocol. The adapted, barcoded library was washed with standard fragment buffer, eluted in 15 µL of elution buffer and quantified on a Nanodrop 8000 spectrophotometer. Fifty femtomoles of eluted DNA was mixed with sequencing buffer, loading beads, and elution buffer before being loaded onto a prepared R10 flow cell. Sequencing was managed on a GridION x5 device by the MinKNOW software (version 23.07.12) and electrical signals were converted to basecalls by Guppy (version 7.1.4). Initial read quality control was managed by the MinKNOW software with a minimum quality score of 10. Reads at or above this threshold were binned into barcode specific folders as fastq files. Barcodes were not removed from raw reads to allow subsequent filtering at higher stringency. Two sequencing runs were conducted with 36 *kdr* amplicon samples in the first run and 32 *kdr* amplicon samples in the second run. Raw reads are available as aligned .bam files under accession: PRJNA1163914.

Bioinformatic analysis

Barcode binned raw reads that met initial quality filters were subsequently filtered at higher stringency to reduce spurious barcode misassignments. The sequencing summary file for each run was filtered using command line tools to require a barcode match of > 97% and more than 37 bases in length at each end of the amplicon. A maximum of 4k reads meeting these requirements were selected for each barcode using seqtk[52] and then subsequently mapped to the *M. domestica* VGSC using Minimap2[53]. The resulting pileups were examined using IGV[54] to determine the presence of SNPs for the 1014, 918, 929 and 600 *kdr* mutations.

Statistics and Reproducibility

Frequency of *kdr* genotypes was ordinated alone or as an aggregate representation of the trapping sites from which they were collected using non metric multidimensional scaling in the 'vegan' package[55] in R version 4.3.2[56]. Centroids (i.e., means) of genotype frequencies were summarized at state and regional levels as 95% confidence interval ellipses. Regions were defined according to the US Census Bureau as "Midwest" (Minnesota, Kansas, Wisconsin), "South" (Texas, Florida, North Carolina, Georgia), and "West" (California). The Jaccard dissimilarity index method was used with NMDS done in three dimensions (i.e., k = 3), which produced the lowest stress. Stress is an important indicator of ordination fit in the assigned number of dimensions, with values < 0.2 being acceptable representation, < 0.1 being good representation, and < 0.05 being excellent representation[57]. Stress plots were visually inspected and both non-metric and linear fit R^2 values are reported. Then, a permutational type II ANOVA was done using the 'adonis.II' function in the 'RVAideMemoire' package[58]. Posthoc comparisons of F statistics were done using the 'pairwiseAdonis' package[59], with a Holm-Sidak P-value correction to account for familywise error rate. Due to the number of pairwise comparisons among states, relatively low sample size, and the associated P-value adjustment, we only report the global permutational type II ANOVA for the state comparisons. For all statistical comparisons, $\alpha = 0.05$. Samples sizes from each of the 22 locations ranged from 32–89 individuals tested from each location. Individual samples that did not produce a clear genotype by rhPCR were excluded from further testing.

Declarations

Data Availability

Experimental data not included in the manuscript are available from USDA AgData Commons at: doi: 10.15482/USDA.ADC/25044329. Sequencing data is available from NCBI under BioProject ID PRJNA1163914.

Competing interests

The author(s) declare no competing interests.

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Author Contribution Statement

ASE and ERB conceived the project and performed data analysis. ASE, NDS and AAC conducted experiments. All authors contributed to the manuscript draft, edits and revisions. All authors have

approved submission of the manuscript.

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Tables

Tables 1 to 3 are available in the Supplementary Files section.

Figures

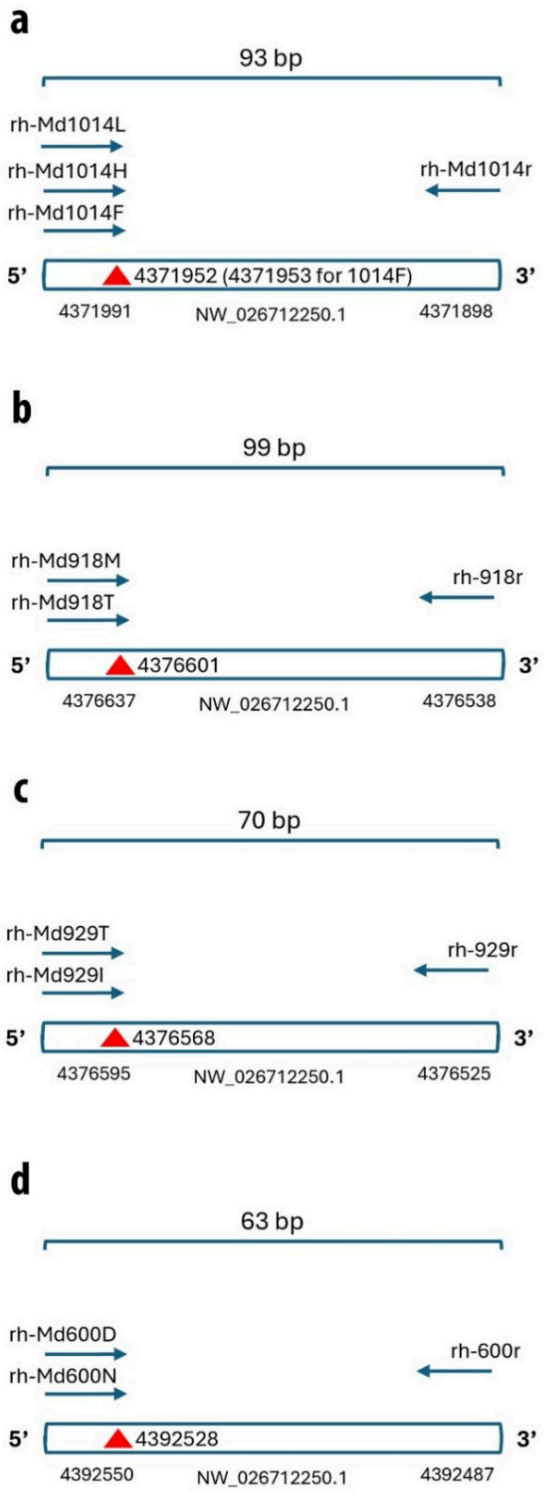


Figure 1

Schematic of rhPCR assays. Genomic locations, amplicon sizes, and base coordinates of the SNP for the rhPCR assays for house flies for the (a) 1014L, 1014H and 1014F alleles, (b) 918M and 918T alleles, (c) 929T and 929I alleles and (d) the 600D and 600N alleles.

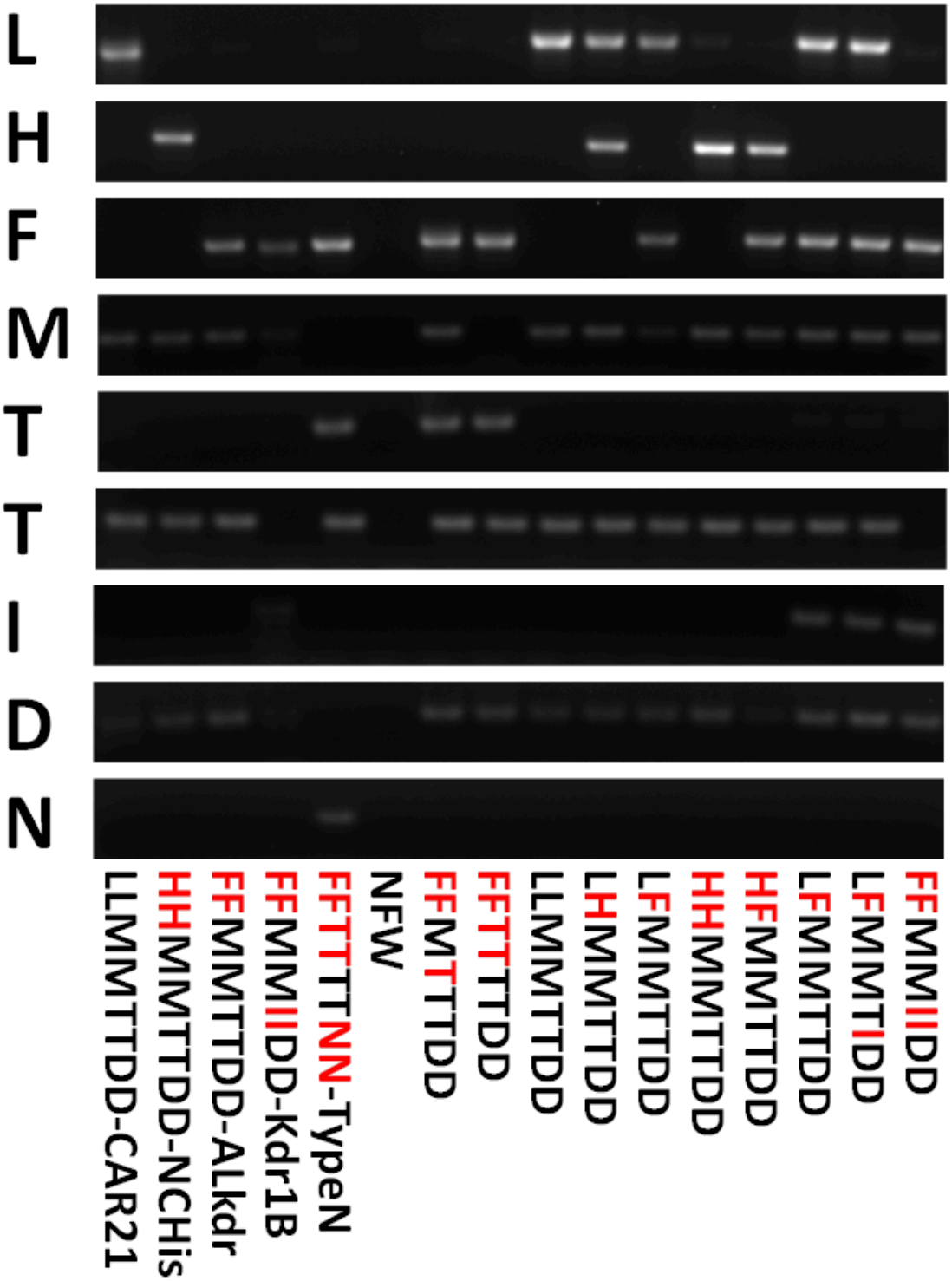


Figure 2

Verification of specificity of rhPCR assays on *Musca domestica*. The rhPCR reactions for each of the 9 kdr alleles were run on the same 16 samples with the genotypes shown. This includes laboratory strains ALkdr (1014F), NCHis (1014H), TypeN (1014F & 918T & 600N) and Kdr1B (1014F & 929I) kindly provided by Dr. Jeffrey Scott. CAR21 (no *kdr* mutations) was provided by Carolina Biological Supply. Samples were amplified using the standard conditions as specified in Table 3. Five microliters of each standard 10 µl

reaction was visualized on a 1.5% agarose gel with 1:10000 GelRed. Gel images were trimmed for presentation. Full images including MW standards are available in the Supplemental Information as Supplemental Images 1-4.

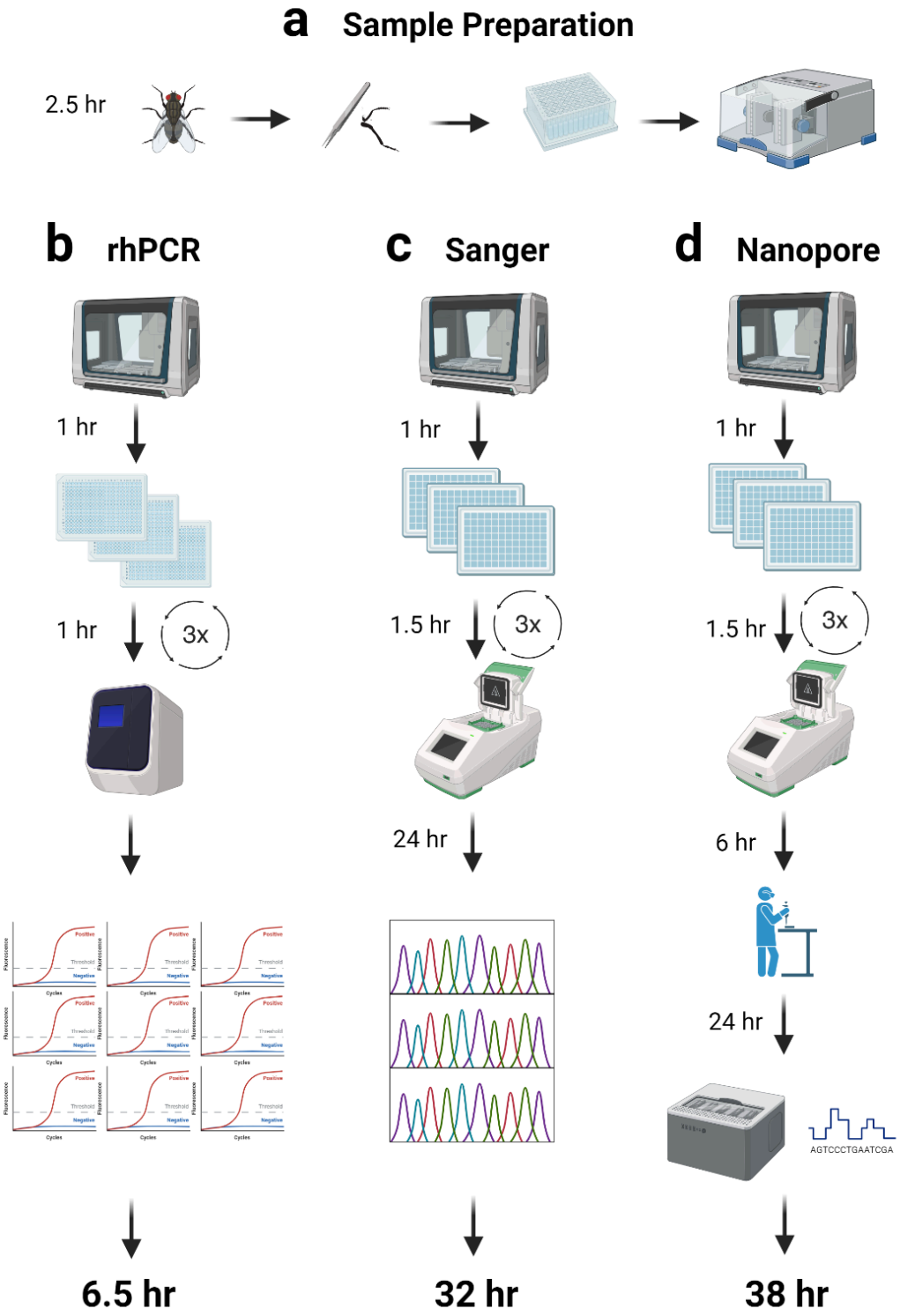


Figure 3

Visualized comparison of the time and major steps in the methods for assessing knockdown resistance SNPs. Initially homogenization (a) is similar for all three procedures but diverge for (b) rhPCR , (c) Sanger, and (d) Nanopore based methods for 96 samples used to determine knockdown resistance genotypes in Musca domestica. Created in BioRender. Estep, A. (2024) BioRender.com/h16l713

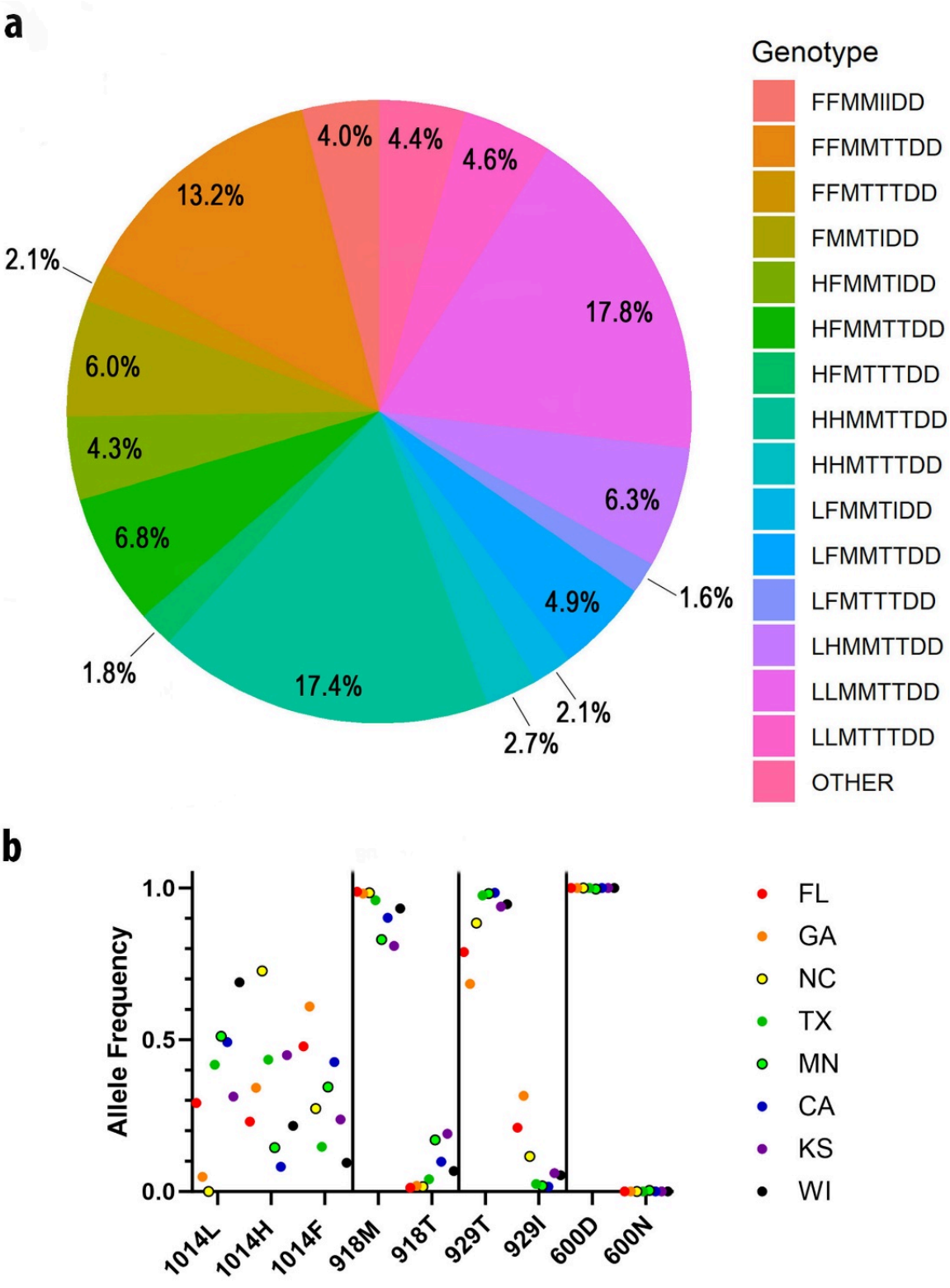


Figure 4

Assessment of *kdr* genotypes in US dairies. (a) Fifteen most common genotypes across all sites. Remaining genotypes combined into “OTHER.” Genotypes are abbreviated as the coding amino acid at positions 1014, 918, 929, and 600. (b) Allele frequencies by state for the 1014, 919, 929 and 600 *kdr* mutations. Total number of samples genotyped is 1209 from 22 locations. Samples per location and site information is found in the data repository.

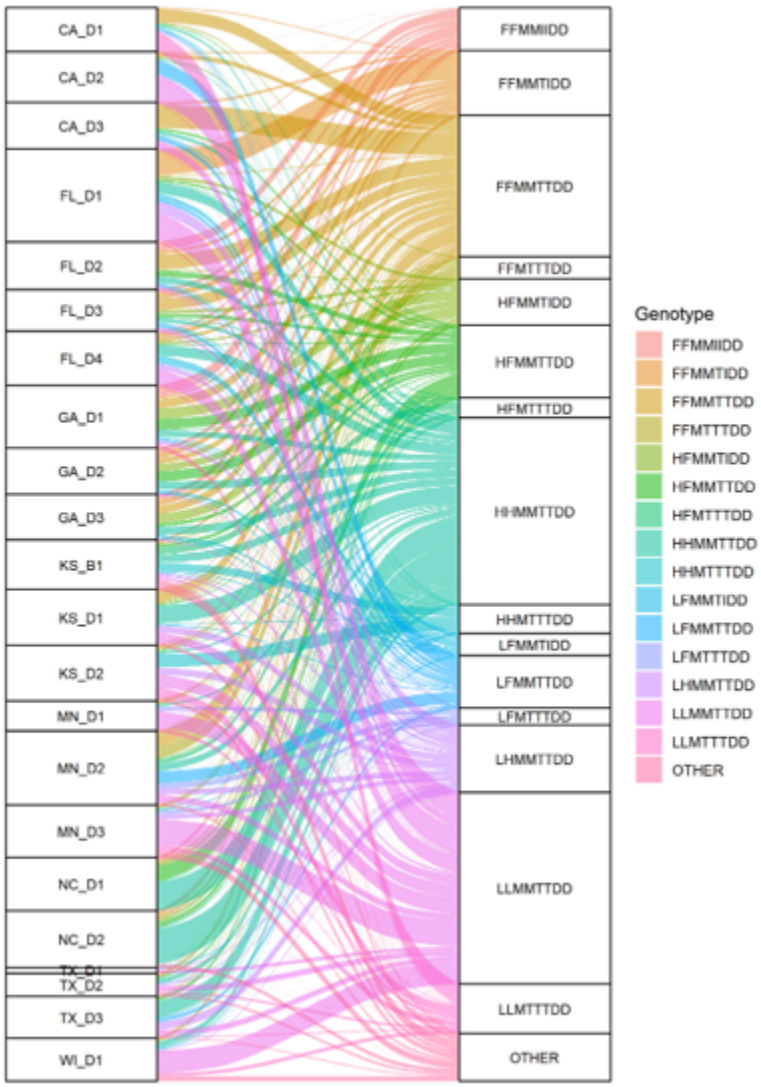


Figure 5

Alluvial plot of genotypes (frequency greater than 0.01%) for individual locations. Genotypes present at a frequency of less than 0.01 are included in the “OTHER” category. Alluvial plot was constructed in R using ggplot2 and ggalluvial[60,61]. Total number of samples for all sites is 1209.

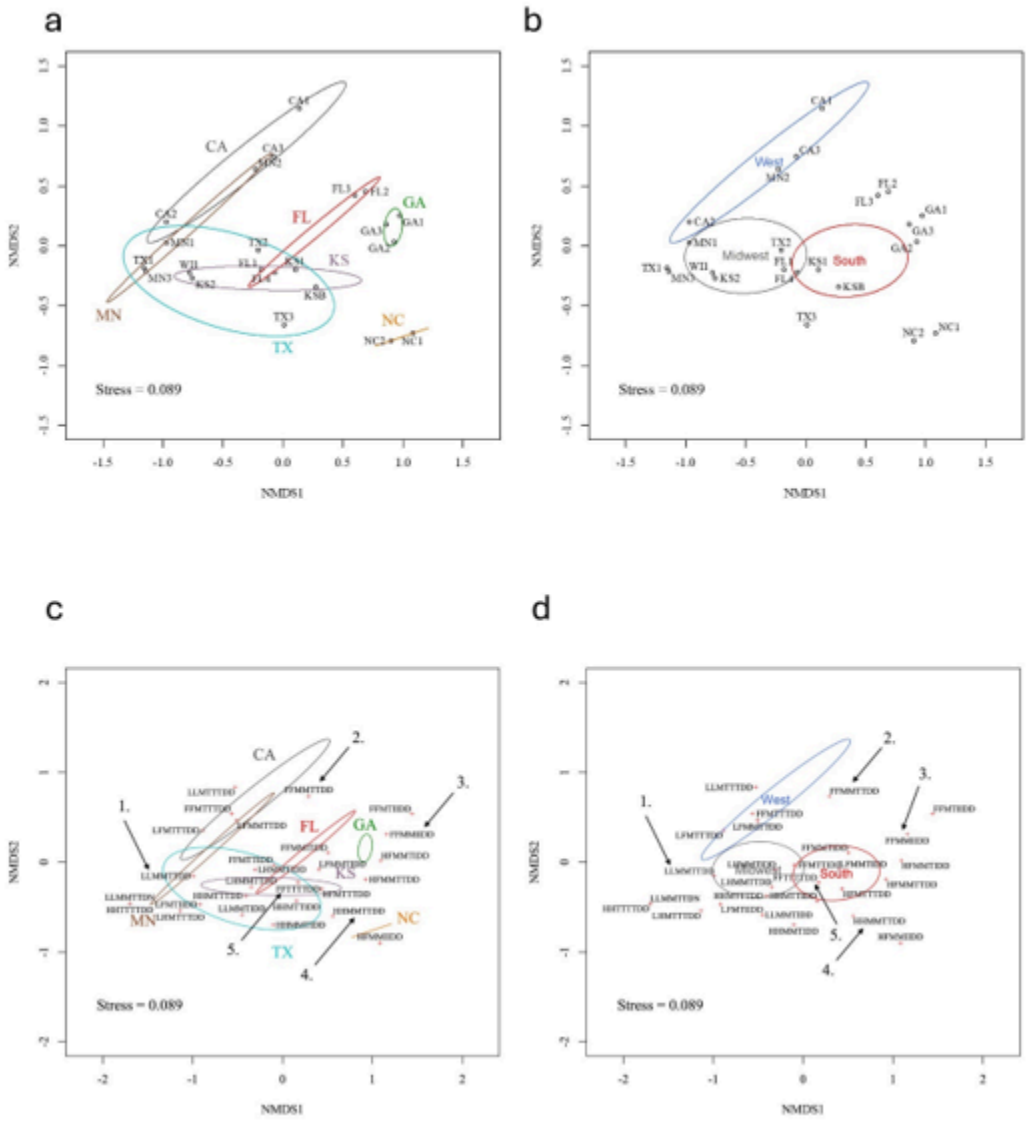


Figure 6

(a) Non-metric multidimensional scaling (NMDS) of Jaccard dissimilarity among house fly *kdr* sample sites. (a) Ellipses represent 95% confidence intervals of sample sites organized by state. The first two letters of each site represent the two-letter abbreviation of each state, with the number at the end to distinguish between different sites within states. (b) Ellipses represent 95% confidence intervals of sample sites organized by region according to the US Census Bureau. The first two letters of each site represent the two-letter abbreviation of each state, with the number at the end to distinguish between different sites within states. (c) Non-metric multidimensional scaling (NMDS) of Jaccard dissimilarity among house fly *kdr* genotypes. Ellipses represent 95% confidence intervals of genotypes in relation to states. Arrows and numbers correspond to the following homozygous *kdr* type according to Freeman et al.[4]: 1) "wildtype no *kdr*" 2) "*kdr*" 3) "*1B kdr*" 4) "*kdr-his*" 5) "*super-kdr*." Very little "*Type N kdr*" (i.e., LLMMTTDN) was observed and was most closely associated with Minnesota. (d) Non-metric multidimensional scaling (NMDS) of Jaccard dissimilarity among house fly *kdr* genotypes. Ellipses

represent 95% confidence intervals of genotypes in relation to geographical regions according to the US Census Bureau. Arrows and numbers are as described in (c).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Tables.docx
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