ABSTRACT

Title of Dissertation:	GENOMIC ANALYSIS OF INSECTICIDE RESISTANCE CANDIDATE GENES IN THE COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA*
	Dongxu Chen, Doctor of Philosophy, 2025
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Since the advent of modern agriculture, humans have continuously developed and deployed chemical compounds with novel modes of action to manage pest populations, while insects have repeatedly responded by evolving resistance. To date, at least 625 arthropod species have evolved resistance to over 360 pesticidal technologies. As a global super pest, the Colorado potato beetle (CPB) has developed resistance to at least 56 different insecticidal active ingredients. Comprehensive genomic scanning of resistance candidate genes can facilitate downstream applications, including mode-of-action analysis, functional interpretation of resistance mutations, development of molecular resistance monitoring tools, and the formulation of sustainable pest management strategies. However, I note that the cys-loop ligand-gated ion channel (cysLGIC) gene superfamily, encoding important target sites, such as nicotinic acetylcholine receptors (nAChRs) and γ-aminobutyric acid (GABA) receptors remain poorly characterized in CPB. In addition, previously suspected resistance-associated mutations have not

been systematically surveyed across U.S. CPB populations. In the current research, leveraging the chromosome-level genome assembly of the Colorado potato beetle (CPB), I systematically characterized the cys-loop ligand-gated ion channel (cysLGIC) gene superfamily in the Colorado potato beetle. This work provides a foundational reference for the annotation of DNA and protein sequences of these receptors and can support future functional and comparative studies. In particular, I highlighted unique patterns observed in specific subunits, such as nAChR α4 and nAChR β1, and discussed their potential roles in mediating insecticide resistance. Furthermore, I conducted high-throughput genomic scanning of 31 resistance candidate genes using whole-genome sequencing data from 74 CPB samples, aiming to identify resistance-associated mutations. My results include the first detection of a *super-kdr-like* mutation (T929I in the voltage-sensitive sodium channel) conferring pyrethroid resistance in U.S. CPB populations, and the first identification of the A301S mutation in the *Rdl* gene, associated with resistance to both dieldrin and fiproles. Additionally, I provide evidence of selection acting on neonicotinoid resistance candidate genes and report novel genetic variants statistically associated with resistance phenotypes.

GENOMIC ANALYSIS OF INSECTICIDE RESISTANCE CANDIDATE GENES IN THE COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA*

by

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List of Abbreviations

ace-2	*acetylcholinesterase-2*
Aaeg	*Aedes aegypti*
ABCT	ATP-binding cassette transporter
ACh	acetylcholine
AChE	acetylcholinesterase
Amel	*Apis mellifera*
Apis	*Acyrthosiphon pisum*
Atum	*Aethina tumida*
Bger	*Blattella germanica*
BLAST	Basic Local Alignment Search Tool
Bt	*Bacillus thuringiensis*
CBs	carbamates
cDNA	complementary DNA
CI	confidence interval
CLGC/cysLGIC	cys-loop ligand-gated ion channel
CNS	central nervous system
CPB	Colorado Potato Beetle
CWG	Coleoptera Working Group
CYP	cytochrome P450
DDT	dichlorodiphenyltrichloroethane
Dmel	*Drosophila melanogaster*
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
GABA	gamma-aminobutyric acid
gDNA	genomic DNA
GluCl	glutamate-gated chloride channel
GRD	GABA/glycine-like receptor of *Drosophila*
HisCl	histamine-gated chloride receptors
IRAC	Insecticide Resistance Action Committee
IRM	insecticide resistance management
kdr	knocking-down resistance
LC_{50}	lethal concentration 50%
LCCH3	ligand-gated chloride channel homologue 3
LD_{50}	lethal dose 50%
Ldec	*Leptinotarsa decemlineata*
LI	Long Island
MD	Maryland
MI	Michigan
MoA	mode of action

mRNA	messenger ribonucleic acid
nAChR	nicotinic acetylcholine receptor
NCBI	National Center for Biotechnology Information
Nvit	*Nasonia vitripennis*
NY	New York
OPs	organophosphates
Pame	*Periplaneta americana*
pHCl	pH-sensitive chloride channels
RACE	rapid amplification of cDNA ends
Rdl	resistance to dieldrin
RNA	ribonucleic acid
RNAi	RNA interference
Tcas	*Tribolium castaneum*
TM	transmembrane
UGT	UDP-glucuronosyltransferase
vssc	voltage-sensitive sodium channels

Chapter 1 Introduction

1.1 Arms war between humans and insect pests

1.1.1 Definition of insecticides resistance

Since the advent of modern agriculture, the war between human beings and insect pests has never stopped. The most potent weapon against pest species is insecticidal chemicals, from Paris Green in the 19th century to the very recent vesicular acetylcholine transporter inhibitor (Ma *et al.* 2025), hundreds of compounds have been assessed and registered for control of insect pests. While insecticides are constantly upgraded, insect pests respond by evolving resistance. Until 2022, at least 625 arthropod species evolved resistance to 360 pesticidal technologies (Fritz 2022).

Insecticide resistance, defined by the Insecticide Resistance Action Committee (IRAC), is "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (http://www.irac-online.org/about/resistance/). Several insect species, such as the Colorado potato beetle, *Leptinotarsa decemlineata* (Chen *et al.* 2023), the diamondback Moth, *Plutella xylostella* (Sun *et al.* 2022), the fall armyworm, *Spodoptera frugiperda* (Zhao *et al.* 2023), and the whitefly, *Bemisia tabaci* (Yan *et al.* 2024), has been described as super-pests by researchers, of which one reason is their ability to evolve resistance to insecticides.

1.1.2 Colorado potato beetle as a super pest

As a global super pest, the Colorado potato beetle (CPB), provides an excellent example of coevolution between insecticides and resistance against them. CPB is the most important insect pest in some major potato-producing areas worldwide. Often with the description of

"notorious," or "infamous,", this beetle can consume about 40 cm^2 of potato foliage throughout its larva stage and continues to consume 10 cm^2 per day in the adult stage (Ferro *et al.* 1985, Logan *et al.* 1985). Beetles may also feed on stems and exposed tubers after consuming all the leaves. Without effective control methods, this insect may destroy the potato field. CPB causes more than $150 million in economic losses annually in North America due to crop yield losses and chemical control costs (Suszkiw 2000). For other regions, the annual losses caused by the CPB are estimated to be $2–2.5 billion in Russia alone (Skryabin 2010), and this number in China is estimated to be $3.2 million (Liu *et al.* 2012).

According to the Arthropod Pesticide Resistance Database (https://www.pesticideresistance.org), CPB has evolved resistance to at least 56 active pesticide ingredients, including all historically widely used synthetic insecticide groups, such as organochlorines (such as, dichlorodiphenyltrichloroethane, DDT), organophosphates (OPs), carbamates (CBs), synthetic pyrethroids, and neonicotinoids.

As the first modern insecticide, DDT successfully controlled CPB by opening voltage-sensitive sodium ion channels in neurons, causing them to fire spontaneously and leading to spasms and eventual death (Bloomquist 1996). However, the complete effectiveness of DDT against CPB lasted only eight years, with the first failure of DDT control noted in 1952 in Long Island (LI), New York (Silcox *et al.* 1985). DDT was widely applied in 1946 in Minnesota, and resistance was noted in 1953. It took approximately 14-24 generations for CPB to develop DDT resistance (Casagrande 1987). Shortly after DDT resistance, cases of resistance by CPB to other early chemical groups were reported, including arsenic-containing compounds in 1955, dieldrin in 1958 (Zichová *et al.* 2010), and other chlorinated hydrocarbon insecticides in 1967 (Kostic *et al.* 2016).

Since the 1970s, two acetylcholinesterase (AChE) inhibitors, organophosphates (OP) and carbamates (CB) have been heavily used as insecticides (Margus *et al.* 2021). However, cases of azinphos-methyl (an OP insecticide) and carbofuran (a CB insecticide) resistance in the United States were reported in 1980 in New York and Connecticut, respectively (Hare 1980). Initial indications were that OP and CB resistance in CPB are autosomal, essentially monofactorial, due to a significant target-site insensitivity associated with altered AChE (Inceoglu *et al.* 2001). Two mutations (S291G and R30K) on the CPB *ace-2* gene were later identified to be associated with CPB's resistance to CBs and/or OPs (Zhu *et al.*, 1996; Zhu & Clark, 1997; Kim *et al.*, 2007).

Synthetic pyrethroids, including permethrin and deltamethrin, were first registered for use in the 1970s. Compared to previous insecticides, the pyrethroids were effective at lower doses and had reduced vertebrate toxicity (Miyamoto 1976). Although pyrethroids share a target site with DDT, the voltage-sensitive sodium channels (vssc), they were initially very effective. CPB resistance to fenvalerate and permethrin was documented in 1981 and 1990, respectively (Tisler and Zehnder 1990). Target site insensitivity was later identified as the primary mechanism in CPB pyrethroid resistance. In 1999, a mutation equivalent to the L1014F mutation in house flies, conferring knockdown resistance (kdr) to DDT and cross-resistance to pyrethroid, was identified in the CPB *vssc* gene (Lee *et al.* 1999). Later, the CPB *vssc* gene was mapped to the X chromosome and was further confirmed to have significant effects on CPB pyrethroid resistance and inherited in an incomplete recessive manner (Hawthorne 2001, 2003).

In the 1990s, genetically modified potatoes shortly became an option for growers. Monsanto introduced the genomic-modified NewLeaf™ to the market in 1995, expressing Cry3A proteins (Balaško *et al.* 2020). However, due to a controversy over the marketing of

genetically modified potatoes and shrinking potato acreage, Monsanto discontinued the sale of NewLeaf™ seed in 2001. CPB did not develop resistance to NewLeaf™ potatoes.

In the early to mid-1990s, resistance in the CPB rendered nearly all modes of insecticidal action ineffective in the eastern potato-growing regions of the U.S. With the loss of the Bt varieties, very few options remained for control of CPB. This situation reversed with the advent of imidacloprid. As the first neonicotinoid chemical, imidacloprid was registered for potato use in 1995. It quickly became a desirable candidate for chemical control due to a novel mode of action (Sparks and Nauen 2015), broad spectrum of activity, low toxicity to vertebrates (Tomizawa and Casida 2005), and favorable environmental fate. Imidacloprid achieved great success in the initial years of CPB management. For example, Michigan started the imidacloprid application right after its registration, increasing yields of potato by $10 million/yr in 1995 and 1996 compared to 1991-1994 (Zhao *et al.* 2000).

Unfortunately, as with previous insecticides, the repeated use of imidacloprid led to regional pockets of resistance to neonicotinoid chemicals. The reduced control efficacy was first reported in a Long Island population in 1997 (Zhao *et al.* 2000), and resistance appeared for the first time in the north-central production region in 2004 (Byrne *et al.* 2005). Resistance to thiamethoxam, another neonicotinoid, was first found in 2003 (Byrne *et al.* 2004).

1.2 Resistance mechanisms and their implications in CPB

Insecticide resistance is hypothesized to occur through evolutionary changes in four main phenotypes (Ffrench-Constant 2013): target-site insensitivity, reduced penetration, enhanced detoxification and transport and behavioral avoidance of toxic compounds.

1.2.1 Target site insensitivities

1.2.1.1 Mutation of the Rdl, the vssc and the ace genes

Target site insensitivity, also called target site resistance, occurs when genetic changes alter the site where the insecticide normally binds, often resulting in reduced sensitivity or complete insensitivity to the compound (Sparks and Nauen 2015). Target-site resistance to conventional insecticides, primarily those in IRAC Groups 1–3, has been well reviewed elsewhere, including mutations in the *vssc* gene that confer resistance to pyrethroids and DDT (Dong *et al.* 2014), mutations in the *Rdl* (stands for Resistance to Dieldrin, a legacy term from when the gene was first linked to insecticide resistance in *Drosophila melanogaster*) gene conferring resistance to cyclodienes and fipronil (Feyereisen *et al.* 2015), and mutations in the *ace* gene associated with resistance to organophosphates and carbamates (Fournier 2005). Mutations of these genes also played an important role in insecticide resistance in multiple CPB populations. Three mutations in the CPB *ace-2* gene—S291G, R30K, and Y45H—are associated with resistance to carbamates (CBs) and organophosphates (OPs) (Zhu *et al.* 1996, Zhu and Clark 1997, Kim *et al.* 2007). The L1014F-equivalent mutation in the *vssc* has been characterized as a major mechanism of pyrethroid resistance across several CPB populations (Lee *et al.* 1999, Kim *et al.* 2004). Later, two additional mutations, T929I and T929N, were found in Bulgarian CPB populations but have not been detected in U.S. CPB populations (Rinkevich *et al.* 2012b). Reports also indicate the detection of mutations in Rdl subunits that confer resistance to endosulfan in an Iranian CPB population (Malekmohammadi *et al.*, unpublished)

1.2.1.2 Mutations on nAChR

Neonicotinoids target nicotinic acetylcholine receptors (nAChRs) (Matsuda *et al.* 2001). Insects encode both α and β type of nAChR subunits, and the neuronal nAChRs are usually heteropentamers of α and β subunits (Matsuda *et al.* 2020). Each subunit possesses a long N-terminal extracellular domain containing six Loops (Loops A to F). On the subunits interface, loops A, B, and C of an alpha subunit and loops D, E, and F of non-alpha subunits form the orthosteric site for acetylcholine or neonicotinoids binding (Corringer *et al.* 2000). Each subunit also possesses four transmembrane domains (TM1-4).

Neonicotinoids target the nicotinic acetylcholine receptor (nAChR), and in all insect species where the nAChR gene family has been well-characterized, it comprises at least eight α subunits and one β subunit (Wang *et al.* 2024). To date, field-evolved resistance-conferring mutations have been identified only in the nAChR β1 subunit, and are limited to three pest species. The first such mutation was R81T in the β1 subunit of *Myzus persicae* (Bass *et al.* 2011), which conferred resistance to imidacloprid. A subsequent mutation, V101I, also located in the M. persicae β1 subunit, was found to confer resistance to the neonicotinoids imidacloprid, thiamethoxam, and notably, dinotefuran (Xu *et al.* 2022). The identical R81T mutation was later detected in a field population of *Aphis gossypii* (Hirata *et al.* 2015). Additionally, an L80S substitution in the β1 subunit of *A. gossypii* was identified and is potentially associated with imidacloprid resistance (Kim *et al.* 2015). In *Bemisia tabaci* (whitefly), dual mutations were discovered which confer target-site resistance to multiple neonicotinoid insecticides: R79E, functionally equivalent to the R81T mutation in *M. persicae* and *A. gossypii* and a novel mutation, A58T (Yin *et al.* 2024).

As an alternative target site-related mechanism, alteration of nAChR subunit gene expression was demonstrated to be associated with neonicotinoid resistance in several insect species, primarily due to a reduced expression, including α2 in *Musca domestica*, α8 in *Nilaparvata lugens*, β1 in *Aphis gossypii*, α1 and β1 subunits in *Bradysia odoriphaga* and multiple α and β1 subunits in *Rhopalosiphum padi* (Markussen and Kristensen 2010, Zhang *et al.* 2015, Chen *et al.* 2017, Wang *et al.* 2018, Shan *et al.* 2020). On the other hand, the expression level of α1 in *Laodelphax striatellus* and α8 in *B. odoriphaga* were upregulated in imidacloprid-resistant strains (Zhang *et al.* 2018, Shan *et al.* 2020).

1.2.1.3 CPB nAChR studies

Considerable studies have investigated the interaction between neonicotinoids and CPB nAChR and the involvement of specific nAChR subunits in neonic resistance. The neural actions of imidacloprid on CPB CNS showed a dose-dependent biphasic relationship, with neuroexcitatory activity in low doses and neuroinhibitory activity in high doses. Although no significant difference was observed between resistant and susceptible strains in the initial excitation dose, a significant difference in the threshold of inhibitory actions was observed, indicating a possible change in the sensitivity of at least one subgroup of nAChRs (Tan *et al.* 2008). Qu *et al.* (2016) suppressed the mRNA level of CPB nAChR α1 in both adults and larvae, leading to a considerable decrease in the sensitivity to imidacloprid and thiamethoxam in adults, and the sensitivity to thiamethoxam in larvae (Qu *et al.* 2016). This study indicates nAChR α1 in one of the subunits comprising the native CPB nAChR and the expression level of nAChR α1 can affect neonicotinoid chemical response. qPCR results showed that the CPB nAChR α3, 8 and β1 were significantly downregulated in the presence of thiamethoxam and imidacloprid (Shi *et al.* 2023). Function expression of the CPB nAChR α1 and α8, and their interaction with

neonicotinoids (thiamethoxam, imidacloprid, and clothianidin) and acetylcholine were evaluated, which supporting the important role of these subunits in the sensitivity of CPB to neonicotinoids (Wang *et al.* 2022).

1.2.2 Enhanced detoxification

Biodegradation of xenobiotics, such as natural toxins produced by host plants and insecticide, is usually executed by a series of metabolic enzymes, such as cytochrome P450 monooxygenases (P450), glutathione S-transferase (GSTs), and carboxy/cholinesterase (CCEs) (Heidel-Fischer and Vogel 2015). Overexpression of these genes—whether through transcriptional upregulation, gene duplication, or altered enzyme activity—can enhance detoxification processes and thereby contribute to insecticide resistance.

Over-expression of P450 genes was also evidenced to be an important resistance mechanism to neonicotinoids in multiple species, including *Bemisia tabaci* (Karunker *et al.* 2008, Jones *et al.* 2011, Yang *et al.* 2013), *Trialeurodes vaporariorum* (Karatolos *et al.* 2010), *Nilaparvata lugens* , *M. persicae* (Puinean *et al.* 2010), and the elevated expression of esterase and glutathione-S-transferases in *Amrasca biguttula* (Halappa and Patil 2016), *Diaphorina citri* (Tiwari *et al.* 2011) and *Aphis gossypii* (Wang *et al.* 2002).

The role of metabolic detoxification in CPB's resistance to neonicotinoids has been widely analyzed (Table 1-1) (Clements *et al.* 2016, Kaplanoglu *et al.* 2017, Molnar and Rakosy-Tican 2021). The preliminary evidence of metabolic factors in CPB imidacloprid resistance was supported by synergism studies. Pretreatment of tolerant insects with the P450 inhibitor, piperonyl butoxide (PBO) and an esterase inhibitor, S, S, S-tributyl phosphorotrithioat (DEF), lowered the imidacloprid resistance ratio of a Long Island resistant population from 110.8 to 20.0

and 40.7, respectively, indicating the involvement of both types of enzymes (Naqqash *et al.* 2020). The imidacloprid resistance ratio of a Michigan field collected CPB population was reduced from 300 to 108 after PBO treatment (Mota-Sanchez *et al.* 2006a).

Associations between enhanced detoxification and neonicotinoid resistance in CPB were further supported by a series of molecular and bioinformatic analyses, including transcriptome comparison, RNA interference, and qRT-PCR (Clements *et al.* 2016, Zhu *et al.* 2016, Kaplanoglu *et al.* 2017, Dively *et al.* 2020). Research on the master regulatory factor CncC/Maf suggested a trans-regulation manner of multiple P450 genes in the CPB, which contributes to detoxifying imidacloprid and potato plant allelochemicals (Kalsi and Palli 2017). CncC/Maf trans-regulatory role is further evidenced by transcriptome comparison, RNAi, and rt-PCR analysis (Gaddelapati *et al.* 2018).

Table 1-1 Overview of studies reporting metabolic resistance mechanisms to neonicotinoids in the Colorado potato beetle

Gene Types	Insecticide	Research Method	Resistance Candidate Gene	Reference
P450s/CCEs	Imidacloprid	Synergism		(Zhao *et al.* 2000)
P450s	Imidacloprid	Synergism		(Mota-Sanchez *et al.* 2006b)
P450s	Imidacloprid	Transcriptome analysis		(Zhu *et al.* 2016)
CncC/P450s	Imidacloprid	Transcriptome analysis/RNAi	CncC, CYP6BJa/b, CYP6BJ1v1, CYP9Z25, and CYP9Z29	(Kalsi and Palli 2017)
CncC/ABC transporters	Imidacloprid	RNAi/RT-qPCR		(Gaddelapati *et al.* 2018)
P450s	Imidacloprid	Transcriptome analysis		(Clements *et al.*, 2017a)
P450s/UDPs	Imidacloprid	Transcriptome analysis		(Clements *et al.* 2018)
P450s/GSS/ABC transporters	Imidacloprid	Transcriptome analysis		(Clements *et al.* 2016)
A P450 and a GSS	Imidacloprid	RNAi		(Clements *et al.*, 2017b)

1.2.3 Behavior resistance

Behavioral resistance, also known as behavior avoidance, was defined as "evolved behaviors that reduce an insect's exposure to toxic compounds or that allow an insect to survive in what would otherwise be a toxic and fatal environment" (Sparks *et al.* 1989, Zalucki and Furlong 2017). For example, resistant mosquitoes could evade lethal insecticides on impregnated nets (Sokhna *et al.* 2013). The avoidance behavior of insecticide bait in cockroaches was due to the aversion to D-glucose in the attractant rather than the insecticide itself (Silverman and Bieman 1993). A later study indicated that the resistance cockroaches had altered response to D-glucose stimulation, which makes the sugar taste bitter (Wada-Katsumata *et al.* 2013).

The genetic basis of behavioral resistance to neonicotinoids was studied in cockroaches and house flies. The house fly showed behavioral resistance to imidacloprid by choosing to feed on only the non-toxic food source when the imidacloprid-containing bait co-presents, resulting in survival even without physiological resistance traits (Gerry *et al.* 2009). Linkage analysis indicated that factors conferring behavioral resistance to imidacloprid in house flies are located on autosomes 1 and 4 (Hubbard and Gerry 2021). Hubbard and Gerry (2020) suggested that behavioral resistance-conferring factors were preadapted in the wide housefly population, presenting in a contact-dependent and specific to the imidacloprid rather than a non-insecticidal component of a bait matrix (Hubbard and Gerry 2020). Neonicotinoid-resistant green peach aphids (*M. persicae*) were found to disperse from neonicotinoid-treated leaves to untreated leaves, showing their behavioral avoidance of insecticides (Fray *et al.* 2014).

In contrast, there is only limited evidence of CPB behavior resistance to imidacloprid. Compared with susceptible populations, a delayed peak of spring-emergence from overwintering sites in the soil was observed in a resistant population of CPB, helping them avoid fatal

neonicotinoid exposure after its application. A recent study indicated that imidacloprid resistant and susceptible strains displayed no difference in innate feeding avoidance to systemically applied imidacloprid at both larval and adult stages, while the susceptible strain preferred to lay eggs on insecticide-free plants than the resistant one (Clements *et al.* 2020).

1.2.4 Effects of cuticle proteins

Evidence supporting the role of cuticular proteins in *Leptinotarsa decemlineata* resistance to neonicotinoids remains limited. However, one cuticular protein gene (National Center for Biotechnology Information (NCBI) accession: GEEF01064138) has been repeatedly implicated in genetic studies. Knock-down of this gene via RNA interference (RNAi) restored susceptibility in an imidacloprid-insensitive population of *Leptinotarsa decemlineata*. The LC_{50} values of RNAi-treated groups were significantly lower than those of control groups (Clements *et al.* 2017). In a separate study, larval feeding on double-stranded RNA (dsRNA) targeting this gene negatively affected multiple biological parameters, resulting in 50.6% to 100.0% mortality, reduced weight gain, and prolonged larval and pupal development (Naqqash *et al.* 2020). This study also conducted a synergism experiment using second-instar larvae from a laboratory-selected resistant population, which exhibited 25.6-fold resistance to imidacloprid. Larvae were first fed dsRNA targeting the cuticular protein gene, and surviving individuals were subsequently treated with imidacloprid. After 72 hours, the mortality rate in the experimental group reached 100%, whereas the mortality in the control group—initially fed on potato leaflets treated with an empty vector—was only $4.23 \pm 1.07\%$ (Naqqash *et al.* 2020).

1.3 Regional specific pattern of insecticide resistance in CPB

Historically, CPB populations in certain potato-growing regions have consistently exhibited a higher rate of resistance evolution (Chen *et al.* 2023) and elevated baseline resistance

levels across multiple insecticide classes (Dively *et al.* 2020). For instance, Long Island is referred to as the "resistance capital of the potato kingdom," where many insecticides have exhausted their control efficiency, and new chemicals usually lose efficacy within eight years (Alyokhin *et al.* 2015). Populations in the northeastern United States, such as those in Maine and Massachusetts, and in the Midwest, including Michigan and Wisconsin, exhibit intermediate rates of resistance evolution. In contrast, northwestern populations, such as those in Oregon and Idaho, have remained susceptible to most insecticides despite prolonged exposure (Huseth *et al.* 2015, Scott *et al.* 2015, Tebbe *et al.* 2016). A similar geographic pattern has also been observed with respect to resistance to neonicotinoid insecticides.

1.3.1 The Long Island population

Imidacloprid resistance evolved rapidly in Long Island CPB populations, with the first anecdotal control efficacy reduction observed only three years after the introduction of imidacloprid in 1995 (Zhao *et al.* 2000). In the Long Island population, imidacloprid was applied four times yearly between 1995 and 1996, and an anecdotal control failure, with less than 50% efficacy, was reported in 1997 (Zhao *et al.* 2000). Bioassays on adult beetles from this population demonstrated an over 100-fold reduction of susceptibility to imidacloprid (LD_{50} resistance 2.422 µg/beetle vs. susceptible 0.024 µg/beetle) (Zhao *et al.* 2000).

The Long Island populations also tend to have a higher baseline tolerance to imidacloprid. Olson *et al.* (2000) conducted a multi-year imidacloprid susceptibility study from 1995-1998, including over 130 field-collected CPB populations across the United States, Canada, and multiple European countries (Olson *et al.* 2000). Bioassay results on first instar larvae indicated that in all tested years, populations from Long Island exhibited the highest tolerance to imidacloprid. In 1995, two populations (Wells and Wulforst) from Long Island had

the highest LC_{50} (4.08 and 4.40 ppm), which was 14.6 and 15.7-fold greater than the most sensitive population, respectively, indicating a higher baseline tolerance to imidacloprid, even before its first application.

1.3.2 The northeast populations

Neonicotinoid resistance evolution in the northeast area was less rapid than in Long Island, except for a small southeast valley in Maine. Potato growers in the valley applied imidacloprid intensively after its introduction in 1998, including one in-furrow and one foliar application per growing season. In 2002, some resistance beetles were observed in two valley farms, followed by a valley-wide decline in imidacloprid control in 2003, representing a new hotspot of imidacloprid resistance instance (Alyokhin and Dwyer 2005). Field trials conducted in 2005 on a commercial potato farm in southern Maine suggested that using neonicotinoid insecticides postponed the development of imidacloprid-resistant beetle populations by approximately 1 to 2 weeks and led to increased potato yields. However, the level of control achieved needed to be improved to avert severe crop losses (Alyokhin *et al.* 2007). The quick resistance evolution in this valley compared to other parts of Maine was attributed to its isolated geographical location, two successive years of potato production, and a higher number of generations per year due to a warmer temperature in the South (Alyokhin *et al.* 2015).

1.3.3 The midwest populations

Populations in the midwest illustrate moderate baseline tolerance to imidacloprid. Seven of ten populations collected in 1997 in Michigan showed an average of 93.6% mortality at 6-7 days of 0.1 ug per beetle imidacloprid treatment. Another three populations showed a slight tolerance with an average of 46.3% mortality; however, it was much lower than the 5.5% mortality of the Long Island strain (Zhao *et al.* 2000) . Grafius and Bishop (1996) found that a

Michigan population of Colorado potato beetles demonstrated lower sensitivity in topical assays and recovered more quickly from previous exposure to imidacloprid (Grafius and Bishop 1996). However, diet incorporation bioassays in 1997 on the first instar larva of this population suggested its imidacloprid sensitivity (LC_{50} 1.19 ppm) remained within the baseline range (designed as LC_{50} of 0.39 ppm and a discriminating concentration of 8 ppm) (Olson *et al.* 2000).

The first imidacloprid resistance case, defined as a higher than ten-fold resistance ratio, was identified in the midwestern region in 1994. The imidacloprid LD_{50} values of adult beetles from two Michigan populations (0.828 and 0.572μg $beetle^{-1,}$ respectively) were 23 and 16 times higher compared with the susceptible strain (0.036 μg $beetle^{-1}$) (Szendrei *et al.* 2012). In 2010, the resistance ratio of the second instar larvae population collected from Mecosta Co., MI, reached 26.7 (Baker *et al.* 2014).

Several studies bioassay the same field population for years, providing insight into dynamic change in resistance evolution. For example, in 2014, the LD50 value for a Wisconsin field population referred to as Systemic-3 was 52.68 ppm, which is significantly higher than in years from 2007 to 2011 and 2013 (with no overlapping of the 95% CI) (Huseth and Groves 2013, Clements *et al.* 2016).

1.3.4 The northwest populations

Despite anecdotal reports of reduced control efficiency, the northwest populations illustrate a relatively low resistance evolution, including neonicotinoids. For example, the baseline susceptibility to imidacloprid of the northwest CPB population was not significantly different from that of Maine in 1995 and 1996 (Olson *et al.* 2000). In 2003, the imidacloprid

LC50 in one Idaho population only increased by 2-fold, which was 20-36 times more sensitive than Maine populations (Alyokhin *et al.* 2006).

Crossley *et al.* (2018) evaluated the imidacloprid LD_{50} of the first instar larva from ten northwest and ten Midwest populations, respectively (Crossley *et al.* 2018). Their data illustrated that the resistance ratio of Midwest larvae was between 9 - and 340-fold higher than that of Northwestern sites. Yang *et al.* (2021) evaluated adult sensitivity to imidacloprid of three CPB populations from the Columbia Basin of Oregon and Washington, with a Wisconsin population as a resistant reference. The Wisconsin reference population showed a 20-fold increase compared to LD50 estimates for Columbia Basin populations (Yang *et al.* 2021).

1.3.5 Resistance factors and genetic basis

Resistance variation across CPB populations could be a comprehensive result of multiple factors such as the number of generations in one growing season, crop rotations, volunteer potatoes, and the initial resistance allele frequency (Alyokhin *et al.* 2015). As mentioned, resistance evolution in Washington state is much less than in other areas in the United States. Alyokhin *et al.* (2015) pointed out that volunteer potatoes might be a reason retarding the evolutionary rate. Potato beetles infesting volunteer potatoes escape from the heavy insecticide selection, limiting the likelihood of insecticide resistance-conferring allele accumulation. These volunteer potatoes can be regarded as natural *refugia*, an important insecticide resistance management (IRM) theory (Comins 1977), where selection pressure is absent, enabling the survival and reproduction of susceptible individuals. The genetic basis of regional differences was discussed in Dively *et al.* (2020) indicating that variation in gene regulation might cause baseline resistance difference between a New York and an Oregon CPB population to novel insecticides (Dively *et al.* 2020). Genomic comparisons across CPB populations in the United

States indicate that although enhanced detoxification mechanism is shared across regions in response to imidacloprid, the specific genes upregulated various regionally (Pélissié *et al.* 2022). The results of this regional pattern have its practical significance: the formulation of integrated pest and insecticide resistance management practices must accord with local pest populations.

1.4 Current IRM perspective and complementary suggestions

According to the Colorado potato beetle (CPB) resistance management poster published by the IRAC Coleoptera Working Group (CWG) in April 2016, "It is recommended that the rotation of effective insecticides with different modes of action are used to provide insect control whilst at the same time reducing the risk of insecticide resistance development." While rotation among insecticides with different modes of action (MoA) is a foundational principle of resistance management, this approach may underestimate the impact of metabolic resistance mechanisms, which can confer cross-resistance across chemically unrelated insecticides. For example, an early study suggested that Colorado potato beetle (CPB) populations with high tolerance to esfenvalerate and azinphos-methyl also exhibited reduced baseline sensitivity to imidacloprid, particularly in the Long Island population (Olson *et al.* 2000). This was presumed to result from preexisting metabolic and excretion mechanisms that had been selected by previous insecticide exposure. More recently, a study involving eight field-collected Colorado potato beetle (CPB) populations across the United States revealed cross-resistance between imidacloprid and isocycloseram, a novel insecticide classified under IRAC Group 30 (γ-aminobutyric acid (GABA)-gated chloride channel allosteric modulators). Transcript abundance analysis showed consistent upregulation of genes belonging to the carboxylesterase, cytochrome P450 (CYP), and ATP-binding cassette transporter (ABCT) families across populations, highlighting the critical role of metabolic detoxification in mediating cross-resistance (Terris 2024). Therefore, in

addition to rotating insecticides based on their MoA, it is important to consider their metabolic detoxification pathways when designing rotation schemes—particularly when managing neonicotinoid resistance.

1.5 Knowledge gaps and opportunities

1.5.1 Gaps

1.5.1.1 Genes encoding key target sites are not well characterized in CPB

Knowing the sequence of target site genes can assist downstream analysis, including mode of action examination, mutation effects interpretation, molecular resistance monitoring tool development, and pest management strategies. I noticed that genes encoding nAChRs and GABA receptors are not well characterized in the CPB. To date, cDNA sequences of several CPB nAChR genes have been cloned (Li *et al.* 2014, Qu *et al.* 2016, Xie *et al.* 2018), while I noticed that amino acid sequences displayed in these studies are not complete or missing annotations. The CPB nAChR α4 protein sequence deposited in the database (NCBI accession KP090398.1) is significantly shorter than expected. The CPB *Rdl* gene described in a previous study (Glueck 1998) was also fragmented, at a length of 176 AA, which is much shorter than the expected length (>500 AA).

1.5.1.2 Limited knowledge of genetic basis of neonicotinoid resistance

Although numerous studies have investigated the molecular basis of neonicotinoid resistance in the Colorado potato beetle and have identified several candidate resistance-associated genes, the genetic basis of resistance remains poorly defined. Specifically, for neonicotinoid resistance, genetic variations—such as mutations that alter the amino acid sequence of target-site proteins or affect the regulation of resistance-related genes—have yet to be conclusively identified.

1.5.1.3 A lack of knowledge of regional-specific genotypic resistance status

The effectiveness of insecticide resistance management programs depends on accurate knowledge of local resistance patterns. The same IRAC CWG poster suggests it is essential to "evaluate the current insecticide resistance situation in the area." Determining the resistance allele frequency in CPB populations across different regions provides critical insights into localized resistance dynamics and informs region-specific insecticide choices. However, comprehensive studies assessing genotypic resistance status, such as the frequence of resistance alleles, of CPB at the regional level remain lacking.

1.5.2 The chromosome-level CPB genome assembly as a platform to facilitate resistance research

A chromosome-level genome assembly for the Colorado potato beetle was recently published by Yan *et al.* (2023). This new assembly spans 1,008.42 Mb and maps to 18 chromosomes (17 autosomes + XO sex chromosomes). Compared with the current genome assembly, Ldec_2.0, from which most of the annotation is based (Schoville *et al.* 2018), it shows substantial improvements in key metrics such as contiguity (N50) and completeness of genes (BUSCO scores) (Table 1-2).

Table 1-2 Comparison of key metrics of two CPB genome assembly

Genome assembly	Ldec_2.0	Chromosome level
Total size (Mb)	642	1008.42
Busco genes (%)	C: 93.0%	C: 98.0%
Scaffold N50 (Mb)	0.139	58.32
Number of Contigs	26,908	18

The availability of a high-quality, chromosome-level genome offers a valuable platform for resistance-gene research. Notably, it enables more accurate and complete gene annotation. For example, when aligning the red flour beetle nAChR α4 protein sequence to the fragmented Ldec_2.0 CPB assembly, only sequence after the 315th amino acid aligned well to contig

NW_019290052.1, while most of the N-terminal region either mapped to separate contigs or produced no significant hits. In contrast, alignment against the chromosome-level assembly (contig CM045695.1) revealed a robust, contiguous match with higher sequence identity across the full length of the protein. Additionally, when combined with regional CPB sequencing data, this assembly facilitates the detection and surveillance of resistance allele frequencies across populations. The improved contiguity of the assembly also allows investigation into the genomic linkage of resistance-associated genes, which may provide new insights into the architecture and evolution of resistance mechanisms

1.6 Aims of this dissertation

The overarching aim of this dissertation is to advance the understanding of the genetic basis of insecticide resistance in the Colorado potato beetle and to provide insights that inform resistance monitoring and pest management strategies. In Chapter 2, I will annotate the genomic and protein sequences of key insecticide target sites, with a focus on nicotinic acetylcholine receptors (nAChRs) and GABA receptors. This foundational work is intended to support future functional analyses of these receptor families. In Chapter 3, I will investigate the frequencies of resistance alleles associated with known mutations across CPB populations. Additionally, I will examine potential novel mutations in the nAChR genes characterized in Chapter 2, along with other candidate resistance genes, to identify their associations with neonicotinoid resistance.

Chapter 2 The cys-loop ligand-gated ion channel gene superfamily of the Colorado potato beetle, *Leptinotarsa decemlineata*

2.1 Abstract

The insect cys-loop ligand-gated ion channel (cysLGIC) superfamily includes nicotinic acetylcholine receptors (nAChRs), γ-aminobutyric acid (GABA) receptors, glutamate- or histamine-gated chloride channels (GluCls and HisCls), pH-sensitive chloride channels (pHCls) and several other functionally uncharacterized receptors. Several of these receptors are target sites of neonicotinoids and other insecticides. Characterizing sequences of cysLGIC genes can facilitate the study of functional expression of subunits allowing insecticide/receptor interaction research, provide candidate targets for RNAi interventions, and can promote molecular resistance monitoring tools development. The Colorado potato beetle (CPB) is an agricultural pest that threatens the production of solanaceous crops. Although this insect shows frequent evolution of insecticide resistance, its cysLGIC superfamily is not well characterized. Twenty-two candidate CPB cysLGIC subunit genes were identified, and the functional regions of their protein sequences were annotated. CPB possesses 22 candidate cysLGIC subunit genes such as *nAChR α4*, *nAChR α6*, *Rdl*, and *GluCl* subunits, with similar sequence, structure, and alternative exon use as that in other insects. RNA A-to-I editing was observed of nAChR α6. Two copies of the pHCl subunit gene were identified, the first duplication of this gene observed in insects. The number of cysLGIC superfamily genes is similar to that of other insect species. Alternative splicing and RNA editing conserved in insect species were also identified in expected subunits, potentially contributing to structural and functional diversity of the receptor. Evidence of naturally truncated nAChR α4 and duplicated pHCl was observed, which invites future validation.

2.2 Introduction

Members of the cys-loop ligand-gated ion channel (cysLGIC) superfamily mediate both fast excitatory and inhibitory synaptic transmission in the nervous system of vertebrates and invertebrates. The cysLGIC are formed by the assembly of five subunits that share similar structural features: a large extracellular N-terminal region containing the signature cys-loop (two cysteines separated by 13 amino acids and bonded by disulfide bonds), ligand-binding motifs (loops A–F), and four transmembrane (TM) domains that form the ion-selective pore (Connolly and Wafford 2004) (Figure 2-1). Insect cysLGICs include the cation-permeable nicotinic acetylcholine receptors (nAChRs) (Tomizawa and Casida 2001), the γ-aminobutyric acid (GABA) receptor with both cationic and anionic (chloride) channel properties (Buckingham *et al.* 2005), the glutamate- or histamine-gated chloride channel (GluCl and HisCl) (Gisselmann *et al.* 2002, Wolstenholme 2012), the pH-sensitive chloride channel (pHCl) (Schnizler *et al.* 2005), and the insect group1 subunits and several other functionally uncharacterized receptors (Knipple and Soderlund 2010) (Table 2-1 and Appendix A). In addition to their roles in the insect nervous systems, these receptors are of particular interest as target sites of widely used insecticides. For example, nAChRs are target sites of the neonicotinoid and spinosyn insecticides, and GABA receptors are target sites of cyclodienes and the phenyl pyrazoles. According to the insecticide resistance action committee (IRAC) mode of action classification, members of the cysLGIC superfamily are target sites of seven insecticide groups (Table 2-1).

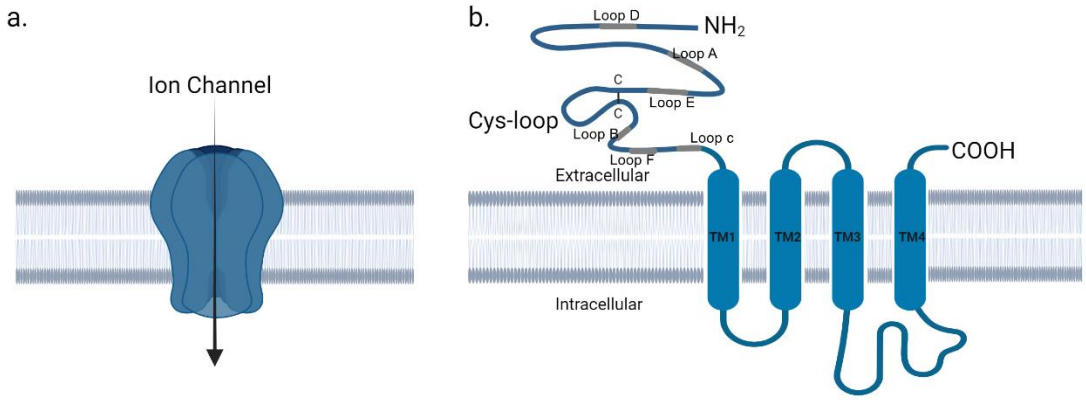


Figure 2-1 Structure of a typical cys-loop receptor

a). The pentameric receptor with ion permeability; b). Topology of a cys-loop receptor subunit. The extracellular domain contains the signature cys-loops and Loops A-F (Gray shaded region) for ligand binding. Sequences connecting transmembrane domains 1 to 3 are usually short and can be longer between TM3 and TM4. Figure created with BioRender.com.

Table 2-1 Sub-category of insect cys-loop ligand-gated ion channels and their role as insecticide target sites

Receptor Name/Description	Endogenous ligand	Nomenclature of subunits	Targeted Insecticide Group
nicotinic acetylcholine receptor	Acetylcholine (ACh)	α (possessing two consecutive cysteines in loop C) and β	Group 4: neonicotinoid, Sulfoximines, etc. Group 5: Spinosyns Group 14: Nereistoxin analogues Group 32: GS-omega/kappa HXTX-Hv1a peptide
γ-amino butyric acid-gated anion channels	γ-amino butyric acid (GABA)	Rdl, LCCH3 and GRD	Group 2: Cyclodiene Organochlorines & Fiproles Group 30: Meta-diamides & Isoxazolines
Glutamate-gated chloride channel	Glutamate	GluCl	Group 6: Avermectins & Milbemycins)
Histamine-gated chloride channel	Histamine	HisCl	
pH-sensitive chloride channel	unclear	pHCl	
Insect group 1 cysLGIC. Initially annotated in *D. melanogaster* as CG6927, CG7589 and CG11340	unclear	CLGC	
Ungrouped subunits. Initially annotated in *D. melanogaster* as CG8916 and CG12344	unclear	8916 or 12344	

Insect nAChRs respond to acetylcholine (ACh) and mediate fast excitatory transmission at cholinergic synapses in the central nervous system (CNS). They involve processing sensory signals, locomotion, and cognitive processes (Matsuda 2021). Insect nAChR subunits are of two types, α (characterized by the two consecutive cysteines in Loop C) and β (Tomizawa and Casida 2001). Most insect nervous nAChRs are hetero-pentameric, containing both α and β subunits, while in some rare cases, particular α subunits can form homomers (Lansdell *et al.* 2012). Imidacloprid acts at the ACh binding site (the orthosteric site) on insect nAChRs, thereby activating the integral cation-permeable ion channel of the receptor (Ihara and Matsuda 2018) (Figure 2-1). GABA receptors are widespread in insect CNS, mediating inhibitory and excitatory neurotransmission. The first insect GABA subunit gene was identified from a *D. melanogaster* strain resistant to dieldrin (a cyclodiene insecticide), named *Rdl* (Ffrench-Constant *et al.* 1991). GluCls have a wide range of functions in invertebrates' nervous systems associated with locomotion, feeding, and sensory inputs (Wolstenholme 2012). The first insect GluCl subunit cDNA was cloned from *D. melanogaster* (Cully *et al.* 1996). Most insect species, including *Apis mellifera*, *Tribolium castaneum*, and *Nasonia vitripennis*, have a single GluCl gene. Histamine is the primary neurotransmitter of arthropod photoreceptors, and these histamine-gated chloride channels likely play a role in vision, although their function remains unclear (Cully *et al.* 1996, Semenov and Pak 1999). The pH-sensitive chloride channel was initially identified in *D. melanogaster*. While its native ligand has not yet been identified, its chloride channel is opened in response to shifts in extracellular pH (Schnizler *et al.* 2005). Insects possess 1 - 3 group1 subunits. In early studies, these subunits were labeled by numbers, consistent with the first discovered *D. melanogaster* orthologs (CG6927, CG7589, and CG11340); later, subunits belonging to this group, were labeled as CLGC, standing for Cys-loop Ligand Gated ion Channel

(Jones and Sattelle 2007). Finally, there are two functionally uncharacterized subunits, initially annotated as CG8916 and CG12344, in the *D. melanogaster* genome assembly.

Insect species typically possess 21-26 cysLGIC subunit genes (Littleton and Ganetzky 2000, Jones and Sattelle 2006, 2007, Jones *et al.* 2010, Knipple and Soderlund 2010, Del Villar and Jones 2018, Matthews *et al.* 2018, Rinkevich and Bourgeois 2020), except for two cockroach species showing over 30 members, mainly because of expansion of nAChR β subunits (Jones *et al.* 2021). These numbers are relatively small compared with other arthropod and nematode cysLGIC superfamilies; for example, the spider *Pardosa pseudoannulata*, the spider mite *Tetranychus urticae*, and the nematode *Caenorhabditis elegans* have 34, 29, and 102 cysLGIC genes respectively (Jones and Sattelle 2008, Dermauw *et al.* 2012, Wang *et al.* 2024).

Genomic analysis of these proteins in pest insects has several benefits. Variation in cysLGIC receptor genes conferring target site insensitivity have been identified in insects (Table 2-2). Characterizing this variation provides the basis for molecular resistance monitoring. Similarly, sequence knowledge allows the development of nucleic acid-based control methods such as RNA interference. Furthermore, RNAi sequences can be designed based on variant sites between beneficial species and pests to achieve effective pest management while minimizing risks to non-target species.

Table 2-2 Target site insensitivity mutations on cys-loop receptors

Subunits	Mutations	Species	Resistance to	Reference
nAChR β1	R81T	*Myzus persicae*	Neonicotinoid	(Bass *et al.* 2011, Mottet *et al.* 2016, Xu *et al.* 2022)
		Aphis gossypii	Neonicotinoid	(Hirata *et al.* 2015, Munkhbayar *et al.* 2021)
	V101I	*Myzus persicae*	Neonicotinoid	(Xu *et al.* 2022)
	L80S	*Aphis gossypii*	Imidacloprid	(Kim *et al.* 2015)
	A58T&R79E	*Bemisia tabaci*	neonicotinoid	(Yin *et al.* 2024)
nAChR α6	G275E	*Frankliniella occidentalis*	Spinosad	(Puinean *et al.* 2013)
Rdl	A301S/G	*A. aegypti*	Fiproles	(Collins *et al.* 2022)
		Nilaparvata lugens	Fipronil	(Zhang *et al.* 2016, Garrood *et al.* 2017)
		Blattella germanica	Fipronil and dieldrin	(Hansen *et al.* 2005, Gondhalekar and Scharf 2012)
	T350M T350M+A301 G	*D. simulans*	fipronil	(Le Goff *et al.* 2005)
GluCl	L315F	*M. domestica*	Ivermectin	(Nakata *et al.* 2017)
	L319F	*B. mori*	Ivermectin	(Furutani *et al.* 2017)

Characterizing cysLGIC subunit sequences also aides the study of subunit function, including the expression of subunits in well-established expression vehicles such as *Xenopus laevis* oocytes and gene editing in multiple insect species (Grauso *et al.* 2002, Henry *et al.* 2020, Ihara *et al.* 2020, Matsuda 2021).

The Colorado potato beetle (CPB) is a damaging pest, threatening the production of solanaceous crops, including potatoes, tomatoes, and eggplants. Intensive insecticide use has led

to resistance to cysLGIC targeting insecticides, such as dieldrin (Hofmaster *et al.* 1967), organochlorines (Balaško *et al.* 2020), neonicotinoids (Grafius and Bishop 1996, Alyokhin and Dwyer 2005) and spinosyns (Schnaars-Uvino and Baker 2021) in CPB populations. Except for a few cysLGIC genes (Li *et al.* 2014, Qu *et al.* 2016, Xie *et al.* 2018), this gene superfamily has not been studied in the CPB despite the importance of insecticides targeting cysLGICs for its control. A recently published chromosome-level CPB genome assembly (Yan *et al.* 2023) provides an opportunity to annotate and characterize the cysLGIC superfamily in this species. Here, I report the first systematic study of the cysLGIC gene superfamily in Colorado potato beetles. The protein coding regions of all subunits were successfully identified in the CPB genome. Characterizing the full complement of CPB cys-loop LGIC subunits represents a critical step in identifying key components of the CPB nervous system and pinpointing particular molecular targets underlying responses to insecticides.

2.3 Materials and Methods

2.3.1 Bioinformatic analysis

To identify potential CPB cysLGIC genes, a tBLASTn (McGinnis and Madden 2004) search was executed against the chromosome-level CPB genome (Genbank accession number JANJPO000000000.1) (Yan *et al.* 2023), with all *T. castaneum* and *Periplaneta americana* cysLGIC subunit protein sequences as queries (see Appendix B for NCBI accession number of query sequences)". *T. castaneum* was chosen because, among all species with well-characterized cysLGIC, it is most closely related to CPB. *P. americana* was chosen because it has the largest number of insects cysLGIC subunits. Candidate CPB cysLGIC genes were determined based on their identity with queries, particularly in the N-terminal ligand-binding region and the four transmembrane domains. In the second round, *T. castaneum* cysLGIC subunits were mapped to

the CPB genome using the protein2genome model in Exonerate (Slater and Birney 2005) to predict the intron and exon boundaries. To find the complete protein coding sequence of each CPB cysLGIC subunit, as well as to validate its expression in CPB, chromosomal hits identified by tBLASTn and Exonerate were extracted and manually assembled to obtain the genomic DNA (gDNA) sequence of each subunit. Then, the assembled gDNA sequences were used as queries to Blastn against a CPB transcriptome (NCBI accession GGNV00000000.1) (Clements *et al.* 2018). Transcript hits with a score higher than 900 were collected.

2.3.2 Sequence selection for protein analysis

Transcripts from each cysLGIC subunit, were translated to their corresponding protein sequences, aligned, and manually screened for completeness of "cysLGIC subunit signature regions" (including the N-terminal ligand-binding motifs and transmembrane domains). Protein sequences proceeding to alignment were selected according to the following rule: for a subunit of which only one of the transcript-translated proteins shows all "signature regions", the transcript-translated sequence will be selected; if multiple transcript-translated proteins can display all "signature regions", the longest one was used; For a subunit of which none of the transcript-translated proteins can show complete "signature regions" or no transcript hit has a score higher than 900, a manual search was executed in the NCBI database to find any of its transcripts, cDNA or protein coding sequence predicted by automated computational analysis.

In order to further investigate the truncated sequence of nAChR α4, I compared the aligned nAChR α4 N-terminal extracellular region protein sequences of five insect species, including the red flour beetle, small hive beetle, western honeybee, American cockroach, and fruit fly (Appendix C). Analysis of these aligned sequences revealed a high degree of structural conservation in this region, with no observed insertions or deletions, also suggesting that use of a

closely related species' nAChR α4 protein sequence as a query to map the CPB genome could be an effective strategy for locating exon regions encoding the N-terminal region.

To investigate the possible cause of highly fragmented nAChR α4 transcripts, I mapped RNA-seq data, obtained from previous studies (Appendix D) to the CPB genome assembly and calculated the mean depth of coverage at each expected nAChR α4 exon region. I also collected the exon specific mean depth of coverage of nAChR α1, which is located on the same chromosome, as a reference.

2.3.3 Protein sequence alignment and annotation

CPB nAChR subunits and non-nAChR subunits were aligned separately. Protein alignment was conducted by Clustal X2 (Larkin *et al.* 2007). The pairwise alignment was set with a gap-opening penalty of 10 and a gap-extension penalty of 0.1, applying the Gonnet 250 protein weight matrix. Sequence alignment was visualized and annotated using JalView version 2.1.1 (J. Wang *et al.* 2020). Transmembrane domains of protein sequences were predicted by TMHMM - 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). Protein signal cleavage sites were predicted by SignalP 5.0. Genedoc calculated identities/similarities between protein sequences.

2.3.4 Phylogenetic analysis

Phylogenetic analyses were run for nAChR and non-nAChR subunits separately. In MEGAX (Kumar *et al.* 2018), the MUSCLE algorithm aligned cysLGIC subunits of CPB, *D. melanogaster*, *A. mellifera*, *P. americana,* and *T. castaneum* (See Appendix B for NCBI accession number for sequence used for phylogeny), and the phylogenetic tree was then reconstructed using the maximum-likelihood method and Dayhoff matrix-based model with 1000

bootstraps replication. The tree file was visualized and manipulated by TreeViewer (Bianchini and Sánchez-Baracaldo 2024).

2.3.5 Alternative splicing and post-transcriptional modification identification

Alternative splicing of a subunit gene was identified by comparing its transcript isoforms. In addition, alternative exons can also be evidenced by tBlastn results, as a region of a protein query can hit multiple genomic regions with a reasonably high identity of each hit, and these alternative regions are tandemly arranged in the genome. Pre-mRNA editing was analyzed by comparing gDNA and transcripts of each subunit and their translated protein sequences.

2.4 Results

2.4.1 Overview of CPB cysLGICs

CPB possesses 22 candidate cysLGIC subunit genes, including 11 nAChR subunit genes and 11 non-nAChR subunit genes. Sixteen subunit genes are found on autosomes and six are located on the X chromosome (Appendix E). A total of 21 subunit sequences were aligned and subsequently used in phylogenetic analysis (Appendix F). Subunits are labelled with a one-letter genus code plus a three-letter species code and the subunit types. For example, the nAChR α6 subunit in *Tribolium castaneum* was denoted as Tcas_ α6 and the Rdl ortholog of CPB is Ldec_Rdl.

2.4.2 CPB nAChR subunits

Eleven nAChR subunits were identified in CPB. Based on characteristic features such as the presence or absence of two consecutive cysteines in loop C (Figure 2-2), ten subunits were categorized as α type and one as β type (Appendix E). Most CPB nAChR subunits had amino acid sequence identities over 60% with their *T. castaneum* counterparts (Table 2-3) except for

Ldec_α9 with 43% identity with Tcas_α9. This relatively low identity between nAChR α9 orthologs is not surprising because previous studies found this subunit to be highly variable across species, such as 28% and 20% identity between the *T. castaneum* nAChR α9 and its counterparts in *A. mellifera* and *P. americana*, respectively (Jones and Sattelle 2007, Jones *et al.* 2021). In addition, a GEK motif preceding TM2, which is conserved among insect nAChR subunits, was present in all CPB nAChR subunits except Ldec_ α9 (Figure 2-2). Similarly, replacements of the GEK motif were observed in multiple nAChR subunits of two cockroach species (Jones *et al.* 2021), silkworms (Shao *et al.* 2007), *N. vitripennis* (Jones *et al.* 2010), as well as the nAChR α9 in *T. castaneum*. Ldec_α1 - Ldec_α4 and Ldec_α8 have insertions in Loop F (Figure 2-2), also seen in other insect species, such as cockroaches (Jones *et al.* 2021), parasitoid wasp *N. vitripennis* (Jones *et al.* 2010) and the red floor beetle *T. castaneum* (Jones and Sattelle 2007), which may contribute to interactions with the neonicotinoid, imidacloprid (Shimomura *et al.* 2004).

Table 2-3 CPB nAChR subunits (identity/similarity) to *Tribolium castaneum*

NCBI accession numbers mentioned in this figure are listed in Appendix B. Potential orthologs are highlighted in bold.

	Ldec_α1	Ldec_α2	Ldec α3	Ldec_α4	Ldec_α5	Ldec_α6	Ldec_α7	Ldec_α8	Ldec_α9	Ldec_α10	Ldec β1
Tcas_α1	**90/94**	53/69	55/66	52/65	29/48	33/51	34/52	53/69	15/32	18/37	38/56
Tcas_α2	51/68	**90/94**	50/65	46/62	26/48	33/52	33/51	50/66	16/33	19/34	38/56
Tcas_α3	55/67	50/65	**87/92**	67/76	28/48	32/49	34/51	55/67	15/32	19/34	41/59
Tcas_α4	52/65	46/62	66/76	**91/95**	27/45	32/48	33/51	55/70	16/32	18/35	40/56
Tcas_α5	23/40	21/40	23/40	22/38	**63/67**	26/41	25/41	21/38	13/27	15/28	24/40
Tcas_α6	34/51	33/51	32/49	33/49	34/53	**89/94**	64/75	33/52	18/36	21/37	33/53
Tcas_α7	33/51	34/52	33/49	34/50	32/51	62/74	**89/94**	33/51	16/33	19/37	32/51
Tcas_α8	52/67	50/66	55/67	51/67	29/50	33/53	34/53	**85/90**	15/34	21/39	40/58
Tcas_α9	15/30	15/32	15/32	14/31	16/36	15/34	14/33	14/32	43/66	23/47	14/33
Tcas_α10	19/36	19/35	18/35	18/35	19/40	20/38	20/39	20/37	26/47	**63/80**	19/38
Tcas_α11	52/66	50/66	55/66	52/66	28/48	33/51	33/50	84/90	15/34	21/38	39/57
Tcas_β1	38/55	38/55	40/57	38/54	29/51	34/52	32/51	39/57	16/31	20/39	**94/96**

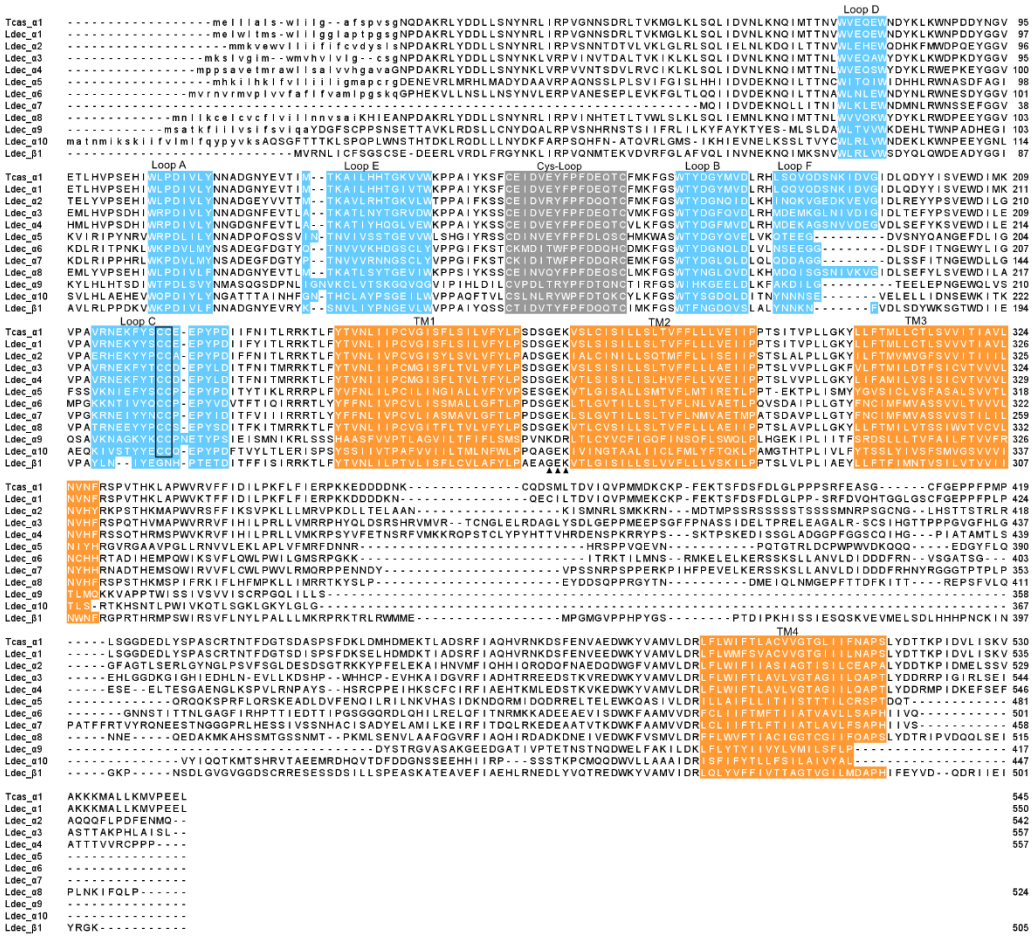


Figure 2-2 Protein sequence alignment of CPB nAChR subunits

The *T. castaneum* nAChR α1 was included as a reference (NCBI accession NP_001103245.1). N-terminal signal leader peptides are indicated by lowercase letters. Loops A-F are highlighted in blue; the cys-loops are highlighted in gray, and transmembrane domains are suggested by orange. The two consecutive cysteines in Loop C, characteristic of α subunits, are enclosed in a box. Three amino acids (GEK) preceding TM2 are indicated by solid triangles underneath. NCBI accession numbers mentioned in this figure are listed in Appendix B.

In the nAChR amino acid sequence-based phylogeny (Figure 2-3), α1 - α8 and β1 subunits are relatively conserved across insect species (Jones *et al.* 2007). The "nAChR α5 group" only includes non-dipteran orthologs, since dipteran α5 subunits were named following different nomenclatures (Shao *et al.* 2007). For example, the *Bombyx mori* α5 shared 17% identity with *D. melanogaster* α5 (Shao *et al.* 2007). Instead, *D. melanogaster* α5 subunits are clustered with non-dipteran α7 subunits, named here as "α7-like group". In addition, two CPB nAChR, Ldec_α9, and Ldec_α10, clustered in the large divergent group.

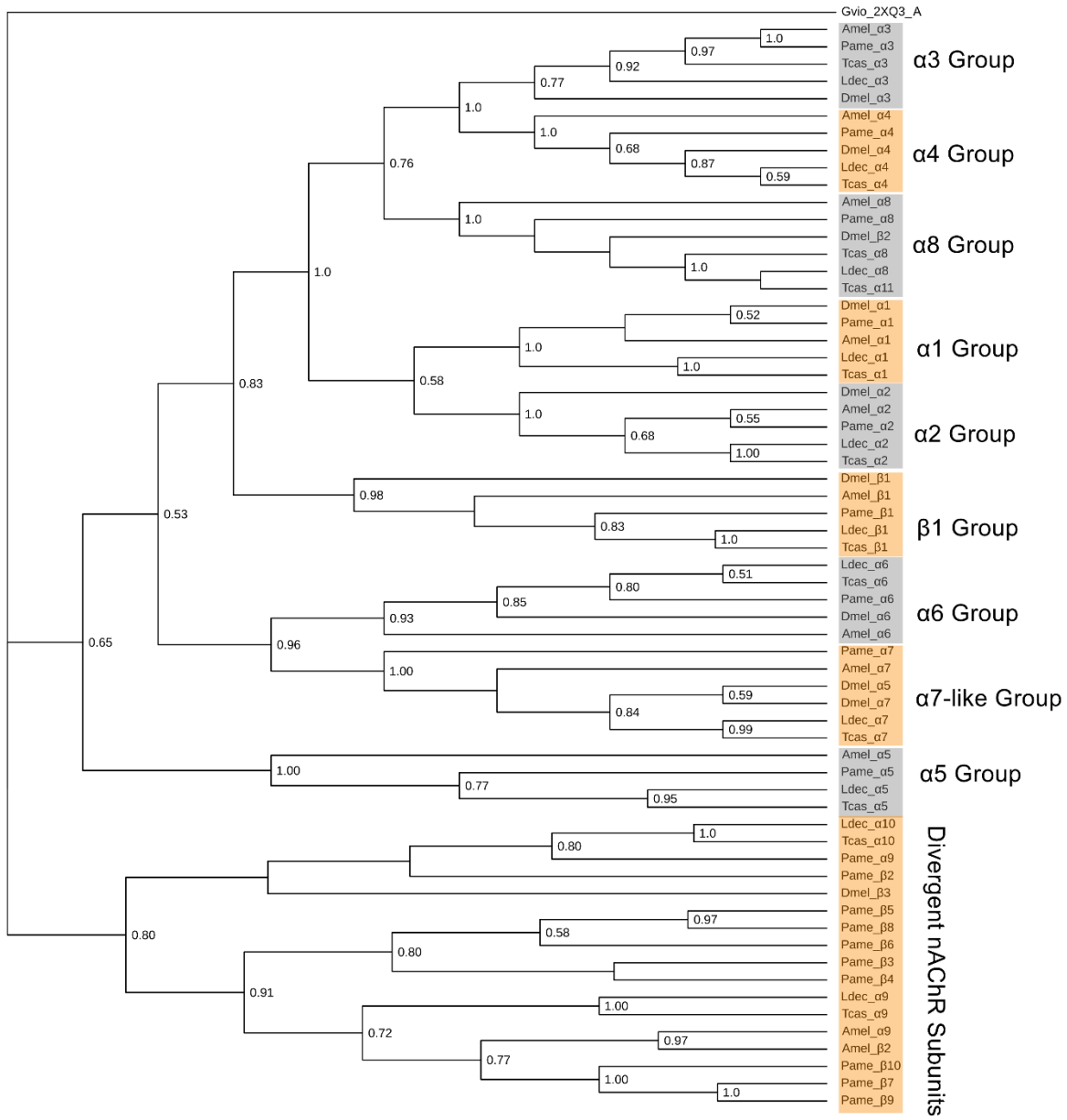


Figure 2-3 Phylogeny of nAChR subunits in five species

Species name annotations: Ldec-*L. decemlineata*; Dmel-*D. melanogaster*; Amel-*A. mellifera*; Tcas-*T. castaneum*; Pame-*Periplaneta americana*. Gvio_2XQ3_A, a bacterial ancestor of cysLGICs from *Gloebacter violaceus* was an outgroup, to which the tree was rooted. Bootstrapping values higher than 0.5 are displayed. NCBI accession numbers mentioned in this figure are listed in Appendix B.

The *Ldec_α7* and *Ldec_β1* genes are located within 474 kb of each other on the X chromosome NCBI Accession number CM045700.1 (www.ncbi.nlm.nih.gov). Clustering of nAChR α7 and β1 subunits have similarly been observed in the genomes of *B. germanica, P.*

americana, A. mellifera, A. gambiae, and *T. castaneum* (Jones *et al.* 2005, 2021, Jones and Sattelle 2006, 2007), suggesting conserved syntony and the possibility that their expression may be coordinated.

Preliminary evidence of alternative splicing in several subunits was supported by tBlastn results. Two alternatives were identified for *Ldec_α4* exon4, labelled exon4a and exon4b respectively (Figure 2-4a). Alternative splicing leads to amino acid polymorphism in agonist binding pocket forming region, Loop E and Loop B, which may contribute to the variable function of protein isoforms. The *Ldec_α6* has two potential alternatives in exon 8 (Figure 2-4b), encoding TM2, which forms the pore of the channel, possibly contributing to functional diversity (Connolly and Wafford 2004). Alternative splicing of exon 4 in the nAChR α4 and exon 8 in nAChR α6 has also been observed in other insect species (Jones and Sattelle 2010).

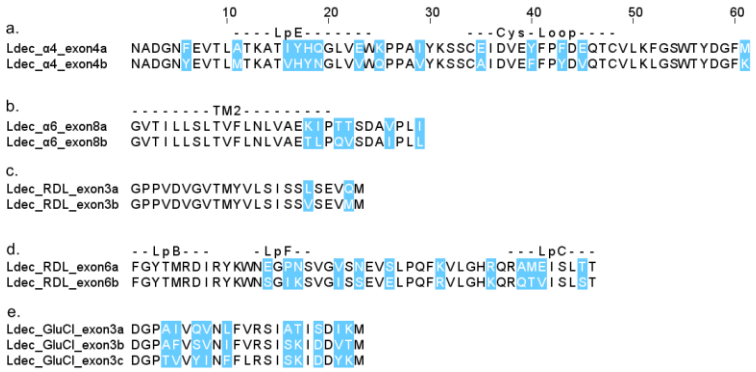


Figure 2-4 Potential alternative splicing

Variable sites are highlighted. a. Ldec_α4 exon four splicing variants. Both exon 4a and 4b were translated from genomic DNA extracted from the chromosome CM045695.1. b. Ldec_α6 exon eight splicing variants. Exon 8a was translated from genomic DNA extracted from the chromosome CM045700.1; exon 8b was translated from the transcript GGNV01207620.1. c. Ldec_Rdl exon three splicing variants. Both exon 3a and 3b were translated from genomic DNA extracted from the chromosome CM045709.1; d. Ldec_Rdl exon six splicing variants. Both exon 6a and 6b were translated from genomic DNA extracted from the chromosomeCM045709.1. e. Ldec_GluCl exon three splicing variants. All three alternative exons were translated from genomic DNA extracted from the chromosome CM045700.1.

A comparison of the CPB nAChR α6 gDNA and a transcript (GGNV01207620.1) sequence indicate three potential cases of adenosine (A) to guanosine (G) editing in gDNA sequence positions 27, 415, and 468 (Figure 2-5a). This conversion results in an S-to-L, N-to-D, and I-to-M amino acid substitution on protein positions 10, 139, and 156, respectively (Figure 2-5b). The S-to-L substitution may not affect the protein product since it is in the signal peptide region (refer to Figure 2-2), which will be trimmed in the final protein. The N-to-D substitution removed a potential N-glycosylation site, which may affect receptor maturation, channel desensitization, and conductance (Gehle *et al.* 1997, Nishizaki 2003). It is worth noting that nucleotide differences between reference genomes may be due to genomic DNA variation rather than RNA A-to-I editing since these two sequences came from different CPB samples.

Figure 2-5 Potential RNA A-to-I editing on CPB nAChR α6

a). A-to-G conversion between the gDNA and the transcript at site 27, 415, and 468 respectively (indicated by solid triangles) b). Amino acid replacement is due to RNA editing, indicated by solid triangles.

2.4.3 GABA receptor subunits

In CPB, one ortholog of each GABA receptor subunit was identified and named Ldec_Rdl, Ldec_GRD, and Ldec_LCCH3, respectively. The Ldec_Rdl shows a high identity with its *T. castaneum* counterpart (Table 2-4) and an anion channel signature PAR motif preceding TM2 which is conserved across insect species (Figure 2-6). Initially, I found no evidence of an alternative Ldec_Rdl exon 3 based on transcript data or tBLASTn results. Subsequently, because *Rdl* is known to have splice variants of exon 3 in other species (Ffrench-Constant and Rocheleau 1993, Jones and Sattelle 2006, 2007, Del Villar and Jones 2018), I mapped all three *Rdl* protein isoforms of *T. castaneum* (NCBI accession numbers NP_001107808.1, NP_001107809.1, and NP_001107764.1) to the CPB genome using Exonerate and successfully identified alternative exon 3 variants (Figure 2-4c). Exon 3 of the CPB *Rdl* gene encodes 23 amino acids preceding Loop D. Additionally, I identified two alternative exon 6 variants, which introduce 12 amino acid polymorphisms (Figure 2-4d). Some of these variations occur between Loops F and C, within the ligand-binding pocket, potentially affecting receptor function. By combining exon 3 and exon 6 alternatives, CPB could theoretically possess three Rdl isoforms, potentially contributing to the functional diversity of GABA receptors

.

Table 2-4 CPB non-nAChR cysLGIC subunits homology statistics (identity/similarity) to *Tribolium*

NCBI accession numbers mentioned in this figure are listed in Appendix B.

	Ldec_12344	Ldec_8916	Ldec_CLGC	Ldec_GluCl	Ldec_GRD	Ldec_HisCl1	Ldec_HisCl2	Ldec_LCCH3	Ldec_pHCl	Ldec_Rdl
Tcas_12344	**70/81**	17/31	16/30	21/41	23/39	26/45	28/48	21/40	17/36	23/40
Tcas_8916	19/33	**77/85**	14/27	23/40	37/53	20/36	21/36	27/43	17/30	26/43
Tcas_CLGC1	17/34	16/30	52/67	20/37	18/32	18/34	19/36	19/38	21/35	18/34
Tcas_CLGC2	18/34	15/28	51/66	20/35	17/30	17/33	17/35	17/35	18/33	17/33
Tcas_CLGC3	21/41	15/30	35/53	21/39	17/37	19/39	19/38	17/37	21/37	18/35
Tcas_GluCl	22/41	22/38	20/33	**95/97**	25/44	28/44	27/45	27/46	24/43	30/44
Tcas_GRD	20/36	39/53	16/28	24/41	**72/81**	21/38	21/38	30/46	17/31	30/45
Tcas_HisCl1	27/47	20/34	15/31	28/45	22/39	**82/89**	54/67	24/42	19/37	24/41
Tcas_HisCl2	27/50	21/36	16/33	28/45	24/42	54/67	**84/89**	26/42	20/39	26/42
Tcas_LCCH3	23/40	26/42	17/34	27/46	29/47	25/42	26/43	**85/92**	18/35	32/48
Tcas_pHCl	18/36	16/30	19/32	25/44	17/33	18/37	18/38	18/35	**86/93**	20/36
Tcas_Rdl	23/40	26/43	17/31	30/45	30/46	24/40	26/41	32/50	20/36	**94/95**

*Potential orthologs are highlighted in bold.

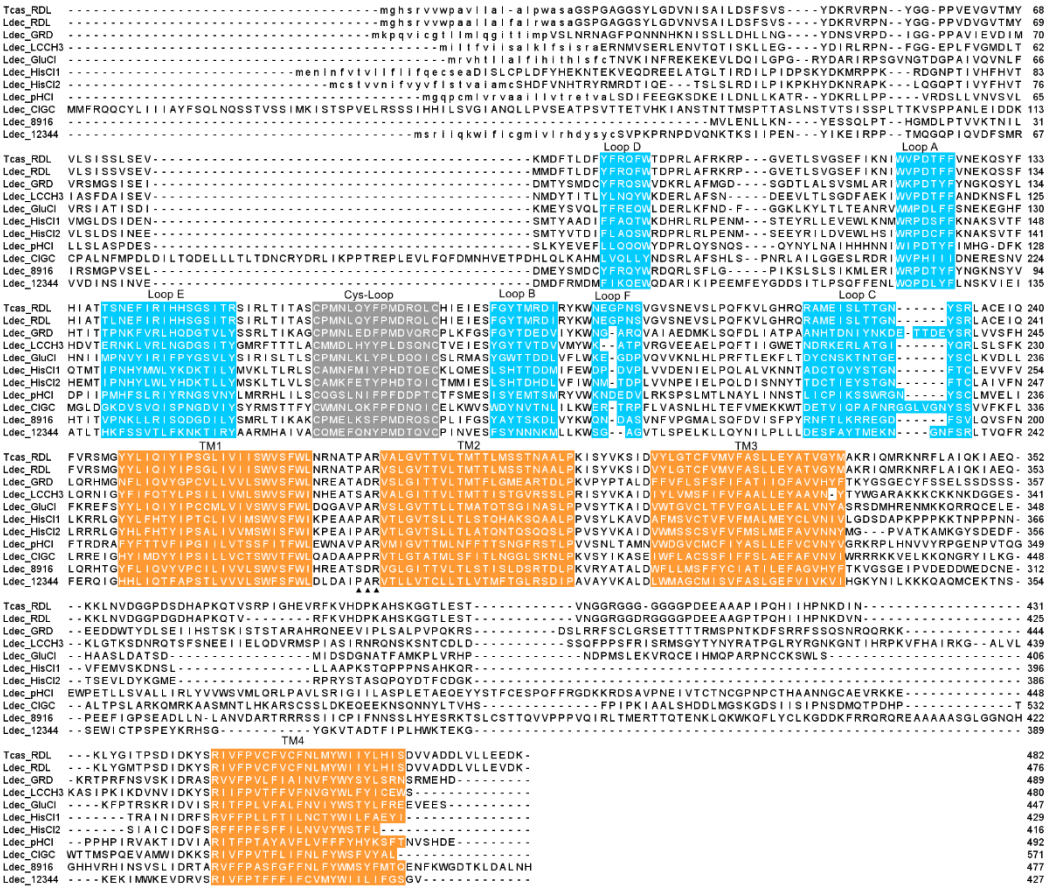


Figure 2-6 Protein sequence alignment of CPB cysLGIC subunits other than nAChR

T. castaneum Rdl subunit (NCBI accession NP_001107809.1) is included as a reference. N-terminal signal leader peptides are indicated by lowercase letters. Loops A-F are highlighted in blue; the cys-loops are highlighted in gray, and transmembrane domains are suggested in orange. Three amino acids preceding TM2 are indicated by solid triangles underneath. NCBI accession numbers mentioned in this figure are listed in Appendix B.

The Ldec_GRD and Ldec_LCCH3 lack the PAR motif (Figure 2-6), indicating their potential role as a cation channel. Two alternative exons 6, leading to 12 amino acid polymorphisms of the Ldec_Rdl were detected by the tBlastn result (Figure 2-4c). Some of the amino acid variation occurs between loops F and C, within the ligand binding pocket, which may cause functional effects on receptors.

2.4.4 CPB glutamate and histamine-gated chloride ion channels

One glutamate ion channel gene (*Ldec_GluCl*) and two histamine-gated chloride ion channel genes (*Ldec_HisCl1*, and *Ldec_HisCl2*) were identified in the CPB genome. *T.*

castaneum and CPB have high sequence similarity (over 82%) of these orthologs (Table 2-4). These three subunits display a PAR motif before TM2, consistent with their role as anion channels. Comparison among transcript-translated protein sequences indicates three alternative splicing products of exon 3 of the *Ldec_GluCl* gene (Figure 2-4d). Numbers of alternative GluCl exons 3 vary among species, with honeybees, fruit flies and two cockroach species having two (Jones and Sattelle 2006, Knipple and Soderlund 2010, Jones *et al.* 2021) and the red flour beetles having three (Jones and Sattelle 2007). *Ldec_GluCl* exon 3 encodes the region proximate to loop D, which participates in the formation of ligand-binding pockets; therefore, amino acid polymorphisms due to alternative splicing may lead to variable ligand-binding properties among isoforms.

2.4.5 CPB pH-sensitive chloride channel subunits

The Tcas_pHCl query matched two very similar, tandemly-arrayed CPB genomic regions on chromosome CM045712.1. Protein sequences deduced from these two genes are equal in length (426AA), having only one amino acid variant at position 356 (S/A), resulting in a 99.8% identity. This suggests that these two genes may be recently duplicated homologues of CPB pHCl. Therefore, they were named *Ldec_pHCl copy1* and *Ldec_pHCl_copy2* respectively (Appendix E). However, only one CPB pHCl protein isoform was validated via transcript sequence and this isoform was used for phylogenetic analysis (Figure 2-7) and protein annotation (Figure 2-6).

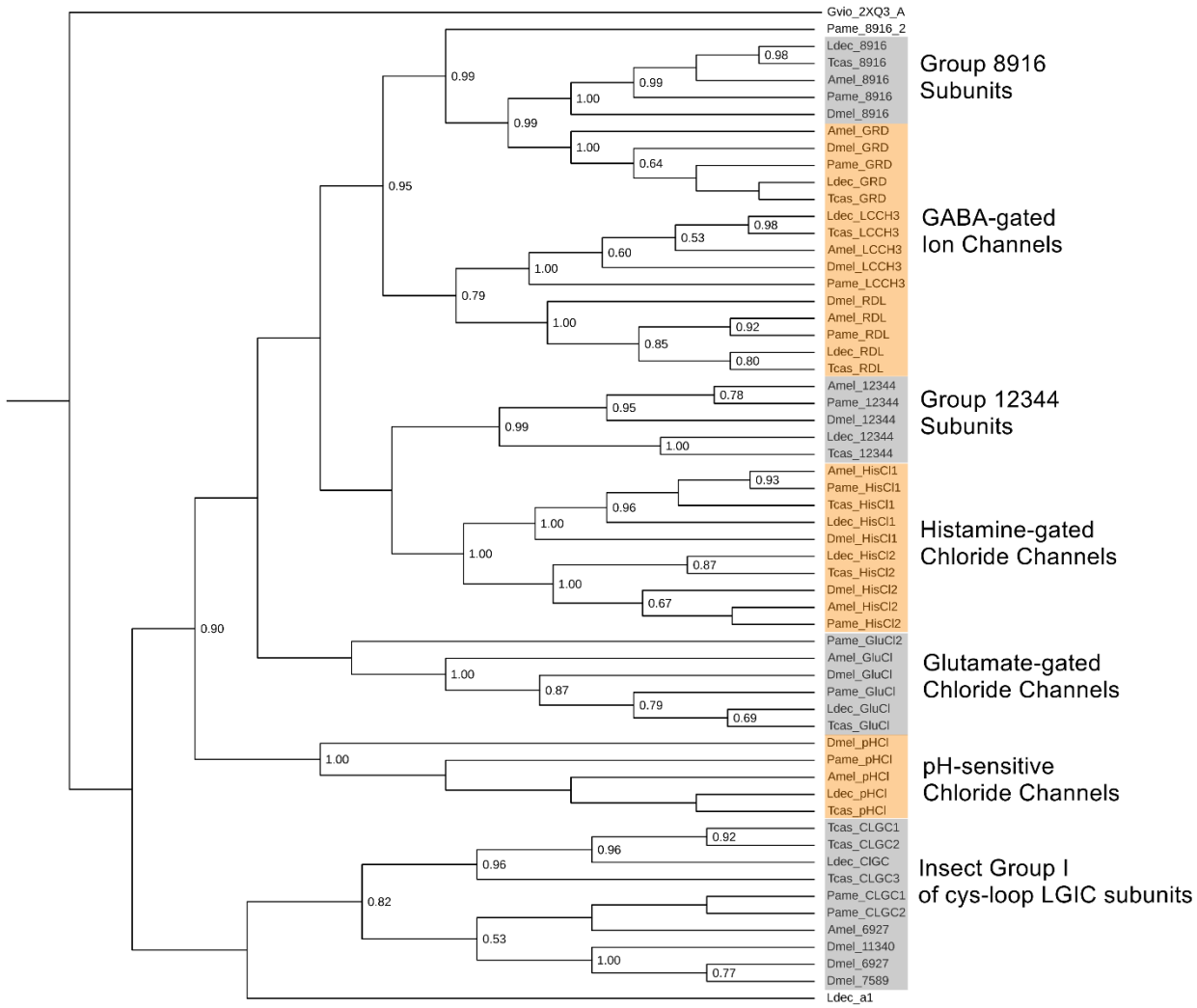


Figure 2-7 Phylogeny of non-nAChR cysLGIC subunits in five species

Gvio_2XQ3_A was an outgroup, and the tree was rooted in it. Ldec_α1 was also included as a reference. Bootstrapping values higher than 0.5 are displayed. Accession numbers of sequence used for phylogeny can refer to Appendix B.

2.4.6 Other CPB cysLGIC subunits

In CPB, only one gene belonging to Insect Group 1 cysLGICs was identified. Its position in the phylogeny reflects its relative similarity with three *T. castaneum* CLGCs (Figure 2-7). Therefore, it was named Ldec_CLGC. Insect Group 1 subunit homologs form an independent group in the phylogeny (Figure 2-7).

Orthologs of *Drosophila* CG8916 and CG12344 were identified in the CPB genome and named Ldec_8916 and Ldec_12344, respectively. The Ldec_12344 displays a PAR motif before TM2 (Figure 2-6), indicating its potential role as an anion channel. The phylogeny shows that the Ldec_12344 and its orthologs grouped with HisCl subunits, while the 8916 group clustered with GABA-gated ion channel subunits (Figure 2-7).

2.5 Discussion

I characterized the CPB cys-loop LGIC gene superfamily, which encodes for receptors that play major roles in the nervous system and are also targets of widely used insecticides. CPB has a similar number (22) of receptor subunit genes as other sequenced insects with greatest similarity to another beetle, *Tribolium*. Mechanisms increasing subunit diversity, such as alternative splicing and RNA A-to-I editing, are observed in nAChR α4, nAChR α6, Rdl, and GluCl subunits.

2.5.1 Ldec_α4, Ldec_α9 subunits of nAChR and Ldec_pHCl have unique features

Compared to other subunits, the Ldec_α9 subunit has low similarity with its *T. castaneum* counterpart, though *T. castaneum* remains the most similar of those included in the phylogram (Table 2-3). Ldec_α9 also lacks the GEK motif preceding TM2, which is usually conserved in nAChR subunits. To test whether the Ldec_α9 is properly categorized as a nAChR subunit I calculated the protein sequence identity between Ldec_α9 and all other CPB cysLGIC subunits. I observed that Ldec_α9 shares 9% to 14% identity with other CPB non-nAChR subunits, while its identity with other CPB nAChR can reach 25%. Further, the relative similarity to the *Tribolium* α9 in Figure 2-3, indicates that Ldec_α9 is more likely a nAChR subunit than another non-nAChR cysLGIC subunit. Whether Ldec_α9 possesses the cation selectivity of other GEK-motif-containing nAChR subunits remains to be determined.

Of the nAChR subunits, α4 is the only one whose complete expression was not confirmed by either transcripts or cDNA. Figure 2-8 displays an alignment of all potential CPB nAChR α4 protein sequences deduced from different sources, including three transcripts obtained from a pest population in Wisconsin (GEEF01180990.1, GEEF01026894.1, and GEEF01038441.1), one cDNA from Xinjiang, China (KP090398.1) and the gDNA sequence (CM045695.1, also from Xinjiang) used in this study, in addition to the *T. castaneum* nAChR α4 for reference. According to Tcas_α4 and the Ldec_α4 gDNA deduced sequence, a typical beetle nAChR α4 protein is expected to be over 550 amino acids; however, the longest transcript-deduced CPB protein (GGNV01187321.1_deduced) possesses only 182 amino acids. The cDNA-deduced sequence is slightly longer but still missing almost all N-terminal regions. This cDNA sequence was obtained in a previous study by mRNA RT-PCR, followed by a 5'- and 3'- Rapid amplification of cDNA ends (RACE) (Li *et al.* 2014). Because of this, the short nAChR α4 cDNA may not be due to incomplete sequencing coverage or mis-amplification. Additional evidence of truncation of the nAChR α4 transcripts is offered by the depth of coverage analysis. Appendix G and figure 2-9a show the mean depth of coverage of RNA reads across the investigated exon regions. For nAChR α4, the mean depth of coverage for exons 6–11, encoding the membrane-spanning region, is at least twice that of exons 1–4, encoding for the N-terminal extracellular region. Notably, exon 1 and exon 3 exhibit few mapped reads, with an average coverage below 1×, and no reads were mapped to exon 2 across all RNA-seq datasets. It is surprising since for each analysis, I used pooled RNA-seq data from multiple samples from a population (Dataset 1-3), or even pooled data from multiple populations (Dataset 4), to map the CPB genome. Excluding exon 2, the ratio of the highest depth of coverage (exon 11) to the lowest (exon 1) is 27.15. This extremely low expression of exons encoding for the N-terminal extracellular region may explain

the inability to assemble a complete transcript. Furthermore, the boxplot (Figure 2-10a) indicates that the interquartile range for exons 1–4 does not overlap with that of exons 6–11.

For nAChR α1, more reads mapped to exons encoding the membrane spanning region (exon 6-8) than the N-terminal coding exons (exon 1-4), the ratio of the highest (exon 8) to the lowest mapped exon (exon 1) was only 2.62. Additionally, the boxplot did not reveal a distinct difference in depth of coverage across exons, as the interquartile ranges overlapped well.

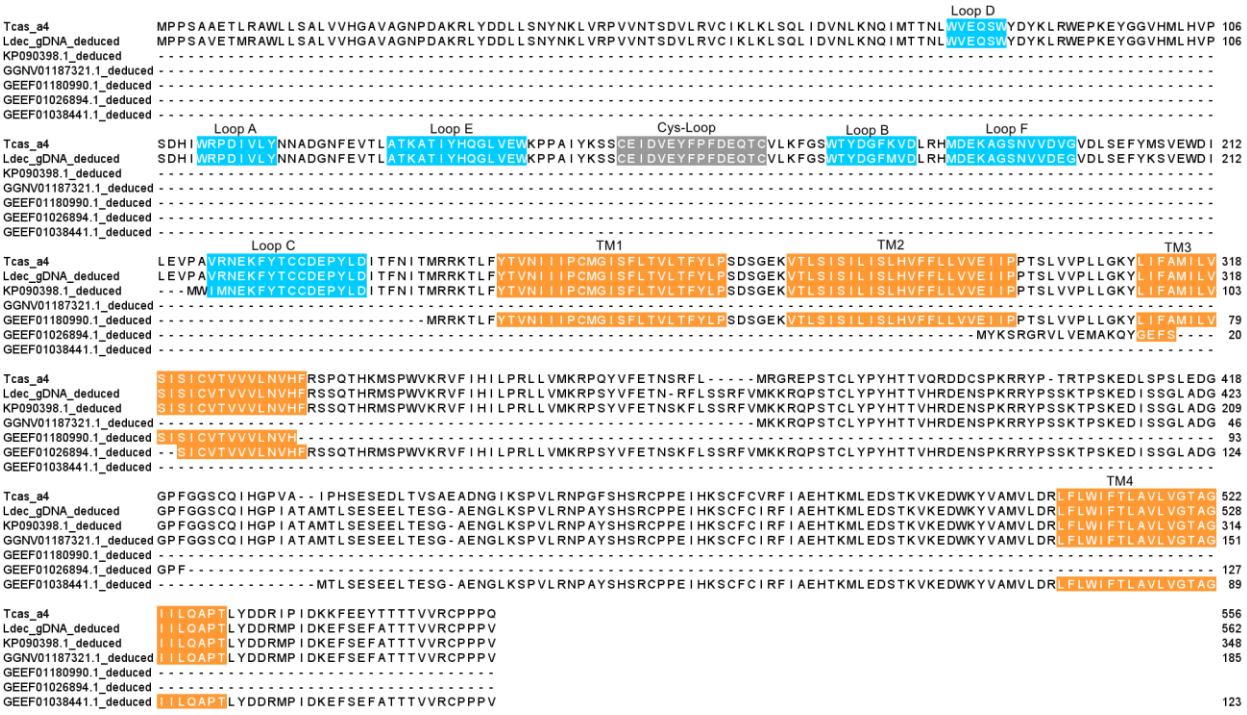


Figure 2-8 Protein sequence alignment of all potential CPB nAChR α4 isoforms

T. castaneum nAChR α4 (NCBI accession NP_001103246.1) is included for reference. Respectively, GGNV01187321.1, GEEF01180990.1, GEEF01026894.1, and GEEF01038441.1 are transcript sequences, KP090398.1 is a cDNA sequence.

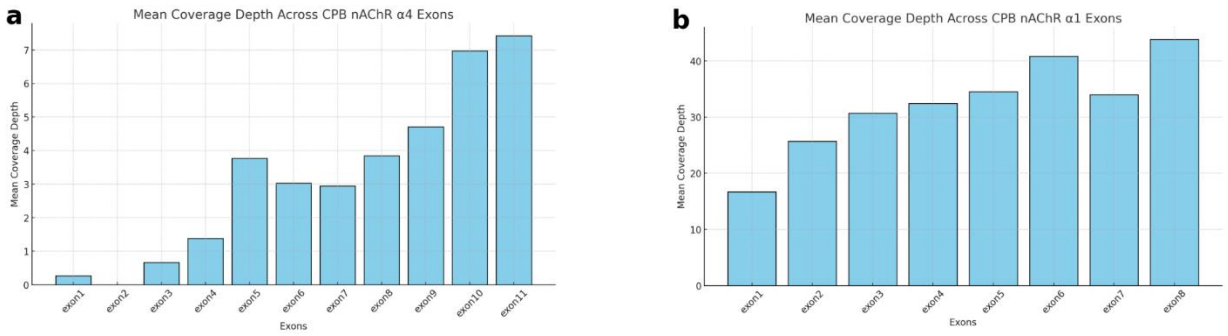


Figure 2-9 Exon-specific mean depth of coverage

a. the depth of coverage of nAChR α4 exons; b. the depth of coverage of nAChR α1 exons.

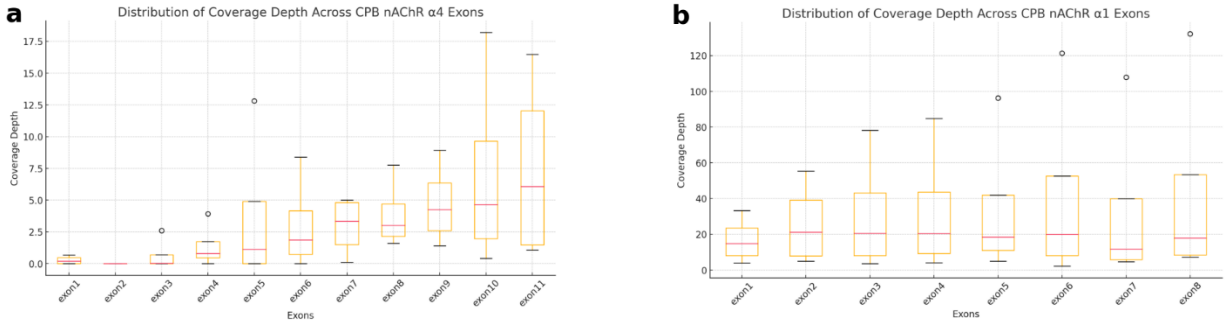


Figure 2-10 Distribution of coverage depth across investigated exons

a. nAChR α4; b. nAChR α1.

These much shorter protein sequences deduced from transcripts and cDNA may indicate that CPB nAChR α4 are naturally truncated and since the cDNA and transcripts are from five different CPB populations, this truncated version of CPB nAChR α4 may be geographically widespread. Truncation of nAChR is also seen in other insect species, such as the truncated α4 and α5 transcripts found in *D. melanogaster* and *B. mori* (Shao *et al.* 2007, Knipple and Soderlund 2010). However, these truncated transcripts lead to a partial loss of transmembrane regions or ligand binding loops, rather than a nearly complete loss of N-terminal extracellular region seen in CPB nAChR α4.

Whether this nAChR α4 truncation alters CPB's natural biological processes and insecticide interactions remains to be determined as does the geographic and phylogenetic range

of this truncation. Importantly, knocking out the nAChR α4 in a *D. melanogaster* strain led to a 1.491, 1.262, and 1.343-fold resistance ratios to sulfoxaflor, imidacloprid, and nitenpyram, respectively (Perry *et al.* 2021). It is possible therefore that this structural change is in response to the exposure of these diverse populations of CPB to neonicotinoid insecticides. Analysis of the roles of truncated nACh4 α4 subunits in the structure and function of nACh4 receptors would clearly be of value.

For non-nAChR cysLGIC subunits, the expressions of Ldec_Rdl and Ldec_8916 were not validated by transcripts or database cDNA sequence. A previous study sought to amplify the CPB Rdl mRNA using PCR (Glueck 1998) while only a fragmented product was obtained. The complete expression of these two subunits in the CPB population still needs to be validated.

I show preliminary evidence of the first *Ldec_pHCl* gene duplication observed in an insect species. This observation deserves additional validation because insect pHCl have potential as insecticide targets due to its exclusive occurrence in invertebrates (Schnizler *et al.* 2005).

2.5.2 Chromosome level genome assembly indicates more genomic features

Improved CPB genome assemblies contributed here to revealing genomic features which, because genomic architecture has implications for adaptation to insecticide exposure, may provide insight into insecticide resistance (Yan *et al.* 2023). To date, all known field-evolved neonicotinoid resistance-conferring mutations (not identified in CPB yet) are found on the β1 subunit, among which the R81T mutation is the most widely reported, detected in multiple *M. persicae* and *A. gossypii* populations worldwide (Hirata *et al.* 2015, Mottet *et al.* 2016, Toda *et al.* 2017, Munkhbayar *et al.* 2021, Xu *et al.* 2022). However, no homozygous 81TT individuals have yet been reported in any field population (Xu *et al.* 2022), possibly due to fitness costs associated with the 81T genotype (Homem *et al.* 2020). Colorado potato beetle is an XX-XO

sex-determination system (Hawthorne 2001), of which the male individuals have a single X chromosome. Therefore, alleles present on the X chromosome of males would be functionally dominant for both resistance and fitness traits. If there were significant fitness costs of that allele, perhaps due to pleiotropic effects on neuronal function, this resistance mutation may not persist in CPB populations because of its deleterious effects on all male carriers of the resistance alleles.

Second, the improved completeness and contiguity of this newer genome assembly revealed the close physical proximity of some nAChR subunit genes. This may facilitate their coordinated expression and co-assembly into the native channel. For example, *D. melanogaster* nAChR α1, α2, and β2 subunit genes are clustered within 200kb of each other on chromosome 3; these three subunits were later shown to form robust functional nAChRs in *X. laevis* oocytes. Here, the protein-coding regions of *Ldec_α7* and *Ldec_β1* were observed to cluster within 470kb on the chromosome CM045700.1 (Appendix E), suggesting that they are likely to co-assemble in native CPB nAChR.

Chapter 3 Characterizing the genetic basis of CPB resistance to insecticides

3.1 Abstract

Mutations can arise in insecticide target sites, disrupting chemical–target site interactions, conferring resistance. Well-characterized target-site mutations include those in the voltage-sensitive sodium channel gene (*vssc*), which confer resistance to DDT and pyrethroids; mutations in the acetylcholinesterase (*ace*) gene, conferring resistance to carbamates and organophosphates; mutations in the GABA receptor subunit gene (*Rdl*), conferring resistance to dieldrin and fipronils; and mutations in nicotinic acetylcholine receptor (nAChR) subunits, associated with resistance to neonicotinoids and Spinosad. Mutations can also occur in detoxification enzymes, altering their catalytic activity and contributing to insecticide resistance.

As a globally significant super-pest, the Colorado potato beetle (CPB) has evolved target-site mutations in *vssc*, *ace-2*, and *Rdl* genes. However, mutations associated with neonicotinoid resistance in CPB remain unidentified. As insecticides representing multiple modes of action continue to be widely applied in U.S. potato fields, understanding the current status of resistance-associated mutations, as well as identifying novel variants, is essential for guiding resistance monitoring and pest management strategies.

In this study, I investigated known target-site mutations in both pest and non-pest CPB populations across the U.S. Presence of frequencies of resistance allele varies across pest CPB populations, and notably, non-pest samples also tested positive for resistance-associated mutations on *Rdl* and *ace-2* genes. I further examined nucleotide diversity and genetic variation in previously identified neonicotinoid resistance candidate genes. Pest populations showed population-specific patterns in these candidate genes, with several populations consistently

identified as outliers. In addition, I discovered potential resistance-associated alleles whose functional effects can be investigated in the future.

3.2 Introduction

The genetic basis of insecticide resistance has historically been a complex issue. Debates and controversies can be traced back as early as 1957, centering on discrepancies between monogenic and polygenic observations of *Drosophila* resistance to DDT (Dapkus 1992, Dapkus and Merrell 1997). Other key questions that have been widely discussed include but are not limited to the initial frequency of resistance-associated mutations in pest populations; the fitness costs associated with resistance; the number of mutations occurring within a particular resistance gene; the number of times a given mutation has independently evolved; and the diversity of mechanisms contributing to resistance.

Theory suggests that the number and effect size of resistance-conferring mutations depend on the available genetic variation within a pest population, fitness cost of mutations, and the strength of selection imposed by pesticides (Mckenzie 2000). Selection pressures acting within the phenotypic distribution of a susceptible population tend to favor the joint selection of pre-existing resistance factors with small sizes effect, resulting in polygenic resistance, while selection pressures outside of this distribution typically favor rare mutations in single genes with major effects (Ffrench-Constant *et al.* 2004).

Target-site mutations leading to resistance often have large effect sizes, and their critical role in resistance has been extensively reviewed in pest species (Scott 2017, Auteri *et al.* 2018). Examples including mutations in the voltage-sensitive sodium channel (vssc) conferring resistance to pyrethroids and DDT (Dong *et al.* 2014), mutations in the GABA receptor

conferring resistance to cyclodienes and fipronil (Feyereisen *et al.* 2015), and mutations in the acetylcholinesterase associated with resistance to organophosphates and carbamates (Lee *et al.* 2015). It was not until 2011, 16 years after the initial commercial use of that insecticide, that the first field-evolved mutation in the nicotinic acetylcholine receptor (nAChR) β1 subunit (R81T), conferring resistance to neonicotinoids, was discovered in *Myzus persicae* (Bass *et al.* 2011).

According to the theory of selection outside the phenotypic distribution, mutations with large effect sizes are initially rare within pest populations before the application of selection pressure. These mutations may arise *de novo* after insecticide application or may exist as low-frequency polymorphisms within the standing genetic variation of the population. However, the frequency of such mutations depends on many factors including the relative fitness benefits or costs of that mutation in the absence of insecticide.

To investigate this issue, the ideal experimental design would involve genotyping historical samples collected prior to the first application of an insecticide. One study employed PCR to examine the esterase-3 (E3) gene from pinned blowflies collected before the introduction of organophosphorus insecticides (OPs) in the 1950s and successfully detected a point mutation associated with malathion resistance (Hartley *et al.* 2006). This finding suggests that resistance-associated mutations can predate the introduction of their corresponding insecticide, potentially serving other biological functions before being co-opted for resistance.

An accompanying assumption of the *de novo* mutation model is that resistance mutations typically carry a fitness cost, and thus, the frequency of resistance alleles will decline when either a different compound (with a different mode of action (MoA)) or no insecticide is used (Ffrench-Constant and Bass 2017). This concept forms the theoretical foundation of current

resistance management strategies, such as those advocated by the insecticide resistance action committee (IRAC), which emphasize rotation based on the MoA.

Experimental evidence supports this assumption. For example, the competitive fitness of *Aphis gossypii* was negatively associated with the initial resistant allele frequency (RAF) of the target-site mutation S431F in ACE1 under laboratory cage conditions (Tieu *et al.* 2017). Similarly, target-site resistance mutations (on *vssc* and *Rdl*) were found to severely impair male mating competitiveness in *Anopheles gambiae* (Platt *et al.* 2015). In *Drosophila melanogaster*, females homozygous for the R81T substitution produced fewer fertile eggs and exhibited impaired mobility at both larval and adult stages, while both homozygous and heterozygous males showed a reduced median lifespan (Homem *et al.* 2020). However, it is important to note that these studies were all conducted under laboratory conditions. Given that resistance is typically defined by repeated control failures in the field, there is an argument that fitness costs must also be validated under field conditions (Ffrench-Constant and Bass 2017). Moreover, the assumption that resistance necessarily entails a fitness cost does not always hold. The story of blowflies suggests that some resistance-associated mutations may not carry a fitness penalty in the absence of insecticide pressure. Supporting this hypothesis, certain resistance alleles, such as a single allele of a single cytochrome P450 gene, *Cyp6g1*, carrying an *Accord* transposon in its 5′ end and conferring DDT resistance, have even been associated with a fitness advantage rather than a cost in pesticide-free environments (McCart *et al.* 2005). Under certain circumstances, target-site mutations can confer a fitness advantage. The S291G mutation in acetylcholinesterase (AChE) may provide such an advantage on potato varieties with high levels of α-chaconine. Potatoes and other solanaceous plants naturally contain high concentrations of glycoalkaloids, such as α-chaconine and α-solanine, which are potent AChE inhibitors. Studies have shown that

an azinphosmethyl-resistant strain (AZ-R)—in which the S291G mutation was later detected—was 1.7- to 1.8-fold more sensitive to α-solanine, but 1.3-fold less sensitive to α-chaconine inhibition, compared to the susceptible strain (SS) (Zhu and Clark 1995). As a result, the AZ-R strain carrying the AChE mutation was hypothesized to have a selective advantage on potato varieties with high α-chaconine concentrations. This hypothesis was supported by later studies, which found that the AZ-R strain exhibited a higher intrinsic rate of increase on such potato plants, but a reduced rate when α-chaconine levels were low (Zhu *et al.* 1996).

Resistance-associated mutations can arise among different nucleotide positions within the same resistance gene. Given the large effective size of insect populations, it is expected that equivalent mutations will arise repeatedly (Ffrench-Constant 2013). Mutations in the voltage-sensitive sodium channel (*vssc*) gene provide an excellent example of these multi-locus and multi-origin observations. To date, more than 30 unique resistance-associated mutations or combinations of mutations in *vssc* have been detected across multiple insect species, with the single L1014F mutation reported in over 20 species (Rinkevich *et al.* 2013) and multiple times independently in the CPB (Rinkevich *et al.* 2012b). Similarly, since the first discovery of the R81T mutation in the nAChR β1 subunit in 2011in the aphid *Myzus persicae* (Bass *et al.* 2011), additional mutations and combinations of mutations have been identified in the β1 subunit in various pest species (Hirata *et al.* 2015, Xu *et al.* 2022, Yin *et al.* 2024).

Another important question concerns how many times the same mutation has independently arisen across different populations. If the same resistance mutation evolved independently multiple times, one would expect to detect the susceptible progenitor allele for each resistance allele, potentially retaining similar flanking sequences (Ffrench-Constant 2013). This can be assessed by investigating the resistance haplotypes across different populations.

Supporting this, Rinkevich *et al.* (2012b) provided evidence for multiple independent origins of kdr (L1014F), super-kdr (M918T+L1014F), and kdr-his (L1014H) mutations in House Fly, *Musca domestica* (Rinkevich *et al.* 2012a). Similarly, the kdr mutation (L1014F) in the Colorado potato beetle (CPB) has also been shown to have multiple independent origins (Rinkevich *et al.* 2012b).

Pest species may evolve multiple resistance mechanisms to a single insecticide, a phenomenon well illustrated by neonicotinoid resistance in the whitefly, *Bemisia tabaci*. Enhanced detoxification has been characterized as a primary mechanism of resistance to neonicotinoids in *B. tabaci*, with overexpression of the cytochrome P450 gene, *CYP6CM1*, most frequently implicated (Karunker *et al.* 2008, 2009). Subsequently, a single amino acid substitution, A387G, located within a predicted P450 substrate recognition site, was identified and shown to enhance the ability of CYP6CM1 to metabolize neonicotinoids (Pym *et al.* 2023). More recently, dual mutations (A58T and R79E) in the nAChR β1 subunit were found to confer target-site insensitivity to neonicotinoids in a whitefly population from Urumqi, Xinjiang (Yin *et al.* 2024).

The Colorado potato beetle was first detected in the United States in 1811, feeding on *Solanum rostratum* (Casagrande 1985). It was not recognized as a pest until a major outbreak on cultivated potatoes (*Solanum tuberosum*) occurred in 1859 (Casagrande 1987). Following this outbreak, CPB rapidly expanded eastward, reaching potato-growing regions along the East Atlantic coast within 15 years, and spread to the western United States and Canada by the 1920s. By the 1950s, CPB had reached Europe and Central Asia, advancing through Eastern Europe, Russia, and Kazakhstan, and eventually arriving in China in the 1990s (Wang *et al.* 2020).

Target-site insensitivity has played an important role in insecticide resistance in multiple CPB populations. Two mutations in the *ace-2* gene—S291G and R30K—are associated with resistance to carbamates (CBs) and organophosphates (OPs) (Zhu *et al.*, 1996; Zhu & Clark, 1997; Kim *et al.*, 2007). The L1014F-equivalent mutation in the voltage-gated sodium channel (vssc) has been characterized as a major mechanism of pyrethroid resistance across several CPB populations (Lee *et al.* 1999, Kim *et al.* 2004). Later, two additional mutations, T929I and T929N, were found in Bulgarian CPB populations but have not been detected in U.S. CPB populations (Rinkevich *et al.* 2012b). The T929I mutation has also been associated with pyrethroid resistance in other insect species, including onion thrips (Toda and Morishita 2009), diamondback moths (Schuler *et al.* 1998), maize weevils (Lee *et al.* 2000), head lice (Lee *et al.* 2000), house flies (Sun *et al.* 2017), and Bagnall thrips (Gao *et al.* 2024). T929I alone can decrease sensitivity to multiple pyrethroid compounds and DDT (Usherwood *et al.* 2007, SupYoon *et al.* 2008, Toda and Morishita 2009). Moreover, the L1014F + T929I double mutation in diamondback moths confers super-kdr-like resistance, resulting in the highest resistance levels among tested genotypes (Schuler *et al.* 1998). Additionally, an alanine (A) to serine (S) substitution in the Rdl subunit has been reported to confer resistance to endosulfan in an Iranian CPB population (unpublished).

Table 3-1 Ranking of insecticide use in fall potato field

This survey was conducted in nine states that together accounted for over 92% of the 901,000 acres planted to potatoes in the United States in 2022: Colorado, Idaho, Maine, Michigan, Minnesota, North Dakota, Oregon, Washington, and Wisconsin. Insecticides are ranked from highest to lowest according to the percentages of acreage applied. United States Department of Agriculture, 2022

Insecticide	**Groups**	**Fall-planted potato acreage treated (%)**
Oxamyl	Carbamate	26
Lambda-cyhalothrin	Pyrethroid	24
Abamectin	Avermectin	22
Imidacloprid	Neonicotinoid	22
Thiamethoxam	Neonicotinoid	20
Chlorantraniliprole	Diamide	10
Fipronil	Phenylpyrazole	10
Spirotetramat	Tetramic Acid	10
Clothianidin	Neonicotinoid	9
Bifenthrin	Pyrethroid	8
Beta-cyfluthrin	Pyrethroid	7
Esfenvalerate	Pyrethroid	7
Methomyl	Carbamate	7
Sulfoxaflor	Sulfoximine	7
Cyantraniliprole	Diamide	5
Cyfluthrin	Pyrethroid	5
Spinetoram	Spinosyn	4
Novaluron	IGR (Benzoylurea)	3
Permethrin	Pyrethroid	3
Flonicamid	Flonicamid group	2
Piperonyl butoxide	Synergist	2
Spinosad	Spinosyn	2
Tolfenpyrad	Pyrazole	2
Pymetrozine	Pymetrozine group	1
Zeta-cypermethrin	Pyrethroid	1

I analyzed insecticide application data from the USDA's 2022 Fall Potato Field Survey (Table 3-1) and found that traditional insecticide groups—such as carbamates, pyrethroids, and fiproles—remain widely used. Based on this observation, I propose to conduct an allele frequency survey of all known CPB target-site mutations across various CPB populations and groups, for the following reasons. First, earlier studies examining U.S. CPB populations collected around 1998 did not detect the presence of super-kdr mutations. Given the continued

use of pyrethroids (Table 3-1) and the known independent origins of resistance-associated mutations, I hypothesize that super-kdr-like mutations may be detectable in more recent U.S. CPB samples. Second, although unpublished data have suggested the presence of Rdl mutations, none have yet been reported in U.S. CPB populations. I aim to assess both the presence and frequency of these mutations. Given the sustained use of carbamates and fiproles, I anticipate the positive detection of resistance alleles associated with these insecticide groups. In addition, I plan to include non-pest CPB samples in my analysis to evaluate the frequency of resistance alleles in populations that are not under strong insecticide selection pressure. Based on the assumption that resistance alleles are associated with fitness costs, I hypothesize that these alleles will be absent or present at very low frequencies in non-pest populations.

I also plan to screen the nAChR subunits of CPB, to screen for resistance associated mutations known in other species not previously identified in CPB before (Table 3-2). I didn't see such an effort in previous studies, potentially due to a negative detection result or due to the poor annotation of the nAChR gene family in CPB. Thorough characterization of CPB nAChR family (Chapter 2) allows this screen for functionally significant mutations to be performed here. I have specific interests in finding mutations on the ligand binding motifs encoding region of nAChR genes. Based on current knowledge of ligand-nAChR interactions, the neonicotinoid binding site is located at the interface of a β subunit and a α subunit (Matsuda *et al.* 2020), with the binding pocket formed by Loop D, E, and F of the β subunit and Loop A, B, and C of the α subunit (Corringer *et al.* 2000). Missense mutations in these functional loops are more likely to alter ligand binding sensitivity, thereby contributing to resistance. This hypothesis is strongly supported by the loop specific location of all field-evolved mutations in nAChR β1 and lab-

selected mutations on nAChR α1 and nAChR α3 that conferring resistance to neonicotinoids (Table 3-2), with only one exception of the V101I mutation between Loop D and Loop A.

Table 3-2 Insecticide resistance-associated mutations on nAChRs in other pest species

Subunits	Mutations & Location	Species	Affected insecticide Group	Reference
nAChR β1	R81T/Loop D	*Myzus persicae*	Neonicotinoids	(Mottet *et al.* 2016, Xu *et al.* 2022)
		Aphis gossypii		(Hirata *et al.* 2015, Munkhbayar *et al.* 2021)
	V101I/Between Loop D and Loop A	*Myzus persicae*		(Xu *et al.* 2022)
	L80S/ Loop D	*Aphis gossypii*		(Kim *et al.* 2015)
	A58T/Loop G & R79E/Loop D	*Bemisia tabaci*		(Yin *et al.* 2024)
nAChR α1 & α3	Y151S/Loop B	*N. lugens*		(Liu *et al.* 2006)
nAChR α6	G275E	*Frankliniella occidentalis*	Spinosad	(Puinean *et al.* 2013)

While CPB resistance to neonicotinoids is predominantly linked to metabolic alterations, relatively little is known about metabolic or target site mutations contributing to this resistance. Transcriptomic analyses have identified sets of metabolic enzymes that are either constitutively overexpressed (Clements *et al.* 2016, Zhu *et al.* 2016, Dively *et al.* 2020) or induced upon exposure (Zhu *et al.* 2016, Clements *et al.* 2018) in imidacloprid-resistant populations. Additionally, the transcription factor Cap-n-Collar isoform C (CncC) has been shown to play a key role in regulating gene networks involved in metabolic resistance (Kalsi and Palli 2017, Gaddelapati *et al.* 2018). However, the profile of overexpressed metabolic genes varies regionally (Dively *et al.* 2020, Pélissié *et al.* 2022), suggesting a potential contribution of *cis*-regulatory factors. Neonicotinoid resistance-conferring mutations can involve (1) non-synonymous single nucleotide polymorphisms (SNPs) and indels in pesticide target genes, (2) SNPs and indels that affect gene regulation, and (3) indels leading to gene copy number

variation. In this study, I focused on investigating the first type—non-synonymous mutations—in candidate resistance genes of CPB.

I first calculated nucleotide diversity (π) across CPB populations/groups in the open reading frames (ORFs) of selected resistance candidate genes. Theoretical models predict that when a beneficial mutation is selected under insecticide pressure in a protein-coding gene, neutral or even slightly deleterious allele increases in frequency because it is physically linked to a beneficial allele under positive selection, known as hitchhiking (Kaplan *et al.* 1989). When selection is strong and acts on a rare or de novo mutation, it can drive the rapid fixation of a single haplotype, resulting in a characteristic reduction in nucleotide diversity in the surrounding genomic region — a pattern referred to as a hard selective sweep. Thus, I hypothesized that certain resistance genes would exhibit reduced nucleotide diversity, providing preliminary evidence for positive selection. Additionally, by comparing geographically proximate resistant and susceptible populations, I aimed to identify specific genetic variations associated with resistance. However, the nucleotide diversity evaluation method has limited power to detect soft seletive sweep from standing genetic varations, where nucleotide diversity could be retained (Messer and Petrov 2013).Whole-genome resequencing (WGS) data across U.S. CPB populations provide an excellent opportunity to achieve these objectives. Compared to PCR-based approaches, which typically target specific loci or genes, WGS offers high-throughput capabilities for simultaneously monitoring changes at multiple genomic loci and identifying novel resistance-associated variations outside of previously targeted regions. This approach has been successfully applied to resistance monitoring in *Anopheles gambiae* mosquitoes (Kientega *et al.* 2024) and the fall armyworm (*Spodoptera frugiperda*) (Guan *et al.* 2021).

3.3 Materials and Methods

3.3.1 Genes of interest and ORF annotation on genome assembly

I included a total of 30 genes in this study, three genes (*ace-2*, *Rdl*, and *vssc*) with known target site mutations (Table 3-3) and 27 candidate neonicotinoid resistance genes, consisting of 14 encoding metabolic enzymes (eight cytochrome P450 (CYPs), three glutathione S-transferase (GSTs), two ABC transporters (ABCTs), and one UDP-glucuronosyltransferase (UGT)), one encoding a trans-regulatory factor, one for cuticle proteins, and 11 nAChR subunits, some of which comprise the putative target site of these insecticides (Table 3-4). For both Table 3-3 and Table 3-4, a notes column was manually annotated to clarify gene descriptions, as the information in the NCBI database can be confusing. Additionally, for Table 3-4, a reference column was added to indicate the studies providing evidence for each gene's association with neonicotinoid resistance. Although not all nAChR subunits had previously documented associations with neonicotinoid resistance, they were still included due to their potential role as target sites for neonicotinoid compounds.

Table 3-3 Known target-site mutations analyzed

Gene ID	Gene Description	Notes	Query Sequence Accession
LOC111502360	Resistant to dieldrin	*Rdl*	XP_023012203.1
LOC111511286	sodium voltage-gated channel paralytic	*vssc*	XP_023023069.1
LOC111502056	acetylcholinesterase-like	*ace-2*	AAB00466.1

Protein sequences or transcripts of these genes were collected as queries (See table 3-3 and 3-4 for NCBI accession numbers), and mapped to the chromosome-level genome assembly (see chapter 2) using Exonerate 2.4.0 (Slater and Birney 2005) with the -showtargetgff command. This generated an annotation file of genes in GFF3 format, which will be used for downstream analysis such as depth coverage calculation, regions for variant calling and mutation type interpretation.

3.3.2 CPB samples

Genomic DNA sequencing reads of 74 CPB samples (Illumina short reads) were downloaded from the NCBI SRA database (Appendix H). Samples include 17 non-pest CPBs collected from a native host plant, Buffalo burr (*Solanum rostratum*) in Mexico, Arizona and the US plains area (Kansas, Colorado, Texas, Missouri, Nebraska and New Mexico), which were presumed not to have been exposed to insecticide stress, and 52 pest samples collected from potato fields covering major potato production areas in the United States, and 5 New Jersey samples which is widely used as an insecticide-susceptible reference population that has been in colony for >20 years, perhaps as early as 1983. Pest samples were further categorized as resistant (n=30) or susceptible (n=22) to imidacloprid according to bioassays, and the 5-lab maintained beetle was also susceptible to imidacloprid (Appendix H). Overall, resistant beetles were active after exposure to over 500 ppm imidacloprid, while susceptible beetles were moribund or dead following exposure to approximately 50 ppm imidacloprid.

3.3.3 Read filtering, genome alignment, and variant calling

Illumina sequencing reads were downloaded from SRA and adapters were removed by BBMap version 39.06 (sourceforge.net/projects/bbmap). BBMap was also used to trim and filter

low quality reads. The processed paired-end reads were aligned against the chromosome using the BWA-MEM algorithm (Li 2013) with default settings. The SAMtools version 1.13 (Li *et al.* 2009) was used to compress, sort, and index alignment files. Average depth of coverage for each sample across analyzed genes was calculated using the SAMtools --depth command and custom scripts. The bcftools were used to call both invariant and variant sites across all 74 CPB samples in analyzed genomic regions. The output will be referred as “raw variant calling file (VCF)” here and after. Invariant sites were included as they are required for downstream nucleotide diversity calculation. Specifically filtering steps were applied to the raw VCF for specific analysis purposes and will be discussed later.

3.3.4 Resistant allele survey and nAChR mutation detection

The raw VCF was first filtered to retain only biallelic sites. Variant sites were then further filtered to include only those with less than 50% missing data, a minor allele count greater than 2, and a variant calling quality score greater than 30. After evaluating different genotype quality (GQ) filtering thresholds, I selected a threshold of GQ > 10 to balance maintaining confident genotype calls while minimizing the loss of informative mutations. Mutation effects were annotated using SnpEff (Cingolani *et al.* 2012), producing a VCF file with all mutations annotated. This file is hereafter referred to as the "Annotated Dataset." Based on the annotated file, focusing on the genomic positions of known target-site mutations, I manually calculated allele frequencies across surveyed populations. Additionally, I collected information on any non-synonymous mutations detected in CPB nAChR subunits to investigate the presence of neonicotinoid-resistance-associated mutations that have been reported in other pest species but not previously observed in CPB (Table 3-2).

3.3.5 Nucleotide diversity calculations

The raw VCF file was filtered to retain both invariant and variant sites with more than 80% genotype calling, a quality score above 30, and a mean depth of coverage greater than 2. Nucleotide diversity per gene for designated CPB groups was calculated using pixy (Korunes and Samuk 2021). For gene families comprising multiple members, such as the nAChR and CYP families, I calculated the mean nucleotide diversity across all included genes within each CPB population or group, along with the standard deviation (SD) and standard error (SE).

To visualize nucleotide diversity at the individual gene level, I generated boxplots to examine distribution patterns (Grubbs 1969, Nuzzo 2016). Since my analysis focused on identifying pest populations that are outliers relative to their counterparts, I excluded non-pest samples (e.g., Mexico, Arizona, and the Plains) and the lab-selected strain (that is, the New Jersey samples) from this comparison.

3.3.6 Mutation type interpretation and association test

In this analysis, I compared imidacloprid-resistant and susceptible beetles both within geographically proximate population pairs and among pooled resistant and susceptible groups. For the NY-R population, I selected the MD-S population as its counterpart, as the NJ-S beetles are highly inbred and could introduce bias. The --keep command in VCFtools was used to filter the raw VCF and retain only the desired sub-samples for each comparison.

Each sub-sampled VCF was further filtered to exclude sites that were no longer polymorphic (i.e., with an alternative allele frequency of 0 or 1). The filtered VCFs were then annotated using SnpEff to determine the mutation types. The annotated VCFs were subsequently processed in PLINK v1.90 (Purcell *et al.* 2007), where association testing was conducted

between phenotyped samples using Fisher's exact test to calculate p-values. I focused on identifying genetic variants—including SNPs, insertions, and deletions—that (1) resulted in non-synonymous changes, and (2) exhibited significant differences in allele frequency ($p < 0.05$) between resistant and susceptible groups.

3.4 Results

3.4.1 Resistance allele frequence across the US CPB populations

I investigated the presence of target-site insensitivity mutations previously reported in the Colorado potato beetle, including mutations on the voltage-sensitive sodium channel (*vssc*) gene, the *Rdl* gene, and the acetylcholinesterase (*ace-2*) gene (Table 3-5).

Among the samples analyzed I observed the A301S on the *Rdl* gene (conferring resistance to the cyclodiene insecticide dieldrin, the R30K and S291G on the *ace-2* gene, and the T929I and L1014F mutations in the *vssc* gene (Table 3-5). The T929I mutation was previously identified in CPB populations from Bulgaria (Rinkevich *et al.* 2012b), but this is the first record of it in the US CPB population. I identified the T929I mutation in two female beetles, one from a Michigan population (ID CPBWGS_52, Appendix H), and another from a New York population (ID CPBWGS_85). Both individuals were heterozygous at position 929, carrying the 929TI genotype. Notably, both also carried the L1014F mutation in a heterozygous state. Previous studies incorporating the T929I+L1014F combination into *Drosophila* sodium channels expressed in *Xenopus* oocytes demonstrated a 10,000-fold reduction in sensitivity to pyrethroids compared to wild-type channels (Vais *et al.* 2001). However, due to the unphased genotype calling, I am unable to determine whether these two mutations are on the same chromosome.

One surprising finding was the fixed 291G genotype in the Plains and Mexico+AZ samples, despite the assumption that these beetles had not been exposed to insecticide stress. In addition, the A301S mutation was also identified in a non-pest Colorado sample (CPBWGS_35).

Table 3-5 Genomic location of known target-site mutations and their allele frequency in CPB populations

Number in parenthesis indicates the number of genotypes called at the variant site. Populations were named uniquely by state abbreviation and field number, e.g., two populations collected from Oregon are named OR-1 and OR-2.

Target Site	Amino Acid Change	Chromosome ID	Position	Resistant Allele	Wild Allele	OR-1	OR-2	WI-1	WI-2	MI-1	MI-2	MD-1	MD-2	NJ	NY	VT	ME	Plains	MX+AZ
Rdl	A301S	CM045709.1	22294834	T	G	0 (2)	0 (8)	0.375 (8)	0.375 (8)	0.5 (4)	0.5 (4)	0 (6)	0.67 (6)	0 (2)	1 (2)	/	0.333 (6)	0.25 (4)	0 (6)
AChE	R30K	CM045707.1	16653571	A	G	0 (6)	0 (8)	0.125 (8)	0 (8)	0 (8)	0.25 (8)	0.125 (8)	0 (4)	0 (6)	0 (4)	0 (6)	0.33 (6)	0 (12)	0 (18)
	S291G		16654353	G	A	1 (4)	1 (6)	0.875 (8)	0.75 (4)	0.875 (8)	0.5 (4)	0.667 (6)	1 (4)	0 (2)	1 (8)	0.83 (6)	1 (8)	1 (8)	1 (14)
vssc	L1014F	CM045700.1	22977445	A	G	0 (2)	1 (6)	0.857 (7)	1 (3)	0.8 (5)	0.4 (5)	1 (2)	1 (5)	1 (5)	0.5 (2)	0.5 (2)	1 (3)	0 (5)	0 (5)
	T929I		22978314	T	C	0 (2)	0 (7)	0 (8)	0 (9)	0.17 (6)	0 (7)	0 (8)	0 (9)	0 (6)	0.1 (10)	0 (7)	0 (9)	0 (6)	0 (14)

3.4.2 Non-synonymous mutations on nAChRs

Across the 74 samples analyzed, I did not detect known insecticide resistance associated mutations on nAChR subunits. Instead, I compiled a comprehensive list of 33 non-synonymous mutations identified in CPB nAChR subunit genes (Table 3-6), including 32 SNPs and one deletion. Thirty SNPs can only lead to a single amino acid change, one SNPs is in the splice donor region, and one SNP has multiple potential effects, which is to potentially affect splicing or single amino acid substitution. The deletion is detected in the nAChR α5 subunit and was interpreted as a frameshift variant & splice region variant mutation. To further examine the effects of this deletion, I manually modified a nAChR α5 transcript (NCBI accession GGNV01170968) at the relevant site and inspected the products. This mutation only makes the sequence one amino acid longer, where the last amino acid T was replaced by SE.

I didn't find mutations on the ligand-binding loops, which may affect the affinity of insecticide with receptors. Interestingly, I detected five missense mutations within the transmembrane (TM) domain nAChR α10 subunit. Since nAChR TMs form ion channels, mutations in these regions could potentially affect ion permeability. In nAChR α9, I detected a C-to-T nucleotide substitution occurring two nucleotides downstream of the last nucleotide of exon 6 (the #760 nucleotide in the CDS). This mutation may disrupt normal splicing by altering splice-site recognition, potentially affecting gene function. Also, in nAChR α9, I found a mutation located in the first transmembrane domain.

Table 3-6 non-synonymous mutations identified on CPB nAChR subunits

Chromosome ID	Subunits	Position	R	ALT	Mutation Effects	SNPs	Amino Acid Change	Locations
CM045696.1	nAChR α2	3150711	G	C	missense_variant	1160C>G	Ser387Cys	between TM3 and TM4
		3170779	A	G	missense_variant	58T>C	Phe20Leu	Signal Peptide
CM045698.1	nAChR α5	63396375	CG	C	frameshift_variant&splice_region_variant	1442delC	Thr481fs	
		63406667	C	T	missense_variant	994G>A	Gly332Arg	Before Loop D
		63480998	G	T	missense_variant	107C>A	Ala36Glu	Signal Peptide
		63481049	A	G	missense_variant	56T>C	Val19Ala	Signal Peptide
		63549758	A	C	missense_variant	41T>G	Ile14Ser	Signal Peptide
		63549771	C	T	missense_variant	28G>A	Val10Ile	Signal Peptide
		63549783	G	A	missense_variant	16C>T	His6Tyr	Signal Peptide
CM045700.1	nAChR β1	49515627	T	C	missense_variant	13T>C	Phe5Leu	Signal Peptide
		49515630	A	G	missense_variant	16A>G	Ile6Val	Signal Peptide
		49571594	C	T	missense_variant	715C>T	Leu239Phe	In TM1
		49596917	C	T	missense_variant	1205C>T	Ser402Phe	between TM3 and TM4
	nAChR α6	61872103	T	C	missense_variant	29T>C	Leu10Ser	Signal Peptide
CM045702.1	nAChR α10	7759634	T	G	missense_variant	1294A>C	Ile432Leu	TM4
		7759709	G	A	missense_variant	1219C>T	Pro407Ser	TM4
		7760072	G	C	missense_variant	991C>G	Leu331Val	TM3
		7763398	G	C	missense_variant	866C>G	Thr289Arg	TM3
		7763462	T	G	missense_variant	802A>C	Ile268Leu	TM3
		7803075	C	G	missense_variant	54G>C	Gln18His	Signal Peptide
		7803112	A	T	missense_variant	17T>A	Ile6Lys	Signal Peptide
	nAChR α8	40937249	T	G	missense_variant	1316A>C	Glu439Ala	between TM3 and TM4
CM045703.1	nAChR α3	13858842	C	A	missense_variant	9C>A	Ser3Arg	Signal Peptide
		13968891	A	C	missense_variant	1394A>C	His465Pro	between TM3 and TM4
CM045705.1	nAChR α9	44824129	A	G	missense_variant	1226T>C	Leu409Ser	between TM3 and TM4
		44824178	C	A	missense_variant	1177G>T	Ala393Ser	between TM3 and TM4
		44824684	T	C	missense_variant	995A>G	Lys332Arg	between TM3 and TM4
		44828541	G	A	splice_donor_variant&intron_variant	760+2C>T		

		44833468	T	C	missense_variant	332A>G	Asn111Ser	Between LoopD and Loop A
		44833641	C	T	missense_variant&splice_region_variant	214G>A	Glu72Lys	Before Loop D
		44836289	T	A	missense_variant	178A>T	Ile60Phe	Before Loop D
		44843603	G	A	missense_variant	38C>T	Ser13Phe	Signal Peptide
		44843607	T	A	missense_variant	34A>T	Ile12Leu	Signal Peptide

3.4.3 Nucleotide diversity

I observed that some CYP genes are closely located on the genome and share the same annotation (Table 3-7), suggesting the possibility of duplicated gene copies. To investigate this, I compared the protein identity and similarity among these genes.

Table 3-7 Genomic locations of analyzed neonicotinoid resistance candidate genes

Gene ID	**Gene Description**	**Notes**	Chromosome	ORF	
LOC111517755	probable cytochrome P450 6a23		CM045712.1	20851231	20860676
LOC111517753	probable cytochrome P450 6a23		CM045712.1	20835868	20842440
LOC111507913	cytochrome P450 9e2-like		CM045695.1	18250578	18400705
LOC111508874	cytochrome P450 9e2-like		CM045695.1	18488624	18494345
LOC111518298	cytochrome P450 9e2-like		CM045695.1	18035668	18057476
LOC111507098	cytochrome P450 9e2-like		CM045695.1	18655867	18684667
LOC111504218	cytochrome P450 4c3-like		CM045710.1	25652775	25688379
LOC111503064	Cytochrome P450 4g15		CM045698.1	10491840	10501177
LOC111509471	glutathione S-transferase 1-like		CM045702.1	34627881	34645301
LOC111515223	glutathione S-transferase 1-like		CM045709.1	27288204	27292028
LOC111502309	glutathione synthetase-like		CM045698.1	54840165	54886764
LOC111517229	ABC transporter G family member 23-like		CM045708.1	42082610	42110131
LOC111502767	multidrug resistance-associated protein 4-like		CM045710.1	29959481	29986596
LOC111510953	UDP-glucuronosyltransferase 1-5-like, partial		CM045710.1	10403208	10408471
LOC111506156	NFE2 like bZIP transcription factor cap-n-collar		CM045700.1	50552614	50704496
LOC111514343	cuticular protein 92F		CM045710.1	12654824	12655609
LOC111506564	nicotinic acetylcholine receptor alpha1	nAChR α1	CM045695.1	67746761	67768291
LOC111511639	nicotinic acetylcholine receptor alpha2	nAChR α2	CM045696.1	3138756	3170836
LOC111512651	acetylcholine receptor subunit alpha-like	nAChR α3	CM045703.1	13858834	13970422
LOC111505535	acetylcholine receptor subunit alpha-like	nAChR α4	CM045695.1	41018307	41754638
LOC111502431	neuronal acetylcholine receptor subunit alpha-7-like	nAChR α5	CM045698.1	63396375	63549798
LOC111512033	neuronal acetylcholine receptor subunit alpha-7-like	nAChR α6	CM045700.1	61872075	62180805
LOC111504729	nicotinic Acetylcholine Receptor alpha7	nAChR α7	CM045700.1	49200034	49427814
LOC111513755	acetylcholine receptor subunit beta-like 2	nAChR α8	CM045702.1	40916130	41021780
LOC111506567	neuronal acetylcholine receptor subunit alpha-3-like	nAChR α9	CM045705.1	44824104	44843640
LOC111513669	acetylcholine receptor subunit alpha-type acr-16-like	nAChR α10	CM045702.1	7759587	7803128
LOC111506321	nicotinic acetylcholine receptor beta1	nAChR β1	CM045700.1	49515615	49597496

Pairwise comparisons of the four genes annotated as "cytochrome P450 9e2-like" revealed protein identities as high as 60%, suggesting they are not likely the result of recent gene duplications. In contrast, the two genes annotated as "probable cytochrome P450 6a23" shared a 92% identity, indicating they may represent recently duplicated, but not identical, copies of the same gene.

Pélissié *et al.* (Pélissié *et al.* 2022) calculated nucleotide diversity (π) for each CPB population using a 10 kb sliding window approach on the same dataset. Their results showed that susceptible individuals from Michigan exhibited approximately 20% higher nucleotide diversity compared to resistant individuals ($\pi = 0.0031$ vs. 0.0025, respectively), although the standard errors overlapped.

Next, I calculated population-specific nucleotide diversity for each candidate resistance gene. For gene families such as CYPs, nAChRs, GSTs, and ABC transporters, I also computed the mean nucleotide diversity (π) across all genes for each population, along with their corresponding standard deviations (SDs) and standard errors (SEs).

Figure 3-1 presents the nucleotide diversity of each population across eight CYP genes. The MX+AZ group exhibited the highest level of nucleotide diversity, followed by the pooled Plains samples. Interestingly, the laboratory-maintained NJ-S group displayed nominally higher nucleotide diversity than eight field-collected pest populations, although those differences were not significantly different. Among all pest populations, only the 95% CI of MD-R doesn't overlap with the Plain's samples. A Welch's t-test revealed a nearly significant difference between MD-R and MD-S ($p = 0.0599$), and a statistically significant reduction in diversity in MD-R compared to the Plains group ($p = 0.0149$).

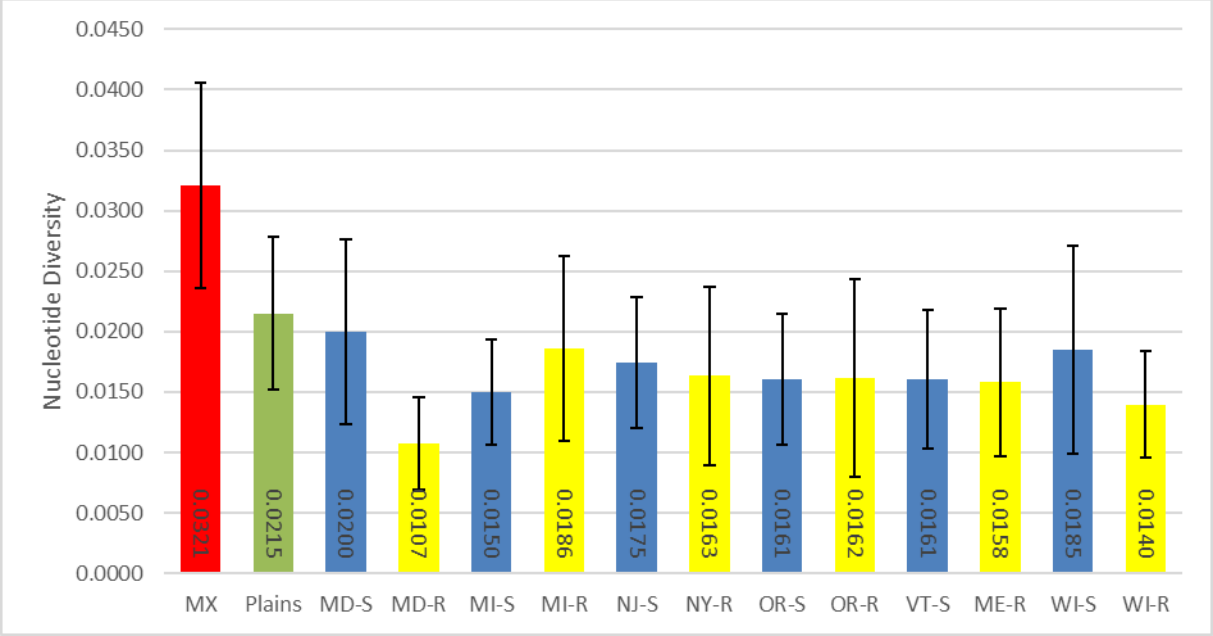


Figure 3-1 Population specific nucleotide diversity across eight CYP genes

The height of the bar indicates the average nucleotide diversity (π) of eight populations across eight genes, and the corresponding values are displayed at the base of each bar. Error bars indicate 95% confidence intervals. Red represents the MX+AZ group, green represents the Plains samples, blue represents imidacloprid susceptible populations/strain, and yellow represents imidacloprid resistant populations.

The box plot of pest populations on each CYP gene indicates outliers (Figure 3-2). Nucleotide diversity of MI-R is an outlier on the gene CYP 4g15 (ID LOC111503064), and the MD-R is an outlier on the CYP 9e2-like gene (ID LOC111507913).

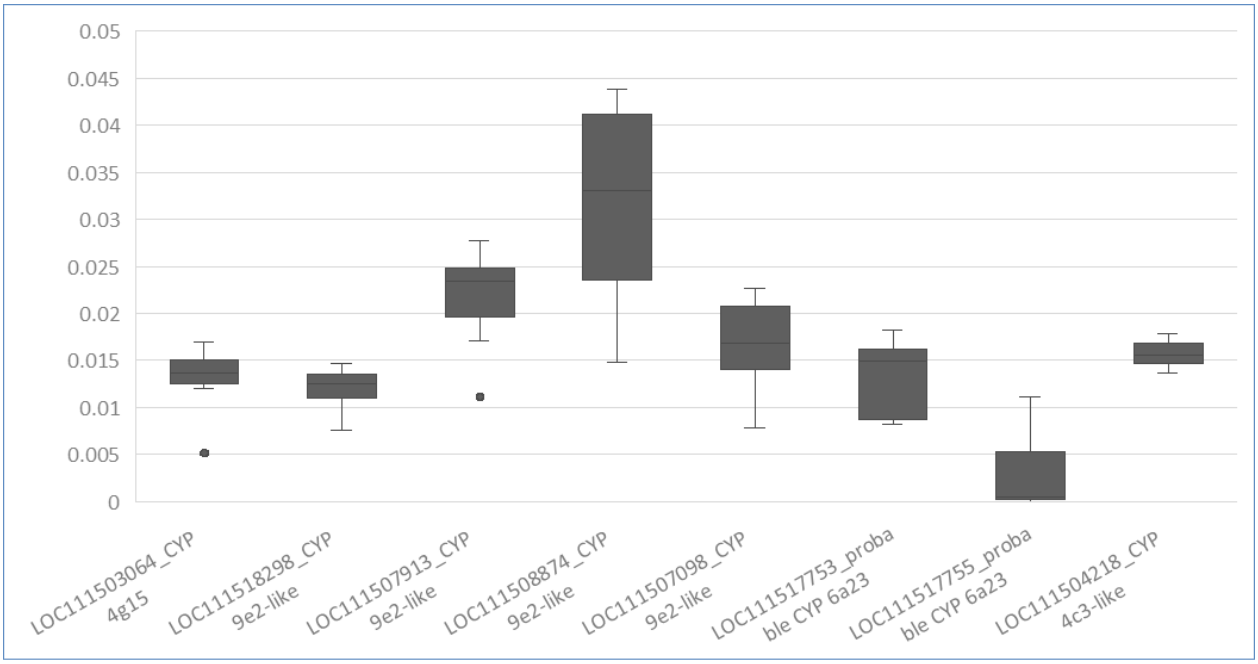


Figure 3-2 Population specific nucleotide diversity of candidate CYPs

Center line in the box indicates the median of the data set; Edges of the box show the middle 50% of the data. Lines extending from the box show the range of the remaining data excluding outliers and points (if present) beyond the extending line are considered outliers. The outlier population on the gene *LOC111503064_CYP 4g15* is the MI-R, and the population outlier on the *LOC111507913_CYP 9e2-like* gene is MD-R.

Figure 3-3 illustrates the population-specific nucleotide diversity across 11 nAChR genes. All field-collected pest populations exhibited similar levels of nucleotide diversity, ranging from 0.01383 to 0.01713, and none differed significantly from the pooled Plains samples ($\pi = 0.0179$). As expected, the NJ-S population showed the lowest nucleotide diversity ($\pi = 0.0111$), which was significantly lower than that of the Plains population ($p = 0.0047$).

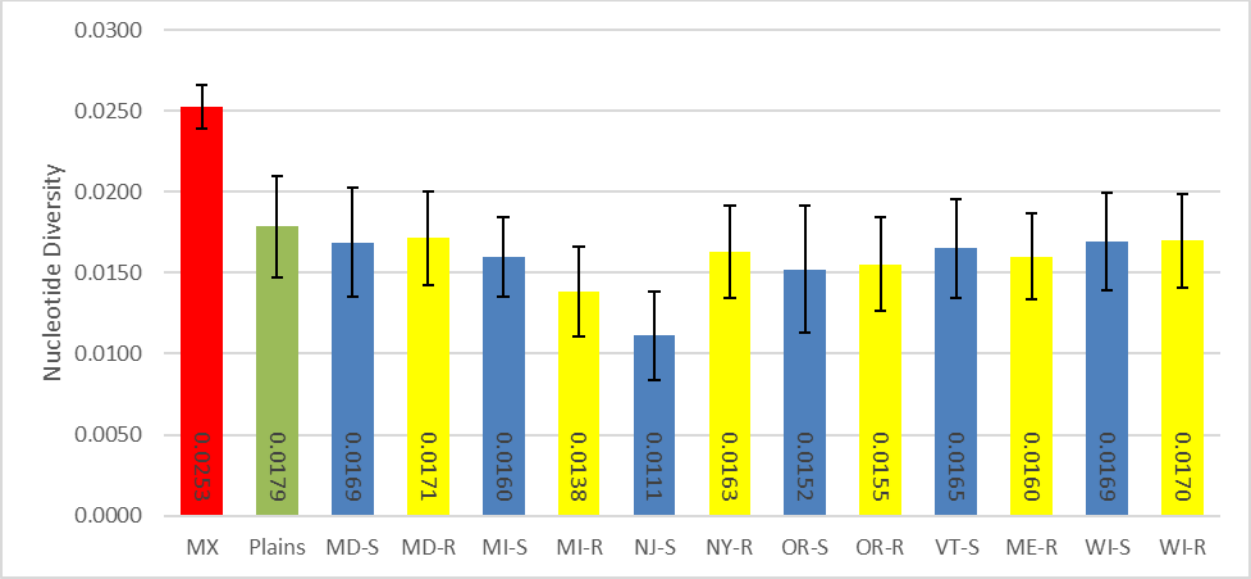


Figure 3-3 Population specific nucleotide diversity across 11 nAChR genes

The height of the bar indicates the average nucleotide diversity (π) of eight populations across 11 nAChR subunit genes, and the corresponding values are displayed at the base of each bar. Error bars indicate 95% confidence intervals. Red represents the MX+AZ group, green represents the Plains samples, blue represents imidacloprid susceptible populations/strain, and yellow represents imidacloprid resistant populations.

I also examined nucleotide diversity per population for each nAChR gene (Figure 3-4). The nucleotide diversity of the MI-R population on nAChR α1, α4, α6, α7, and β1 genes were recognized as lower outliers. In contrast, the OR-S population showed lower outlier values for nAChR α8 and higher outlier values for nAChR α9 and β1.

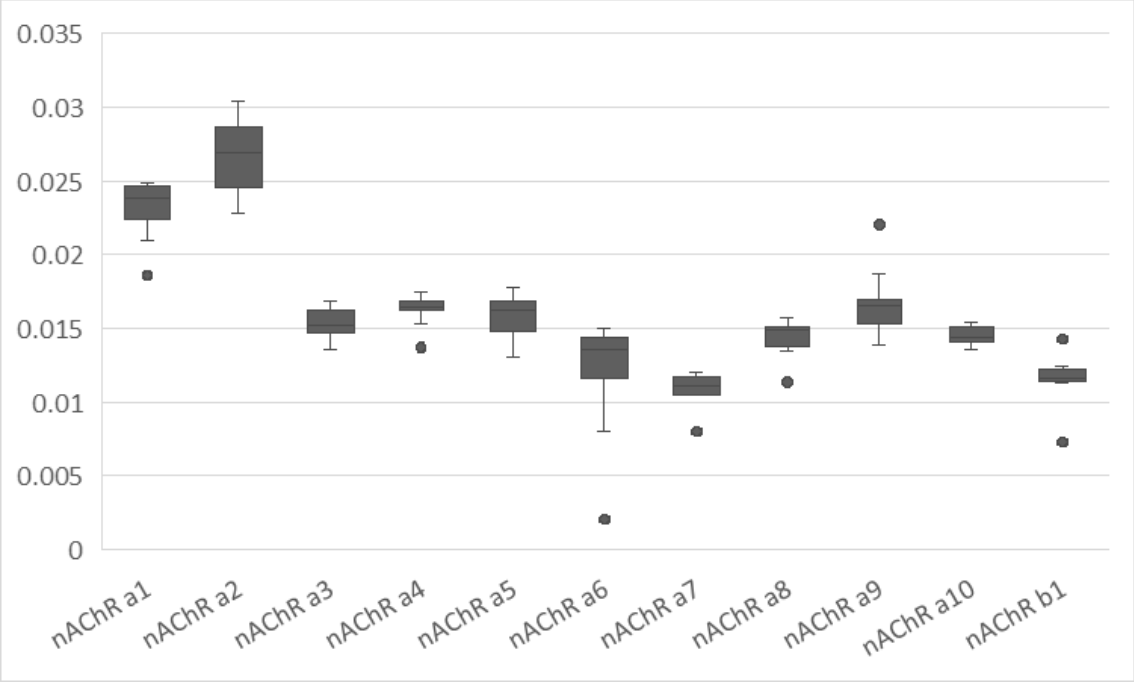


Figure 3-4 Population specific nucleotide diversity on each nAChR gene

The lower outlier on nAChR α1, α4, α6, α7 and β1 are the MI-R population; the lower outlier at α8, and higher outlier are α9 and β1 are the OR-S population.

I also see a sign of selection on glutathione S-transferase (GST) genes (Figure 3-5). Even though no sign of change in nucleotide diversity in the grouping level, the OR-S population was detected as outliers in two GSTs genes.

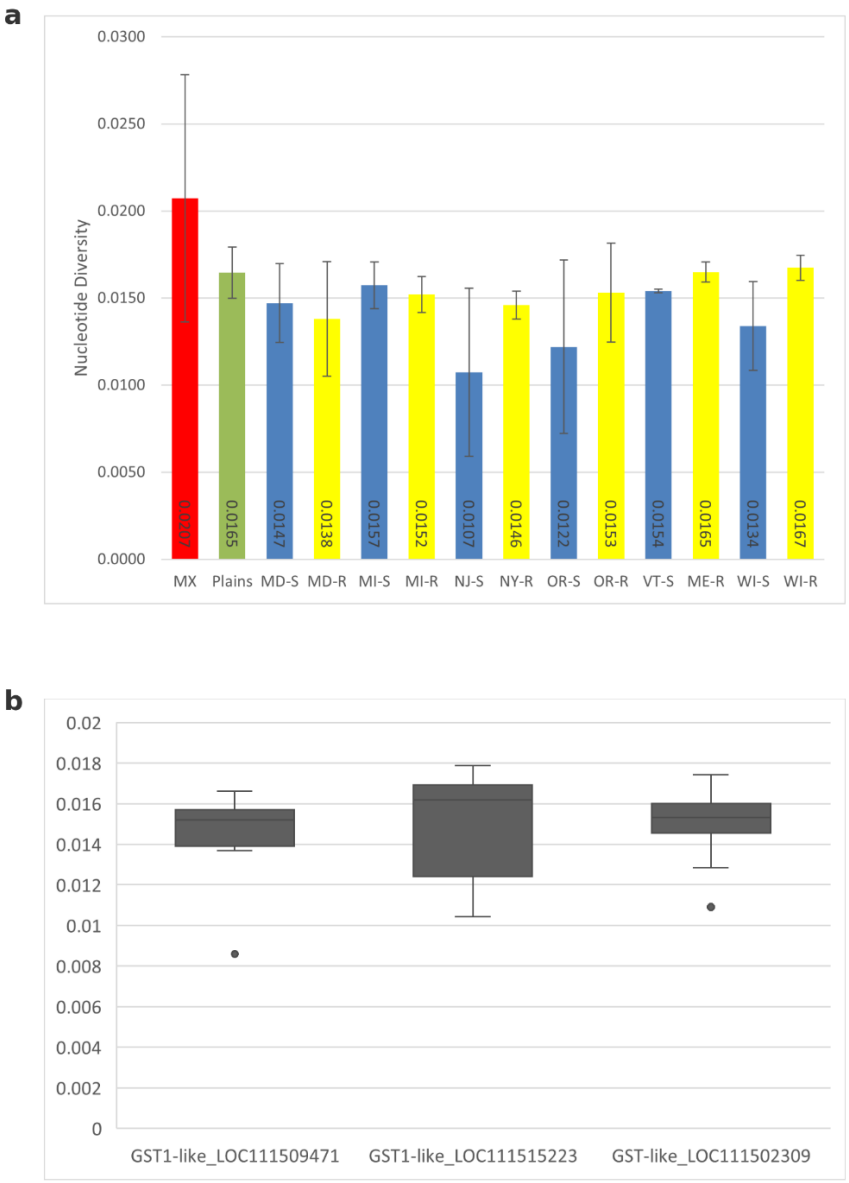


Figure 3-5 Nucleotide diversity on three glutathione S-transferase genes

a. Population specific nucleotide diversity across 3 GSTs gene analyzed; b. Boxplot of population specific nucleotide diversity (π) on each GST gene. Two outliers displayed in b. are the OR-S population.

For all other genes analyzed, I plot each population nucleotide diversity as a bar chart (with error bar indicating 95% CI if multiple genes involved) and the boxplot of π of all pest populations side by side (Appendix I). Except for the UDP-glucuronosyltransferases (UGT) gene, the laboratory-maintained NJ-S population consistently exhibited the lowest level of

nucleotide diversity. Across these genes, no pattern of reduced nucleotide diversity was observed in resistant populations compared to their susceptible counterparts or the pooled Plains samples.

3.4.4 Resistance associated genetic variation detection

Within each geographically proximate imidacloprid-resistant/susceptible pair, as well as between pooled resistant (n = 30) and susceptible (n = 27) samples, I sought to identify genetic variants leading to structural or single amino acid changes in proteins, from alleles showing differential frequencies between resistant and susceptible groups. When such alleles more frequent in the resistant group they are called a Potential Resistance-Associated Allele (PRAA).

A total of six PRAAs were identified from the geographically proximate pairs (Table 3-8), all caused by SNPs resulting in single amino acid substitutions. These included two in the MD pair, three in the MI pair, and one in the OR pair. Among these, four mutations were in CYP genes, and one each in the *CncC* and *vssc* gene. Notably, the kdr mutation on the voltage-gated chloride channel, causing target site insensitivity to DDT and pyrethroid, absent in the Oregon susceptible population but fixed in the OR-R population (Table 3-5), was among these six PRAAs, highlighting the sensitivity of my mutation detection pipeline. No nucleotide variants meeting the sequence-quality and frequency-difference criteria were detected in the WI-RS, ME-R/VT-S, and NY-R/MD-S pairs.

Within the cytochrome P450 9e2-like gene (ID LOC111508874), I identified two distinct PRAAs. One results in a His-to-Tyr substitution at amino acid position 162 in the MD pair, with the Tyr allele fixed in the resistant population. The second is a Lys-to-Glu substitution at position 10 in the MI pair, with the Lys allele fixed in the resistant beetles.

From the pooled analysis of resistant and susceptible samples, I identified nine PRAAs. These included three variants in two CYP genes, three in two ABC transporter genes, and one each in nAChR β1, CncC, and UGT. The SNP in CncC matched the one found in the MD pair and results in an Asn-to-Ser substitution at position 659. The Asn allele (PRAA) was fixed in the MD-R population (AF = 1.0, supported by six genotype calls) and nearly fixed in the pooled resistant samples (AF = 0.974, supported by 55 genotype calls). Three SNPs were identified within the cytochrome P450 9e2-like gene (ID LOC111507098). The first causes a Leu-to-Met substitution at position 196, with the Met allele (PRAA) showing a 0.2157 higher frequency in resistant samples compared to susceptible ones. The second leads to a Thr-to-Asn substitution at position 211, with the Asn allele showing a 0.23 higher frequency in resistant beetles. The third mutation, identified within the NY/NJ pair, results in a Phe-to-Cys substitution at position 146. The Phe allele was fixed (AF = 1.0) in the resistant population and present at a 50% frequency in the NJ-S strain. A SNP in nAChR β1 was significantly different between the pooled resistant and susceptible samples, resulting in a Phe/Leu substitution at the fifth amino acid. This mutation was not in the region forming the ligand binding sites, therefore is unlikely to have a direct functional role in receptor activity.

Table 3-8 Potential resistance-associated alleles

Comparisons	Gene ID	Type	Chromosome ID	Position	SNP	Amino Acid Polymorphism	Potential Resistant Associate Allele	PRAA in R	PRAA in S	R-S	P	Odds Ratio
MD	LOC111508874	cytochrome P450 9e2-like	CM045695.1	18488968	484C/T	His162Tyr	T/Tyr	1	0.5	0.5	0.02288	0
	LOC111506156	Cap-n-collar isoform C	CM045700.1	50557998	1973A/G	Asn658Ser	A/Asn	1	0	1	0.03571	0
MI	LOC111507913	cytochrome P450 9e2-like	CM045695.1	18250588	1571G/C	Arg524Pro	C/Pro	0.5	0	0.5	0.03251	
			CM045695.1	18396396	277G/A	Ala93Thr	A/Thr	1	0.5	0.5	0.03251	0
	LOC111508874	cytochrome P450 9e2-like	CM045695.1	18494318	28A/G	Lys10Glu	A/Lys	1	0.5	0.5	0.03571	0
OR	vssc	vssc	CM045700.1	22977445	2980T/C	Phe994Leu	T/Phe	1	0	1	0.00202	0
Pooled	LOC111518298	cytochrome P450 9e2-like	CM045695.1	18042109	1076A/T	Gln359Leu	A/Gln	1	0.91667	0.08333	0.03917	0
	LOC111507098	cytochrome P450 9e2-like	CM045695.1	18672477	586C/A	Leu196Met	A/Met	0.7407	0.525	0.2157	0.0484	0.3868
			CM045695.1	18672523	632C/A	Thr211Asn	A/Asn	0.6667	0.4318	0.2349	0.03537	0.38
	nAChR β1	nAChR β1	CM045700.1	49515627	13T/C	Phe5Leu	C/Leu	0.2727	0.0625	0.2102	0.009676	5.625
	LOC111506156	NFE2 like bZIP transcription factor cap-n-collar	CM045700.1	50557998	1973A/G	Asn658Ser	A/Asn	0.97368	0.8056	0.16808	0.0263	0.112
	LOC111517229	ABC transporter G family member 23-like	CM045708.1	42087211	2078C/T	Ser693Phe	T/Phe	0.1667	0	0.1667	0.006391	
	LOC111510953	UDP-glucuronosyltransferase 1-5-like, partial	CM045710.1	10408193	235A/C	Ile79Leu	A/Ile	1	0.92593	0.07407	0.04739	0
	LOC111502767	multidrug resistance-associated protein 4-like	CM045710.1	29975493	2303T/C	Phe768Ser	T/Phe	1	0.91304	0.08696	0.03549	0
			CM045710.1	29981969	2989A/G	Lys997Glu	A/Lys	0.7143	0.4091	0.3052	0.04422	0.2769

For each PRAA, I also investigate its distributions in all CPB populations and groups (Table 3-9). In this table, I am more interested in those PRAA with an increasing allele frequency from the non-pest groups (MX+AZ and Plains) to the susceptible groups and to the imidacloprid resistant groups. Four PRAAs meet this criterion. Including one in the gene cytochrome P450 9e2-like (LOC111508874), two in the CYP450 9e2-like gene (ID LOC111507098) and one in the nAChR β1 subunits.

Table 3-9 Allele frequency of PRAAs in CPB populations/groups

Comparisons	Gene ID	Gene Description	PRAA	PRAA in CPB populations/groups															
				MI-R	**MI-S**	**OR-R**	**OR-S**	**ME-R**	**VT-S**	**MD-R**	**MD-S**	**NY-R**	**NJ-S**	**WI-R**	**WI-S**	**R**	**S**	Plains	MX+AZ
MD	LOC111508874	cytochrome P450 9e2-like	T/Tyr	0.5	1	0.6	NA	0.8	0.75	1	0.5	1	0.5	1	0.5	0.8	0.67	0.6	0.167
	LOC111506156	NFE2 like bZIP transcription factor cap-n-collar	A/Asn	1	0.83	1	1	0.875	0.75	1	0	1	1	1	0.67	0.974	0.806	1	0
MI	LOC111507913	cytochrome P450 9e2-like	C/Pro	0.5	0	0	0	0	0	0.125	0.2	0.3	0	0	0.1	0.155	0.056	0.0714	0
		cytochrome P450 9e2-like	A/Thr	1	0.5	0.625	0.75	0.625	0.833	0.9	0.8	0.6	0.5	0.5	0.5	0.722	0.65	0.6	0.875
	LOC111508874	cytochrome P450 9e2-like	A/Lys	1	0.5	0.8	1	0.5	0.75	0.9	0.9	0.5	1	0.5	0.8	0.731	0.812	0.857	0.857
Pooled	LOC111518298	cytochrome P450 9e2-like	A/Gln	1	1	1	1	1	1	1	0.9	1	0.625	1	1	1	0.917	0.929	1
	LOC111507098	cytochrome P450 9e2-like	A/Met	0.625	0.6	0.5	1	0.7	0.5	0.9	0.625	0.833	0.25	0.9	0.625	0.741	0.525	0.5	0
			A/Asn	0.5	0.6	0.5	1	0.7	0.5	0.875	0.5	0.333	0.833	0.833	0.375	0.667	0.432	0.167	0
	nAChR β1	nAChR β1	C/Leu	0.6667	0	0	0	0.375	0.125	0.1667	0.1	0	0	0.4	0.1	0.272	0.0625	0	0
	LOC111517229	ABC transporter G family member 23-like	T/Phe	0.4	0	0.375	0	0.1667	0	0	0	0	0	0	0	0.167	0	0.167	0
	LOC111510953	UDP-glucuronosyltransferase 1-5-like, partial	A/Ile	0	0	1	1	1	0.75	1	1	1	0.8	1	1	1	0.926	1	1
	LOC111502767	multidrug resistance-associated protein 4-like	T/Phe	1	1	1	1	1	0.833	1	0.8	1	1	1	0.9	1	0.913	0.929	1
			A/Lys	0.75	0.375	1	1	0.5	NA	0.5	0.5	0.5	1	0.833	0.5	0.714	0.409	0.5	1

3.5 Discussion

New mutation may arise and increase in frequency due to selective pressure. Here, I detected the T929I mutation on the voltage-sensitive sodium channel gene in two samples analyzed, suggesting the first report of this mutation in the US CPB population. The T929I mutation has been previously detected in multiple pest species, including onion thrips (Toda and Morishita 2009), diamondback moths (Schuler *et al.* 1998), maize weevils (Araújo *et al.* 2011), head lice (Lee *et al.* 2000), house flies (Sun *et al.* 2017), and Bagnallia thrips (Gao *et al.* 2024). The L1014F + T929I double mutation in diamondback moths confers super-kdr-like resistance, resulting in the highest resistance levels among tested genotypes (Schuler *et al.* 1998). The two CPB samples hosting this mutation also carry the L1014F mutation, indicating for the first time a potential super-kdr-like phenotype in North American CPB populations.

The organophosphate and carbamate resistance conferring mutation S291G on the CPB *ace-2* gene was at high allele frequency (>0.5) across all surveyed pest populations (Table 3-5). Several populations, including WI-2, MI-1, and ME, showed the presence of all known resistance-associated alleles, excluding T929I, highlighting the importance of resistance profiling prior to selecting effective insecticides or designing a rotation scheme for resistance management.

I initially hypothesized that target-site mutations with major resistance effects would be rare in non-pest populations due to potential fitness costs in a common sitation. Contrary to this expectation, the S291G mutation appeared fixed in all non-pest CPB samples, including Plains, Mexico and Arizona. This observation suggests either a lack of fitness cost associated with mutation. As previously discussed, the S291G mutation may confer a fitness advantage when

beetles feed on potato plants with high concentrations of α-chaconine. To further investigate this possibility, one could sample the natural host potatoes of these CPB populations. The high resistance allele may also be due to the possibility of gene flow from pest to non-pest populations, resulting in its introgression.

It is important to note that allele frequencies in this analysis were estimated from a relatively small number of genotypes calls due to limited sample sizes and low sequencing depth. These factors may reduce the accuracy of frequency estimates. Future work should incorporate larger sample sizes and deeper sequencing to more reliably characterize the genetic variation in each population.

In my analysis of nAChR genes, I did not identify any resistance-associated mutations previously reported in other pest species. Despite my particular interest in mutations affecting ligand-binding domains, no such variants were detected. I had anticipated discovering mutations in the nAChR α4 subunit, especially given that Chapter 2 presented evidence of a truncated α4 transcript. However, this analysis did not support that finding. The absence of mutations may suggest that the previously observed truncation was a false positive, potentially due to sampling bias, or that it reflects a post-transcriptional modification rather than a genomic change. Instead, I cataloged all detected mutations within nAChR genes and conducted a preliminary assessment of their potential effects based on their genomic locations. Several mutations were located within the predicted signal peptide or transmembrane domains. Mutations in the signal peptide region are not well characterized in the context of insecticide resistance. However, such mutations may disrupt protein targeting or processing, potentially resulting in receptor mis localization, reduced surface expression, or altered receptor function (Gao *et al.* 2020).

I investigated the potential effects of selection on CPB populations by analyzing nucleotide diversity across 28 candidate resistance genes. My genome mapping revealed that several previously identified genes are in proximity on the genome—an observation that was previously unrecognized, likely due to the use of an annotation based on the highly fragmented Ldec_2.0 genome assembly. Four physically clustered genes annotated as cytochrome P450 9e2-like were all found to be overexpressed in imidacloprid-resistant populations. Although sequence identity analysis did not support the hypothesis of a recent duplication of a single gene, the coordinated overexpression of these genes suggests potential regulatory linkage. In addition, two genes annotated as probable cytochrome P450 6a23 were repeatedly reported to be overexpressed and associated with imidacloprid resistance. Their high sequence identity suggests they likely arose from a recent duplication event. These findings highlight the potential role of gene duplication of detoxification enzymes in the evolution of imidacloprid resistance.

The nucleotide diversity results indicate population-specific differences on candidate resistance genes. The MD-R showed decreased nucleotide diversity in CYP genes, the MI-R as lower outlier π in 5 nAChR genes, and the OR-S π as lower outlier of one nAChRs and two GST genes and high outlier on two nAChR genes. In contrast, I observed limited changes in nucleotide diversity across other gene families, implying that selection on protein-coding regions of these genes may not play a major role in the evolution of imidacloprid resistance in these populations. Alternatively, resistance may involve selection on multiple loci with small effect sizes that do not strongly impact nucleotide diversity at individual genes. Interestingly, nucleotide diversity of CYP and UGT genes in field populations was like that of the laboratory-maintained NJ-S strain, which consistently exhibited the lowest diversity among all other analyzed genes. The reasons behind this observation remain unclear and suggest additional

investigation. My analysis focused on comparisons among pest populations. Although comparing pest and Plains populations could provide additional insights, interpreting such differences is challenging. Pest populations may exhibit reduced variation due to bottlenecks during the colonization of potato crops. Alternatively, pest populations could have increased diversity due to hybridization of previously isolated populations, or reduced diversity driven by selection from insecticides or exposure to novel host plant chemistries.

My results identified single nucleotide polymorphisms (SNPs) that lead to amino acid polymorphisms and exhibit significant allele frequency differences between resistant and susceptible phenotypes. These SNPs were found in genes encoding detoxification enzymes, insecticide target sites, and key regulatory factors. It is worth noting that some SNPs were detected as significant in the pooled comparison between resistant and susceptible samples and some SNPs showed identical allele frequencies across different comparisons but were significant in one comparison and not in another. For example, the mutation on cytochrome P450 9e2-like (ID LOC111508874) (Table 3-9) exhibited the same allele frequency in both the MD and WI population pairs (Table 3-10, contig CM045695.1, position 18,488,968), yet it was only statistically significant in the MD pair. This discrepancy arises because I used Fisher's exact test, which calculates p-values based on allele counts rather than frequencies. As a result, the sample size, in this case, the allele count, can influence the significance of the results.

Notably, the cytochrome P450 9e2-like genes, *LOC111508874* along with the transcription factor *CncC*, were repeatedly identified across multiple comparisons (cytochrome P450 9e2-like genes *LOC111508874* were intersect by MD pair and MI pair comparison, and the CncC intersect by the MD pair and pooled sample comparison). The association between altered expression levels of these genes and insecticide resistance has been well documented in previous

studies. The cytochrome P450 9e2-like genes *LOC111508874* were consistently upregulated in a Long Island CPB population compared to an Oregon population (Dively *et al.* 2020). The Long Island CPB population has for decades been shown to have a higher insecticide tolerance than the Oregan population, and this is further supported by the imidacloprid dose–response curves presented in the study. In addition, their bioassay showed that feeding on 1.25 μg mL^{-1} imidacloprid treated leaves for 4 hours caused 8% mortality in the OR population but 0% in the LI population after 24 hours, suggesting reduced sensitivity in the latter. The gene *LOC111508874* was also identified as a candidate resistance gene under positive selection in CPB's rapid adaptation to agricultural environments, as shown by both outlier-based and environmental-association genome scans (Pélissié *et al.* 2022). For the transcription factor *CncC* (gene ID: *LOC111506156*), expression was dramatically increased—42-fold during the pupal stage and 14-fold during adulthood—in imidacloprid-resistant strains compared to susceptible ones (Kalsi and Palli 2017). In the same study, *CncC* was shown to regulate the expression of a suite of downstream *CYP* genes associated with neonicotinoid resistance. Previous transcriptomic studies emphasize the role of overexpression of detoxification genes, especially CYPs genes, in terms of neonicotinoid analysis. The discovery of PRAA in the protein coding region of these genes indicates in addition to altered gene expression, amino acid changes may also contribute functionally to resistance. One limitation of the current study is I cannot tell from these data whether either or both substitutions alter the rate of hydrolysis of the insecticide by these proteins.

In the future, the effects of these amino acid substitutions on protein structure and the intersection with substance can be predicted computationally or through functional expression of

different protein genotypes. Broader sampling may also be needed to assess their prevalence across geographically diverse CPB population

Chapter 4 Outlook and conclusions

4.1 Investigation of sex-linked CPB nAChR β1

To date, although insect species possess at least nine genes encoding nAChR subunits, all field-evolved mutations have been discovered in the β1 subunit. Studies indicate that mutations in nAChR β1 can carry significant fitness costs. CRISPR/Cas9 was used to introduce an nAChR β1 R81T analogous mutation into the genome of *Drosophila melanogaster*, indicating that the R81T mutation imposes a notable fitness cost in homozygous *D. melanogaster* strains but not in heterozygotes. Homozygous females lay fewer eggs, and those eggs exhibit reduced fertility. Additionally, larvae with the homozygous R81T mutation display slower crawling behavior, and adults show diminished performance in climbing assays, with a median lifespan reduced to just 16 days. In the case of CPB, mutations equivalent to the R81T mutation would be functionally dominant in males, since CPB males possess only one X chromosome, which harbors the nAChR β1 subunit. The CPB nAChR β1 subunit can be mutated at the relevant site, and the effects can be investigated. A high fitness cost—or even lethal effects—may indicate a low likelihood of such mutations accumulating in CPB populations

4.2 Highly species-specific insecticides can be designed targeting divergent nAChR or divergent sites

Species-specific control agents, such as RNAi, can be designed on divergent sites of vital nAChR subunits, or divergent nAChR groups, to achieve accurate pest control. For example, the successful expression of nAChR β1 is potentially vital for insect survival, as homozygous nAChR β1 knockout *D. melanogaster* strains are either lethal (Lu *et al.* 2022) or exhibit significantly reduced viability (Perry *et al.* 2021). Therefore, nAChR β1 could serve as an ideal target for designing pest-specific RNAi products. Based on this premise, I compared the nAChR

β1 mRNA sequences encoding the region from Loop D to Loop C in western honeybees, Colorado potato beetles, and small hive beetles (Figure 4-1). I highlighted the first ten sites that are unique to honeybees but conserved among the beetle species. An ideal nucleotide-based control method, such as RNAi, could be designed based on these signature sites to selectively manage pest species while minimizing adverse effects on honeybees.

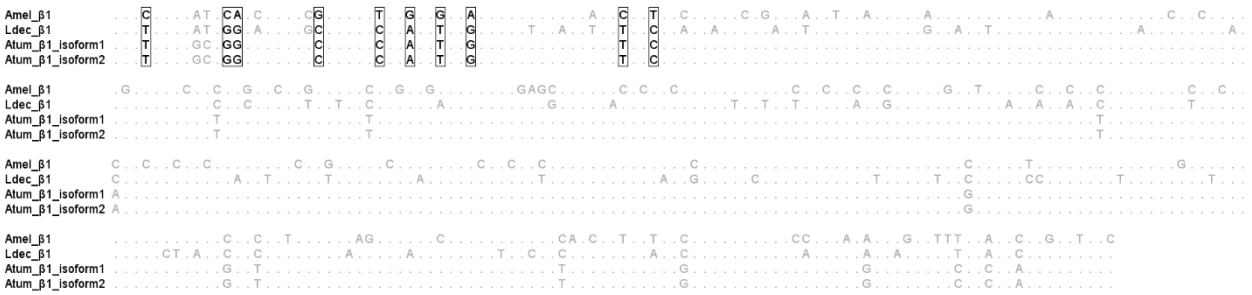


Figure 4-1 Comparison of nAChR β1 mRNA sequences among selected insect species

The displayed sequences encode the region from Loop C to Loop D. NCBI accession numbers for the mRNA sequences shown: *A. mellifera* (Amel) - NM_001079560.1; *L. decemlineata* (Ldec) - KP339870.1; *Aethina tumida* isoform 1 - XM_049961093.1, isoform 2 - XM_020024745.2.

Furthermore, as discussed in Chapter 2, insect species possess divergent nAChR subunits with low interspecific identity, which may serve as ideal target sites. For example, dsRNA targeting the β2 subunit alone has demonstrated insecticidal effects on aphid larvae without affecting the efficacy of imidacloprid at either larval or adult stages (Ligonniere *et al.* 2024). Additionally, it did not exhibit significant acute toxicity to *Apis mellifera*. As noted, CPB also possesses divergent nAChR subunits, such as α9 and α10, which may serve as potential targets for DNA-based control strategies.

4.3 The involvement of historical samples to detect the initial frequency of resistance-associated mutations

In Chapter 3, I investigated non-pest CPB samples to test the hypothesis of "rare initial resistance allele" assumption. However, my results indicate the allele frequency, especially the

S291A conferring resistance to OP and CB on *ace-2* gene are high. To most accurately evaluate the initial resistance allele frequency, one may need to involve historical samples.

4.4 Functional validation of PARR

I identified potential resistance-associated alleles by comparing phenotyped samples and conducting statistical analyses. These mutations may serve as candidates for further validation of their functional effects. Although such cases are relatively limited, mutations in detoxification enzymes can alter catalytic activity and, when combined with gene overexpression, contribute to resistance. For example, in *Anopheles funestus*, a single point mutation (Leu119Phe) in the GSTe2 gene confers resistance to both DDT and pyrethroids, with the resistance further enhanced by gene overexpression (Riveron *et al.* 2014). Similarly, in whiteflies, both overexpression of the enzyme CYP6CM1 and a mutation (A387G), present in two alleles, lead to increased resistance to several neonicotinoids (Pym *et al.* 2023). Gene editing tools, such as CRISPR/Cas9, provide a powerful approach to directly assess the functional impact of specific mutations by introducing them into model organisms or cell lines. The potential resistance-associated alleles (PRAAs) identified in this study can be introduced and their role in insecticide resistance can be investigated.

4.5 Association of copy number variation and resistance should be investigated

Genome mapping results indicate that two previously identified neonicotinoid resistance candidate genes—both annotated as probable cytochrome P450 6a23 (LOC111517755 and LOC111517753)—are physically linked on the same chromosome and share 93% sequence identity. This suggests they likely arose from a recent gene duplication event, highlighting duplication as a potential alternative mechanism of resistance. Copy number variation (CNV), as a source of genetic diversity and a contributor to gene overexpression, has been associated with

insecticide resistance in multiple pest species (Nam *et al.* 2019, Núñez-Acuña *et al.* 2023, Lucas *et al.* 2024). However, I was surprised to find that the role of CNV in resistance has not yet been characterized in the super-pest, the Colorado potato beetle (CPB). This gap presents an opportunity for future research to explore CNV as a potential resistance mechanism in CPB.

4.6 Conclusions

The Colorado potato beetle (CPB) possesses a typical number of cys-loop ligand-gated ion channel (cysLGIC) superfamily genes compared to other insect species, with representatives from all major subunit groups. Widespread alternative splicing and RNA editing were observed in CPB cysLGIC transcripts, potentially contributing to the structural and functional diversity of these subunits. Notably, the Ldec α4, Ldec α9, and Ldec β1 subunits of nAChR exhibit species-specific features that may influence receptor function and insecticide interactions.

Resistance allele frequencies varied across CPB populations, and population-specific patterns of selection on candidate neonicotinoid resistance genes were evident. In this study, I report for the first time the presence of a super-kdr-like allele combination (T929I + L1014F) in the vssc gene of U.S. CPB populations, highlighting the ongoing evolution of target-site resistance. My result also confirms the first observation of the A301S mutation in Rdl in the U.S CPB populations. Furthermore, I identified specific alleles significantly associated with neonicotinoid resistance, though their functional impacts warrant further investigation.

Appendix A. Numbers of cys-loop ligand-gated ion channel gene superfamily gene in insect species, including Colorado potato beetles characterized in the current study.

	Dmel	Amel	Tcas	Nvit	Apis	Aaeg	Atum	Bger	Pame	Ldec
nAChR										
α type	7	9	11	12	9	10	11	10	9	10
β type	3	2	1	4	2	4	1	7	10	1
GABA										
Rdl	1	1	1	1	2	1	1	1	1	1
GRD	1	1	1	1	0	1	1	1	1	1
LCCH3	1	1	1	1	0	1	0	1	1	1
Others										
8916	1	1	1	1	0	0	1	2	2	1
Insect group1 (CLGC)	3	1	3	1	2	1	1	2	2	1
HisCl	2	2	2	2	2	2	2	2	2	2
GluCl	1	1	1	1	2	1	1	2	2	1
pHCl	1	1	1	1	2	1	1	1	1	1
12344	1	1	1	1	1	0	1	1	1	1
Total	22	21	24	26	22	22	21	30	32	21

Species name annotations: Dmel-*D. melanogaster*; Amel-*A. mellifera*; Tcas-*T. castaneum*; Nvit-*Nasonia vitripennis*; Apis-*Acyrthosiphon pisum*; Aaeg-*Aedes aegypti*; Atum-*Aethina tumida*; Bger-*Blattella germanica*; Pame-*Periplaneta americana*; Ldec- *L. decemlineata*

Appendix B. List of NCBI accession numbers of sequences used for analysis.

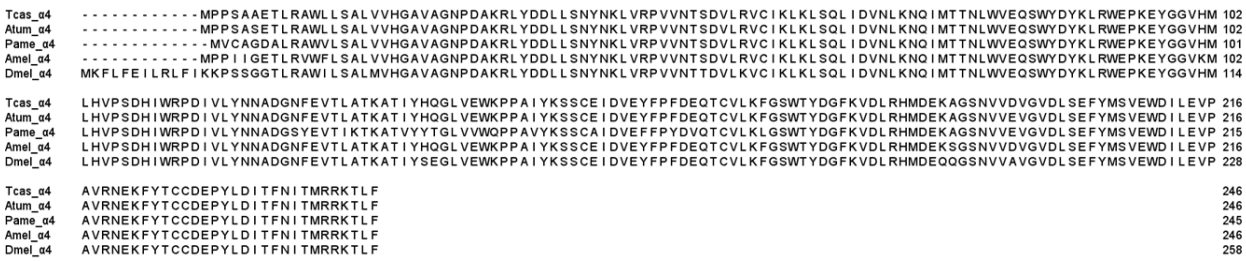
Subunits	Genomic DNA	Transcripts	mRNA	Proteins
Ldec_α1		GGNV01022290.1		
Ldec_α2		GGNV01160150.1		
Ldec_α3		GGNV01295170.1		
Ldec_α4	CM045695.1*			
Ldec_α5			MF197919.1	AWC68049.1
Ldec_α6		GGNV01207620.1		
Ldec_α7		GGNV01057173.1		
Ldec_α8		GGNV01106564.1		
Ldec_α9		GGNV01304972.1		
Ldec_α10		GGNV01314871.1		
Ldec_β1			KP339870.1	AKL79440.1
Ldec_12344		GGNV01048522.1		
Ldec_8916	CM045709.1*			
Ldec_GRD		GGNV01130196.1		
Ldec_LCCH3		GGNV01135992.1		
Ldec_Rdl				XP_023012203.1
Ldec_HisCl1		GGNV01338955.1		
Ldec_HisCl2		GGNV01159996.1		
Ldec_GluCl				XP_023018980.1
Ldec_pHCl		GGNV01044124.1		
Ldec_CLGC		GGNV01134008.1		
Amel_α1			NM_001098220.1	NP_001091690.1
Amel_α2			NM_001011625.2	NP_001011625.1
Amel_α3			NM_001079561.1	NP_001073029.1
Amel_α4			NM_001098221.1	NP_001091691.1
Amel_α5				AAS75781.1
Amel_α6			DQ026035.1	AAY87894.1
Amel_α7			NM_001011621.1	NP_001011621.1
Amel_α8			NM_001011575.1	NP_001011575.1
Amel_α9			NM_001098224.2	NP_001091694.1
Amel_β1			NM_001079560.1	NP_001073028.1
Amel_β2			NM_001098229.1	NP_001091699.1
Amel_12344			DQ667194.1	ABG75746.1
Amel_6927			XM_006563702.3	XP_006563765.1
Amel_8916			NM_001077822.1	NP_001071290.1
Amel_GRD			DQ667183.1	ABG75735.1
Amel_LCCH3			NM_001077812.1	NP_001071280.1
Amel_Rdl			DQ667182.1	ABG75734.1
Amel_HisCl1			NM_001077811.1	NP_001071279.1
Amel_HisCl2			DQ667188.1	ABG75740.1
Amel_GluCl			NM_001077809.1	NP_001071277.1
Amel_pHCl			NM_001143878.1	NP_001137350.1

Dmel_α1			X07194.1	CAA30172.1
Dmel_α2			NM_079758.3	NP_524482.1
Dmel_α3			Y15593.1	CAA75688.1
Dmel_α4			AJ272159.1	CAB77445.1
Dmel_α5			AF272778.1	AAM13390.1
Dmel_α6			NM_164874.3	NP_723494.2
Dmel_α7			NM_001298507.1	NP_001285436.1
Dmel_β1				P04755.1
Dmel_β2			X55676.1	CAA39211.1
Dmel_β3			NM_080359.4	NP_525098.1
Dmel_12344			NM_001299369.1	NP_001286298.1
Dmel_6927			NM_131966.2	NP_572194.1
Dmel_8916			NM_132862.3	NP_573090.3
Dmel_11340			NM_001316541.1	NP_001303470.1
Dmel_7589			NM_001275057.1	NP_001261986.1
Dmel_GRD			NM_079407.3	NP_524131.1
Dmel_LCCH3			NM_206746.2	NP_996469.1
Dmel_Rdl			M69057.2	AAA28556.1
Dmel_HisCl1			NM_079682.3	NP_524406.1
Dmel_HisCl2			NM_141859.4	NP_650116.2
Dmel_GluCl			AF297500.1	AAG40735.1
Dmel_pHCl			NM_001038936.4	NP_001034025.2
Pame_α1			JQ585634.1	AFJ04793.1
Pame_α2			KP725464.1	AKV94621.1
Pame_α3			KR021292.1	AKR16132.1
Pame_α4			JN390946.1	AFA28129.1
Pame_α5		GFCQ01005211.1		
Pame_α6			JF731243.1	AEA40429.1
Pame_α7			MW201211.1	QQH14653.1
Pame_α8			MW201212.1	QQH14654.1
Pame_α9			MW201214.1	QQH14656.1
Pame_β1			MW201213.1	QQH14655.1
Pame_β2		GFCQ01032711.1		
Pame_β3		GFCQ01027461.1		
Pame_β4		GAWS02039241.1		
Pame_β5		GFCQ01009686.1		
Pame_β6		GFCQ01010089.1		
Pame_β7		GFCQ01012153.1		
Pame_β8		GFCQ01034959.1		
Pame_β9		GBJC01015771.1		
Pame_β10		GFCQ01027794.1		
Pame_12344		GFCQ01007480.1		
Pame_8916		GFCQ01012789.1		
Pame_8916_2			MW206636.1	QQH14658.1

Pame_GRD			MW201215.1	QQH14657.1
Pame_LCCH3		GFCQ01015543.1		
Pame_Rdl			LC171474.1	BAW87781.1
Pame_HisCl1		GFCQ01022534.1		
Pame_HisCl2			MW206637.1	QQH14659.1
Pame_GluCl			LC171470.1	BAW87777.1
Pame_GluCl2		GFCQ01031120.1		
Pame_pHCl		GAWS02040818.1		
Pame_CLGC1		GAWS02050116.1		
Pame_CLGC2		GFCQ01015521.1		
Tcas_α1			NM_001109775.1	NP_001103245.1
Tcas_α2			NM_001109953.2	NP_001103423.1
Tcas_α3			NM_001114298.1	NP_001107770.1
Tcas_α4			NM_001109776.2	NP_001103246.1
Tcas_α5			NM_001109782.1	NP_001103252.1
Tcas_α6			NM_001114301.1	NP_001107773.1
Tcas_α7			NM_001109950.1	NP_001103420.1
Tcas_α8			NM_001109949.1	NP_001103419.1
Tcas_α9			NM_001109954.1	NP_001103424.1
Tcas_α10			NM_001109777.1	NP_001103247.1
Tcas_α11			NM_001114299.1	NP_001107771.1
Tcas_β1			NM_001109948.1	NP_001103418.1
Tcas_12344			NM_001109773.1	NP_001103243.1
Tcas_8916			NM_001109955.1	NP_001103425.1
Tcas_GRD			NM_001114300.1	NP_001107772.1
Tcas_LCCH3			NM_001109781.1	NP_001103251.1
Tcas_Rdl			NM_001114337.1	NP_001107809.1
Tcas_HisCl1			NM_001109952.1	NP_001103422.1
Tcas_HisCl2			NM_001109951.1	NP_001103421.1
Tcas_GluCl			NM_001114303.1	NP_001107775.1
Tcas_pHCl			NM_001114303.1	NP_001107777.1
Tcas_CLGC1			NM_001109780.1	NP_001103250.1
Tcas_CLGC2			NM_001109779.2	NP_001103249.1
Tcas_CLGC3			NM_001109778.1	NP_001103248.1

*As indicated in the result section, protein sequences of these two subunits were translated from the manually assembled genomic DNA sequences based on Exonerate results. Accession numbers were linked to the chromosomes from which the genomic DNA was extracted

Appendix C. N-terminal extracellular region protein sequence alignment nAChR α4



NCBI accession number of sequence: Tcas_α4, NP_001103246.1; Dmel_α4, CAB77445.1; Pame_α4, AFA28128.1, and Amel α4, NP_001091691.1

Appendix D. RNA sequencing samples used for exon specific depth of coverage analysis, grouped by study.

Dataset #	Population	Year Sampled	N[a]	Imidacloprid Resistance	Induction	Tissue	Millions of reads, library type, and technology	NCBI SRA Accession#	Reference
1	French Biolabs-USDA-New Jersey Department of Agriculture colony, West Trenton, New Jersey (NJ)	2013	6	Susceptible	No	Larvae	11.1-12.7, PE, HiSeq 2000	SRR1820770, SRR1820776, SRR1820785, SRR1820843, SRR1820867, SRR1820877	(Schoville *et al.* 2018)
2	Hancock, Waushara County, Wisconsin (WI)	2013	3*	Resistant 1st generation	No	Adult body	57.6-102.3, PE, HiSeq 2500	SRR2600374, SRR2600376, SRR2600438	(Clements *et al.* 2016)
3	Holly, Colorado (CO)	2017	6	Susceptible	No	Adult body	27-35, PE, NovoSeq 6000	SRR12121888-SRR12121893	(Pélissié *et al.* 2022)
4	Hermiston, OR (OR)	2014	3*	Susceptible	No	Larvae body	4.4-23, PE, HiSeq 2500	SRR13510812-SRR13510823	(Dively *et al.* 2020)
			3*	Susceptible	Yes	Larvae body			
	Riverhead (Long Island), NY (NY)		3*	Resistant	No	Larvae body			
			3*	Resistant	Yes	Larvae body			

Notes: a. Number indicates the number of RNA sequencing libraries involved. Number with a * indicate a library was made by pooled sample of multiple CPB.

Appendix E. Location of CPB cysLGIC subunit genes on the chromosome.

Subunit gene	Chromosomal ID*	CDS start	CDS end
Ldec_α1	CM045695.1	67746761	67768291
Ldec_α2	CM045696.1	3170836	3138756
Ldec_α3	CM045703.1	13858834	13970425
Ldec_α4	CM045695.1	41018307	41754641
Ldec_α5	CM045698.1	63481056	63396375
Ldec_α6	CM045700.1	61872075	62180805
Ldec_α7	CM045700.1	49090340	49655565
Ldec_α8	CM045702.1	41014845	40916109
Ldec_α9	CM045705.1	44836427	44824104
Ldec_α10	CM045702.1	7803128	7759587
Ldec_β1	CM045700.1	49515645	49597496
Ldec_12344	CM045702.1	23277189	23316372
Ldec_8916	CM045698.1	41150513	41092358
Ldec_CLGC	CM045706.1	4769722	4747013
Ldec_GluCl	CM045700.1	16070642	16150978
Ldec_GRD	CM045702.1	48892562	48874817
Ldec_HisCl1	CM045702.1	53884993	53871120
Ldec_HisCl2	CM045700.1	51999541	51966458
Ldec_LCCH3	CM045698.1	41229977	41274666
Ldec_pHCl copy1	CM045712.1	24506564	24420011
Ldec_pHCl copy2	CM045712.1	25371690	25459705
Ldec_Rdl	CM045709.1	22156746	22310306

*Chromosome ID are as indicated by the chromosomal assembly deposited at GenBank with accession number JANJPO00000 0000

Appendix F. Blastn results and the selection of protein sequence for alignment.

Receptor Name	Subunit ID	Number of transcript hits with bit score > 900)	ID of the transcript producing the longest protein isoform	Completeness of "signature regions" on the longest isoform	Treatment for protein alignment
nicotinic Acetylcholine receptor	Ldec_α1	2	GGNV01022290.1	Complete	No treatment
	Ldec_α2	3	GGNV01160150.1	Complete	No treatment
	Ldec_α3	9	GGNV01295170.1	Complete	N/A
	Ldec_α4	3	GGNV01187321.1	Only TM4 present	Replaced with gDNA-translated protein
	Ldec_α5	1	GGNV01170968.1	Missing Loops D, A, E, and part of Loop B	Replaced with AWC68049.1
	Ldec_α6	6	GGNV01207620.1	Complete	No treatment
	Ldec_α7	1	GGNV01057173.1	Complete	No treatment
	Ldec_α8	1	GGNV01106564.1	Complete	No treatment
	Ldec_α9	1	GGNV01304972.1	Complete	No treatment
	Ldec_α10	4	GGNV01314871.1	Complete	No treatment
	Ldec_β1	1	GGNV01068309.1	TM4 partially missing	Replaced by AKL79440.1
GABA-gated ion channels	Ldec_Rdl	NA	GGNV01394567.1	Missing ligand binding loops and TMs	Replaced by XP_023012203.1
	Ldec_GRD	4	GGNV01130196.1	Complete	No treatment
	Ldec_LCCH3	2	GGNV01135992.1	Complete	No treatment
glutamate-gated chloride channels	Ldec_GluCl	6	GGNV01268739.1	N-terminal loops missing	Replaced by XP_023018980.1
Histamine-gated chloride channels	Ldec_HisCl1	3	GGNV01338955.1	Complete	No treatment
	Ldec_HisCl2	4	GGNV01159996.1	Complete	No treatment
pH-sensitive chloride channel	Ldec_pHCl_copy1 and copy2*	2	GGNV01044124.1	Complete	No treatment

insect group 1 ligand-gated ion channels	Ldec_CLGC	3	GGNV01134008.1	Complete	No treatment
Others	Ldec_8916	1	GGNV01117992.1	Missing Loops D, A, E, B, and F	Replaced by gDNA-translated protein
	Ldec_12344	1	GGNV01048522.1	Complete	No treatment

XP_023012203.1 and XP_023018980.1 are predicted by automatic computational analysis; AKL79440.1 and AWC68049.1 are deduced from cDNA obtained by mRNA RT-PCR and 5'- & 3'- RACE.

*Protein sequence translated from Ldec_pHCl_copy1 and Ldec_pHCl_copy2 only display one amino acid variance. GGNV01044124.1 were the best hit transcript for both, and show a identical amino acid as Ldec_pHCL_copy2 at the polymorphism site. Therefore, protein sequence from GGNV01044124.1 was selected to represent both gene, and named Ldec_pHCl in the alignment.

Appendix G. Exon specific depth of coverage of RNA seq data.

Chromosome ID	Start	End	Exon #	Dataset 1	Dataset 2	Dataset 3	Dataset 4	Average
nAChR α4								
CM045695.1	41018307	41018510	exon1	0.41	0.00	0.68	0.00	0.27
	41206210	41206254	exon2	0.00	0.00	0.00	0.00	0.00
	41240941	41241050	exon3	0.00	0.00	2.61	0.06	0.67
	41445469	41445649	exon4	0.00	1.01	3.92	0.61	1.38
	41550858	41550973	exon5*	0.00	2.26	12.82	0.00	3.77
	41561504	41561672	exon6	0.00	2.75	8.38	0.99	3.03
	41618616	41618752	exon7	0.10	4.74	5.00	1.95	2.95
	41674793	41674939	exon8	1.60	3.69	7.77	2.34	3.85
	41693584	41693718	exon9	3.00	5.50	8.93	1.40	4.71
	41731215	41731452	exon10	0.41	18.21	6.79	2.50	6.98
	41754450	41754638	exon11	1.07	16.47	10.54	1.61	7.42
nAChR α1								
CM045695.1	67746761	67746955	exon1	20.35	3.81	9.39	33.26	16.70
	67749241	67749285	exon2	33.68	4.98	8.80	55.41	25.72
	67749346	67749455	exon3	31.52	3.47	9.58	78.22	30.70
	67751505	67751685	exon4	29.97	4.02	10.97	84.73	32.43
	67754374	67754755	exon5*	23.84	4.99	12.94	96.34	34.53
	67757418	67757712	exon6	29.72	2.15	10.05	121.35	40.82
	67764909	67765149	exon7	17.23	6.30	4.60	107.83	33.99
	67768091	67768291	exon8	27.20	7.22	8.72	132.18	43.83

Exons with an * indicate it encodes the sequence connecting the N-terminal extracellular region and the membrane spanning regions.

Appendix H. CPB samples and sequencing data information

Sample ID	Sex	Pest Status	Group Code	Location	Sequencing Effort	NCBI Accession
CPBWGS_11	Female	Non-pest	MX+AZ	Copper Canyon S. of Huachuca Mtn., Cochise Co., Arizona	6-7X	SRR10388399
CPBWGS_14	Male	Non-pest	Plains	Ellis Co., Kansas	6-7X	SRR10388396
CPBWGS_15	Male	Non-pest	Plains	Yuma, Yuma Co., Colorado	6-7X	SRR10388395
CPBWGS_16	Male	Non-pest	Plains	Burleson Co., Texas	6-7X	SRR10388394
CPBWGS_17	Male	Non-pest	Plains	Boone Co., Missouri	6-7X	SRR10388393
CPBWGS_18	Male	Non-pest	MX+AZ	Guerrero, Mexico	6-7X	SRR10388392
CPBWGS_19	Male	Non-pest	MX+AZ	Jalisco, Mexico	6-7X	SRR10388391
CPBWGS_20	Male	Non-pest	MX+AZ	Morelos, Mexico	6-7X	SRR10388390
CPBWGS_21	Male	Non-pest	MX+AZ	Oaxaca, Mexico	6-7X	SRR10388388
CPBWGS_22	Female	Non-pest	MX+AZ	Puebla, Mexico	6-7X	SRR10388387
CPBWGS_23	Male	Non-pest	MX+AZ	Texcoco, Mexico	6-7X	SRR10388386
CPBWGS_24	Female	Non-pest	MX+AZ	Saltillo, Mexico	6-7X	SRR10388385
CPBWGS_25	Female	Non-pest	MX+AZ	Durango, Mexico	6-7X	SRR10388384
CPBWGS_26	Female	Non-pest	MX+AZ	Chihuahua, Mexico	6-7X	SRR10388383
CPBWGS_28	Male	Non-pest	Plains	Caprock, Lea Co., New Mexico	6-7X	SRR10388381
CPBWGS_35	Female	Non-pest	Plains	Vogel Canyon, Otero Co., Colorado	6-7X	SRR10388373
CPBWGS_36	Female	Non-pest	Plains	Kearney Co., Nebraska	6-7X	SRR10388372

CPBWGS_39	Male	Pest; Imidacloprid Susceptible	WI-1/WI-S	Arlington, Columbia Co., Wisconsin	6-7X	SRR10388369
CPBWGS_40	Female	Pest; Imidacloprid Susceptible		Arlington, Columbia Co., Wisconsin	6-7X	SRR10388368
CPBWGS_41	Female	Pest; Imidacloprid Susceptible		Arlington, Columbia Co., Wisconsin	6-7X	SRR10388366
CPBWGS_42	Female	Pest; Imidacloprid Susceptible		Arlington, Columbia Co., Wisconsin	6-7X	SRR10388365
CPBWGS_43	Male	Pest; Imidacloprid Susceptible		Arlington, Columbia Co., Wisconsin	6-7X	SRR10388364
CPBWGS_44	Female	Pest; Imidacloprid Resistant	WI-2/WI-R	Hancock, Waushara Co., Wisconsin	6-7X	SRR10388363
CPBWGS_45	Female	Pest; Imidacloprid Resistant		Hancock, Waushara Co., Wisconsin	6-7X	SRR10388362
CPBWGS_46	Female	Pest; Imidacloprid Resistant		Hancock, Waushara Co., Wisconsin	6-7X	SRR10388361
CPBWGS_47	Female	Pest; Imidacloprid Resistant		Hancock, Waushara Co., Wisconsin	6-7X	SRR10388360
CPBWGS_48	Male	Pest; Imidacloprid Resistant		Hancock, Waushara Co., Wisconsin	6-7X	SRR10388359
CPBWGS_49	Male	Pest; Imidacloprid Susceptible	MI-1/MI-S	Allegan Co., Michigan	6-7X	SRR10388358
CPBWGS_50	Male	Pest; Imidacloprid Susceptible		Allegan Co., Michigan	6-7X	SRR10388357
CPBWGS_51	Male	Pest; Imidacloprid Susceptible		Allegan Co., Michigan	6-7X	SRR10388355
CPBWGS_52	Female	Pest; Imidacloprid Susceptible		Allegan Co., Michigan	6-7X	SRR10388354
CPBWGS_53	Female	Pest; Imidacloprid Susceptible		Allegan Co., Michigan	6-7X	SRR10388353
CPBWGS_54	Male	Pest; Imidacloprid Resistant	MI-2/MI-R	Allegan Co., Michigan	6-7X	SRR10388352
CPBWGS_55	Female	Pest; Imidacloprid Resistant		Allegan Co., Michigan	6-7X	SRR10388351
CPBWGS_56	Male	Pest; Imidacloprid Resistant		Allegan Co., Michigan	6-7X	SRR10388350
CPBWGS_57	Female	Pest; Imidacloprid Resistant		Allegan Co., Michigan	6-7X	SRR10388349

CPBWGS_58	Female	Pest; Imidacloprid Resistant		Allegan Co., Michigan	6-7X	SRR10388348
CPBWGS_60	Male	Pest; Imidacloprid Susceptible	OR-1/OR-S	Hermiston, Umatilla Co., Oregon	6-7X	SRR10388346
CPBWGS_61	Male	Pest; Imidacloprid Susceptible		Hermiston, Umatilla Co., Oregon	6-7X	SRR10388344
CPBWGS_62	Male	Pest; Imidacloprid Susceptible		Hermiston, Umatilla Co., Oregon	6-7X	SRR10388343
CPBWGS_64	Female	Pest; Imidacloprid Resistant	OR-2/OR-R	Hermiston, Umatilla Co., Oregon	6-7X	SRR10388341
CPBWGS_65	Male	Pest; Imidacloprid Resistant		Hermiston, Umatilla Co., Oregon	6-7X	SRR10388340
CPBWGS_66	Male	Pest; Imidacloprid Resistant		Hermiston, Umatilla Co., Oregon	6-7X	SRR10388339
CPBWGS_67	Male	Pest; Imidacloprid Resistant		Hermiston, Umatilla Co., Oregon	6-7X	SRR10388338
CPBWGS_68	Female	Pest; Imidacloprid Resistant		Hermiston, Umatilla Co., Oregon	6-7X	SRR10388337
CPBWGS_69	Female	Pest; Imidacloprid Susceptible	MD-1/MD-S	Prince George's Co., Maryland	6-7X	SRR10388336
CPBWGS_70	Female	Pest; Imidacloprid Susceptible		Prince George's Co., Maryland	6-7X	SRR10388335
CPBWGS_71	Female	Pest; Imidacloprid Susceptible		Prince George's Co., Maryland	6-7X	SRR10388333
CPBWGS_72	Male	Pest; Imidacloprid Susceptible		Prince George's Co., Maryland	6-7X	SRR10388332
CPBWGS_73	Female	Pest; Imidacloprid Susceptible		Prince George's Co., Maryland	6-7X	SRR10388331
CPBWGS_74	Female	Pest; Imidacloprid Resistant	MD-2/MD-R	Dorchester Co., Maryland	6-7X	SRR10388330
CPBWGS_75	Female	Pest; Imidacloprid Resistant		Dorchester Co., Maryland	6-7X	SRR10388329
CPBWGS_76	Female	Pest; Imidacloprid Resistant		Dorchester Co., Maryland	6-7X	SRR10388328
CPBWGS_77	Male	Pest; Imidacloprid Resistant		Dorchester Co., Maryland	6-7X	SRR10388327
CPBWGS_78	Female	Pest; Imidacloprid Resistant		Dorchester Co., Maryland	6-7X	SRR10388326

CPBWGS_79	Female	Pest; Imidacloprid Susceptible	NJ/NJ-S	French Biolabs-USDA-New Jersey Department of Agriculture colony, West Trenton, New Jersey	6-7X	SRR10388325
CPBWGS_80	Female	Pest; Imidacloprid Susceptible		French Biolabs-USDA-New Jersey Department of Agriculture colony, West Trenton, New Jersey	6-7X	SRR10388324
CPBWGS_81	Male	Pest; Imidacloprid Susceptible		French Biolabs-USDA-New Jersey Department of Agriculture colony, West Trenton, New Jersey	6-7X	SRR10388322
CPBWGS_82	Male	Pest; Imidacloprid Susceptible		French Biolabs-USDA-New Jersey Department of Agriculture colony, West Trenton, New Jersey	6-7X	SRR10388321
CPBWGS_83	Male	Pest; Imidacloprid Susceptible		French Biolabs-USDA-New Jersey Department of Agriculture colony, West Trenton, New Jersey	6-7X	SRR10388320
CPBWGS_84	Female	Pest; Imidacloprid Resistant	NY/NY-R	Long Island, Suffolk Co., New York	6-7X	SRR10388319
CPBWGS_85	Female	Pest; Imidacloprid Resistant		Long Island, Suffolk Co., New York	6-7X	SRR10388318
CPBWGS_86	Female	Pest; Imidacloprid Resistant		Long Island, Suffolk Co., New York	6-7X	SRR10388317
CPBWGS_87	Female	Pest; Imidacloprid Resistant		Long Island, Suffolk Co., New York	6-7X	SRR10388316
CPBWGS_88	Female	Pest; Imidacloprid Resistant		Long Island, Suffolk Co., New York	6-7X	SRR10388315
CPBWGS_89	Female	Pest; Imidacloprid Susceptible	VT/VT-S	Chittenden Co., Vermont	6-7X	SRR10388314
CPBWGS_90	Male	Pest; Imidacloprid Susceptible		Chittenden Co., Vermont	6-7X	SRR10388313
CPBWGS_91	Female	Pest; Imidacloprid Susceptible		Chittenden Co., Vermont	6-7X	SRR10388311
CPBWGS_92	Female	Pest; Imidacloprid Susceptible		Chittenden Co., Vermont	6-7X	SRR10388310
CPBWGS_94	Female	Pest; Imidacloprid Resistant	ME/ME-R	Presque Isle, Aroostook Co., Maine	6-7X	SRR10388308
CPBWGS_95	Female	Pest; Imidacloprid Resistant		Presque Isle, Aroostook Co., Maine	6-7X	SRR10388307
CPBWGS_96	Male	Pest; Imidacloprid Resistant		Presque Isle, Aroostook Co., Maine	6-7X	SRR10388306
CPBWGS_97	Female	Pest; Imidacloprid Resistant		Presque Isle, Aroostook Co., Maine	6-7X	SRR10388305
CPBWGS_98	Female	Pest; Imidacloprid Resistant		Presque Isle, Aroostook Co., Maine	6-7X	SRR10388304

Appendix I. Nucleotide diversity of populations on ABC transporters, CncC and UDP-glucuronosyltransferase

a. Nucleotide diversity of populations on ABC transporters;
b. Nucleotide diversity of populations on CncC;
c. Nucleotide diversity of populations on UDP-glucuronosyltransferase

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