Australian Government

Cotton Research and Development Corporation

SUMMER SCHOLARSHIP REPORT: 2014-2015 SEASON

1.	**Project Title** (Maximum 85 char)	:	Resistance genes in *Helicoverpa armigera* from Northern Australia.
2.	**Proposed Start Date**	:	Monday, 24 Nov 2014
	Proposed Cease Date	:	Friday 30 January 2015
	Scholarship Type (summer or honours)	:	Summer
3.	**Summer or Honours Scholar (If known) and University**	:	Jaeeun Ryu, Australian National University
4.	**Organisation & Location for the project**	:	CSIRO Biosecurity Flagship, Canberra
5.	**Administrative Contact**	:	Michelle Stuckey
	Telephone	:	02 6246 4192
	Facsimile	:	
	Postal Address	:	GPO BOX 1700, Canberra ACT 2601
	Email	:	Michelle.Stuckey@csiro.au
6.	**Project Supervisor**	:	Tom Walsh
	Position in organisation	:	Research Scientist
	Telephone	:	02 6246 4083
	Facsimile	:	
	Email	:	tom.walsh@csiro.au
	Postal Address	:	Ecosystem Sciences Building, Clunies Ross St., Acton, 2601

Project Collaborators (Name and Organisation):

Dr Wee Tek Tay, CSIRO Biosecurity Flagship
Dr Andreas Zwick, CSIRO Australian National Insect Collection
Dr Lisa Bird, NSW DPI.

SUMMER SCHOLARSHIP REPORT

(Maximum FOUR pages)

1. Executive Summary:

Resistance to insecticides has repeatedly developed in *Helicoverpa armigera* around the world and is of major concern to the cotton industry in Australia. *CYP337B3*, the gene responsible for fenvalerate resistance was identified in 2012 (Joußen *et al*.) Different alleles of this resistance gene have been identified at different frequencies in different regions of the world. Identifying the variants present in Northern Australia gives us an insight into movement of *H. armigera* from Asia in Australia. A total of 91 samples from N. Australia, were tested. In this data set, the majority of individuals collected from the field in N. Australia (80%) were positive for *CYP337B3*, either as a homozygote or a heterozygote. Almost half (47%) of the alleles sequenced from N. Australia were found to be the allele predominantly found in Asia (*CYP337B3v2*) as compared to 20% of alleles from the cotton growing regions identified as Asian. The major implication from this work is that there is gene flow between Asian populations and Australia. F_2 testing of an individual collected from the Ord River showed that this *CYP337B3v2* gene was associated with survival of a discriminating dose of fenvalerate. Further work is required to examine connectivity between these populations and the potential risk of other resistance alleles arriving from Asia.

2. Background:

Helicoverpa armigera, or cotton boll worm, is one of the major pests of agriculture especially cotton. This species has the ability to rapidly develop resistance against insecticides. Cytochrome P450s are a gene family known to be involved in the metabolism of xenobiotics such as pesticides. A cytochrome P450 gene, *CYP337B3* was identified as the cause of pyrethroid resistance in *Helicoverpa armigera* in 2012 (Joußen *et al.*, 2012). This gene is a chimeric gene thought to arise from unequal crossing-over between two parental P450 genes, *CYP337B1* and *CYP337B2*. Different alleles of *CYP337B3* have been observed around the world; *CYP337B3v1* was the first identified and is commonly found in Australia while *CYP337B3v2* has been associated with resistance in Pakistani populations (Rasool *et al.*, 2014), and identified in China and across India. Unpublished data generated in this lab suggests that these different alleles developed in different regions of the world, and can be used as markers of metapopulations.

3. Aims and Objectives:

This project aims to investigate *Helicoverpa armigera* from Northern Australia for evidence of these resistance genes and to show that there is movement of specimens with these pyrethroid resistance alleles from Asia to Australia.

4. Materials and Methods:

4.1 Samples

A total of 91 samples were tested. 43 samples of adult *H.armigera* were collected by Dr Zwick along a transect from Darwin to Kununurra in 2014. The date and location of the sampling can be found in Table 1.

Table 1: Samples collected from N. Australia

Date	GPS coordinates	Description
26.03.2014	130.9344°E 12.3968°S	Holmes Jungle Nature Park, NT
27.03.2014	132.4565°E 14.5839°S	Cutta Cutta Caves Nature Park, NT
28.03.2014	131.7520°E 15.0098°S	4km on road to Flora River Nature Park, NT
29.03.2014	130.2273°E 15.6520°S	30km West of Timber Creek, NT
31.032014	128.7122°E 15.6543°S	Research Station, Kununurra, WA
1.04.2014	128.2598°E 15.5497°S	Marlgu Billabong, Parry Lagoons Nature Reserve, WA
2.04.2014	128.3827°E 15.5808°S	farmland East of Parry Lagoons Nature Reserve, WA
3.04.2014	128.2555°E 15.7144°S	The Grotto, Wyndham, WA
7.04.2014	131.5848°E 15.3359°S	tributary of Brandy Bottle Creek, 4km South on Buntine Highway, NT

A further 48 samples including 24 live and 24 dead are the result of an F_2 cross of *H. armigera* collected near the Ord River in northern Western Australia and screened for pyrethroid resistance The bioassay methods used to screen *H. armigera* for resistance were based on the methods of Forrester et al. (1993). This involved rearing larvae to the third or fourth instar. Larvae within a weight range of 30-40mg were transferred to fresh toxin-free diet and screened by topical

administration of 1µL of a discriminating dose of acetone/insecticide solution applied to the dorsal thorax of larvae using a 50µl micro-syringe in a repeating dispenser (Hamilton Company, Reno, NV, USA). Trays containing tested larvae were covered with heat-sealed perforated lids (Oliver Products, Grand Rapids, MI, USA); acetone alone was used as the control. Bioassays were maintained for 3 days at 25ºC with a 14:10 [L:D] hour cycle, and assessed for mortality using one or more of the following criteria: larvae unable to demonstrate coordinated movement when prodded; paralysis of prolegs; larvae very slow to right themselves (time exceeding 3 seconds).

4.2 Identification of resistance alleles

DNA was extracted using the DNeasy Blood & Tissue Kit from QIAGEN following the standard protocol. The CYP337B genes were amplified by PCR in 25µl reactions using Platinum Taq (Invitrogen) following the manufacturer's recommendations and the primers developed by Joußen *et al*, (2012). The resulting PCR products were examined on an agarose gel and scored for the presence of the resistant gene (*CYP337B3*) or the susceptible alleles (*CYP337B1* and *CYP337B2*)
In order to identify the allele of the *CYP337B3* gene, these PCR products were Sanger sequenced at the Biological Resources Facility at the Australian National University. Sequences were then compared using CLCGenomics (Life Technologies) to the known alleles and a frequency of each was calculated for the samples sequenced.

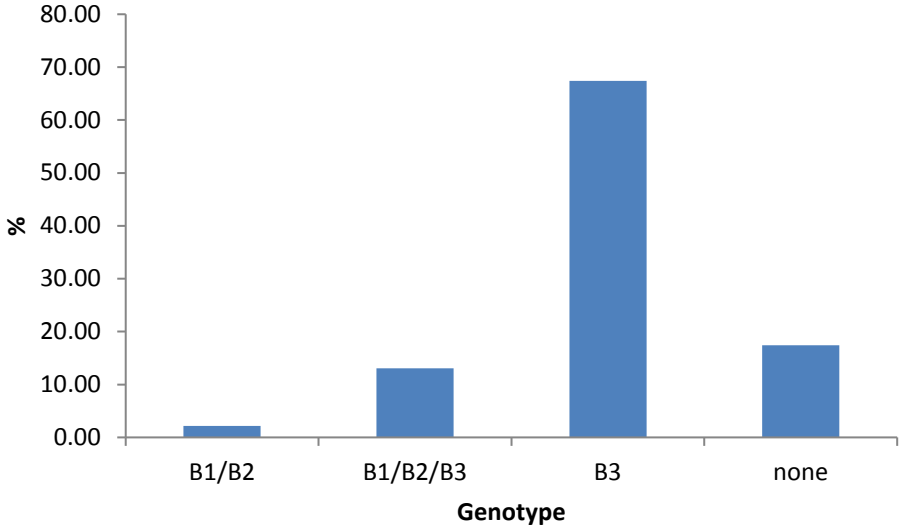
5. Results:

5.1 Field collected insects

Individual insects were scored for the presence of *CYP337B1*, *CYP337B2* and *CYP337B3*. Figure 1 shows the distribution of the genotypes in the insects collected from the field in N. Australia. The majority of individuals were carrying the pyrethroid resistance gene, the majority as homozygote individuals. In a small number of individuals, no amplification product was observed suggesting either a methodological failure or the collection of a different but morphologically similar species.

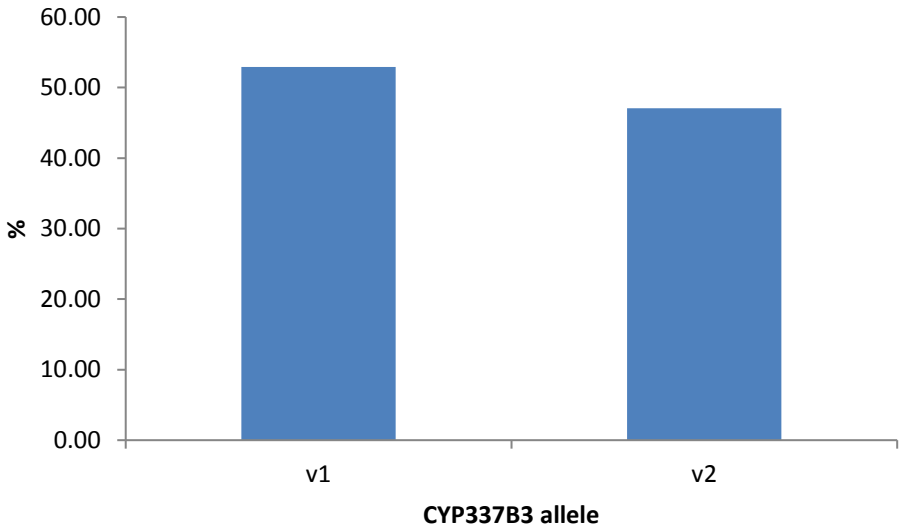
Where the *CYP337B3* gene was identified, the amplified PCR product was sequenced to determine the allele present. Individual alleles have unique DNA sequences and either the allele identified from Australia (*CYP337B3v1*) or an allele found in Asia (*CYP337B3v2*) was identified (Figure 2). This data shows that the Asian allele (47%) is almost as common in Northern Australia as the Australian allele (53%). In insects collected in the cotton growing regions of S. Queensland and NSW, the Asian allele is less common compared to the Australian allele (v2 20%, v1 80% - unpublished CSIRO data). Furthermore, a number of individuals that were homozygous for *CYP337B3* were heterozygotes for *CYP337B3v1* and *v2* suggesting that the Asian allele has introgressed into the native population.

Figure 1: *H. armigera* CYP337 genotypes in moths collected in light traps between Darwin and Kununurra. B1/B2 – susceptible allele, B1/B2/B3 – heterozygous, B3 – homozygous resistant, none – no amplification products.



Where the *CYP337B3* gene was identified, the amplified PCR product was sequenced to determine the allele present. Individual alleles have unique DNA sequences and either the allele identified from Australia (*CYP337B3v1*) or an allele found in Asia (*CYP337B3v2*) was identified (Figure 2). This data shows that the Asian allele (47%) is almost as common in Northern Australia as the Australian allele (53%). In insects collected in the cotton growing regions of S. Queensland and NSW, the Asian allele is less common compared to the Australian allele (v2 20%, v1 80% - unpublished CSIRO data). Furthermore, a number of individuals that were homozygous for *CYP337B3* were heterozygotes for *CYP337B3v1* and *v2* suggesting that the Asian allele has introgressed into the native population.

Figure 2: CYP337B3 alleles present in individuals collected in light traps between Darwin and Kununurra. v1 – *CYP337B3v1*, most common in Australia; v2 *CYP337B3v2*, most common in Asia.



5.2 Bioassayed individuals.

An F_2 bioassay was performed on an insect collected from the Ord River irrigation scheme and a laboratory strain, GR. The survivors and the dead individuals were examined for the presence of *CYP337B3* and for the allele (n = 48). All of the surviving insects were found to possess *CYP337B3* gene while all the dead insects did not. Furthermore, many of the survivors were heterozygotes (83%) containing CYP337B1, B2 and B3 suggesting that this gene is dominant for resistance. All the survivors possessed *CYP337B3v2* suggesting that the field collected parent possesed this allele and that *CYP337B3v2* allows them to survive at the discriminating dose.

6. Discussion and Conclusions:

Resistance to insecticides has repeatedly developed *Helicoverpa armigera* around the world and is a major concern and threat to the cotton industry in Australia. *CYP337B3*, the gene responsible for fenvalerate resistance was identified in 2012 (Joußen *et al*.) Different alleles of this resistance gene have been identified at different frequencies in different regions of the world. Identifying the variants present in N. Australia gives us an insight into movement of *H. armigera* from Asia into Australia.

The results of the F_2 testing show that *CYP337B2v2* enables survival at discriminating dose and that the resistance is dominant. This is thought to be similar to the originally identified *CYP337B3v1* and the presence of heterozygotes for v1 and v2 would tend to support this conclusion. Recent work has cast some doubt on the role of this gene (Han *et al*., *in press*) but in this limited data set it is completely associated with the resistant phenotype.

In this data set the majority of individuals collected from the field in N. Australia (80%) were positive for *CYP337B3*, either as a homozygote or a heterozygote. This suggests that the gene for fenvalerate resistance is very common in N. Australia though not as common as in the cropping regions (94% - CSIRO unpublished data). Almost half of the alleles sequenced from N. Australia were found to be the allele predominantly found in Asia (India, China, Pakistan, Korea – CSIRO unpublished data). If there was movement from Asia into Australia we would expect to see a higher frequency of Asian alleles in N. Australia as compared to central and S. Australia. While this dataset is limited in size, this is indeed what we see with only 20% of alleles from the cotton growing regions identified as Asian (n = 94 - CSIRO unpublished data).

The major implication from this work is that there is recent and/or ongoing gene flow between Asian and Australian populations. Further work is required to examine connectivity between these populations and the potential risk of other resistance alleles arriving from Asia. In particular, there is heavy selection pressure for resistance to Cry1Ac in many parts of Asia and resistant isolates have been identified including a potentially dominant mutation from China (Zhang et al., 2012). Cry1Ac remains the rarest resistance type detected in Bt resistance monitoring in Australia and the potential for selection for resistance elsewhere followed by gene flow from Asia represents a significant risk to the industry in Australia.

7. Highlights:

This work clearly indicates that movement between Asia and Australia is occurring in *H. armigera* and that some individuals at least are carrying resistance genes.

The difference in frequency, even in this small sample size, suggests that at least some of the movement into Australia may be natural rather than human derived.

8. Future Research:

This dataset represents a preliminary examination of gene flow between Australia and Asia in *H. armigera*. Clearly more sampling across N. Australia would add significance to the data and sampling in Indonesia would also be useful. In addition, using higher throughput techniques to examine known resistance genes (conventional and Bt) as well as larger scale independent methods for generating markers to assess gene flow would be extremely useful.

The potential for the development of agriculture in N. Australia also has implications, potentially creating a larger link between Asia and the major cropping regions of Eastern Australia where new pests but also pests with new resistances or diseases can spread through Australia.

9. Presentations and Public Relations:

The intention is that these results will contribute to a larger manuscript currently in preparation on global *H. armigera* movement and resistance to fenvalerate.

10. Reference List:

Forrester, N. W., Cahill, M., Bird, L. J., & Layland, J. K. (1993). Management of pyrethroid and endosulfan resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Australia. *Bulletin of Entomological Research*: Supplement Series, (Supplement 1).

Han, Y., Yu, W., Zhang, W., Yang, Y., Walsh, T., Oakeshott, J. G., & Wu, Y. (2014). Variation in P450-mediated fenvalerate resistance levels is not correlated with CYP337B3 genotype in Chinese populations of *Helicoverpa armigera*. Pesticide Biochemistry and Physiology.

Joußen, N., Agnolet, S., Lorenz, S., Schöne, S. E., Ellinger, R., Schneider, B., & Heckel, D. G. (2012). Resistance of Australian *Helicoverpa armigera* to fenvalerate is due to the chimeric P450 enzyme CYP337B3. *Proceedings of the National Academy of Sciences*, 109(38), 15206-15211.

Rasool, A., Joußen, N., Lorenz, S., Ellinger, R., Schneider, B., Khan, S. A., ... & Heckel, D. G. (2014). An independent occurrence of the chimeric P450 enzyme CYP337B3 of *Helicoverpa armigera* confers cypermethrin resistance in Pakistan. *Insect biochemistry and molecular biology*, 53, 54-65.

Zhang H, Wu S, Yang Y, Tabashnik BE, Wu Y (2012) Non-Recessive Bt Toxin Resistance Conferred by an Intracellular Cadherin Mutation in Field-Selected Populations of Cotton Bollworm. *PLoS ONE* 7(12): e53418. doi:10.1371/journal.pone.0053418

Please email at least 30 days after Summer Scholarship to: research@crdc.com.au

---