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Cotton Research and Development Corporation

**ASSESSING THE LATEST MOLECULAR GENETIC MARKERS
FOR DETECTING POPULATION STRUCTURE AND MOVEMENT
IN *HELICOVERPA***



Project number:

UMON3C

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**A FINAL REPORT PREPARED FOR THE COTTON RESEARCH AND
DEVELOPMENT CORPORATION**

SUMMARISED REPORT

Modern molecular genetic techniques are used to examine *Helicoverpa armigera* for variation amongst individuals from different geographical locations around Australia. The prime objective has been to assess population structure: to find regionally diagnostic variation that will allow us, indirectly, to estimate the magnitude, direction and timing of movement patterns of these pest moths. This information would be valuable to the ecologists in their efforts to better predict moth movement into cropping areas and hence improve control. It would also help in planning strategies to minimise the spread of insecticide resistance and thus benefit the Cotton Industry.

Australia-wide mitochondrial DNA variation in *H. armigera* indicated a relatively homogeneous population, indicative of a species with high levels of mobility and gene exchange. Although some differentiation occurred in frequency of genetic types between samples from the south-east and both north-east and north-west Australia this was not at a magnitude that would be valuable or practical for estimating movement of moths into or out of the south-eastern area. Another genetic marker, a para sodium channel gene revealed a small but significant difference in frequency of genetic types between a Namoi Valley sample and one from St George, indicating some restriction to movement between these sites. Three hypervariable microsatellite loci are currently being sampled for geographic variation by our CRDC scholar, Ms Merrin Spackman, and the data will be reported later in 1996 when she submits her PhD thesis. Thus there are several genetic markers now known, including some described by Daly and Gregg (1985), that each show a level of regional differentiation. Each marker alone providing insufficient differentiation to be useful for the ecologists.

In future a multi-locus sampling programme (sampling individual moths from different localities for several genetic markers) will be the approach to use. The techniques and genetic markers are now available. To test the approach an intense sampling effort will be required in an important, though relatively isolated, cropping area. This would need to be carried out in close collaboration with ecologists and with accompanying ecological and climatic data. Exactly the same methodologies and approach will be valuable for *H. punctigera*.

By the end of 1996 we will know if the three microsatellite loci also hold promise for helping to diagnose moth origins. We will also report whether or not the DNA sequence of the mtDNA fragment used in our studies will be of taxonomic value for helping to sort out the Australian Heliiothines (a recent side benefit of our work that is being carried out in collaboration with Dr Marcus Matthews). This will be of benefit to the Cotton Industry because it would speed up future trials of new insecticides and pest control biocides

This project has laid valuable technique foundations for future *Helicoverpa* research. The techniques can be used to study inbreeding and mating patterns, genetics of migration and in linkage and gene mapping studies. Such information will be valuable for understanding insecticide resistance genetics and mechanisms, that will inturn help in programmes that are designed to prevent development of resistance, or help decrease the spread of resistance.

Introduction

Understanding movement patterns of adult *Helicoverpa* moths is vital to programmes aimed at control of these cotton pests. It enables the ecologists to provide more accurate predictions about where and when infestations are likely to occur. This facilitates earlier control measures, perhaps even in regions where breeding occurs prior to movement into cropping areas. It also helps in developing strategies to minimise the spread of insecticide resistance. In many species with a widespread distribution, marked genetic differentiation occurs among populations in different regions. If we can detect such differentiation, and quantify its magnitude, the information can be related to levels of movement and gene flow in the species. Once basic research has characterised the genetic/geographical distribution of the species, genetic types found in the 'wrong' region at times when the species is 'on the move' are implicated as migrants from specific locations. Thus by indirect methods moth movement patterns can be established.

This project examined Australia-wide variation among populations of pest *Helicoverpa* moths using the latest molecular techniques. Based on findings in other organisms, and on theoretical grounds, the genetic variation revealed by molecular techniques is potentially greater than found using earlier methods. Molecular genetic data has greater potential to detect heritable differences that might exist between moths from different geographical regions. Thus in order to find genetic markers that are of value to ecologists we aimed (i) to develop techniques, and (ii) take small preliminary samples from several widespread Australian populations of *Helicoverpa armigera*. Over the past two to three years it has become clear, from extensive work on population variation of hypervariable DNA regions in many species, that a multi-locus approach is likely to be the practical way to identify associations between geographic regions and genetic types. Thus, determination in single moths of genetic constitution for a number of variable genes is likely to reveal regionally diagnostic genetic types. With an awareness of this we have been attempting to find in *H. armigera* a number of different genes that each show some degree of regional differentiation. This approach also increases our chances of finding any one gene that itself might show high levels of regional differentiation. Methodologies developed for *H. armigera* are likely to be just as suitable for *H. punctigera*. This research was built on foundations laid earlier in an ARC and CRDC-funded joint project using *Helicoverpa punctigera*, jointly with Dr A. A. Hoffmann of LaTrobe University (ULA2C).

In April 1992 the *H. armigera* project became a viable reality with CRDC graduate studentship funding for Ms Merrin Spackman (UMON2C). She has provided the great majority of bench effort since that time, having her scholarship extended by CRDC to December 1995 (giving her a total of 3.75 years of financial support). Only in the last four months of 1995 has the final technique breakthrough occurred, enabling her to examine a new set of hypervariable genetic markers. This development was essential if we were to truly assess the value of the latest markers for understanding *Helicoverpa* movement, and for the work to be recognised as PhD material. For the first few months of 1996 Ms Spackman will be applying these new methods to assess geographic differentiation in *H. armigera* using the existing samples of moth DNA from five diverse Australian localities. With other grants coming to the laboratory this year we are able to financially support the maintenance aspect of the work, thus bringing Ms Spackman's project to completion. She will not have a living allowance during this time. However, she will obtain some remuneration from part-time teaching in our Department. Financial support for her to

assemble her thesis will also be available. Her thesis should be submitted by the end of 1996. Three additional publications will arise from her thesis (see, Communications: To Appear, below).

A side benefit of this project, once particular genes or gene regions have been sequenced, is that the methods will be in place to find the equivalent sequence in related species. This is likely to be valuable as a taxonomic/classification tool for a large group of hard-to-identify Australian native species. In fact, Ms Spackman is involved in collaboration on this with Dr Marcus Matthews, who is currently at CSIRO Entomology and, I understand, supported by CRDC. Thus we anticipate using the technique to 'chemically' define/identify as many of the Australian Heliothine species as possible. These data will be valuable when there are future needs to be confident of how many distinct Australian species might be susceptible to, or threatened by, any new insecticide or biological control agent under development, including genetically engineered 'pest-specific' pathogens. The information will speed up the process of testing and approving new pest control agents for environmental impact, and the Cotton Industry will benefit by earlier introduction of more effective control measures.

The techniques are tricky, expensive and time consuming to adapt to any new species, requiring specialist molecular biological skills. The project has completed this phase and established foundations. A future intense sampling programme, particularly in a single, relatively isolated, cropping area will enable the true value of the multi-locus approach to be ascertained.

Objectives:

- 1) To develop for *H. armigera* the technique to study naturally occurring molecular variation in the control region of mitochondrial DNA (mtDNA) and to use this to survey variation among samples of *H. armigera* from widely dispersed Australian sites.
- 2) To develop for *H. armigera* the techniques for expedient study of naturally occurring molecular variation of microsatellite loci and to use this to survey variation among samples from widely dispersed Australian sites.
- 3) To develop for *H. armigera* the techniques for expedient study of naturally occurring molecular variation of other hypervariable genes and to assess population variation.
- 4) To assess the value of mitochondrial DNA variation for clarifying the taxonomy of Australian Heliothine species that are closely related to *H. armigera* and *H. punctigera*.

Results and Discussion:

1. MtDNA variation:

The technique for examining mtDNA variation in *H. armigera* was developed and applied to samples from eight populations, Kununurra, Biloela, St George, Narrabri, Cowra, Myrtleford, Bairnsdale and Orbost. The final methodology for surveying mtDNA variation involved the PCR reaction and DNA sequencing. It proved to be the case that the common variants of *H. armigera*, unlike those of *H. punctigera*, were not discernable by the more expedient and cheaper methodology (PCR followed by restriction digest and gel electrophoresis). For this

reason our sample sizes remained relatively small. The DNA sequence was determined over 260 bp of the hypervariable control region from each of 148 moths. A total of 35 different genetic types (DNA sequences) were detected and described, making this a particularly variable species for this piece of DNA (compared to *H. punctigera*). Overall, the data suggest Australia-wide homogeneity of genetic types, a pattern consistent with the established high mobility of this species. Some genetic types are relatively common and occur in most regions, while others, usually rarer, are unique to particular samples. The 'uniqueness' of rarer types to certain samples may be largely a consequence of small sample sizes. However there were a few cases of small but significant differences between samples in allele frequency distributions. In an analysis that involved pooling together samples from geographically closer sites, and pooling genetic types that were more closely related, significant heterogeneity was detected between south-eastern and both north-eastern and north-western regions. Thus some degree of population structuring (restricted movement between sample sites) is indicated using mtDNA: particular genetic types being absent/rarer in Victorian samples.

2) **Microsatellite loci:**

Considerable effort was required before this methodology was perfected and three polymorphic loci were revealed. Data will be available shortly for five dispersed Australian sample sites as Ms Spackman finalises her laboratory work (DNA preparations from these sites are already in our freezer).

3) **Other genes:**

We examined molecular variation in a hypervariable part of one other gene, the para sodium channel gene, since it was known to show marked genetic differences among different southern populations of the American pest *Heliothis virescens*. After perfecting the technique for *H. armigera* 16 variants of the gene were found in samples of *H. armigera* from St George, Qld, and Namoi valley, NSW, with sampling help from Dr Neil Forrester. A significant difference in frequency distributions of these variants from the two areas occurred suggesting some restriction to gene flow. Since this gene has been shown to be associated with resistance to pyrethroid insecticides in the American species we also tested for an association with resistance in *H. armigera*, but no hint of an effect was detected.

4) **Molecular systematics using mtDNA:**

Collaboration with Dr Marcus Matthews has helped markedly in our awareness of important/cryptic species to sample, and helped with the actual collection of these moths. Preliminary analysis of the DNA sequence of the hypervariable part of the mtDNA control region has indicated that this sequence is too variable to be taxonomically useful. Fortunately however the other end of the large mtDNA fragment (the fragment that was amplified to study control region variation) contains part of a more conserved ribosomal RNA gene. Preliminary analysis of variation in this sequence indicates that it will be taxonomically useful. Ms Spackman is finishing this work at present and we are confident it will make a small publication of international and practical significance.

Conclusions:

1. A large number of mtDNA variants were found in *H. armigera*, many more than from a similar-sized sample of *H. punctigera*. Australia-wide mitochondrial DNA variation in *H. armigera* indicated a relatively homogeneous population indicative of a species with high levels of mobility and gene exchange. This marker alone will be of little value for estimating movement patterns. Although some differentiation occurred in frequency of genetic types between samples from the south-east and both north-east and north-west Australia (7,8,11) this was not of a magnitude that would be practical for estimating movement of moths into or out of the south-eastern area.
2. Another genetic marker, a para sodium channel gene, has been sampled, and three other loci are being sampled for geographic variation. For the channel gene a small but significant difference in frequency of genetic types occurred between a Namoi valley sample and one from St George, indicating some restriction to movement between these sites (10). We will report on the other loci later this year.
3. Earlier work on mtDNA variation of *H. punctigera* (largely ULA2C funded) indicated a pattern of variation that also suggested extensive gene exchange and thus moth movement among Australian sample sites for this species. Two samples, especially one from Mareeba in northern Queensland, had different unique variants at high local frequency, suggesting a restriction on movement of moths in these areas (3,4,11).
4. Since low levels of geographic variation at several loci, when these are examined together in individual moths, has real potential to reveal patterns of genetic differentiation that are regionally diagnostic, a multi-locus approach will be of future value (see Narang *et al.* 1994, Beltwide Cotton Conferences 2:780-90). Several gene loci are now available, including some reported by Daly and Gregg (1985, Bull. ent. Res. 75:169-184), and others will soon be available (see 6. below), making this an imminently feasible approach. Testing the idea will require an intense sampling effort in an important, though relatively isolated, cropping area. This would need to be a study in close collaboration with ecologists, where ecological data, known movement data and data on concurrent local climatic conditions, including wind patterns, are available. Exactly the same methodologies and approach would be possible for *H. punctigera*.
5. A gene that has been implicated as the underlying cause of pyrethroid insecticide resistance in a related species of moth was assessed for its possible role in resistance in *H. armigera*. The data suggested that this gene was not a major causal factor for resistance in the two Australian populations sampled (9,10).
6. Pending results:

By the end of 1996 Ms Spackman will have completed her research and submitted her PhD thesis. We will then know if the microsatellite loci hold promise for helping to diagnose moth origins (and whether a more extensive investigation of regional variation of these would be of value), and we will know if the mtDNA ribosomal gene region is of taxonomic value for helping to sort out the Australian Heliiothines.

7. Laboratory populations of *Helicoverpa* are often studied to better understand life history traits that are important for prediction of field population dynamics. In these efforts usually large mass-bred populations are established in the laboratory from many field caught individuals so as not to obtain atypical life-history parameters that might arise if a single cohort/family was studied. Our early work examined maternally inherited, mitochondrial DNA, variation in and between such "mass-bred" laboratory populations. The data indicated, surprisingly, that within 3 or 4 generations of laboratory culture a gross depletion of genetic variation occurred (1,2,3). This strongly suggests that very few of many founding females contributed progeny each generation, and that data from such populations is likely to be equivalent to that from a single family and therefore unrepresentative of field populations. Future work using large laboratory cultures of *Helicoverpa* should take this into account. Work should be on several independently maintained laboratory populations, perhaps 10 or more, in order to obtain reliable mean values for life history traits that are to be used for predicting field breeding performance.
8. This project has laid valuable technique foundations for future research into inbreeding and for linkage and gene mapping studies in these moths. Such studies will be valuable for understanding resistance genetics and mechanisms that will in turn help programmes to prevent development of resistance and/or help decrease the spread of resistance. We have added to the international data-base, for both the species, the DNA sequence of the 650 bp long control region of mtDNA (Genbank accession numbers U02678 and L17343, for *H. armigera* and *H. punctigera*, respectively).
9. An unexpected result in this study has provided valuable data that contributes to our understanding of evolutionary-genetic mechanisms. Very different patterns of molecular mtDNA variation occurred in the two morphologically and ecologically similar species. The data suggests that in the recent evolutionary history of *H. punctigera*, but not in *H. armigera*, a new advantageous mutation occurred and spread quickly through the species. Harmless mutations that are useful as genetic markers have accumulated in the mtDNA of *H. armigera*. In *H. punctigera* insufficient evolutionary time has passed, since the new mutation spread through the species, for this to have occurred.
10. This project has been of enormous value to our Department in providing a background for research training of Honours students, and in providing teaching examples for undergraduates and overseas agricultural research workers, mainly Asian, that participated over three years in an ACIAR teaching programme at Monash. We have continually acknowledged the support from CRDC in these programmes.

Communications:

1. McKechnie, S. W. and Hoffmann, a. a. (1992) Regional genetic divergence in a vagile Australian moth, *Helicoverpa punctigera*. Genetics Society of Australia: 39th Annual Conference. Abstract.

2. Hoffmann, A. A. and McKechnie, S. W. (1992) Native budworm populations may be regionally distinct. Proceedings of the 1992 Australian Cotton Conference, pp 359-363.
3. McKechnie, S. W., Hoffmann, A. A., Kovacs, I. V., Cacoyianni, Z., Naughton, N. E. and Katsabanas, S. (1993) Genetic variation among Australian populations of native budworm, *Helicoverpa punctigera* (Lepidoptera: Noctuidae). In "Pest Control and Sustainable Agriculture" S.A. Corey, D.J. Dahl and W. M. Milne eds. CSIRO Publications. Ch 11, pp 428-431.
4. McKechnie, S. W., Spackman, M. E., Naughton, N. E., Kovacs, I. V., Ghosh, M. and Hoffmann, A. A. (1993) Assessing budworm population structure in Australia using the A+T rich region of mitochondrial DNA. 1993 Proceedings of the Beltwide Cotton Conferences, New Orleans, Vol 2: 838-840.
5. Taylor, M. F. J., McKechnie, S. W., Pierce, N. and Kreitman, M. (1993) The Lepidopteran Mitochondrial Control Region: Structure and Evolution. Mol. Biol. Evol. 10: 1259-1272.
6. McKechnie, S. W. and Spackman, M. E. (1994) Large differences in mitochondrial haplotype diversity between closely related *Helicoverpa* moth species. Genetics Society of Australia: 41st Annual Conference. Abstract.
7. Spackman, M. E. and McKechnie, S. W. (1994) Assessing population structure in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), using variation in the A+T rich region of mitochondrial DNA. World Cotton Research Conference 1. In press.
8. Spackman, M. E. and McKechnie, S. W. (1995) Assessing the value of mitochondrial DNA variation for detecting population subdivision in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), in Australia. Proceedings of the Beltwide Cotton Conferences, San Antonio, Vol 2: 811-814.
9. McKechnie, S. W., Stokes, N. H. and Forrester, N. W. (1995) Natural allelic variation in a sodium channel gene from *Helicoverpa armigera* detected by temperature gradient gel electrophoresis. Genetics Society of Australia: 42nd Annual Conference. Abstract.
10. Stokes, N. H., Forrester, N. W. and McKechnie, S. W. (1996) Multiple allelic variation in a sodium channel gene from *Helicoverpa armigera* (Lepidoptera: Noctuidae) suggests geographical differentiation but no association with resistance to pyrethroid insecticide. Submitted to J.Aust. Ent. Soc.

To appear:

11. Spackman, M. E. and McKechnie, S. W. Contrasting levels of DNA sequence variation of mitochondrial control region in two closely related *Helicoverpa* moths. In preparation for Genetics.

12. Spackman M. E. and McKechnie, S. W. Australia-wide microsatellite variation in the pest moth *Helicoverpa armigera* (Lepidoptera:Noctuidae). In preparation for Aust. J. Zoo.
13. Spackman, M. E., Matthews, M. and McKechnie, S. W. Mitochondrial DNA sequence variation as an aid in the taxonomy of Australian Heliothine moths. In preparation for Biochemical Systematics.

Appendix:

<u>Budget:</u>	Contribution for Dr S.W. McKechnie to attend the 1993 Beltwide Cotton Conference	\$ 1,000
	UMON3C - Maintenance July 1993 - June 1995	\$ 25,000
	UMON2C - Graduate Studentship for Ms Merrin Spackman April 1992 - March 1995 Studentship extended to Dec 1995	\$ 69,600 \$ 19,000

<u>Total</u>	: April 1992 to December 1995 (About \$31,000 per year over 4 years)	\$ 114,600

No special considerations, no further supporting data, and no Royalty and Intellectual property arrangements are relevant.

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