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Management Research Unit
1926-05-00-Beneficial Insect Introduction Research Unit

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Newark, Delaware

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Genetics of host specificity and climatic adaptation in biological control agents introduced for control of arthropod pests and weeds

Investigators
Keith R. Hopper, Lead Scientist……..90%

Scientific Staff Years
0.9

Planned Duration
60 months

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SIGNATURES

Keith R. Hopper, CRIS 1926-22000-013-00D, Genetics of host specificity and climatic adaptation in biological control agents introduced for control of arthropod pests and weeds

This project plan was revised, as appropriate, according to the peer review recommendations and/or other insights developed while considering the peer review recommendations. A response to each peer review recommendation is attached. If recommendations were not adopted, a rationale is provided.

_____ _____

Research Leader Date

This final version of the project plan reflects the best efforts of the research team to consider the recommendations provided by peer reviewers. The responses to the peer review recommendations are satisfactory.

_____ _____

Center, Institute, or Lab Director Date

This final version of the project plan reflects the best efforts of the research team to consider the recommendations provided by peer reviewers. The responses to the peer review recommendations are satisfactory.

_____ _____

National Program Leader Date

The attached plan for the project identified above was created by a team of credible researchers and internally reviewed and recognized by the team's management and National Program Leader to establish the project's relevance and dedication to the Agricultural Research Service's mission and Congressional mandates. The project plan has completed a scientific merit peer review in accordance with the Research Title of the 1998 Farm Bill (PL105-185) and was deemed feasible for implementation. Reasonable consideration was given to each recommendation for improvement provided by the peer reviewers.

_____ _____

Area Director (original signature required) Date

PROJECT SUMMARY

Host specificity is crucial for biological control of insect pests and weeds. The likelihood that a host-specific insect will evolve to attack a novel host species depends on how many genes are involved, how these genes interact, how much change in each gene is needed to cause a shift in host use. The first objective is to determine the genetic basis for host specificity in certain parasitoids and herbivores. Using crosses, quantitative trait loci (QTL) mapping and differences in gene expression, we will determine the genetic basis for a host shift in the specialist herbivore, *Heliothis subflexa*, and for differences in host specificity between species in the *Aphelinus varipes* complex. We will confirm gene function by silencing with RNA interference.

Climate matching is frequently used to decide where to collect biocontrol agents for introduction. However, three hypotheses can explain climatic adaptation: (1) populations in different regions are adapted to local climates, (2) single populations have the full range of genetic variation in traits affecting climatic adaption, (3) physiological plasticity is sufficient for local adaptation. These hypotheses have very different implications for collection strategies. The second objective is to test these hypotheses using the *A. varipes* complex.

In the third objective, we will use the knowledge and methods developed under objectives 1 and 2 to screen candidates proposed for introduction to control *Diuraphis noxia* and *Aphis glycines*, introduce the most promising candidates, evaluate their impact on target and non-target species, and determine whether the screening was useful in improving the success and safety of biocontrol introductions. This results of this research will increase establishment and efficacy of introduced natural enemies and decrease damage by pests, while increasing the safety of biocontrol introductions.

OBJECTIVES

Host specificity is crucially important for the efficacy and safety of introductions of biological control agents to control insect pests and weeds. In *Objective 1 (Determine the genetic and evolutionary basis for host specificity of insect parasitoids and herbivores)*, we will test the hypothesis that host use is determined by many genes of small effect interacting epistatically, rendering saltational changes in host range unlikely.

Climate matching is frequently used to decide where to collect biocontrol agents for introduction. In *Objective 2 (Determine the importance of climatic adaptation for establishment and growth of introduced populations of insects)*, we will test three competing hypotheses about climatic adaptation: (1) populations in different regions are irreversibly adapted to local climates, (2) single populations have essentially the full range of genetic variation in traits involved in climatic adaption, or (3) physiological plasticity is sufficient for local adaptation.

In *Objective 3 (Screen, introduce, and evaluate impact of candidates for biological control introductions, based on host specificity and climatic tolerances)*, we will use the knowledge and methods developed under objectives 1 and 2 to screen candidates proposed for biocontrol introductions, introduce the most promising candidates, evaluate their impact on target and non-target species, and determine whether the screening was useful in improving the success and safety of biocontrol introductions.

NEED FOR RESEARCH

Description of Problem to be Solved: Unintentional introductions of exotic species that become pests is an increasing problem for agriculture, human health, and the environment. Increasing cost of pesticide development, rapid evolution of target resistance to pesticides, pest resurgence after destruction of natural enemies by pesticides, and public concern about health and environmental impact of pesticides demand alternatives for pest management. For introduced pests, biological control by introduction of exotic natural enemies is an important way of reducing pest abundance and thus impact. Although biological control introductions have been practiced for over 100 years, not enough is known about the factors that determine success. Furthermore, controversy has arisen concerning the risk of impact on non-target species. This project will use past and new introductions to test the effects of host specificity and climatic adaptation on natural enemy establishment, efficacy, and impact on non-target species.

Relevance to ARS National Program Action Plan: This research addresses components III 'Plant, pest, and natural enemy interactions and ecology', V 'Pest control technologies', and IX 'Biological control of weeds' of ARS National Program 304 on 'Crop Protection and Quarantine'.

Potential Benefits: This research will increase establishment and efficacy of introduced natural enemies and thus decrease damage by pests, while increasing the safety of biocontrol introductions. This will benefit U.S. farmers by reducing their losses to pests and costs for production, and it will benefit the general public by decreasing costs of food and fiber and reducing adverse impacts on human health and the environment. Target pests for this project were chosen because of their high impact on U.S. agriculture. *Diuraphis noxia*, the Russian wheat aphid, is a major pest of wheat and barley, causing over $1 billion damage since 1987. *Aphis glycines*, the soybean aphid, infested 42 million acres of soybean in the US, causing $100 million in damage in 2003 alone. Weeds in the US are estimated to cost American agriculture $13 billion annually in control expenditures and yield losses (ARS National Program Plan). Environmental impacts of weeds are difficult to quantify, but the President's Executive Order on Invasive Species (3 February 1999) states that they are a major threat to our parks, forests, rivers, and shores.

Anticipated Products: This research will provide knowledge and methods for

practitioners of biological control, both public (university, state, and Federal) and private (commercial vendors, farmers, and farmers' cooperatives, and land managers), to improve the establishment and efficacy of exotic natural enemies for control of arthropod pests and weeds and reduce risks of non-target impacts. Results will be published in peer-reviewed scientific journals and thus be accessible to the scientific community. The third objective addresses introduction of candidates that will control major pests.

Customers: Two categories of customers will benefit from results of this program. The first is users of published research, who are scientists at universities, state departments of agriculture and natural resources, federal agencies, and conservation organizations, and decision-makers at state departments of agriculture and natural resources, federal agencies, conservation organizations. The second includes farmers growing crops attacked by target pests, who benefit from reduced production costs and reduced exposure to toxic substances, and the general public, who benefit from reduced prices, reduced health risks from pollution, reduced impact on non-target species.

SCIENTIFIC BACKGROUND

Objective 1: Determine the genetic and evolutionary basis for host specificity of insect parasitoids and herbivores. Host specificity is crucially important for biological control of insect pests and weeds. First, host specificity determines whether natural enemies from one host species can be used against other host species and whether reservoirs of natural enemies can persist in the field when the target pest becomes rare. Second, host specificity determines whether unwanted shifts of biocontrol agents to attack non-target species are likely to occur after introduction. The safety of biocontrol introductions could be increased by improved host specificity testing and better understanding of the evolution of host specificity [1]. Although host specificity screening of candidates for insect control has been implemented and such screening of weed biocontrol agents is well developed [2], concerns have been raised about the potential for evolution of host range [3]. The evidence about such evolution in species introduced for biocontrol is weak and controversial (e.g., [4] versus [5]). Research is needed on the genetics and evolution of host range in various taxonomic and functional groups of biocontrol agents (e.g., parasitoids versus herbivores). Experiments on the mechanisms of host specificity and its underlying genetic basis are crucial. Such experiments will be most efficient with **model organisms** that are easy to rear and cross in the laboratory and for which some genetic data are available. The likelihood that a parasitoid or herbivore would evolve to attack a novel host species depends on: (1) how many genes are involved, (2) how these genes interact, and (3) how much change in each gene is needed for a shift in host use. We have begun to address these questions using the generalist herbivore *Heliothis virescens* and the closely related specialist *Heliothis subflexa* (Lepidoptera: Noctuidae). We chose this system because (1) the general biology and especially rearing and crossing techniques are well known, (2) although they are closely related, they differ greatly in host range, (3) the two species can be hybridized. **We are using *H. subflexa* as a model for evolutionary host shifts in agents introduced for biological control of weeds.** We introgressed *H. virescens* genes for feeding on cotton (a plant far outside the host range of *H. subflexa*) into the *H. subflexa* background by hybridizing the two species and repeatedly backcrossing the progeny to *H. subflexa*, while selecting for larval feeding on cotton. We used amplified fragment-length polymorphism (AFLP) markers [6], to map quantitative trait loci (QTL) involved in feeding on the novel host plant [7]. QTL mapping is a powerful technique for determining the number and nature of genes involved in quantitative genetic traits [8] and has been used to study the genetics of physiological, behavioral and life-history traits in *Drosophila melanogaster* (Diptera: Drosophilidae) [9], *Apis mellifera* (Hymenoptera: Apidae) [10], and *H. virescens* [11], among others. After three generations of backcrossing with selection, genes on 2 out of 31 chromosomes in the *Heliothis* genome explained 62% of the variance in larval feeding on cotton (F=15.31; df = 2,19; P<0.0001). This

is a much smaller number than anticipated and suggests that evolutionary shifts in host use in this species complex may be rather likely. Although the laboratory population of *H. subflexa* we used for the hybrid crosses described above showed almost no larval feeding on cotton, field-origin *H. subflexa* from Texas and North Carolina do feed on cotton. Indeed, some field-origin *H. subflexa* ate as much as *H. virescens* in our 24 hour assay. As measured by response to one generation of selection, 48% of variation in feeding on cotton by 5th instar *H. subflexa* was heritable. We crossed lines from Texas selected for feeding on cotton with insects from the laboratory colony which did not feed on cotton and backcrossed the F_1 progeny in both directions. Backcross larvae showed segregation for genes involved in feeding on cotton.

Parasitoid species may consist of distinct host races that switch little between host species in the field [12-17]. Differences in host use among populations may often be explained by unrecognized sibling species. Recent evidence suggests that sibling species of parasitoids may be far more common than previously realized [18-20]. For example, although *Aphelinus varipes* has been reported from 40 host species across several genera of aphids [21], we found distinct patterns of host use among *A. varipes* from different hosts and regions (Fig. 1). However, most of these populations showed fixed differences in DNA sequences (Fig. 2), subtle but highly significant differences in morphology (data not shown), and were reproductively incompatible (Table 1). Thus, the host range reported in the literature for *A. varipes* is incorrect because sibling species have been confounded. Although closely related species sometimes show similar patterns of host specificity, phylogenetic affinity has not proven to be a reliable indicator of host specificity. Even among the rather closely related species and populations in the *A. varipes* complex, use of some host species roughly maps onto the parasitoid phylogeny, but use of other species does not (Fig. 3). Therefore, we need to examine the genetic basis of host switches if we are to predict when they will occur. Two populations in the *A.varipes* complex, one from *D. noxia* in Georgia ('Georgia-*D. noxia*') and the other from *A. glycines* in Japan ('Japan-*A. glycines*') were reproductively compatible, despite differences in DNA sequences, morphology, and host use. 'Japan-*A. glycines*' parasitoids do not parasitize *D. noxia*, whereas 'Georgia-*D. noxia*' parasitoids readily parasitize this host (Fig. 1). **This difference results from differences in oviposition, not survival, in *D. noxia*.** The most direct test of post-introduction evolutionary shifts in host specificity involves retrospective analyses of the host ranges of previously introduced natural enemies. Parasitoids in the genus *Aphelinus* spp. have been introduced and established in North America to control several aphid species [22-26]. These parasitoids have been exposed to new aphid species since their introduction and may have evolved new patterns of host use. Because we have measured host specificity among species and populations in the *A.varipes* complex from their source regions and we have good molecular markers for each species/population, allowing us to identify sources of the populations established in the US, we can compare host ranges between introduced and source populations to determine whether there have been evolutionary host shifts.

Objective 2: Determine the importance of climatic adaptation for establishment and growth of introduced populations of insects. Failure to adapt well to a new climate after introduction has often been suggested as a major reason why introduced natural enemies have failed to establish or to control target pests [27]. In response, recommendations concerning biocontrol introductions include both a match between source and target climates and genetic diversity in material being introduced [28]. However, three competing hypotheses can be invoked concerning climatic adaptation: (1) populations in different regions are genetically adapted to local climates, (2) single populations have essentially the full range of genetic variation in traits involved in climatic adaption, (3) physiological plasticity is sufficient for local adaptation so there is little genetic variation in climatic adaptation traits either between regions or within populations. Under the first hypothesis, introduced insects would only establish and flourish where the source and target climates were similar, and no post-introduction evolution would occur. Under the second, climate matching would be unnecessary because a sufficient sample of insects from single population would be able to establish in a wide range of climates

through post-introduction evolution. Under the third hypothesis, climate matching would be unnecessary, and even a very limited sample of insects from a single population would be able to establish in a wide range of environments without post-introduction evolution. Unfortunately, evidence concerning these hypotheses is weak for biocontrol introductions. The true usefulness of climate matching is ambiguous, recommendations about how to actually match climates are rare and often obscure. Thus, some researchers are skeptical about the value of climate matching [29, 30]. In a retrospective analysis of published cases, species introduced from the tropical to temperate zones established less often than other sorts of introductions (temperate-temperate, temperate-tropical) [31], but this is a very coarse level of climate matching and unlikely to be useful for most introductions. Many apparent cases of within-species geographic variation appear to actually involve sibling species [19] that may differ in traits other than climatic adaptation. Furthermore, introduction of new material often has no effect on establishment or efficacy [19]. On the other hand, well-documented examples of post-introduction evolution of insects are rare [32-34]. Although extensive and elegant theoretical analyses and empirical observations on the population genetics of colonization have been published, almost all of this work has dealt with selectively neutral alleles, and not with traits affect fitness [35, 36]. Most studies on traits affecting fitness have been on introduced plants, not insects [34, 37], and the results on plants can rarely be extrapolated to insects.

Objective 3: Screen, introduce, and evaluate impact of candidates for biological control introductions, based on host specificity and climatic tolerances. One of the most promising options for sustainable management of *D. noxia* and *A. glycines* is introduction of natural enemies from source regions [25, 38]. Experiments and field surveys have shown that natural enemies reduce density and population growth rate in source regions of both pests [39-41]. Exploration and introduction of natural enemies of *D. noxia* during 1989-1994 resulted in establishment of three parasitoid species, one in the *A.varipes* complex, one in the *A.asychis* complex, and *Aphidius uzbekistanicus* (Hymenoptera: Braconidae) [26], although they have not provided substantial control of this pest [42]. Subsequent exploration has provided another parasitoid species, with a very narrow host range, which is a promising candidate for release against *D. noxia*. During exploration for natural enemies of *A. glycines* in 2001-2004, we obtained 15 populations/species of parasitoids and predators from China, Japan, and Korea, with collaboration of colleagues at state universities (Profs. George Heimpel and David Ragsdale, University of Minnesota; Prof. Robert O'Neil, Purdue; Prof. David Voegtlin, Illinois Natural History Survey), overseas ARS laboratories (Drs. Kim Hoelmer and William Meikle, European Biological Control Laboratory, ARS-USDA), and foreign institutes (Prof. Wu Kongming, Chinese Academy of Agricultural Sciences). These natural enemies are promising biological control agents because they were collected in regions of Asia very similar climatically to those where *A. glycines* has established in the U.S. and they were found where *A. glycines* density was low, suggesting that they will be effective at low host densities. So far, we have measured the host range of eight *A.varipes* complex populations from China and Japan and an *A. asychis* population from China. *Aphelinus asychis* from China parasitized all 7 aphid species to which it was exposed, and the populations of *A.varipes* complex parasitoids parasitized 6 out of 7 aphid species in four of the five genera to which they were exposed (the exception being *D. noxia*). The host specificities of the species and populations tested so far show that we should continue the search for parasitoids with narrower host ranges. The methods used for measuring impacts on target pests are well developed [43]. These techniques fall into three categories: (1) field surveys; (2) exclosure experiments; and (3) pre/post introduction experiments. The same methods can be used to measure impacts of introduced natural enemies on non-target species. Mathematical models are useful when based on data sets collected over sufficient space and time and including measurements of climate and habitat so that interpolation is possible. Without experimental manipulations, field survey data can be difficult to translate into estimates of population level impacts. However, models developed from data collected in one set of places or times may successfully predict dynamics in other

places or times. Such models may provide estimates of impact or lack thereof impossible to achieve with experimental manipulations because of the mobility of arthropod species. Exclosure experiments have been found to be powerful tools for testing the impact of biocontrol agents on target weeds [44] and insects [39], especially when used in conjunction with analyses of field survey data [40] and other manipulations [45]. These techniques should be just as powerful for tests of impacts on non-target species. The probability of impacts on non-target species is extremely difficult to evaluate, given the current state of ecological theory and its thin experimental foundation. Unfortunately, possible impact and actual evidence for impact have sometimes not been carefully separated in discussions about the risk of biocontrol introductions. Despite a long history of concern about impacts of introduced natural enemies on non-target species [46-48] and heightened debate over the issue during the last 15 years [3, 49-56], there appear to be no rigorous studies testing whether arthropods intentionally introduced for biocontrol have reduced abundances, let alone caused extinctions, of native non-target species [57, 58]. Although several studies document attack on native non-target species [59, 60], almost none have tested even local changes in abundances.

CSREES-CRIS Search - Research results from this project will enhance the following projects, either directly through screening candidates for biological control introductions or indirectly by developing principles for carrying out such screening: CRIS 1926-22000-012-00D - Development of Biological Control and Related Technologies for Invasive Insect Pests with Emphasis on The Asian Longhorn Beetle (Newark, Delaware); CRIS 1926-22000-014-00D - Biological Control of Gypsy Moth and Other Tree Pests and Quarantine Service for Beneficial Insects (Newark, Delaware); CRIS 1926-22000-015-00D - Classical Biological Control Insect Pests of Crops (Newark, Delaware); CRIS 4012-22000-017-00D – Foreign Exploration for Natural Enemies of Insects, Weeds, and Plant Pathogens (Montpellier, France); CRIS 4012-22000-019-00D – Discovery, Biology & Ecology of Natural Enemies of Insect Pest of Crops, Urban & Natural Areas (Montpellier, France); CRIS 6204-22000-016-00D - Biological Control of Exotic and Invasive Pests (Weslaco, Texas); CRIS 6217-22000-012-00D – Biorational Cereal Aphid Management (Stillwater, Oklahoma). An additional 8 projects are somewhat related.

APPROACHES AND PROCEDURES

Objective 1: Determine the genetic basis and evolutionary basis for host specificity of parasitoids and herbivores being introduced for biological control.

Sub-objective 1a – Determine the genetic architecture and evolution of host specificity in the Aphelinus varipes complex.

Experimental Designs *Approaches* - We will test the hypothesis that parasitism of a particular host species is determined by many genes of small effect that interact epistatically. If this hypothesis is true, changes in genetic host range are unlikely. To test this hypothesis, we will determine the genetic basis for differences between two species in the *A. varipes* complex in parasitism of *D. noxia*. The 'Georgia-*D. noxia*' parasitoids and 'Japan-*A. glycines*' readily hybridize (Table 1), but 'Japan-*A. glycines*' does not parasitize *D. noxia*, whereas 'Georgia-*D. noxia*' readily parasitizes this host (Fig. 1). Using a positional cloning approach, we will map QTL and identify genes involved in parasitism of *D. noxia*. We will cross males and females from the two species, backcross F_1 hybrid females to 'Japan-*A. glycines*' males and measure parasitism of *D. noxia* by backcross females. Using the results, we will map QTL affecting parasitism of *D. noxia*. We will test whether gene expression differs backcross females that do and do not parasitize *D. noxia*. We will sequence genes that are differentially expressed and compare sequences with those in Genbank to see if they resemble genes of known function. We will develop markers from differentially expressed genes to test whether they segregate with QTL associated with parasitism of *D. noxia*. Assuming we identify genes involved in parasitism of *D. noxia*, we will use them to estimate a phylogeny and test how well the phylogeny based on

these genes matches that based on putatively neutral sequence differences (see 'Scientific Background'). To do this, we will sequence the parasitism genes in all seven members of the complex for which we have measured patterns of host use and use the sequences to construct a phylogeny. We will test concordance of the two phylogenies.

Procedures - The parasitoid colonies for this research originated from parasitized *D. noxia* collected in the Republic of Georgia in 1998 and from parasitized *A. glycines* collected in Japan in 2001. We will rear the parasitoids on their original hosts on wheat or soybean as replicated colonies (6 replicates per species with a population size of >200 adults parasitoids per replicate) at 20 or 25^{o}C, 50-70 % RH, and 16 hour photoperiod. The aphid colonies for this research originated from *D. noxia* collected in Wyoming in 1996, and *A. glycines* collected in Delaware in 2004. We will rear the aphids under the same environmental conditions as the parasitoids. To generate fine-scale maps of QTL for parasitism of *D. noxia*, we will assay 2000 virgin parasitoid females from backcrosses of F_1 females to 'Japan-*A. glycines*' males. Because each F_1 female will provide 25-50 backcross females, this will require about 50 F_1 females, which we should be able to obtain from 1-2 hybrid crosses. We will assay parasitism of *D. noxia* by exposing each backcross female to *D. noxia* nymphs for 24 hours and scoring the number of aphids parasitized. We will use >50 aphids per female to ensure the parasitoids do not exhaust the supply of unparasitized aphids (daily fecundity of these parasitoids is 8-13). Both 'Georgia-*D. noxia*' and 'Japan-*A. glycines*' accept and develop in *A. glycines*. Thus to avoid selection on parasitism of *D. noxia*, we will rear F_1 and backcross females on *A. glycines*. Assaying 2000 backcross females should allow us to map to <1 cM. To estimate the number of markers needed for mapping with this precision, one needs estimates of physical and recombination genome sizes. The most closely related species for which genome size data are published is *Nasonia vitripennis* (Hymenoptera: Pteromalidae), whose physical genome is 340 Mb [61] and recombination genome is 765 cM [62], giving 445 Kb/cM. Other bees and wasps have physical genomes between 160 and 350 Mb [63]. Assuming similar values for species in the *A. varipes* complex, mapping to within <1 cM would require about 1500 markers which would require analysis with about 100 selective AFLP primer pairs (assuming 15 polymorphic markers per primer pair). To reduce the number of primer pairs to a reasonable number (e.g., 10-20), we will first map QTL to chromosome, of which there are 4 in the species in the *A. varipes* complex whose relative lengths are 0.4, 0.25, 0.19, and 0.16 (unpublished data). If the QTL are on the largest chromosome, we will map QTL crudely within that chromosome. We will use markers flanking the QTL at the chromosomal or crude sub-chromosomal level in bulk-segregant analysis to find more markers for fine-scale mapping. Using SAGE (serial analysis of gene expression [64]) or SSH (suppression subtractive hybridization [65]), we will test for differences in gene expression between backcross females that do or do not parasitize *D. noxia*. If we find differentially expressed genes, we will sequence them, develop markers from the sequences, and determine whether they are linked to the QTL identified in the analysis of backcross insects. We can use the same backcross females for this linkage analysis, which will save much labor and time. We will compare differentially expressed genes with sequences in Genbank to see if they resemble genes of known function. More details on molecular methods are given in the Appendix.

The difference between 'Japan-*A. glycines*' and 'Georgia-*D. noxia*' in parasitism of *D. noxia* results from differences in oviposition in this host (see 'Scientific Background'). However, differences in parasitism of *D. noxia* by backcross females might also involve differences in progeny survival. We will test this with two approaches. First, we will test whether backcross females that fail to reproduce have laid eggs in the host. To do this, we will compare egg loads in females that have reproduced to those that have not. Although *Aphelinus* spp. are synovigenic (i.e., continue to produce eggs during adulthood), the full egg load for species in this complex is 10-20, which can quickly be exhausted if hosts are abundant and takes time to replace, so that females that have recently laid eggs will have a lower egg load than those that have not. If the egg load of backcross females that reproduce is consistently lower than those

that do not reproduce by an amount comparable to the number of progeny per female, this will indicate that failure to reproduce results from failure to oviposit rather than low survival after oviposition. If egg load in backcross females that do not reproduce is bimodal, with some females having egg loads similar to females that reproduce and others having egg loads similar to the full complement, this will suggest segregation of genes affecting survival, independently of genes affecting acceptance. In the latter case, we will map QTL affecting survival, using egg load as surrogate. To further distinguish between QTL associated with host acceptance and progeny survival, we will expose each backcross female to *A. glycines* as well as *D. noxia*. For females that reproduce in both hosts, we will test whether markers that occur in these females differ in frequency between progeny from each host. Markers associated with QTL affecting survival in *D. noxia* would be more common in progeny from *D. noxia* compared to those from *A. glycines.*

Contingencies - Genes involved in parasitism of *D. noxia* are may not be expressed in adult females. For example, they might be genes that control development of pathways of interneurons. In this case, identifying genes by differential expression will fail. An alternative would be to use a BAC (bacterial artificial chromosome) library. One could sequence flanking markers for each QTL to develop probes and then probe the library for BAC(s) which contain flanking markers. After determining which BAC(s) are likely to contain QTL, one could sequence the BAC(s) and analyze the sequences for candidate genes by searching for open reading frames and by comparing sequences those in GenBank. Thus, it would be useful to have a BAC library for the *Aphelinus-varipes* complex. We will apply to the NIH BAC Resource Network for grant support to develop an *Aphelinus* BAC library. Assuming we obtain support, we will commission a BAC library from the Texas A&M BAC Center or one of the other centers that specialize in developing them.

Collaborations - *Outside ARS:* James Woolley (Texas A&M University) and John Heraty (University of California, Riverside) will collaborate on phylogenetics and morphometrics.

Objective 1b: Determine whether there has been post-introduction evolution of host range in the Aphelinus varipes complex.

Experimental Design *Approach* - We will test whether host specificity of introduced species in the *Aphelinus-varipes* complex have evolved since introduction. To do this, we will first determine the sources of populations in this complex that have established in the U.S. on *D. noxia* and other aphid species by comparing DNA sequences to those from the species for which we have measured host specificity. For the established populations for which we have host range data from the source population, we will compare host specificity of the population established in the US with that of the source population.

Procedures - We will collect aphids parasitized by populations of the *A.varipes* complex established on *D. noxia* in the western US (Colorado, Wyoming, and Washington). Besides collecting parasitized *D. noxia*, we will also collect other aphid species parasitized by aphelinids on cereals and surrounding host plants. **We will compare DNA sequences from these parasitoids with those from Eurasian populations/species, using genes that have been diagnostic for distinguishing among populations and species in this complex (*ArgK, COI, COII, ITS1, ITS2*). Adults will be killed directly in 95% EtOH and stored at -80C. They will be dried in open Eppendorf tubes at 32^{o}C for 30 minutes prior to extraction. Total genomic DNA will be purified using either a phenol-choroform extraction for minute insects [18] or the Chelex® extraction method [66]. Although Chelex extractions provide a greater volume of extract, two gene regions, ITS1 and ArgK, could not be amplified from this material. Three nuclear (*ITS1, ITS2, ArgK*) and two mitochondrial (*COI, COII*) gene regions will be PCR-amplified. The following forward (F) and reverse (R) primer combinations will be used: 28S-D2, (F) 5'-CGT GTT GCT TGA TAG TGC AGC-3' and (R) 5'-TCA AGA CGG GTC CTG AAA GT-3'; ITS1, (F) 5'-GTT TCC GTA GGT GAA CCT GC-3' and**

(R) 5'-GAG AAC AGC AGG AAC ACA GAA-3'; ITS2, (F) 5'-TGT GAA CTG CAG GAC ACA TG-3' and (R) 5'-TCT CGC CTG CTC TGA GGT-3'; ArgK, (F) 5'-GAT CAT CTT CGT ATT ATT TCC ATG-3' and (R) 5'-GTA CCC AAG TTA GTC GGG CA-3'; COI, (F) 5'-TAT ATT TTA ATT YTW CCW GGA TTT GG-3' and (R) 5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3'; COII, (F) 5'-ATT GGA CAT CAA TGA TAT TGA-3' and (R) 5'-CCA CAA ATT TCT GAA CAT TGA CCA-3'. PCR products will be purified using QIAgen PCR Purification Kit, direct sequenced in both directions, and analyzed on a CEQ 8000.

We will cross the introduced populations with the putative source population to confirm reproductive compatibility. In laboratory experiments, we will test host specificity using eight aphid species: *Acyrthosiphon pisum* (on alfalfa), *Aphis glycines* (on soybean), *Aphis gossypii* (on cotton), *Myzus persicae* (on radish), *Diuraphis noxia*, *Rhopalosiphum maidis*, *Rhopalosiphum padi*, and *Schizaphis graminum* (on barley). We will measure the propensity of each parasitoid species to parasitize each aphid species by exposing them in no-choice experiments and recording the number of mummified aphids and adults emerged. For each combination of parasitoid and aphid species, we will expose 10-20 two-day-old, sugar-fed, mated females individually to 100 aphids of mixed stages for 24 hours. We will rear the aphids to determine the number of mummies formed, number of adult parasitoids emerged, and adult sex ratio. We will use analyses of variance to test the effects of parasitoid source and host species on number of mummies, adult emergence, and progeny sex ratio.

Contingencies - Currently, we have three species in the *A.varipes* complex from Eurasia in culture which parasitize *D. noxia*. If none of these match established populations, we will compare DNA sequences from established populations with those from voucher material from exploration for natural enemies of *D. noxia,* but for which host specificity was not measured. If sequences match, we will collect live material from the source population and measure host-specificity as described above. If we are unable to identify the source of established populations in the *A.varipes* complex, we will switch to studying populations which will be introduced under Objective 3.

Sub-objective 1c: Determine the genetic basis of host use shifts in Heliothis spp.

Experimental Designs *Approaches* - We will test the hypothesis that host plant use by a specialist herbivore is determined by many genes of small effect that interact epistatically, so that saltational changes in host range are unlikely. We will continue to study the genetic basis of host shifts in *H. subflexa*. Using a positional cloning approach [11], we plan to identify the genes involved in larval feeding on cotton, and we plan to expand the traits examined to include adult oviposition. We will make a fine-scale map of QTL involved in larval feeding on cotton using fifth-generation backcross progeny from the hybridization of *H. virescens* and *H. subflexa* described under 'Scientific Background'. We will sequence flanking markers for each QTL to develop probes and then probe the *H. virescens* BAC library (publically available from Texas A&M University) for BACs which contain the markers. After determining which BAC(s) are likely to contain QTL, we will sequence the BAC(s) and analyze the sequences for candidate genes by searching for open reading frames and by comparing sequences from the BAC(s) with sequences in GenBank. Once we have identified candidate genes, we will silence gene function by transforming *H. virescens* [67, 68] to express hairpin RNA homologous to mRNA for candidate genes [69]. We will assay feeding on cotton by larvae from the transformed lines to determine whether gene silencing has changed phenotype. Using the backcross progeny from crosses of high and low feeding selection lines of *H. subflexa* described under 'Scientific Background', we will also map QTL and identify genes in *H. subflexa* that control larval feeding on cotton. Using probes developed from cotton-feeding genes identified in *H. virescens*, we will test whether the genes from *H. virescens* are in the regions where QTL are found in *H. subflexa,* suggesting that they may be the same genes. If so, we will silence function of these genes in *H. subflexa*, using the same approach as in *H. virescens*. We will also study oviposition on a novel host plant species. Because larvae are relatively immobile, oviposition by adult females

determines to a great extent which host plants are available for larval feeding. Thus for a switch to a new host plant species, changes must occur in both oviposition and in larval feeding. To determine the genetic changes required for a shift in oviposition, we will take the same positional cloning approach as for identifying genes involved in larval feeding. We will introgress genes for oviposition on cotton from *H. virescens* into *H. subflexa* by hybridizing the two species and repeatedly backcrossing the progeny to *H. subflexa* and while selecting for oviposition on cotton. Using AFLP markers, we will map QTL for oviposition on cotton, develop probes from the flanking markers, probe the *H. virescens* BAC library, identify candidate genes, and silence gene function with transformation to express hairpin RNA homologous to mRNA for candidate genes [69].

Procedures – To map the QTL for larval feeding on cotton, we introgressed genes for larval feeding on cotton from *H. virescens* into the *H. subflexa*. To assay larval feeding on cotton, we exposed newly molted fifth instars for 24 hours to excised cotton leaves placed on moist filter paper in Petri dishes. Fifth instar *H. virescens* will eat most to all of a 100 cm^2 cotton leaf in this period; fifth instar *H. subflexa* from the NCSU colony will eat none to less than 25 mm^2 of a 100 cm^2 cotton leaf in this period. For leaves with any feeding, we captured their images using a flatbed scanner and analyzed the images for area eaten. All leaves where traced or scanned prior to exposure to larvae so that where necessary we could determine amount eaten by subtracting area remaining after feeding from area present before feeding. Larvae were reared on artificial diet before and after feeding, allowed to pupate (when they were sexed), and frozen as adults, either immediately or after have being used in matings. In the *H. virescens* x *H. subflexa* hybridization, we used a single F_1 cross so one set of AFLP markers can be used for the whole progeny set and each generation can be treated as a single large family for QTL mapping. For the first 4 generations (BC_{1-4}), we backcrossed hybrid females to *H. subflexa* males. Because recombination does not occur in female *Heliothis*, there was no recombination between *H. virescens* and *H. subflexa* chromosomes and QTL in BC_{1-4} can only be mapped to chromosome. To produce BC_5 , we backcrossed BC_4 males to *H. subflexa* females so that recombination between *H. virescens* and *H. subflexa* chromosomes occurred, allowing us to map with greater precision. We assayed over 2000 BC_5 larvae for feeding on cotton so we should be able to map to 1 cM (about 100 Kbp in *H. virescens*) if we have sufficient density of markers. Because only two linkage groups (=chromosomes) in BC_3 explained much of the variation in feeding on cotton (see 'Scientific Background'), we will concentrate on mapping QTL in those linkage groups. The genome for *H. virescens* contains about 403 Mbp in 31 chromosomes [70], or 13 Mbp per chromosome. This means that we will need about 130 markers per chromosome to map to 1 cM, which will require about 10 selective primer pairs (assuming about 15 informative markers per primer pair, which is about what we have found so far with this system). Because there is no recombination on *H. virescens* chromosomes prior to BC_5, we will use bulk-segregant analysis in BC_4 to find the required number of markers for these linkage groups. In the *H. subflexa* selection experiment, we selected lines from material collected in Texas for feeding on cotton. To do this, we assayed 5^{th} instar larvae as described above and mated only those larvae which fed in the range of *H. virescens*. After 1-3 generations of selection, we crossed moths from these lines with moths from the NCSU colony which did not feed on cotton. We then backcrossed the F_1 males and females in both directions and assayed backcross 5^{th} instars for feeding on cotton, using the protocol described above. We used three F_1 families, each of which produced several backcross families, the progeny of which can be pooled by F_1 family for QTL mapping. To map QTL for oviposition, we will again hybridize *H. virescens* and *H. subflexa* and backcross hybrid females to *H. subflexa* while selecting for oviposition on cotton. We will assay hybrid females by putting them in 1 m long x 20 cm diameter tubes covered with surgical gauze at one end and surgical gauze treated with extract of cotton leaves at the other. Females that oviposit preferentially on the gauze with cotton extract will be selected for mating. After 5 generations of backcrossing and selection, we will map QTL for oviposition in BC_5 females using the

techniques described above. To silence function of candidate genes for larval feeding and adult oviposition, we will transform *H. virescens* by injecting two plasmid vectors (pUC) into <3 h old *H. virescens* eggs. One plasmid will express the *piggyBac* transposase. The other will carry the gene for GFP, a universal heat-shock inducible promoter (3x3P nerve-tissue specific promoter from *Drosophila melanogaster*), and a DNA sequence which will express a short RNA subsequence of the candidate gene but which will fold upon itself to form double-stranded RNA [69], all flanked by the transposition targets for the *piggyBac* transposase. Injections will be done by David O'Brochta (University of Maryland) [67, 68]. Insects will be reared and screened for successful transformation (expression of GFP) at the USDA-ARS Beneficial Insect Introductions Research Laboratory. Insects from transformed lines will be assayed for cotton feeding or oviposition. Details on molecular methods are given in the Appendix.

Contingencies - If markers flanking QTL are far apart, assembling a contig of intervening BACs may prove expensive, and in any case, uninformative because there would be too many candidate genes. In this case, we will test whether there are differences in gene expression depending on whether backcross larvae fed on cotton, using SAGE or SSH. We froze BC_5 larvae at 0 and 24 hours after assay in the *H. virescens x H. subflexa* hybridization experiment so we could extract mRNA from them. If we find differentially expressed genes, we will sequence them, develop markers from the sequences, and determine whether they are linked to the QTL identified in the analysis of backcross insects. We can use the same females for this linkage analysis, which will save much labor and time. We will compare differentially expressed genes with sequences in Genbank to see if they resemble genes of known function.

Collaborations - *Outside ARS:* We will collaborate with Fred L. Gould (North Carolina State University), who will provide insects and expertise in QTL mapping, and David A. O'Brochta (University of Maryland), who will provide expertise in genetic transformation and gene silencing.

Objective 2: Determine the importance of climatic adaptation for establishment and growth of introduced populations of insects.

Experimental Designs *Approaches* – We will test the following competing hypotheses concerning climatic adaptation: (1) populations in different regions are genetically adapted to local climates, (2) single populations have essentially the full range of genetic variation in traits involved in climatic adaptation, or (3) physiological plasticity is sufficient for local adaptation so there is little genetic variation in climatic adaptation traits either between regions or within populations. We will test these hypotheses with species and populations in the *A.varipes* complex, several of which have been introduced into North America for biological control. In laboratory experiments, we will determine whether there is phenotypic variation in tolerance to low and high temperature and humidities and in conditions for diapause induction and termination within and between populations of this complex from the most extreme climates, both in source regions and where they have established in the US. We will use information generated under Sub-objective 1b about the sources of populations in this complex that have established in the U.S. on *D. noxia* to match introduced populations with those from Eurasia in culture. If we find phenotypic differences between source and introduced populations, we will choose the pair of populations with the greatest differences and determine the genetic basis of differences by assaying progeny of crosses within and between populations. If we find phenotypic differences within source populations, we will determine the genetic basis using parental half-sib crosses.

If we find genetic differences between source and introduced populations that match predictions based on differences in climate, this will indicate that these wasps have indeed adapted since introduction into the US. On the other hand, if we find that source and introduced populations do not differ, but that established populations are those predicted from climatic matching, this will indicate that pre-adaptation was important for establishment. If we find genetic variation within populations but not among them, this will mean either (1) insufficient

differences in selection among climates, (2) large environmental variance leading to insufficient heritability in the field, or (3) pleiotropism or epistasis leading to counterbalancing selection. If we find little genetic variation either within or among populations or no pattern in the relationship between source and established populations, this will mean that species in the A.*varipes* complex have sufficient phenotypic plasticity to handle differences in climate among the various regions where they originated or established.

Procedures - In the laboratory, we will measure phenotypic variation in tolerances for extremes of temperature and humidity, in response norms for development and behavior versus temperature, and in thresholds for diapause induction and termination within and between populations from the US (which we will collect) and from Eurasia (which we have in culture). We will use a set of small, programmable temperature cabinets, in which we can also manipulate humidity by using various saturated salt solutions [71]. We will measure survival of adults and diapausing and non-diapausing pupae at low and high extremes of temperature (-20-0 and 30-40^{o}C, respectively) and humidity (12-22% and 82-98%, respectively). We will expose parasitoids for periods appropriate to the stage and season when the extremes would be encountered in the field. We will measure the effect of intermediate temperatures (5-30^{o}C) on development time from parasitization to adult emergence and on walking speed [72]. ***Aphelinus spp.*** **search for hosts primarily on foot, so that walking speed should be one of the major factors determining how many hosts it finds. Walking speed is relatively easy to quantify and may be one of the best indicators of searching capacity for species that search on foot [73]. Walking speed has been used extensively for quality assessment of *Trichogramma* spp. [73, 74], another group that does much of its searching on foot.** Lastly, we will determine thresholds of temperature and photoperiod for diapause induction and termination by exposing adults and the aphids they parasitize to a series of low and high temperatures and short and long photoperiods.

Assuming we find phenotypic differences between source and introduced populations, we will reciprocally cross and backcross the extremes and measure backcross progeny responses. If the phenotypic differences have a genetic basis, there should be segregation in phenotypes among backcross progeny.

Contingencies - Currently, we have three populations from Eurasia in culture which parasitize *D. noxia*. If none of these match established populations, we will compare DNA sequences from established populations with those from voucher material obtained during exploration for natural enemies of *D. noxia*. If sequences match, we will collect live material from the source population and measure climatic tolerances as described above. If we are unable to identify the source of established populations in the *A.varipes* complex, we will switch to studying populations which will be introduced under Objective 3. Although we have not yet measured differences in climatic tolerances in the *A. varipes* complex, others have found such differences [75, 76], and we are confident we will find phenotypic differences across the broad range of environments in which these species occur. If we do not, this will be revealing in itself, indicating either (1) adaptation to a wide variety of climates does not involve within species genetic variation and thus pre-adaptation or post-introduction evolution are not needed for establishment or (2) populations in different climates are actually different sibling species. We do not plan to map QTL for climate tolerances during the period of this project. However, if the crosses and backcrosses prove interesting, we may pursue mapping and positional cloning. Alternatively, we could test for differences in expression of mRNA coding for heat shock proteins which are involved temperature tolerance in particular and stress tolerance general in *Drosophila melanogaster* and other organisms [77].

Objective 3: Screen, introduce, and evaluate impact of candidates for biological control introductions, based on host specificity and climatic tolerances.

While introducing agents to control *Diuraphis noxia* and *Aphis glycines*, we will test (1) whether

host specificity evaluated prior to introduction predicts well host specificity after introduction, and (2) species pre-adapted to the climate of introduction are more likely to establish. Biocontrol by introductions involves search for natural enemies likely to control a pest where it has established since introduction and unlikely to affect abundances of non-target species. This search may fail at various points, but with a well-designed program, such failures provide useful information both about a particular pest and its natural enemies, about how to proceed with biocontrol introductions in general, and about the ecology and evolution of host-parasitoid and prey-predator interactions.

Sub-objective 3a - Screen candidates for biological control introductions, based on host specificity and climatic tolerances.

Experimental designs *Approaches* – In cooperation with Kongming Wu, (Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China), we will continue carry out field surveys and exclosure experiments to measure impact of predators and parasitoids on *A. glycines*, identify the most important agents, and measure their use of alternative prey and hosts in the field [41]. We will import parasitoids and predators from Europe and Asia. In our quarantine facility, we will screen for pathogens and hyperparasitoids. We will send adult natural enemies for identification to systematists at the USDA-ARS Systematic Entomology Laboratory or others appropriate for the taxonomic group in question. We will use data from the literature, field experiments overseas, and experiments in quarantine on climatic, habitat, and host specificities to determine the potential geographical and habitat range of each candidate, the likely impact on the target pest, and the likely impacts on non-target species. We will use the information obtained to draft an Environmental Assessment [78] for each candidate for introduction. We currently have one species in the *A. varipes* complex that attacks only *D. noxia* among seven aphid species we have tested. This candidate is from the south of France, and based on climate matching, it would be appropriate to release against *D. noxia* in certain areas of California. However, we will measure climatic tolerances to determine whether this is correct. In any case, we will develop an Environmental Assessment for release of this parasitoid against *D. noxia*. Assuming that the Environment Assessment results in a finding of no significant impact and the candidate is approved for field release, we will proceed to the next sub-objective with that candidate. This procedure will be repeated for each candidate for introduction.

Procedures – Population dynamics of *A. glycines* and associated natural enemies will be measured in field surveys in China. This will involve visual examination of host plants of *A. glycines* (soybean and buckthorn species) and neighboring plants at frequent intervals (e.g., weekly) during spring, summer, and fall to obtain counts of *A. glycines* and associated natural enemies. Impact of natural enemies on *A. glycines* densities in China will be measured using exclosure experiments. This will involve treating soybean plants with insecticide to remove resident insects, infesting the plants with *A. glycines*, and caging some plants while leaving others uncaged. Some cages will be small-meshed to exclude all natural enemies, others will be large-meshed to allow aphid movement and access by large natural enemies. Each treatment will have at least 20 replicates and the experiment will be repeated for at least three years in several locations. Aphids will be counted at frequent intervals (< 1 week) and the effects of treatments will be tested with analysis of variance. If necessary data will be transformed to normalize and homogenize variances. In quarantine, parasitoids will be reared on the host species from which they were collected and on appropriate host plants at 25^{o}C, 50-70% relative humidity, and 16:8 h (L:D) photoperiod. Plants will be grown under natural light (supplemented with halide lamps in winter) in the greenhouse until infested with aphids. Using the methods described under *Sub-objective 1b*, we will test host specificity of candidates for introduction. At present, we have 8 species/populations in quarantine at Newark for which we need to carry out host specificity testing. Given current resources we can test specificity of one parasitoid species in about 3 months. This means that the material on hand will take another 2

years to test. In addition to the eight aphid species we will use for tests in Newark, George Heimpel (University of Minnesota) will test parasitism of *Aphis nerii* and *Aphis fabae*. These species were chosen to cover the host specificity issues raised by natural enemy introductions: attack on other pest aphids, use of potential overwintering or alternative hosts, attack on native aphid species, and phylogenetic distribution of host use. For *A. glycines*, we will test specificity of aphelinids and Prof. Heimpel will test that of braconids and predators. For *D. noxia*, we will test specificity of all parasitoids and any predators that promise to have narrow prey ranges. We will measure the propensity of each parasitoid species to parasitize each aphid species by exposing them in no-choice experiments and recording the number of mummified aphids and adults emerged. For each combination of parasitoid and aphid species, we will expose 10-20 two-day-old, sugar-fed, mated females individually to 100 aphids of mixed stages for 24 hours. We will rear the aphids to determine the number of mummies formed, number of adult parasitoids emerged, and adult sex ratio. We will use analyses of variance to test the effects of parasitoid source and host species on number of mummies, adult emergence, and progeny sex ratio. Using the methods described under *Sub-objective 2*, we will measure climatic tolerances of candidates for introduction. These measurements, along with information on climate in the source area, will allow us (1) to predict where candidates are most likely to establish and test these predictions in experimental releases under *Sub-objective* 3c, and (2) to predict where candidates are likely to spread and thus which non-target species are likely to be at risk.

Contingencies - We will rely primarily on staff of the USDA-ARS laboratories in Montpellier and Beijing and other collaborators for exploration and collection of natural enemies overseas. However, in some cases, we will have to make trips ourselves for this purpose. We currently have 16 species/populations of parasitoids of *D. noxia* and *A. glycines* in culture. We have tested host specificity of 9 of these. One parasitoid of *D. noxia* in the *A.varipes* complex appears sufficiently specific for introduction against this pest. All the species/populations of aphelinids from *A. glycines* tested so far are not sufficiently specific to be introduced at this time. Thus, additional exploration is needed for natural enemies of *A. glycines*. If we are unable to find more host specific natural enemies of *A. glycines* after more exhaustive exploration, we will reconsider introduction of the species/populations already tested. Although the host specificity experiments are labor-intensive, we have already used all the techniques in several experiments with *Aphelinus* spp. (See 'Scientific Background') and have resources to continue with such experiments. However, space and other resource limitations require blocking over time, and we will take this block structure into account in our analyses of variance.

Collaborations - *ARS:* We will collaborate with the overseas laboratories in China and France for collection of candidates for introduction. *Outside ARS:* We will collaborate with George Heimpel (University of Minnesota), who will conduct host specificity tests for braconid parasitoids of *A. glycines*; John Heraty (University of California, Riverside), who will collect parasitoids of *D. noxia* and *A. glycines* in India; and Kongming Wu (Chinese Academy of Agricultural Sciences), who will carry out field surveys and experiments in China.

Sub-objective 3b - Introduce and establish natural enemy species approved for release.

Experimental designs *Approaches* – Once we have obtained a release permit for a new species, we will choose replicated release sites with adequate target host abundance, climatic match, and likelihood that the site will remain undisturbed long enough for the candidate population to establish. To test predictions of establishment based on climatic tolerances and climate matching, we will also choose sites with adequate target host abundance and lack of disturbance, but where we predict the candidate will not establish because of lack of climate match. We will rear and release sufficient large numbers of parasitoids to ensure establishment. We will monitor parasitoid abundances at each site in the same season and in subsequent seasons.

Procedures – To establish introduced species they must be released in sufficient numbers to avoid Allee effects from failure to find mates [79]. The numbers needed depend on

the dispersal rate and mate-finding capacity of the introduced species. Because large numbers of aphid parasitoids can be reared in a very small space, we will have little difficulty rearing and releasing large numbers in replicated sites. Indeed, in studies to measure mating success and dispersal in the field, we have already reared and released over 50,000 parasitoids in a single experiment. Thus, we plan to rear and release 100,000 individuals of a parasitoid in the *A. varipes* complex from *D. noxia* in France. We will release 10,000 individuals per site in 5 sites where we predict this parasitoid will establish and 5 sites where we predict that it will not establish, based on climatic tolerances and climate matching. To test whether the introduced parasitoids overwinter successfully, we set up replicated field cages which high numbers of aphids and release parasitoids in these cages. In the following spring, we will capture emerging adult wasps on sticky traps inside these cages. We will collect mummies and healthy aphids in the fields were we release the parasitoids and in surrounding habitats. We will hold the mummies for parasitoid emergences and the healthy aphids for parasitoid development and subsequent emergence. After identifying parasitoids using morphological traits, we will confirm that they are the ones we released using molecular markers.

Contingencies - We currently have one candidate with a very narrow host range for release against *D. noxia* so we will proceed with releases of this candidate once we obtain a release permit. Releases of other candidates depend on finding ones with narrow host ranges, which could take the duration of the project. Thus, we may not make any additional releases. If so, we will shift resources to the other objectives.

Sub-objective 3c - Evaluate impact on target and non-target species.

Experimental designs *Approaches* – For parasitoid species which establish and reach sufficient densities, we will evaluate impact on target and non-target species, using field exclosures and surveys. For closely related species (or biotypes of the same species) which show differences in climatic tolerances, we will test the relationship between the traits involved and establishment and impact in replicated releases sampled for several subsequent generations. We will use molecular markers to track strains in the field (nuclear markers for thelytokous species; mitochondrial DNA markers for arrhenotokous species), and we will do common-environment field releases to test whether traits measured in the laboratory correlate with parasitism by free-ranging natural enemies.

Procedures – Using the methods described under *Sub-objective 3a*, *Sub-objective 1b* and *Sub-objective 2,* we will evaluate impact on target and non-target species, evolutionary shifts in host specificity, and post-introduction climatic adaptation. We will do this for each introduced species that has or will establish for biocontrol of *D. noxia* and *A. glycines.*

Contingencies - Establishment and spread of the current candidate we plan to release against *D. noxia* may take longer than the period of this project plan. However, three species have already been established against *D. noxia.* Therefore, we will start with research on target and non-target impact of these species, and we will concentrate on the one from the *A.varipes* complex.

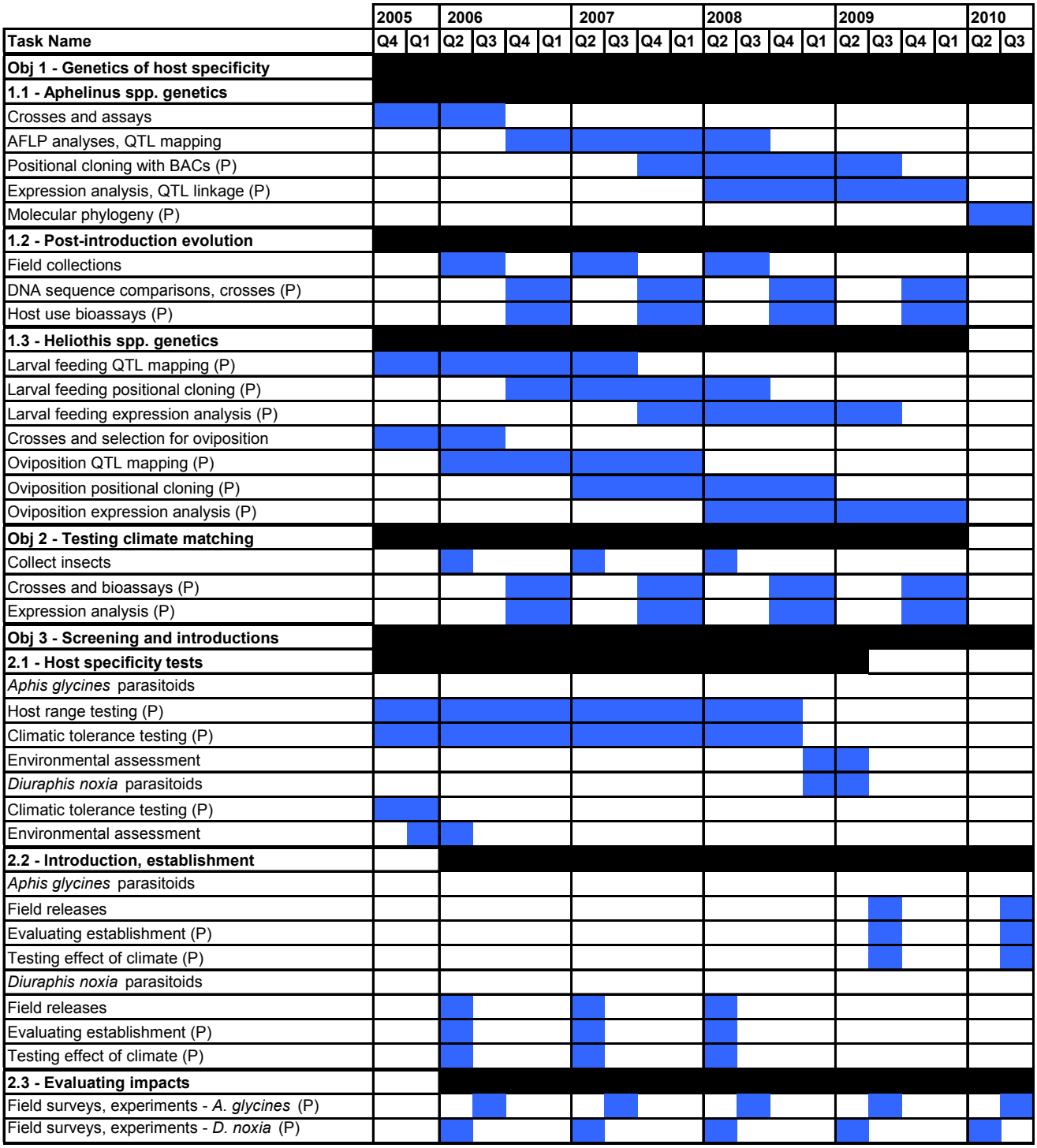
Collaborations - *ARS:* PI is also involved at 0.1 FTE in CRIS 1926-22000-015-00D which includes other work on biocontrol of *A. glycines*, and the PI will collaborate with the participants on that CRIS (Kim Hoelmer and Roger Fuester, USDA-ARS-BIIRU, Newark, Delaware).

PHYSICAL AND HUMAN RESOURCES

Personnel: Working on this project are the PI (0.9 FTE), two research associates, a support scientist, two technicians, and student helpers (6 FTE). The project has has support for an ARS Administrator-funded Research Associate ($100,000), and the PI has received has received over $1 million in grant funding during the last 10 years. ***Equipment:*** *Insect importation and rearing:* Quarantine facility certified by APHIS for the receipt and handling of exotic parasites and predators; 20 reach-in plant-growth and environmental chambers environmental chambers

with temperature, humidity, photoperiod controls. *Behavioral observations:* stereoscope microscopes; high resolution video camera and recorder; compound microscope with dark-field, phase-contrast, fluorescence and video camera; micromanipulators; CO_2 anaesthetic stages. *Molecular genetics:* High-throughput molecular genetics laboratory *Computers:* 10 (0.3-2.7 GigaHz) computers; software for statistical analysis, linkage analysis, QTL mapping, phylogenetic analysis, simulation modeling, image analysis, and GIS. *Other:* >50 m^2 laboratory space, including refrigerators, incubators, analytical and ultramicro balances.

MILESTONES AND OUTCOMES



FIGURES AND TABLES

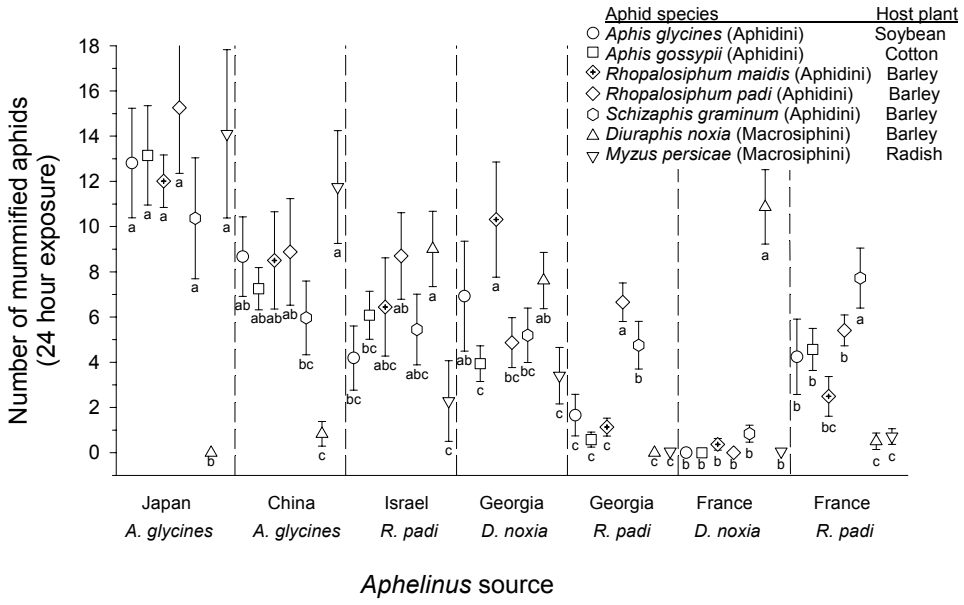


Fig. 1. Parasitism of seven aphid species by species/populations in the *Aphelinus varipes* complex in no-choice laboratory experiment. Error bars are standard errors of the means. Common letters indicate means that do not differ with a single parasitoid species.

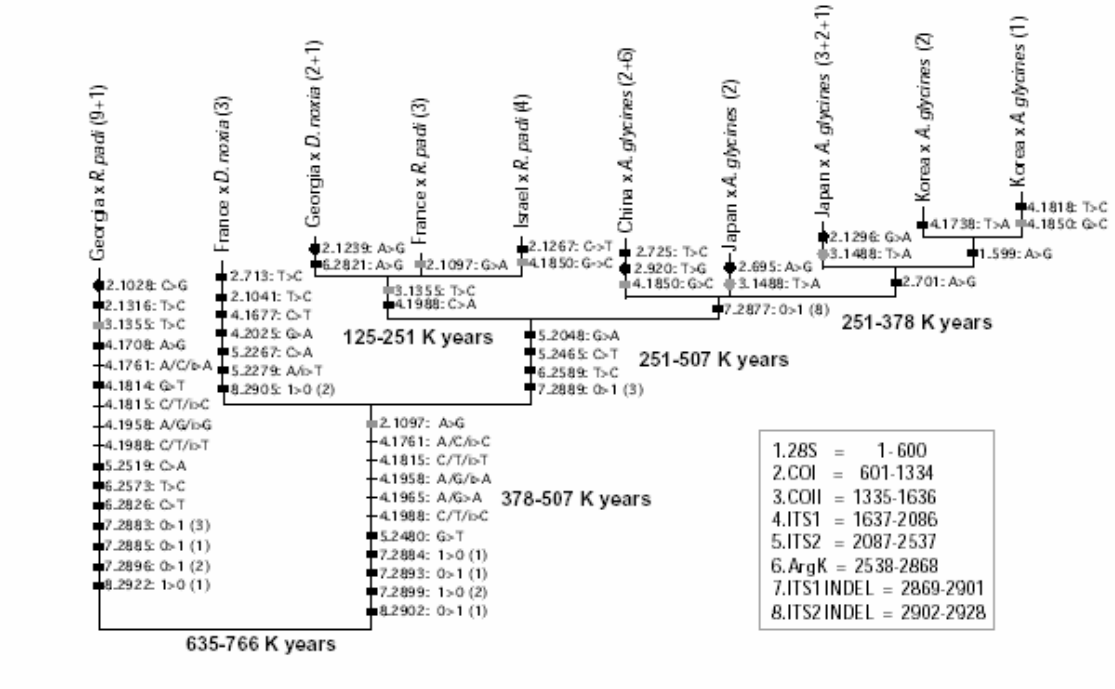


Fig. 2. Molecular phylogeny of species/populations in *Aphelinus varipes* complex. Base and indel changes plotted for single most-parsimonious tree; thick bars are unambiguous changes, thin bars are ambiguous, circles indicate within population variation, gray indicates homoplasy. Estimated divergence times are from changes in COI (thousands of years).

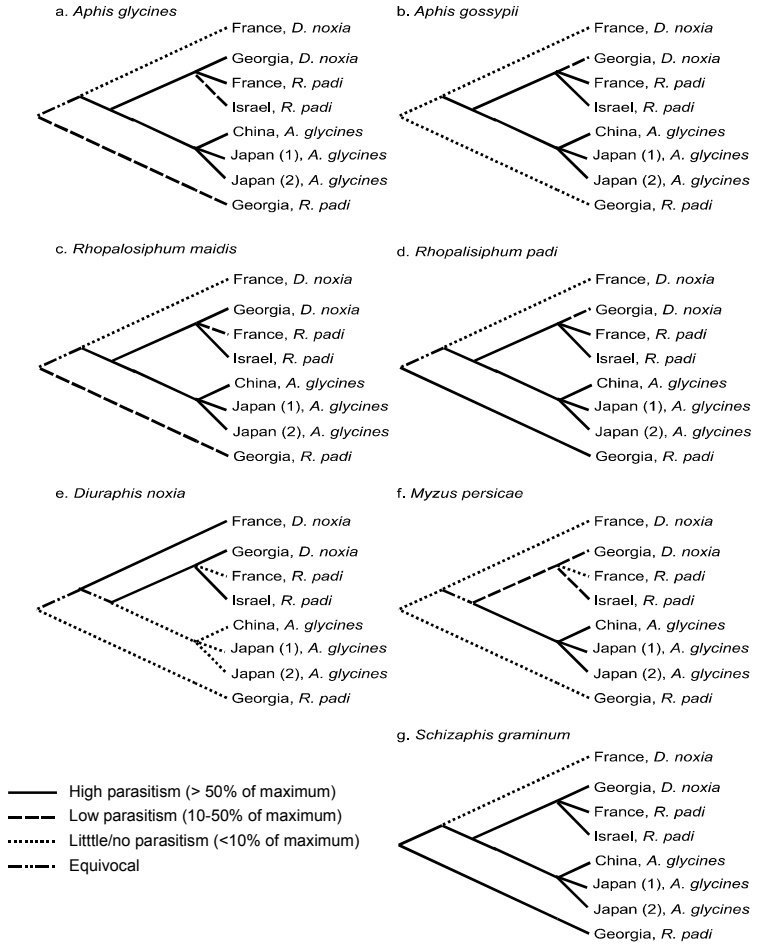


Fig. 3. Parasitism of seven host species mapped onto molecular phylogeny of species and populations in the *Aphelinus varipes* complex.

Table 1. Reproductive compatibility among species/populations in the *Aphelinus varipes* complex. Frequencies of families with female offspring; shade values for differ significantly from within species/population result (Permuted Chi-square test correcting for multiple comparisons, $P < 0.05$).

Female source:		Male source: China *A. glycines*	Japan *A. glycines*	France *D. noxia*	France *R. padi*	Georgia *D. noxia*	Georgia *R. padi*
China	*A. glycines*	6 / 10	5 / 10	0 / 8	0 / 8	4 / 8	0 / 9
Japan	*A. glycines*	8 / 11	4 / 7	0 / 7	0 / 7	6 / 7	0 / 10
France	*D. noxia*	0 / 8	0 / 9	7 / 9	0 / 8	0 / 9	0 / 10
France	*R. padi*	0 / 7	1 / 8	0 / 8	6 / 9	0 / 5	0 / 8
Georgia	*D. noxia*	5 / 6	7 / 7	0 / 8	-	7 / 8	0 / 9
Georgia	*R. padi*	0 / 10	0 / 9	0 / 7	0 / 10	0 / 6	6 / 7

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PAST ACCOMPLISHMENTS

Education Background:
1968-1973 University of California, Santa Barbara; major, Biology; B.A., 1973.
1973-1981 University of California, Davis, major, Ecology; Ph. D., 1981.

Work Experience: A crucial, but rarely tested assumption in biocontrol by introductions is that pest species are controlled by natural enemies in the source region. Field exclosure experiments in France showed the density of *D. noxia* reached peaks 10- to 18-fold higher on wheat where natural enemies were excluded. A two-year field survey in France showed that *D. noxia* population growth rate declined with predator density, but did not vary with plant maturity, rainfall, temperature, aphid density, or parasitoid density. This suggests that predators limited *D. noxia* abundance in France. However, sampling with sentinel plants, artificially infested with *D. noxia*, over a two-year period revealed peak parasitism of 24-72% so that parasitoids may sometimes help limit *D. noxia* abundance in France. Field exclosure experiments in the U.S. showed that indigenous natural enemies reduced growth rate of *D. noxia* populations, although not sufficiently to prevent the aphid from being a pest. Host attraction and defense may affect parasitoid impact on hosts. However, laboratory experiments showed that host attraction and defense could not explain differences in abundances of the two major parasitoids of Russian wheat aphid. On the other hand, analysis of parasitism and superparasitism of European corn borer (*Ostrinia nubilalis*) by one of its major parasitoids, *Macrocentrus grandii*, showed that a spatio-temporal refuge made the majority of hosts unavailable, thus reducing impact of this parasitoid on corn borer abundance. A review of the literature on risk-spreading in insect population biology revealed that spatial risk-spreading is very unlikely to explain low levels of parasitism in the field.

In laboratory and field experiments, male *A. asychis* found mates using a trail pheromone left on substrates by receptive females. In laboratory experiments, female *A. asychis* kept virgin for a period, and thus constrained to produce only male progeny, produced more female-biased sex ratios after mating than females mated at emergence. This sex ratio manipulation matches theoretical predictions qualitatively, but is not large enough to compensate for failure to find mates during introductions. Field surveys of the distribution of parasitized aphids per colony suggest that most females must disperse from their natal colony to find mates and thus mate-search during introductions may be a problem for this species. This research on Allee effects during introductions has inspired several research programs on this issue in the U.S. and abroad, and it is affecting how introductions are carried out.

Natural enemies are often rare in their homeland. Collecting from more abundant host species may cause problems if natural enemies collected from other hosts are less able to attack the target pest than those from the target, but little is known about the genetics of host specificity. In experimental crosses of *Aphelinus varipes* from different host aphids, switching among host species did reduce parasitoid survival. During biocontrol introductions, one is frequently forced to rear natural enemies in the laboratory, but such rearing may select for laboratory-adapted genotypes maladapted to the field. In research on changes during long-term laboratory rearing of *A. asychis*, fitness components did not change in specific directions after 47 generations of laboratory rearing, but replicate populations of the same age did differ significantly in fitness components and in neutral molecular markers, indicating genetic drift among populations. One way to preserve genetic variation under laboratory rearing is to take advantage of drift by maintaining isofemale lines. This research represents one of the few systematic studies of the genetics of natural enemy introductions and should lead to a reevaluation of situations where genetics is likely to affect the success of biocontrol introductions.

List of all publications during last 5 years and those relevant to the proposed research during last 10 years:

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Heimpel GE, Ragsdale DW, Venette R, Hopper KR, O'Neil RJ, et al. **2004**. Prospects for importation biological control of the soybean aphid: Anticipating potential costs and benefits. *Annals of the Entomological Society of America* 97: 249-58
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ISSUES OF CONCERN STATEMENTS

Animal Care - Not relevant.

Endangered Species - Not relevant.

Environmental Impact Statement - An Environmental Impact Statement (EIS) is not normally required for the release of arthropod parasitoids and predators into the environment, but initial field releases of non-indigenous organisms require an Environmental Assessment (EA), unless the candidate organism is in a group having a Categorical Exclusion. Because it is uncertain which of several natural enemies attacking the target pests will be selected for release, and because lines of research proposed in this project statement are designed to assess the risk that candidates for release might present to non-target organisms, it is not appropriate to submit any environmental impact documentation at this time. As candidate species for introduction are identified, supporting documentation will be developed as needed and reviewed by ARS for compliance with the National Environmental Policy Act (NEPA) and Endangered Species Act. This will be done on a case by case basis for each species being considered for release.

Human Study Procedure - Not relevant.

Laboratory Hazards - The only identified laboratory hazards involve ethidium bromide used for staining agarose gels and acrylamide for polyacrylamide gels. These are handled with standard laboratory procedures and disposed of as hazardous waste.

Occupational Safety and Health - Not relevant.

Recombinant DNA Procedures - The recombinant DNA used in cloning genes in *E. coli* and all waste from this and other molecular biological techniques will be autoclaved before disposal. The research on transformation of *H. virescens* to silence gene function has been registered with and approved by the University of Delaware Biosafety Committee.

While preparing the Project Plan, I (Keith R. Hopper) have carefully examined all aspects of the planned research to ensure that appropriate safety concerns are addressed, all necessary permits have been identified, and that environmental issues have been considered in making the National Environmental Policy Act (NEPA) decision documented in the statement. All permits are in hand or have been requested. Documentation supporting NEPA decision is in the MU project file and available for review upon request.

I (Wilda H. Martinez) certify that the proposed research conforms to current regulations and guidelines regarding the above issues and concerns.

APPENDICES

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Molecular Procedures

The AFLP protocol is a technique for generated large number of polymorphic markers that can be used in fingerprinting or linkage mapping [6]. It involves digesting a sample of high-quality genomic DNA with restriction enzymes to produce fragments in the range of 50-500 bp, ligating short (16-18 bp) adaptors to these fragments, amplifying the fragments in two rounds of PCR (polymerase chain reaction), one with primers (in our case labelled with fluorecent dyes) matched to the adaptors extended several (usually 3) base pairs 3'. Each selective primer pair produced about 15 (range 8-27) polymorphic informative markers (present in *H. virescens* and absent in *H. subflexa*) in the hybrid crosses described under 'Scientific Background'. To provide high-throughput analysis using the AFLP protocol, we will use a laboratory automation workstation (Beckman-Coulter Biomek 2000) for DNA extraction (using Qiagen DNeasy 96-well tissue kits), quantitation (using SpectroMax UV spectrometer), and normalization. We will also use this robot for setup of AFLP reactions (restriction-ligation, pre-selective PCR, and selective PCR) and for preparation of PCR product for fragment analysis on an automated capillary electrophoresis DNA analysis system (Beckman-Coulter CEQ 8000).

We will generate linkage maps of AFLP markers in backcross progeny using Mapmaker [80]. We will use these linkage maps for multiple-interval mapping of QTL [81, 82] with QTL Cartographer [83].

To produce probes from AFLP markers, we will sequence markers flanking QTL by running selective amplification product on sieving agarose gels, cutting out and extracting the bands of the desired molecular weight, and sequencing the extracted fragments. Using these sequences, we will develop biotinylated oligonucleotide probes and use them to detect BAC clones by probing to nylon membranes spotted with clones (for the *Heliothis virescens* BAC library, 6144 clones per membrane; ~40,000 clones in the library). We will detect hybridized probes using chemiluminescence. If both flanking markers for a QTL hybridize to the same BAC, we will shotgun sequence that BAC. If the flanking markers hybridize with different BACs, we will sequence the ends of these BACs and use these sequences to probe reciprocally to see if they overlap. If they do, we will shotgun sequence both. If not, we will continue reprobing and BAC-end sequencing until we have a contig of BACs that includes both flanking markers.

SAGE (serial analysis of gene expression) is a method for analysis of gene expression that does not rely on extensive knowledge of the genome of interest [64]. It involves generating short cDNA sequences (tags) at the 3' end of mRNA (taking advantage of the polyA tail and clever use of a restriction enzymes that cuts 20 bp away from its recognition site), linking them together to form ditags, amplifying the ditags with PCR, cutting off the linkers, ligating the freed tags together, ligating the sequence into a plasmid for subsequent cloning and sequencing. SAGE is a relatively cheap method for generating quantitative expression data for wide range of levels of gene expression in non-model organsims.

SSH (suppression subtractive hybridization) is another method for analysis of gene expression that does not rely on extensive knowledge of the genome of interest [65]. Here, cDNA from mRNA from two sources is digested with a restriction enzyme to produce shorter, blunt-ended fragments, the cDNA from one source is split into two pools, different adaptors are ligated onto each pool, and the pools are hybridized separately to abundant cDNA from the other source. This hybridization normalizes the concentration of ss cDNA because abundant cDNA sequences anneal more quickly than rare cDNA sequences. The two pools are mixed, more cDNA from the second source is added, and hybridization is done again. PCR with primers specific to the two adaptor sequences exponentially amplifies cDNA found in the first but not the second source. Unlike SAGE, information about expression level within a source is lost. However, differences between sources in even very rare transcripts can be detected.