CRP Title:
Enabling Technologies for the Expansion of SIT for Old and New World Screwworm

Section/Division: IPC/NAFA-NAAL

Project Officer: Jorge Hendrichs and Alan Robinson

Period Covered: 2001-2007

Objectives of CRP:
(a) Overall
To enhance the efficiency of the implementation of SIT for screwworms and to reduce risk associated with the introduction of screwworm into new areas.

(b) Specific
− To establish genetic relationships between populations of Old and New World screwworms.
− To identify the origins of new outbreaks in order to improve quarantine regulations.
− To develop a genetic sexing strain for New World Screwworm.

Outputs:
(a) Research:
1. Development and optimisation of new genetic markers for OWS and NWS
An optimised suite of molecular genetic markers that include: mitochondrial and nuclear DNA sequence markers, associated RFLP analysis, and microsatellite loci have been developed for these two species. These markers have been used for basic and applied studies of screwworm genetics, including studies of intraspecific phylogenetic variation and population genetics analysis.

2. Population genetics of NWS
NWS populations sampled from across the range of the species, from Uruguay in the south to Cuba in the north, have been subjected to molecular analysis. Populations have been analysed by microsatellite analysis, and by sequence/RFLP analysis of three gene markers (COI+II, Control region/12S rRNA, EF1alpha) in two separate laboratories, UNICAMP, Campinas, Brazil and University of Exeter, UK. Overall, the species appears to have moderate to high genetic variability across the current range of the species. This variability has been characterised by deviations from Hardy-Weinberg and significant disequilibrium, suggesting that populations are not freely interbreeding. Preliminary results suggest the species may be characterised by a range of population structures. Significantly, there does not appear to be any relationship between geographical and genetic distance, suggesting a possible meta-population model. In addition, island populations appear to have distinct genetic signatures, with that of populations from Cuba appearing particularly distinct. The populations from Jamaica appear to cluster with those from other Caribbean islands. In populations from Uruguay significant temporal structuring was also observed. Significantly, the results from all genetic markers (microsatellites and single gene markers) employed in the two laboratories were in general overall agreement with regards to the patterns of variation seen.

3. Analysis of E3 OP resistance gene in NWS
In an additional study, part of the E3 gene in NWS (ChaeE7) was isolated and characterized. This region contains the gene responsible for resistance to organophosphorous (OP) insecticides in Lucilia cuprina. A PCR-RFLP assay therefore provided a fast, reliable DNA-based method for identifying NWS individuals...
with a mutation in this gene. Further bioassays to determine the association of this mutation with OP resistance in NWS should allow the development of more effective strategies for managing this species.

4. DNA database for OWS populations world-wide enabling improved risk assessment protocols for potential new outbreaks to be developed.

Initial cladistic analyses of mitochondrial DNA sequences of OWS gave strong support for recognizing two races of OWS, one from sub-Saharan Africa and the other from Asia. The latter race could be further subdivided into two lineages, i.e., one from mainland Asia (from the Persian Gulf to the Malay Peninsula) and the other from two islands of Papua New Guinea. The work conducted during the period of this RCM concentrated on the latter race, looking at a longer mitochondrial sequence of the Cytochrome b gene (717 bp) and two nuclear sequences, elongation factor 1-alpha and nuclear white eye-colour gene. While some haplotypes are shared in countries from the Gulf region to Malaysia and northwards to Hong Kong, most of the islands of Indonesia showed different haplotypes, some unique to individual islands. The exception is Sumatra, which shares a haplotype with Malaysia, consistent with general “Wallace Line” predictions. The data suggest that the Indonesian island regions would not be subject to much long-distance re-invasion during a SIT trial and that one or more of the islands would be a good candidate for an SIT trial, especially where they could then become a buffer against potential movement of the species to OWS-free Australia.

5. Better laboratory strains of the OWS

Laboratory colonies were developed in Iraq, Iran and Indonesia. These were able to routinely produce up to 20-30,000 adult OWS per generation (Iraq/Indonesia). One of the problems with the sterile flies produced by the Malaysian/Australian sterile OWS facility in Kluang, Malaysia, was the poor mating competitiveness compared to wild males. One of the objectives of future studies should be to determine the mating competitiveness of flies produced in the three research colonies developed in this CRP. Initial studies in Iraq comparing flies colonized for just three generations (i.e. virtually wild male flies) with those colonized for 68 generations showed that those males in colonization for the longer period were just as competitive in mating with wild females as those colonized for the shorter period. This is a positive result in relation to the use of males from the Iraq colony in an SIT programme.

6. Genetic transformation of NWS

Transformation was achieved in the NWS at the ARS Midwest Livestock Insects Lab in Lincoln, Nebraska. A P95 wild lab-strain of NWS was the host for transformation with a piggyBac vector marked with polyubiquitin-regulated enhanced green fluorescent protein (EGFP) (pB[PUb-nls-EGFP]). From a total of 2,180 embryos injected, 49 adults survived of which 22 were fertile. Approximately 4,800 G1 offspring were screened for GFP expression and eight transformant sublines were isolated. PiggyBac-mediated transformation events were verified by TTAA insertion site duplications for all integrations. Due to closure of the ARS Lincoln, NE Biosecure lab, six of the transgenic lines have been cryopreserved with the assistance of Dr. Roger Leopold, ARS Fargo, ND.

7. Fitness of transgenic lines

These tests were performed on eight transgenic strains of NWS with comparison to the wild-type parental laboratory strain (P95) in colony. Measurements of average weight of pupae, percentage of adults emerging from pupae, ratio of males to total emerged adults, and mating competitiveness were analyzed. None of the transgenic colonies exhibited significantly lower fitness characteristics than the control parental colony. One transgenic colony had a higher ratio of adults emerging from pupae, and five colonies had higher average pupal weight; because fitness cost would only be indicated by lower values, the statistical variations were not significant. One transgenic strain was tested in competitiveness tests with wild-type males and the transgenic males mated with equal frequency compared with males of the wild-type strain.
8. Genetically marked NWS

Strains of primary interest include those that are genetically marked with fluorescent proteins for detection in the field, including those for green (EGFP) and red (DsRed) fluorescence. The existing transgenic strains marked with EGFP may be considered for release, but fluorescent protein markers can now be introduced with new transgene vectors that can be stabilized by deletion of terminal sequences. This will enhance the ecological safety of the transgenic strains, and would be preferred.

9. Remote sensing and NWS eradication

NWS was discovered on the island of Aruba in April, 2004. Satellite imaging was used to select preferred habitats of screwworms where ground release stations for sterile insect releases were then located. The screwworm infestation was eliminated in less than 9 months.

10. Diffusion model for movement of sterile NWS

Sterile flies, marked with fluorescent powder, were release from an airplane in a flight-line perpendicular to the centre of a 10 km trap-line. Releases were replicated in three habitats in Panama. Traps were separated by about 500 meters along the trap-line. Sterile flies, marked with a different colour fluorescent powder, were ground-released at the centre of the trap-line. Released flies were captured for about 2 weeks, one following week was used to re-establish traps and allow residual surviving flies to perish. Data showed that the current release rate and pattern were correct for continued use in the Panama barrier but that the width of the barrier-zone could be reduced.

11. NWS are vectors for highly contagious viral diseases

In collaboration with virologists at the Foreign Animal Disease Diagnostics Laboratory of Plum Island, screwworm larvae in larval media with various antibiotics were exposed to viruses causing foot-and-mouth disease as well as hog cholera. Viruses were not detected on the screwworms when reared in the normal larval media containing formalin as the antibiotic. These results help ensure that when developing new strains of screwworm that highly contagious diseases will not be moved to new areas.

12. Improvements to NWS

Cellulose fibre was tested and shown to be superior to previous gelling agents used in the larval medium. Cellulose fibre was adopted by the mass rearing facility and the optimum level of blood protein was determined which, when fed to adults, optimized egg production. The cellulose fibre in spent diet is biodegradable, while the use of the previous gelling agents was not.

Sodium hydroxide was used to separate screwworm egg masses; the egg-hatch was not adversely affected. This allows researchers and mass production personnel to more efficiently monitor egg hatching, an important quality control parameter. Separating the eggs also provides a critical advantage for research in cryopreservation and transgenic applications to screwworm.

The effect and interaction of three temperatures (24.5, 29.5 and 34.5°C), two diets (carbohydrate and carbohydrate + protein) and three population densities (300, 400, and 500 flies/cage) on egg production, egg hatch, number of observable fertilized eggs, mortality (male and female) and ovarian development were determined. Using the protein diet increased egg production at all temperatures, but 24.5°C was the optimal temperature for adult survival. Also, when compared to the normal rearing photoperiod (12 L:12 D), short photoperiod (1 L:23 D) increased egg production, egg hatch and fertility, and lowered mortality.

13. Genetic maps for NWS

Six mutant strains of screwworm were characterized by crosses to wild-type strains. Each mutant was confirmed to be a recessive allele. Classical analyses of $F_2$ individuals from dihybrid and trihybrid crosses are being completed, but preliminary results indicate a linkage group of 5 genes. These same strains were used in backcrosses (test crosses to the male mutant) and then subjected to genetic analysis using Amplified Fragment Length Polymorphism. Results indicated four linkage groups.
14. Polytene chromosome maps for NWS

Polytene chromosome maps have been developed for NWS. These chromosome preparations will be an essential component of the development of a genetic sexing system based on classical genetics.

(b) Others:
- Enhanced collaboration among and between South American, European, and North American Laboratories.
- Capacity building in South America.
- Fellowship support and two young researchers have started their PhD studies at participating institutes.
- Focused international attention on the contribution of genetics to efficacious area-wide screwworm fly control.
- Research studies of genetic diversity, behaviour and control of *Wohlfahrtia magnifica* in the Mediterranean Basin. Stimulation of the write up of past studies of NWS trap development.
- Recruitment of new workers into the screwworm fly community

Effectiveness of CRP:

(a) In reaching Specific Objective 1: To establish genetic relationships between populations of Old and New World screwworms.

Overall, research focusing on genetic relationships between populations of New World screwworm flies suggests moderate to high genetic variability across the current range of the species. This variability has been characterised by deviations from Hardy-Weinberg and significant disequilibrium, suggesting that populations are not freely interbreeding. Preliminary results suggest that NWS may be characterised by a range of population structures. Significantly, there does not appear to be any relationship between geographical and genetic distance, suggesting a possible meta-population model. In addition, island populations appear to have distinct genetic signatures, with that of populations from Cuba appearing particularly distinct. In populations from Uruguay significant temporal structuring was also observed. Current and planned research is investigating the likelihood of local reinvasions in maintaining temporal structuring.

Considerable progress was made in evaluating mitochondrial DNA lineages of OWS from the Gulf region to Indonesia and initial progress was made with examining some nuclear genes. This work will continue beyond the present CRP through the PhD study of Mr April Hari Wardhana from Indonesia. It was disappointing that no progress was made on evaluation of populations from the Horn of Africa, a supplier of livestock to the Gulf region. However, within the period of the CRP no collections of OWS could be made in the Horn of Africa. Collaborative links with the Arab Organisation for Agricultural Development (AOAD) have resulted in the supply of specimens for future analysis from several countries in the Gulf region (Iraq, Saudi Arabia, Oman) and will produce more in 2007.

In reaching the Specific Objective 2: To identify the origins of new outbreaks in order to improve quarantine regulations.

The tools developed and the results obtained to date provide a framework for potential epidemiological tracking of flies from their population of origin to new areas. However, over much of South America, a lack of distinct population differences suggests that this may not be achievable with a high degree of confidence, though tracking of flies within the Caribbean may be possible with a slightly higher degree of confidence. Planned research will focus on the evolutionary origins of the distinct populations of NWS in Cuba and the potential for spread and reinvasion.

For OWS, specific, geographically isolated haplotypes were identified that can be used to indicate the origin of some potential movements of OWS, either by natural dispersion or, more likely over long distance, by human activity (e.g. livestock shipments). In some instances the separation was very clear, e.g., Asian versus Sub-Saharan populations. Populations in the Horn of Africa need to be sampled to determine their relationship to the nearby populations of OWS in the Gulf region. The island fauna of
Indonesia showed especially high genetic diversity and, in view of these fauna being geographically nearest to Australia, this result could have real value to the biosecurity services of Australia in identifying any future potential introductions of OWS.

Studies of the genetics of *Wohlfahrtia magnifica* illustrate the potential application of genetic techniques in identifying or eliminating sources of new infestation, since they were able to unambiguously eliminate Spain as the origin of the new populations of *W. magnifica* on the island of Crete.

**In reaching Specific Objective 3: To develop a genetic sexing strain for New World Screwworm.**

Research on screwworms within the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) has undergone recent changes and these have impacted negatively on the development of a genetic sexing strain using the classical approach. Specifically, the genetic analysis of male linked translocations and selectable markers could not be carried out. This work would have been accomplished in Lincoln, NE but closure of the facility, relocation and reassignment of scientists, and transfer of research responsibility to a new, international location (Panama, where new laboratory facilities are in process of being established) interfered with accomplishing these projects.

However, work on developing transgenic techniques for eventual sexing of screwworms was enhanced as was work in developing understanding of genetic linkage through other contracts and collaborations. Germ-line transformation was achieved using a *piggyBac* transposon-based vector marked with *polyubiquitin*-regulated enhanced green fluorescent protein (PUbnlsEGFP) that can be used for field detection of released flies. The fitness of these strains was assessed and shown to be of an acceptable level.

(b) In contributing towards Overall Objective: To enhance the efficiency of the implementation of SIT for screwworm and to reduce risk associated with the introduction of screwworm into new areas.

The efficiency of the implementation of SIT for screwworm was enhanced by a much better understanding of the genetic differences that exist between the different geographical populations of the OWS and NWS. However, these differences alone do not provide information on the mating compatibility of the different populations (see below under Recommended Future Action by the Agency.

Studies of OWS population genetics demonstrated geographical populations that could rapidly identify the source of introductions of screwworm, thereby enabling action to be taken to close down the route of introduction, reducing the risk of potential introductions to other uninfested areas through the same route.

The question of genetic compatibility of released, sterile mass reared flies and their wild conspecifics has not been investigated directly, but genetic studies of natural populations do not in general show high levels of population structure. In consequence, a high degree of mating incompatibility between released and wild NWS flies would not be predicted, perhaps apart from in Cuba, where local populations show a high degree of genetic separation from other NWS populations. As NWS populations from a number of potentially key areas remain to be studied, e.g. Colombia, Peru, Ecuador, patterns of genetic variation in these populations remain to be investigated thoroughly. For OWS, however, there appears to be a single biological species in Indonesia and the Gulf based on mitochondrial analysis, and this was supported by an analysis of a nuclear gene sequence. Unfortunately no samples could be obtained from the Horn of Africa.

(c) Factors, if any, which adversely affected the effectiveness of the CRP:

Problems in coordinating sampling support early in the project – largely resolved for NWS after the 3rd meeting by the appointment of an IAEA consultant, Mr Rene Garcia. This work is due to continue, which will help to ensure the long-term continuation of research activities associated with the CRP.

It quickly became apparent that we needed political and administrative support for sampling from all relevant countries, e.g. Argentina. In future, ideally the Agency would provide support to talk directly to
relevant personnel (e.g. national veterinary coordinators, health service administrators) within all countries not directly represented in the CRP.

Some difficulties were experienced in Indonesia with inadequate levels of staffing over certain periods to collect samples from the field, as there was no replacement of staff during their secondment on training.

As a result of the breakdown in security in Iraq and problems with the electricity supply, the colony level has presently been reduced to a minimal level of about 500 adults. Indeed it is a testament to the hard work and commitment of colleagues in Iraq that any research work has been completed there under such difficult conditions.

Some problems arose with movement of CRP members to the different countries where the RCMs were held, due to visa issues, e.g., Indonesia to Uruguay.

Maintaining the colony in Iran was made difficult due to the poor quality of some local components of the OWS larval diet (e.g. skimmed milk powder, egg yolk powder, whole dried blood).

IAEA provided additional research grants to some CRP members (research contracts) but no major alternative funding sources were identified. The Arab Organisation for Agricultural Development (AOAD) was able to encourage submission of OWS samples from some countries in the Gulf region, but only towards the end of the CRP and the samples have still to be analysed.

**Impact of the CRP:**

1. Improved decision making for future NWS SIT programmes in South America through an assessment of patterns of genetic variability in contemporary field populations. The CRP has motivated researchers in the region to enter or expand research in the NWS area, and to generate information that will be indispensable for any future eradication programmes.

2. In the Caribbean, SIT programmes will need to establish local mass rearing colonies in view of the significant NWS population differences detected.

3. The existence of two races of OWS, one from sub-Saharan Africa and the other from Asia, has significant implications for quarantines and any future SIT activities in these regions.

4. Several Indonesian islands have been found be good candidates for SIT implementation in view of their genetic isolation and could also become a buffer against potential movement of the species to OWS-free Australia.

5. Successful genetic transformation in NWS opening up the way for a variety of strategies for improved SIT and biocontrol using recombinant DNA. Fundamental genomics analysis will also be possible in NWS.

6. Cryopreservation enables transgenic germplasm to be stored for future use avoiding expense and fitness costs associated with rearing, and the loss of genetic resources during transitions in rearing capabilities.

7. Demonstration that some NWS transgenic strains have fitness equivalent to the non-transgenic host laboratory strains.

8. Several new vectors systems are now available for testing in NWS that should allow more highly efficient and ecologically safe SIT and conditional-lethal based strategies for biocontrol.

9. Sufficient progress on transgenic research has stimulated the potential of collaboration between the ARS-Screwworm Research Unit and ARS-U.S. Livestock Insects Laboratory in Kerrville, TX. Initial work in Kerrville will document techniques using the horn fly (*Haematobia irritans*) as a model insect with further application to the screwworm.
Relevance of the CRP:
The CRP succeeded in focusing attention of investigators on the necessity of understanding the genetic basis of parasite populations and collecting relevant baseline data to inform the management of subsequent SIT control programmes.

Recommended future action by Agency:
There remains much to be done in terms of completing the population genetic analysis of the OWS and NWS over their distributions worldwide. In fact some of these activities will be included with tsetse population genetics and GIS/RS in a new CRP that is in the planning stage. Although as mentioned earlier, the genetic analysis does provide an indication of population variation, it does not answer the question as to whether populations share mating compatibility. This is an important issue and should also be addressed. There are good precedents for this in the Agency where support has been provided to carry out field cage tests in one location of different field populations of the same pest species.

Training
The establishment of reciprocal fellowships between South American and Asian institutions could greatly facilitate interest in the screwworm fly problem to further institutional capacities and scientific progress. Specifically, we recommend establishing a programme to support the training of personnel in the ecology and population dynamics to support and direct the interpretation of genetic data. The programme would cover both theory and practice:

A. Field techniques
- Geographic information systems
- Mark-release-recapture training
- Representative sampling and analytical procedures
- Equipment, software and resources necessary to accomplish the foregoing

B. Laboratory practice
- Microsatellite library development, use of high-throughput automated microsatellite analysis, including training in multiplex design and interpretation of electropherogramme outputs, primer design and DNA sequencing
- Exercises in phylogenetics and population genetic statistics using a range of current software.

Capacity building: regional centres and human resources
Support is required for personnel to undertake microgeographic studies across the range of the NWS. Ideally, three studies would be undertaken at the extremes of the range, e.g. in Cuba, mid-west Brazil, Uruguay/Argentina. Such microgeographic studies would allow a range of population characteristics to be investigated: temporal stability, population structure and population dynamics. At present, the definitive interpretation of population genetics data is being held back by a lack of knowledge concerning these factors. Each study would require a dedicated scientist to undertake and coordinate the research; funding would be necessary for these personnel and all associated equipment, software and consumables. For OWS an expansion of the sampling is required in the different Indonesian islands. The return of a participant following a PhD at the Natural History Museum in London, will add considerable capacity to this effort in the future.

Establishment of a central database
To facilitate sharing and dissemination of information, all data generated by the activities of the CRP (and continuing activities) should be coordinated and maintained on a central database.
Continuing support for population genetic analysis

While coverage of NWS and OWS populations has been adequate to gain an insight into broad patterns of genetic variation within the species, a number of key areas remain to be samples and analysed, e.g. central Brazil, northern and western South America, particularly regions west of the Andes for NWS and India, the Horn of Africa and Indonesia for OWS. Only when populations from these areas have been thoroughly sampled and characterised will we have a true and accurate picture of patterns/levels of genetic variation in NWS and OWS. Accordingly, we encourage the Agency to provide continuing support for this work.

Movement of biological material

Shipping biological materials, such as DNA, ethanol-preserved screwworm flies and cryopreserved materials, is becoming extremely difficult. The application of laws to protect wildlife and endangered species has delayed movement of screwworm material. Laws governing genetically modified organisms may prohibit field trials of transgenic, sterile screwworm flies. If present trends continue, it will soon be quite impossible to carry out research that requires any form of transport of biological specimens. CRP participants therefore recommended that FAO/IAEA keep scientific collaborators and applicators appraised of rules and laws regarding GM organisms. Scientists must be aware of current rules and regulations, and they proposed that FAO/IAEA provide their good offices in encouraging Member States to lessen the red tape that increasingly interferes with needed medical and agricultural research.

A new CRP is required

For all the foregoing reasons, a new CRP would greatly encourage and support progress in the future NWS or OWS suppression or eradication programmes. It would do so by providing research and outreach focus on obtainable goals, disseminating fundamental scientific knowledge, new scientific applications, and, above all, encourage technology transfer to South American, Asian and African countries directly affected by these parasites:

- Application to OWS of microsatellite techniques developed in a microsatellite study of NWS, testing as a first step libraries developed in Brazil for *Chrysomya* species.
- Assistance to improve sample collection in the Horn of Africa, sub-Saharan Africa, and in Asian countries located between the Gulf and Malaysia (especially the Indian subcontinent).
- Assistance to improve sample collection of NWS populations in selected areas of South America and Caribbean.
- Facilitation of discussion of SIT trials in Indonesia (e.g. Sumba Island).
- Application of geographic information systems (GIS) and remote sensing (RS) techniques for mapping population genetic data and other relevant information for facilitated planning and management of intervention campaigns against screwworm and possibly tsetse fly populations.

Resulting Publications

The final proceedings of the CRP are published as full papers in a dedicated issue of the journal Medical and Veterinary Entomology.