

QUEENSLAND FRUITFLY

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Contents

1. INTRODUCTION

Queensland fruitfly (Qfly), *Bactrocera tryoni*, is a key agricultural pest along the East coast of Australia. The current genus name *Bactrocera* is of quite recent standing, following the taxonomic revision by Dick Drew in 1989 [1]. Prior to this Qfly was known as *Dacus tryoni*, which causes some confusion as a substantial amount of the literature has the old name.

The School of Biological Sciences at Sydney University has a long history of involvement with Qfly. It was the principal organism of Charles Birch, the Professor of Zoology for many years, and during his time the CSIRO Division of Entomology under Alan Bateman established a section within the School to work on Qfly.

Most of this work was behavioural and physiological. The only genetical work that I know of was done by Steve McKechnie in a PhD with

Charles Birch, using isozymes identified by electrophoresis, mainly to study the difference between *B. tryoni* and a closely related species *B. neohumeralis*. There had, in fact, been a bit of work along genetical lines by Birch and Dick Lewontin, proposing that it was hybridisation between the two species that had led to the adaptation of *B. tryoni* to cooler climates.

1.1. Founding of the Fruit Fly Research Centre - FFRC.

Sometime around 1990 I was thinking of the possibility of branching out from *Drosophila* work. I have to admit that this was partly because of the difficulties of continued funding for *Drosophila* work, and I wanted to look at the possibility of working on something for which commercial funding might be available. Maybe there was also a desire to do something a bit more applied and/or useful.

I was quite struck at the time at the differences between the approaches taken for Qfly and for another Australian fly that was the subject of much work in CSIRO and universities, the sheep blow fly *Lucilia cuprina*. There was a huge amount of genetical work on the latter, contrasting with the low emphasis on genetical work in Qfly, mainly owing to the scientific background of the people working on the two species.

Around this time Marianne Frommer, my wife, was looking at the possibility of moving out of her work in a cancer lab on the other side of Sydney and moving to Sydney University. Marianne's background was in molecular biology, exactly what was needed at the time for moving into genetical work on a new organism. She put in a proposal for an ARC Senior Post-Doctoral Fellowship to work on Qfly, which, somewhat to her surprise, she was awarded. In retrospect Marianne made one of the worst career moves of all time. Just prior to moving to Sydney University she published the bisulphite method for sequencing of methylated DNA [3], which has had an enormous impact in screening for pre-cancerous states and would have made her rich and famous had she remained in the field.

Neither Marianne or I knew anything much about Qfly. Fortunately a third person, Alfie Meats, was already involved in fruitfly work at Sydney University, and knew almost everything about Qfly. So the combination of the three of us, with our respective backgrounds in population genetics, molecular biology and entomology seemed ideal for the project.

I should also mention a fourth person, Merryl Robson, who had been a fellow undergraduate at Adelaide University. Merryl's great strength, aside from her genetics knowledge, was in fund-raising. She managed to persuade Woolworths to give us seeding funds, which together with matching ARC funds enabled the FFRC to prosper for a number of years. I always hoped that Woolworths got something from the project, mainly in terms of kudos from the growers with whom they worked.

For a history of the various activities of the FFRC see <http://www.bio.usyd.edu.au/fruitfly>.

2. STUDIES ON THE DISTRIBUTION OF QFLY

One of our first aims was to develop genetical markers to be able to differentiate populations. Literally nothing was known about this, except that there was a large base population in northern Australia, mainly Queensland, and smaller populations in NSW and Victoria. Historical documents showed that Qfly had arrived in Sydney around the beginning of the 20th century.

As might be expected, one Qfly looks pretty much like another. Steve McKechnie's isozymes hadn't shown anything at this level. Even physiological studies to show cold tolerance of flies raised from the south as opposed to flies raised from the north were not revealing.

There were a few possibilities for genetical markers at the time. The method on most people's lips was RAPDs, Random Amplification of Polymorphic DNA. This method relied on fingerprinting of flies chosen using fortuitous PCR primers that showed up differences between strains. It was quite easy to set up, and perhaps for this reason very popular at the time. However the variability between samples could be a problem.

Less well known at the time were 'microsatellites'. These are short regions of repeated dinucleotides or trinucleotides that differ between individuals in the number of copies of the repeat. Each such microsatellite needs to be individually amplified by PCR using specially chosen primers. Marianne had the insight to see that despite the much greater amount of work needed to set up the microsatellite system, it had huge advantages in terms of reproducibility when used on the rather degraded DNA samples that one gets from trapped flies. So we started on the right track at the beginning. Nowadays microsatellites are all-pervasive in the field of population genetic studies.

Our original isolation of microsatellite markers produced six good polymorphic markers [4]. This was somewhat less than we had hoped for, but, fortunately, turned out to be enough to get very useful information.

2.1. The CSIRO Double Helix study.

Getting samples is always a problem in field studies of any organism. Fortunately we found out that the CSIRO Double Helix Club to involve children in science was looking for a national project in the year 1994. It took a lot of setting up, but the project turned out to be a triumph. Children were shown how to make a simple trap using a cut soft-drink bottle. A lure that attracts male Qfly and some insecticide were sent to them in the post. They made observations on the weather and the number of flies trapped over a 2-week period, and then sent us the flies preserved in a small bottle containing alcohol.

Many hundreds of samples arrived at the lab. The flies were sorted into species by Rachel Osborne, an Honours student in the lab with an entomology background [7]. The Qfly, the majority species in most cases, were deep-frozen.

The original 1994 sample involved all children in the club. Some from the north trapped hundreds of flies. Others in southern regions collected none (fortunately), although I hope that they felt some satisfaction in being part of the overall project. A subset of the children who had provided the samples from regions where Qfly is endemic were asked to remain part of the project in 1995, and the study was extended up till 1998. In some cases parents took over the sampling, and continued to send us samples beyond that point. We ended up with good samples from several regions for the years 1994 - 1998 (Figure 1).

2.2. The Australia-wide distribution.

The data for six microsatellite loci were produced by Hong Yu [10]. She worked intensively for many months, analysing nearly 25,000 samples. This represented an average of over 40 flies at six loci for each of around twenty sampling sites for each of the five years of the study. There were remarkably few missing values in the data set.

Although initially we had little idea of what sort of population distribution to expect, it soon became clear that some of the samples close to each could be grouped as coming from the same population. I'm a great believer in the old-fashioned χ^2 test statistic (see below), rather

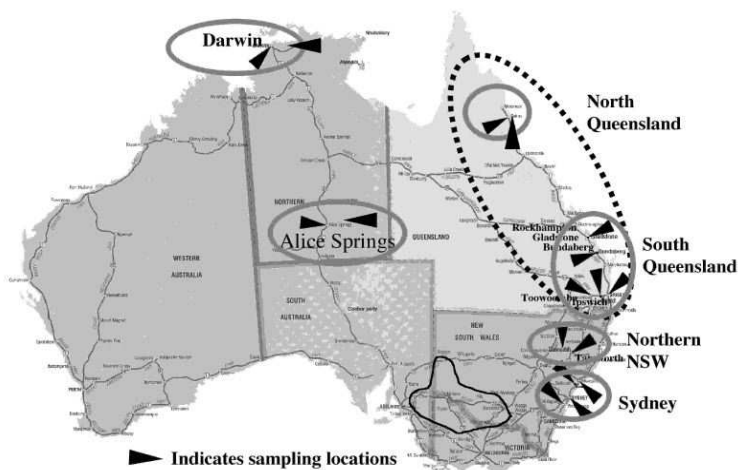


FIGURE 1. Sampling sites for the Australia-wide study

than in some of the fancier new genetic distance statistics. I wrote a small computer program to calculate χ^2 for any combination of samples tested against any other. Repeated use of this showed that the samples could be grouped into six regions as in Figure 1.

The most striking result, however, was the finding that the samples from North and South Queensland were completely homogeneous and could themselves be grouped. As is clear from the map, these regions are a long way from each other. But this is the home range of Qfly, and it seems clear that there is little if any population differentiation within this range.

Outside of this range there is clear differentiation between samples. Four regions outside of Queensland were identified, as seen in the figure. More on this below.

2.3. Variation between years.

The homogeneity of samples from different regions also extended to homogeneity of most samples from different years. In fact the one

exception to this occurred in the sample in Figure 1 labeled 'Northern NSW', where the χ^2 values were quite significant.

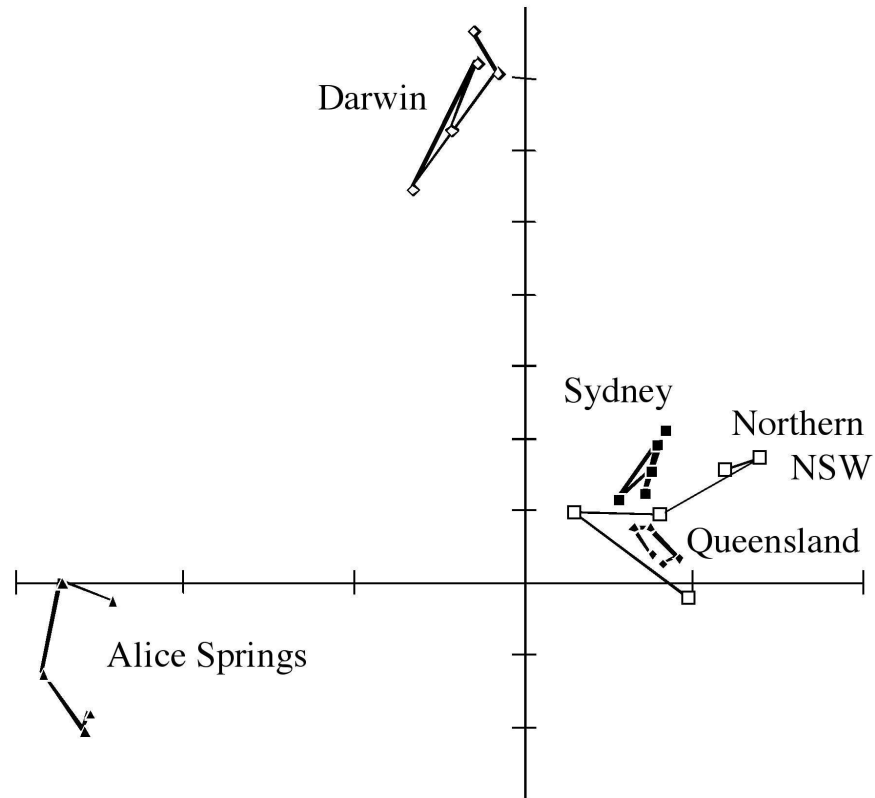


FIGURE 2. 2D analysis of microsatellite frequencies for different years in the five regions

A diagrammatic representation of the distribution is given in Figure 2. This is a 2-dimensional representation of the principal coordinates of the microsatellite frequencies. The diagram has nothing to do with actual physical distances. It simply reduces a set of frequencies of nearly 50 alleles at the six loci to the two dimensions that best summarise the variability in the data.

Clearly the Darwin and Alice Springs samples are well differentiated from the East coast samples. However the important point shown by the figure is that the points remain in the same area for each of the populations except for Northern NSW. The diagram does not indicate

significance, since the variability in this figure depends on the sample size as well as the allele frequencies. Queensland, for example, is the most stable because of the large sample size.

What seems to have happened in the Northern NSW population is that it was well differentiated from Queensland, and to a lesser extent from Sydney, for the years 1994 - 1996. Then in the years 1997 and 1998 the samples became indistinguishable from the Queensland samples. Presumably there was some sort of invasion from the much larger Queensland population in those years. However it is possible that the previous three years were anomalous, since studies since have shown a very low level of differentiation between Queensland and Northern NSW samples.

2.4. An aside on the use of the χ^2 test.

I was raised, statistically speaking, in a department heavily influenced by RA Fisher, often with lectures from the great man himself. Unlike the analysis of variance and many other standard statistical techniques, Fisher didn't actually invent χ^2 , which dated from before his time. He did, however, work out the basis for the heterogeneity or contingency test, the most common use of χ^2 , and personally calculated the probability tables.

His use of χ^2 that I recall most vividly was the analysis of Mendel's data [2], in which he showed that Mendel's data agreed too closely with expectations. The idea that data can agree too closely with expectations may seem a strange one, but when one is doing counts for repeated random events, a certain level of chance deviation is always expected. Many things can happen to make the deviations greater than expected, but it is much more difficult to explain away the lack of deviation. Cynical teachers will often apply such a test to 'data' produced in undergraduate experiments to indicate that the student has invented the data rather than bothering to do the counts.

The χ^2 test shows the expected deviation very clearly. Where there are many classes being tested, and/or a number of tests are combined, the expected value of χ^2 is equal to the number of 'degrees of freedom'. So, for example, if 100 independent tests for agreement with a simple ratio are combined, giving 100 degrees of freedom, then a value of χ^2 significantly greater than 100 indicates some positive deviation from the hypothesis, whereas a value significantly less than 100 indicates

something 'fishy'. Fisher used to claim that it was almost impossible for someone to invent a large data set that a competent statistician couldn't show had been artificially manipulated. He, incidentally, showed not only that Mendel's data agreed too closely with expectations, but that in one case Mendel got the expectations wrong and the data agreed too closely with the incorrect expectations but departed from the correct expectations. Fisher liked to think that Mendel's gardener knew what was expected in the experiments and threw out some of the cases where deviations looked too large.

Anyway all of this is preliminary to looking at Hong's data set for the microsatellites from the Double Helix study. With nearly 25,000 observations, there were many possible χ^2 tests, including heterogeneity within regions, between regions and between years. I'll show, for example, one test that indicates that Northern and Southern Queensland are part of one large population.

| Year | 1994 | 1995 | 1996 | 1997 | 1998 | Total |
|----------|------|------|------|------|------|-------|
| χ^2 | 67.7 | 73.6 | 53.2 | 62.7 | 68.4 | 325.6 |
| d.f. | 76 | 58 | 60 | 58 | 66 | 318 |
| N | 2316 | 2256 | 1980 | 2100 | 2100 | 10752 |

In all cases the χ^2 values are appropriately close to the number of degrees of freedom. The sample size in each case is shown in the bottom row.

Other tables are shown in the publication Yu et al (2001) [10]. The values show a satisfyingly consistent picture of isolated populations stable over years, save for the one example where invasion has occurred from the large nearby core population. Overall it is the cleanest large data set whose analysis I have been involved in.

2.5. Implications for control of Qfly.

The most important conclusion coming from the study is that there is a core population of Qfly in Queensland, and genetically differentiated populations outside of this range. This genetic differentiation may, or may not, indicate adaptation, as discussed below.

From the point of view of control of Qfly, the important populations are those on the East coast of Australia. This control is not necessarily relevant for the Northern NSW and Sydney regions, where the fly is endemic, and where little can be done at present. Where the control is

important is in agricultural regions to the South and inland, particularly in the Fruit Fly Exclusion Zone (FFEZ) shown roughly in outline on the map in Figure 1, where occasional outbreaks occur.

The fact that the endemic NSW populations are so stable and differentiated from the main Queensland population strongly suggested to us that it is these populations, rather than the main Queensland population, that provide the source for outbreaks. Stuart Gilchrist and I [8] analysed this situation further using a more detailed set of populations in the region of the FFEZ. We noted that some of these populations shared alleles that were comparatively rare in the Queensland population, and developed a test for the use of such alleles to show that there must be intermediate population. These conclusions were also supported by heterozygosity calculations, showing where variability has been lost in the founding of new populations.

More recent studies by Stuart Gilchrist using an extended set of microsatellites and analysing samples on a much smaller scale from particular towns and outbreak samples have confirmed these conclusions. It is now getting close to the situation where the source of outbreaks can be pin-pointed to resident populations usually associated with particular towns.

Efforts to control the movement of fruit should evidently concentrate on local movements rather than long distance movements from the North. These policies seem now to have been accepted by the relevant agriculture departments.

The differentiation between Queensland and the rest of the country suggests an interesting possibility. A long-term campaign for the control of Qfly could possibly be based on the complete eradication of flies South of the main Queensland population. If these populations are, indeed, adapted to the more marginal climates, then it is possible that the flies might not easily be able to re-invade. Given that the invasion happened once already around 100 years ago, this may seem a risky proposition. However the original invasion may have occurred over a reasonably long period required for genes for adaptation to be selected.

One area where evolutionary theory is on our side is the likelihood that such genes for adaptation, should they have been selected, are unlikely to have spread to the core Queensland population. The relative sizes of the populations, and the adaptation to the original climate regimes,

make it likely that the core population has been unaffected by the southern invasions.

The chance that complete eradication in southern regions will be attempted, or would be successful, is, unfortunately, low. Total eradication of Medfly has been achieved by Chile, and by some states in Mexico. My colleague Alfie Meats tells me, however, that it would be more difficult to achieve in Qfly. And it is unlikely that the political will exists to even seriously contemplate such an exercise.

2.6. Northern Territory populations.

The Darwin and Alice Springs populations of Figure 1 are both more genetically differentiated from the Queensland population. The origin of the Alice Springs population seems clearly to have been a propagule of around ten flies. Emilie Cameron, who did a PhD on these populations, showed that the Alice Springs population has also led to at least one further invasion, of a small agricultural area called Ti-tree.

Emilie analysed much more widely in Northern and Western regions, and found that flies closely related to the Darwin population occur throughout the region. The origin of these flies, whether by straight invasion or by some sort of hybridisation event, is not really clear.

2.7. The *B. tryoni* complex.

B. tryoni has a closely related sibling species, *B. neohumeralis*. The two species coexist in areas of Queensland, although the range of the latter is much smaller. A third species, *B. aquilonis*, was reported to occur in the Northern Territory. However following Emilie Cameron's study, the status of this species is in some doubt.

There is no similar doubt about *B. neohumeralis*, however. The co-existence of these two species is a matter of considerable interest, and is considered briefly in the next section.

3. THE GENETICS OF QFLY

Our original intention in the Qfly project was to develop a set of visible and molecular markers as the basis for a 'genomics' study. We have had mixed success in this.

3.1. Visible markers.

Our main approach to find useful visible markers was based on in-breeding. This approach has apparently been successful in a variety of organisms, although it is difficult to find references for this. My authority on this was Mel Green, Professor Emeritus at Davis, who used to visit Australia regularly and undoubtedly knew more than almost anybody else post HJ Muller about mutation.

In *Drosophila* it is easy to introduce wild strains. Just getting a single female from the wild and putting her in a tube of food will almost always lead to a line of flies. Unfortunately this rarely works in Qfly.

We developed a compromise technique in which wild males were introduced to laboratory-adapted females. This produces offspring in a reasonable fraction of cases. Single pairs can then be crossed, and their offspring intercrossed to produce sib-mating. In theory if an the original wild type male had a single recessive mutation there should be a reasonable chance that this will show up in recognisable homozygous form.

Sheelagh Cuneen and Pat Maheswaran, both I'm fairly sure astute observers, stared at thousands of F1 and F2 progeny. For whatever reason, the results were meagre. Some eye colour mutations were found. These all turned out to be allelic to each other, and the locus designated as *orange eyes* (*oe*) -probably homologous to *Drosophila scarlet*. But we never found a white-eyed mutation, although these are known in many insects and reputed also to have been seen in Qfly.

Two other mutations were found, *white marks* and *bent wings*. These were both useful in our attempts to produce a strain for 'male only sterile release' (see below). Much effort went into screening for bristle markers, many of which are known in *Drosophila*, but none was found.

3.2. A polytene chromosome map for Qfly.

One of the most useful tools in *Drosophila* has been the detailed chromosome map produced from the giant chromosomes of salivary cells. Such chromosomes are usually available in other insects, although often difficult to prepare. Such turned out also to be the case in Qfly. However after much effort, literally years of effort, JingTing Zhao in our lab learned to prepare and recognise individual chromosomes, and to use *in situ* hybridisation to map individual markers. She did this under the

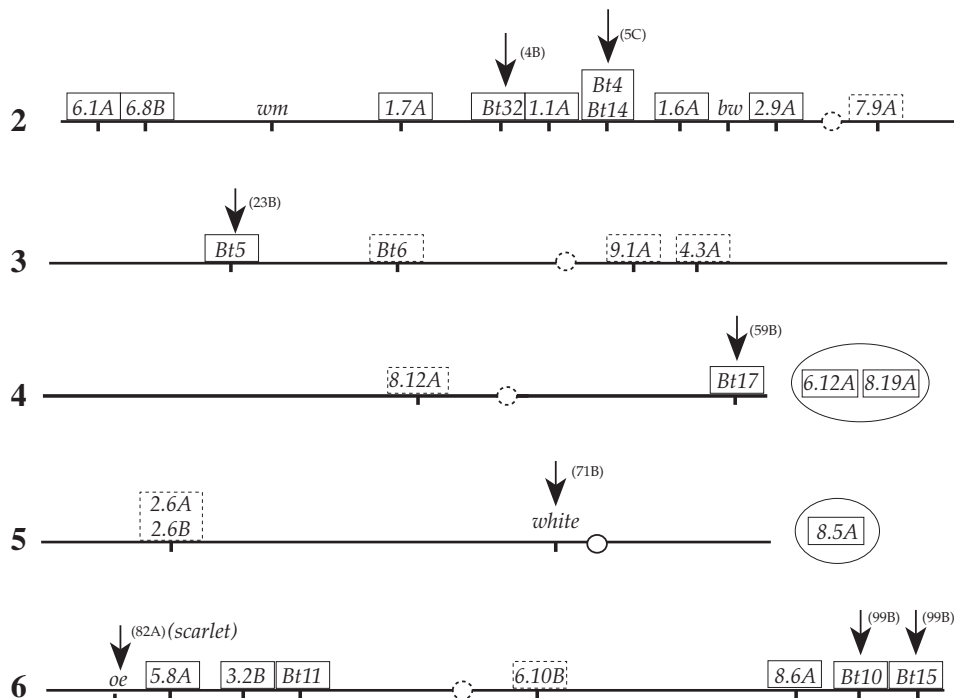


FIGURE 3. Map of microsatellite, visible and molecular markers

tutelage of Antigone Zacharopoulou who similarly spent years working out how to do this in Medfly.

The result of all this is the map shown in Figure 3. This includes the original six microsatellite markers, an additional 20 or so microsatellite markers from a later screen, the three identified visible markers and two molecular markers. Some of this mapping was done using *in situ* hybridisation to particular bands in the polytene chromosomes, shown by arrows on the map. Most was done by standard mapping in crosses, the analysis of which was my small contribution to the effort. For the genetically mapped markers, the positions on the polytene map are only very approximate.

JingTing, who did the chromosome work, has now left the lab. The chances of anyone else learning to do this are very low. I once had a vision of working up a computer system to document banding patterns and identify individual chromosomes, but this was probably pie in the sky. This is a real problem if we ever need to extend the mapping.

3.3. The sterile insect technique (SIT).

The only non-chemical method currently available for control of a pest such as Qfly is through the release of large numbers of sterilised flies. The sterile males mate with wild type females, mate with them, and thereby sterilise them, so the theory goes. There is evidence that SIT works in some cases, the best-known example being screw-worm. SIT is widely used for Medfly. For Qfly, there is a small 'factory' on the outskirts of Sydney where flies are bred, of the order of a few million a week at peak time, the flies are sterilised at a nearby nuclear facility, and then released as pupae.

A problem with SIT in its most basic form is that large scale breeding produces large numbers of females as well as males. Extra irradiation doses need to be given to ensure that fertile females are not released, and the evidence also indicates that the sterile males are less likely to look for wild females if many sterile ones are available at the point of release.

With millions of flies being produced, sexing on an individual scale is impossible. However there are ways of producing so-called genetic sexing strains. In Medfly the Y chromosome, which contains the genes causing the fly to be male, has been joined on to another chromosome containing a particular wild type gene. A mutant form of this gene causes lethality in double dose, but in males, if the mutant gene is protected by the wild type gene, flies are normal. This system needs to be 'conditional', otherwise all females would die. In Medfly the lethal gene is temperature sensitive, so that a burst of high temperature at the early larval stage kills the females. For an account of the procedures see eg. <http://www.ars.usda.gov/is/AR/archive/jun00/flies0600.htm>.

We attempted to produce a similar strain in Qfly. A suitable mutation in our case was the *bent wings* mutation, which is unable to fly, and therefore effectively unfit in the wild. Furthermore we found that the mutation was sensitive to developmental temperature and therefore potentially a fully-fledged temperature sensitive lethal. Therefore our aim was to translocate the Y chromosome onto the chromosome containing this mutant gene, chromosome 2 (Figure 3) to produce a genetic sexing strain. We irradiated the flies and were able to select four cases of Y-2 translocations, the joining that we required. Unfortunately there was so much sterility in the strains produced that we were forced to abandon the exercise. The saga was written up in [5].

Other work in the laboratory has been undertaken to improve the performance of flies released from the factory. The most promising line of work involves hybridisation between established factory strains and

wild type flies to try to nullify some of the weaknesses selected when flies become adapted to factory conditions. This work, undertaken by Stuart Gilchrist, is currently under way.

3.4. Genetic differences between *B. tryoni* and *B. neohumeralis*.

Jennifer Morrow [6] sequenced mitochondrial and *white* DNA in a number of individuals. Surprisingly few differences were found between these two apparently well established species. Even at the level of microsatellite frequencies there are few differences [9].

A major isolating factor in the field is time of mating - *B. tryoni* mates at dusk and *B. neohumeralis* mates during the day. When placed together in the laboratory, however, the two species cross reasonably easily. Hybrids are fertile, can be backcrossed to both parents etc. The co-existence of this species pair is one of the best documented cases of its kind, and work continues to try to understand the genetic and physical factors involved.

4. MOLECULAR STUDIES IN QFLY

The above account covers only briefly the work in the FFRC where I have had some involvement. I have not been involved in the molecular work. Major studies relate to the genetics of sex determination, the study of clock genes, means of transformation etc by Deb Shearman, Kathie Raphael, Marianne Frommer, Cindy An and others. For references to these please see <http://www.bio.usyd.edu.au/fruitfly>.

REFERENCES

- [1] R.A.I. Drew. The tropical fruit flies (diptera: Tephritidae: Dacinae) of the australasian and oceanian regions. *Memoirs of the Queensland Museum.*, 26:1–521, 1989.
- [2] RA Fisher. Has mendel's work been rediscovered? *Annals of Science*, 1:115–137, 1936.
- [3] M Frommer, L E McDonald, D S Millar, C M Collis, F Watt, G W Grigg, P L Molloy, and C L Paul. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual dna strands. *Proc Natl Acad Sci U S A*, 89(5):1827–1831, 1992 Mar 1.

- [4] M. W. Kinnear, H. S. Bariana, J. A. Sved, and M. Frommer. Polymorphic microsatellite markers for population analysis of a tephritid pest species, *bactrocera tryoni*. *Mol Ecol*, 7(11), 1998.
- [5] AW Meats, P Maheswaran, M Frommer, and JA Sved. Towards a male-only release system for sit with the queensland fruit fly, *bactrocera tryoni*, using a genetic sexing strain with a temperature-sensitive lethal mutation. *Genetica*, 116:97–106, 2002.
- [6] J Morrow, L Scott, B Congdon, D Yeates, M Frommer, and J Sved. Close genetic similarity between two sympatric species of tephritid fruit fly reproductively isolated by mating time. *Evolution*, 54(3):899–910, 2000 Jun.
- [7] R Osborne, A Meats, M Frommer, Drew RAI Sved, JA, and MK Robson. Australian distribution of 17 species of fruit flies (diptera: Tephritidae) caught in cue lure traps in february 1994. *Australian Journal of Entomology*, 36:45–50, 1997.
- [8] J. A. Sved, H. Yu, B. Dominiak, and A. S. Gilchrist. Inferring modes of colonization for pest species using heterozygosity comparisons and a shared-allele test. *Genetics*, 163(2), 2003.
- [9] Y Wang, H Yu, K Raphael, and A S Gilchrist. Genetic delineation of sibling species of the pest fruit fly *bactrocera* (diptera: Tephritidae) using microsatellites. *Bull Entomol Res*, 93:351–360, 2003.
- [10] H. Yu, M. Frommer, M. K. Robson, A. W. Meats, D. C. Shearman, and J. A. Sved. Microsatellite analysis of the queensland fruit fly *bactrocera tryoni* (diptera: Tephritidae) indicates spatial structuring: implications for population control. *Bull Entomol Res*, 91(2), 2001.