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Genetic Technologies to Enhance the Sterile Insect Technique (SIT)

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ABSTRACT: The Sterile Insect Technique (SIT) has been used very successfully against range of pest insects, including various tephritid fruit flies, several moths and a small number of livestock pests. However, modern genetics could potentially provide several improvements that would increase the cost-effectiveness of SIT, and extend the range of suitable species. These include (i) improved identification of released individuals by incorporation of a stable, heritable, genetic marker; (ii) built-in sex separation ("genetic sexing"); (iii) reduction of the hazard posed by non-irradiated accidental releases from mass-rearing facility ("fail-safe"); (iv) elimination of the need for sterilization by irradiation ("genetic sterilization"). We discuss applications of these methods and the state of the art, at the time of this meeting, in developing suitable strains. We have demonstrated, in several key pest species, that the required strains can be constructed by introducing a repressible dominant lethal genetic system, a method known as RIDL®. Based on field experience with Medfly, incorporation of a genetic sexing system into SIT programs for other tephritids could potentially provide a very significant improvement in cost-effectiveness. We have now been able to make efficient female-lethal strains for Medfly. One advantage of our approach is that it should be possible rapidly to extend this technology to other fruit fly species; indeed we have recently been able also to make genetic sexing strains of Mexfly (*Anastrepha ludens*).

Key Words: RIDL, genetic sexing, genetic sterilization, genetic marker, genetic containment

The Sterile Insect Technique (SIT) is an effective, species-specific and environmentally friendly method for controlling pest populations (Dyck et al., 2005; Keng-Hong, 2000; Knipling, 1955; Koyama et al., 2004; Lindquist et al., 1992). Modern genetic methods hold out the prospect of significant operational and cost-effectiveness improvements to the SIT (Alphey, 2002; Alphey, 2007; Alphey et al., 2007; Benedict and Robinson, 2003; Gong et al., 2005; Gould and Schliekelman, 2004; Handler, 2002; Heinrich and Scott, 2000; Horn and Wimmer, 2003; Thomas et al., 2000). We have proposed several areas where genetics may provide benefits, including markers, genetic sexing, genetic sterilization and genetic containment. Each of these is discussed below. Recently, we have been able to produce strains of pest species that have the necessary properties. Among tephritids, our primary targets are Medfly (*Ceratitis capitata* Wiedemann) and Mexfly (*Anastrepha ludens* Loew); we are also developing equivalent strains of various other pest insects, including mosquitoes and moths.

There is potentially a great deal of scope for genetic improvements. Fundamentally, SIT programs rear and release sterile insects of the target species to mate with a wild population of the pest. The key property is mating compatibility and competitiveness, which the insects should naturally possess, though this will likely be reduced by genetic or behavioral drift during mass-rearing, by damaging treatment of the strain before release, or potentially by inappropriate strain selection. However, in other respects, wild-type individuals of the target species may be far from ideal agents of biological control. For example, one would like to be able to distinguish the released control agents (sterile insects) from the target wild population. This presently re-

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quires physical modification of the control agents, for example by applying a dye. Genetics could potentially provide an alternative marking system, either by classical genetics, e.g. a visible mutation (Niyazi et al., 2005), or by recombinant DNA methods (e.g. Alphey, 2002; Gong et al., 2005; Handler and Harrell, 2001; Horn et al., 2002; Koukidou et al., 2006). Fluorescent proteins, expressed in the sperm, may also allow more rapid and accurate classification of the mating status of trapped females than present methods. Such strains have been developed for *Drosophila melanogaster* (e.g. Civetta, 1999; Santel et al., 1997) and the mosquito *Anopheles stephensi* (Catteruccia et al., 2005), and significant progress towards developing such strains for Medfly was reported by Marc-Florian Schetelig and Francesca Scolari at this meeting. The use of these various genetic markers may improve the speed and accuracy with which trap catches may be analyzed. In particular, since genetic markers can mark all tissues, and uncertain identifications can be confirmed by molecular analysis (e.g. PCR-based methods), the incorporation of genetic markers into a release strain should reduce the number of false positives (sterile insects mis-identified as wild type) and consequent quarantine responses.

More significantly, genetics may be used to kill selected classes of individual. For example, selective elimination of females would allow the release of a substantially male-only population, rather than a mixture of approximately equal numbers of males and females. This is highly desirable, as discussed below. Such separation of males and females by use of genetics is known as genetic sexing, and strains that permit it are known as genetic sexing strains.

Release of a male-only population is desirable for two independent reasons. First, females may damage fruit, for example by oviposition (so-called sting damage), even if they have been sterilized, thus directly causing some of the damage that the control program is intended to reduce. Second, if males and females are released together, the males may court the wild females, and consequently not seek out the wild females as effectively as if they had been released without sterile females. This distraction effect of sterile females on sterile males has been shown to have a major impact on male effectiveness in the field; in extensive field evaluation, males released on their own were estimated to be 3-5 times more effective than a similar number of males co-released with females (Rendón et al., 2004).

The present genetic sexing strains were made by classical genetics. In these strains a chromosome translocation has moved a selectable marker onto the Y chromosome. In most cases this is the wild type (dominant) allele of a recessive autosomal marker, with a corresponding autosomal mutant or mutant plus deficiency. Such strains have been developed for several species of fruit fly using visible markers such as pupal color (Franz, 2005; Hendrichs et al., 1995; McCombs and Saul, 1995). In the TSL strains developed by the IAEA group at Seibersdorf, Austria, both a pupal color marker (*white pupa*) and a closely linked conditional lethal mutation (*temperature-sensitive-lethal*) are present, with a covering translocation for both on the Y chromosome. Thus the females of the strain show a temperature-sensitive phenotype, but the males do not, so females can be eliminated by a suitable temperature treatment (Franz et al., 1997; Hendrichs et al., 1995; Robinson, 1989; Robinson, 2002; Robinson et al., 1999). Unfortunately, the chromosome aberrations and mutations that are an integral part of these selection systems tend to reduce the overall performance of the flies that carry them, making them less effective agents for biological control. Despite much effort to minimize the problem (Franz et al., 1994; Kerremans and Franz, 1995), the translocations are also unstable; this instability is a significant problem when large populations of in-

sects are reared and rearing methods need to be adapted to compensate. These 'filter rearing systems' add cost, but do appear to adequately manage the issue of strain instability (Caceres, 2002; Franz, 2005). Perhaps most significantly of all, sexing strains made by classical genetics must be developed anew for each new species – genetic tools developed in one species by classical mutagenesis cannot be transferred to another species.

Recombinant DNA methods can provide alternative types of genetic sexing strain. We have developed such strains for both Medfly (Fu et al., 2007) and Mexfly, based on proof-of-principle work in *Drosophila* (Heinrich and Scott, 2000; Thomas et al., 2000). One design goal for these systems was that they should be relatively easy to transfer from one pest species to another – in contrast to classical systems – and this has indeed been our experience. It should now be possible by such methods to develop effective sexing strains of any tephritid suitable for SIT in a relatively short period of time.

Another area of interest is genetic sterilization. All current SIT programs use ionizing radiation as the sterilizing principle, though some early trials and programs used other methods such as inter-species hybrid sterility, cytoplasmic incompatibility or mutagenic chemical sterilants (Benedict and Robinson, 2003; Klassen and Curtis, 2005). However, all that is required is that the progeny of matings between the released individuals and the target population either die or are themselves sterile. For population control, it is sufficient merely that the *female* progeny of such a mating die. In most cases this does not have to be 100% effective; in practice radiation doses are usually adjusted to a level giving high, but not 100% sterility, to give a suitable balance between sterilization and decreased performance of the sterilized fly. Indeed, SIT using considerably lower levels of sterility has been proposed for the mosquito *Anopheles arabiensis* (Helinski et al., 2006).

Genetic systems have the potential to remove the need for irradiation. This was attempted by classical genetics some years ago, for example in the Australian program directed against *Lucilia cuprina*, but the necessary strains are extremely difficult to develop, and tend to suffer from reduced fitness and competitiveness (Whitten and Foster, 1975). Recombinant DNA methods allow a wider range of options. Inducible lethal systems have been proposed where insects die at particular temperatures, for example (Fryxell and Miller, 1995; Schliekelman and Gould, 2000b). However, we have focused on the use of repressible dominant lethal genetic systems (Alphey, 2007; Alphey et al., 2007; Fu et al., 2007; Gong et al., 2005; Thomas et al., 2000), in a method we refer to as RIDL®. In such a system, the lethal effect would be suppressed in the mass-rearing facility by a dietary additive, effectively an antidote to the genetic system. RIDL insects would then be reared and released in the normal way, but without irradiation. Progeny of matings between RIDL insects and wild insects would inherit one copy of the RIDL construct. Lacking the antidote, they would die – much as if the released insects had been irradiated, but without the need to do so. By this approach, the financial cost and direct and indirect (e.g. handling) damage to the insects caused by the irradiation process may be eliminated. We have described the production of such strains of Medfly (Gong et al., 2005), and have equivalent strains for a range of other pest insects.

Though the above discussion assumes the death of all individuals carrying the RIDL system but lacking the antidote, i.e. both males and females, this is not necessary, and may not even be optimal. Killing females only is sufficient, and may in many circumstances be significantly more effective than killing both sexes (Schliekelman and Gould, 2000a; Thomas et al., 2000). Furthermore, this allows the genetic sexing method and

the genetic sterilization to be combined, as follows. A female-specific strain of RIDL insects (i.e. ones homozygous for a RIDL system that, in the absence of the antidote, kills females but not males) would be mass-reared in the presence of the antidote. The insects to be released would simply not be given the antidote – all the females would therefore automatically die, with no further action required from the rearing staff. This is genetic sexing. The remaining RIDL males could be released without irradiation. They would mate with wild females; all the progeny from such a mating would inherit a copy of the RIDL system and so all the female progeny would die. This is sufficient for population control, since the number of females essentially determines the reproductive potential of the population. Furthermore, the males survive and can pass the RIDL system on to at least some of the next generation. This is one of the features that make the RIDL system in principle more efficient than conventional radiation-based methods, even disregarding the cost of irradiation. One potential countervailing issue is that of time of death. At present, this is larval or pupal for published strains. Death at this relatively late stage may be advantageous, compared with earlier lethality, for some species, e.g. mosquitoes (Atkinson et al., 2007; Phuc et al., 2007), however for fruit flies embryo or early larval lethality would be preferable, both for genetic sexing and for radiation replacement. We have recently developed strains with earlier lethality; Ernst Wimmer's group demonstrated a RIDL-like system giving embryonic lethality in *Drosophila* and are attempting to extend this to Medfly (Horn and Wimmer, 2003; Schetelig et al., 2007). It has also been suggested that a control system might be designed around converting females into males, rather than killing them (Saccone et al., 2007; Schliekelman et al., 2005). The performance of female-specific RIDL strains can further be improved by increasing the number of copies of the RIDL system in the mass-reared strain, though here there is a trade-off between the improved effectiveness with increasing copy number, and the likely increased genetic load (Schliekelman and Gould, 2000a).

The above repressible lethal systems have another potential application, in mitigating the consequences of unintended release of mass-reared insects. This application is called genetic containment. At present, mass-rearing facilities for SIT rear very large numbers of dangerous pest insects, which only become beneficial pest control agents once they have been sterilized with a sufficient dose of radiation. Large-scale release of non-irradiated insects would be harmful at best, and potentially catastrophic. In fact no such large-scale releases have yet occurred, though there is clearly a risk that earthquake, hurricane, fire, sabotage or other natural or man-made disaster or accident could have this outcome. However, smaller non-irradiated releases have occasionally happened, for example of New World Screwworm in Mexico and Panama in 2003 (del Valle, 2003). The consequence of such an escape could be greatly mitigated by use of repressible lethal genetic systems such as those described above. Such strains cannot persist in the wild, as they or their progeny require a specific chemical to be added to their diet. As for genetic sterilization, it does not matter whether the system kills just females, or both males and females; either prevents the strain from establishing in the wild. It would seem a wise precaution to employ such genetic containment in mass-rearing, particularly where the facility is located in a region where escape of the pest would be problematic, but also to reduce the negative impact of releasing even small numbers of inadequately irradiated insects in a control program.

The prospective use of recombinant DNA methods in SIT programs will bring to the

foreground regulatory issues relating to the field use of genetically modified organisms (GMOs). This does not apply to conventional methods, despite their use of radiation, and in some cases strains such as TSL, which have highly modified genetic properties. However, though different countries have different regulatory frameworks, often not yet well developed with respect to transgenic insects, the technical aspects do not look insuperable. SIT applications, in which the insects are sterilized before release, by radiation, genetics or other methods, represent a particularly safe, unthreatening, "soft" application of transgenic methods to pest control, and have been advocated as a sensible first application (Alphey, 2002; Benedict and Robinson, 2003). This is in contrast to "gene drive" systems, for example, which are intended to spread themselves through wild populations and therefore raise a number of issues about containment, spread, stability and long-term effectiveness that do not relate to SIT-like applications (Benedict and Robinson, 2003; Braig and Yan, 2001; Gould and Schliekelman, 2004).

One specific technical issue about the environmental safety of transgenic insects has now been solved. This is the hypothetical possibility that the use of non-autonomous transposons as gene vectors (Atkinson et al., 2001; Handler and James, 2000; Li et al., 2005; Spradling and Rubin, 1982; Wimmer, 2003) might somehow destabilize the inserted transgenic construct, perhaps even, in the most improbable scenario, leading to movement of the construct to another species, so-called horizontal gene transfer (Alphey et al., 2002; Handler, 2004; Hoy, 2000; Wimmer, 2003). The basis of this concern is that we know that autonomous transposons (ones that encode their own transposase) are capable of transferring to new species and spreading within them. However, we also know that these are extremely rare events; individual elements probably do not successfully transfer to a new species as often as once or a few times per million years. Such elements are ubiquitous, and they and their derivatives are present in thousands of copies in every insect cell, making up a significant fraction of the entire genome, but their diversity between different insects testify to the rarity of inter-species transfers. For elements that do not encode their own transposase, are present in one or a few copies, are in a species that does not have other transposons of the same type, and where the construct does not provide a fitness advantage, such an event is spectacularly improbable, many orders of magnitude less likely than the transfer and invasion of naturally occurring transposons. However, recently methods have been developed that reduce (Handler et al., 2004) or eliminate (Dafa'alla et al., 2006) this hypothetical risk by eliminating one or both ends of the transposon, respectively. In the method of Dafa'alla et al (2006), all transposon sequence can be eliminated, resulting in an insertion that is no more sensitive to any transposase than is any other gene or sequence in the insect's genome. Perhaps more relevant to real, rather than hypothetical issues, these methods should also allow stable transgenics to be generated even in those insects which do have endogenous copies of the transposon used as the gene vector. For *piggyBac*, the most widely used gene vector, the Oriental Fruit Fly *Bactrocera dorsalis* (Hendel) and some related species are known to have an endogenous version, closely related to that used for transformation (Handler and McCombs, 2000). However, even here such "stabilization" methods may not be necessary – Oriental Fruit Fly was successfully transformed with a *piggyBac*-based gene vector, transformation efficiency was not obviously decreased, nor was there any evidence of instability in the resulting transgenics (Handler and McCombs, 2000).

In conclusion, modern genetic methods are poised to make a series of significant improvements to the SIT. The combined effect should be to improve the cost-effectiveness of the SIT against a variety of pests of agricultural, veterinary and medical importance. This will extend the range of circumstances in which SIT is the preferred pest control strategy, and improve the economics of control for those species, such as several tephritids, where SIT is already the dominant component of an integrated pest management (IPM) strategy.

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