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Autosexing strains to control populations of fruit flies (DIPTERA: Tephritidae): why do they fail to succeed?

Summary

The development of a unique genetic sexing strain to control fruit fly populations has repetedly failed. But why do they fail to succed? We previously demonstrated in *Certitis capitata* (Wied.) that the autosexing mechanism must be developed on the germplasm of the population to be controled. The present integrative study addresses the causes for the lack of success, thus studying: 1- compatibility tests between *Anastrepha fraterculus* (Wied.) germplasms from different geographic origins; 2- the genotype by environment interaction component of phenotypic variation in *A. fraterculus* and 3- essential knowledge on polymorphisms of the Y-chromosome carrying the marker linked to male sex. Our hypothesis: a- chromosomal and morphological variants are associated to different argentinian geographic populations of *C. capitata*; b- chromosomal variants are not randomly distributed in *A. fraterculus* populations. We sampled guava fruits during 30 years to recover larvae and adult flies from both species, in order to study the chromosomal pattern of larvae from wild populations and derived strains. Banding patterns were obtained with routine and molecular cytogenetics. Sexual chromosome variants were associated to different strains. Analysis showed ten sexual chromosome variants in *A. fraterculus*. In *C. capitata* we found sexual chromosome polymorphisms for the X as well as for the Y. Our results -throughout the years- show the necessity of performing periodically genetic samplings of the populations to be treated in order to detect new mutations affecting mating beahaviour between laboratory and wild populations or lack of compatibiliy when applying the sterile male technique. The study of genotype by environment interaction parameter is mandatory to identify the right germplasms on which to develop the autosexing mechanism in order to successfully control populations of both fruit flies species.

Keywords: molecular cytogenetics, genetic sexing, fruit flies, embryogenesis

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Introduction

This work addresses the problem of genetic control of fruit flies populations, particularly the Mediterranean fruit fly *Ceratitis capitata* (Wied) and the South American fruit fly *Anastrepha fraterculus* (Wied). The paper condense 40 years of studies on population genetics structure of both species, to understand the factors underlying the successive failures of genetic sexing strains. Both species belong to the Tephritidae family which groups the true fruit flies. Often, both fruit fly species share fruits: females compete for a common source by punching fruits with their ovipositor to lay their eggs. Then embryogenesis takes place inside fruits, the eggs hatch and larval development begins. The complete cell cycle takes aproximately 30 days for *C. capitata* and 45 days for *A. fraterculus*.[1]

Each Medfly female can oviposite up to 35 eggs/day,[2] while each *Anastrepha* female is reported to oviposit an average of 11-15 eggs/day.[3] Consequently fruit flies control is based on inundative strategies by releasing millions of laboratory sterile flies which must compete with fertile flies of the natural population to be controlled. Genetic control was first based on sterilization of millions of laboratory insects followed by their release in the wild: this is known as the sterile insect technique or SIT. The Sterile Insect Technique (SIT) is an inundative technique based on irradiation and sterilization of millions of laboratory pupae (males and females) to be released in the natural population to be controlled where they will compete with wild flies. It is a flood technique that requires: 1- representative sampling of the natural population and rearing the insects in the laboratory; 2- gamma irradiation of first generation pupae or the sampled insects (dose of radiation must be adjusted according to insect species and stage of development); 3- release of millions of sterile males and females to raise the possibilies of matings with those in nature. This method increases the number of females in wild populations, which although sterile, they maintain the habit of oviposition, thus increasing damage to fruits. An improvement of this technique, is the Sterile Male Technique (SMT) where only irradiated males (sterilized or parcially sterilized) are released. The "only males production" depends on the development of a "genetic sexing strain".

Genetic sexing strain (GSS) concept- A genetic sexing strain is a genetic construction based on 1) an induced point mutation to generate and isolate a selectable genetic marker and 2) a chromosomal translocation in order to link the Y-chromosome -carrying the male determining factor- to the autosome carrying the genetic marker.[4–7] The genetic marker proved and selected to be linked to the sexual Y-chromosome was based on the sw (slow) mutation which maps on chromosome 2 of *C. capitata*.[8] This gene sw affects both the rate of development as well as the eye colour and iridescence in genetic sexing strains (GSS), so it allows separation of fast-developing males from slow-developing females.[9]

Objectives

The purpose of this study is to dissect the reasons for the failure of genetic sexing strains.

1-Production of poor basic knowledge on the genetic composition of the populations to be controlled

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2-Selection of a good genetic marker (poor fitness mutation: white pupa with developmental problems of sclerotization provided BY IAEA)

3- Lack of compatibility studies among different gemplasms

4- Lack of genotype by environment interaction studies

5- The use of a unique genetic sexing strain (GSS) or autosexing strain (male) to copule with all female flies in all populations in the world.

Materials and methods

I. Basic knowledge

a) Representative samplings of wild populations and establishment of laboratory colonies;

b) Transmision of chromosomal, morphological and behavioural polymorphisms within genetic strains throught generations;

II. Selection of a good genetic marker

a) Detection of genetic markers in laboratory colonies and their subsequent isolation in laboratory families and strains to evaluate its fitness.

III. Not enough compatibility studies among different germplasms

a) Germplasms from different geographic origins ;

b) Germplasms carrying different genetic markers

c) These studies were performed in both species[3,9] (and this paper)

IV. Lack of Genotype x environment interaction studies

a) Studies of G x E interaction were developed in *Anastrepha fraterculus*.[10–12]

I. The use of a unique genetic sexing strain to control most populations in the world

Periodically representatitve samplings to monitor the genetic variability in wild populations of each one species and to isolate strains carrying different chromosomal variants are imperative. This allowed us to study the compatibility between:

a) different populations or different derived laboratory strains [13–18]

b) germplasms from different populations including laboratory and field populations. These studies and crossings produced knowledge about the magnitude of the genetic variation that could be present within natural populations occupying different ecological niches.[19]

c) compatibility tests among germplasms from different strains in *Anastrepha fraterculus* in order to know if flies from different ecological niches and with different germplasms are reproductively compatible.

d) Study of chromosome x site interaction in *Anastrepha fraterculus* (DIPTERA: Tepritidae).

Selection of a good genetic marker to be linked to the Y

Pioneer studies in *C. capitata* performed by Ernesto Lifschitz and Fanny Manso[5] were related to selection of a marker to be linked to the Y- sexual chromosome. The limit of tolerance to temperature treatments that a developing embryo of *Ceratitis capitata* is able to withstand without suffering a reduction in hatchability or an increase in late abortions was investigated. This late abortion production was investigated in three different embryo stages and using two pulse lengths. It was found that an exposure of embryos aged 16 hr after egg laying (right before the beginning of the head involution) to 35°C for a period of 15 hr is close enough but below the limit of tolerance for the strain Translocated (Y-nig+) 5038 used in the investigation.

Two male-linked translocation systems, one based on pupal colour, black pupae *bp*,[13] and the other based on temperature sensitivity, *tsl,*[13] have been used in medfly SIT programmes and they have quite different impacts on mass rearing strategy. In strains based on temperatura sensitive lethal mutation tsl, female zygotes are killed using high temperature and for black pupae strains, female and male pupae are separated based on their colour. In all these systems the colony females are homozygous for the mutation requiring that the mutation is not too deleterious and the males are also semi-sterile due to the presence of a male-linked translocation. Zzz

The male sterile technique (MST) requeries a) knowledge on mutagenic processes to generate point mutations and b) knowledge on doses of radiation must be developed on the germplasm to be used: high doses produces reduction of fitness of the genetic sexing strain.

Knowledge on the system of sex determination in *C. capitata*.

Localization of the sex determination factor through *in situ hybridization* to elucidate -along with the study of aneuploids- the system of sex determination in *Anastrepha fraterculus*.[14]

A limiting factor for the success of the *sterile male technique* is compatibility between natural and laboratory populations.[10,14]

The purpose of this work is to dissect the reasons why these methods recurrently fail to succed.

The objective of non contaminant methods, is not "to erradicate fruit flies populations" but to reduce their number to a lower one so that they don´t generate significant economic losses.

The success of this technique requieres deep knowledge on the genetic composition of the populations to be controlled.

Results

Why do Genetic Sexing Strains fail to succeed?

1- Lack of knowledge on the genetic composition of fruit flies populations

2- Lack of knowledge on compatibility among different genotypes and/or germplasms.

3- Lack of knowledge on the selectable marker to be linked to the genetic sexing strain.

4- Lack of knowledge and/or studies about the sublehtal mutant "White pupa"

Since 1982, the *wp* locus was successfully produced by IAEA as a mutant.[15,16]

The *wp* marker (white pupa—mutant, Wappner[17]) is defective in the mechanism that provides hemolymph catecholamines to the puparial cuticle; this defect prevents normal sclerotization and pigmentation and has reduced reproductive fitness.

Where is the key problem? 1- The GSS (see introduction)- obtained

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by applying gamma radiation -to induce a translocation between the chromosome carrying the "*wp* "marker (white pupae has reduced fitness) and the Y-chromosome- accumulates further reduction of fitness. This GSS needs to be sterilized and -at the same time- must compete with wild males of the population to be controlled. This is the first reason for the failures of SIT in Mendoza-Argentina facilities since 1986.

The second reason is lack of monitoring Genetic structure of wild populations.[10] The first essential step to control a population is to develop knowledge on its genetic composition.[20]

a. Knowledge on the genotypic structure of a particular population is based on representative samplings. We studied chromosomal polymorphisms of natural populations from both tephritid species: *C. capitata*.[18,21]

b. Availability of materials with genetic, morphological, enzymatic or behavioral mutations in the laboratory, represents a great advantage to study the genetics, development, behaviour etc.

c. The development of a unique genetic sexing strain to control fruit flies populations has repetedly failed. Our previous studies using cytogenetics along with compatibility tests demonstrated that the autosexing mechanism to produce only males must be developed on the germplasm of the population to be controlled.[10] Thus, we studied the chromosome per site interaction component of phenotipyc variation.

The study of *A. fraterculus* populations based on their chromosome compositions showed variation among localities (Figure 1); the patterns of chromosomal variants established through the analysis of C-banding, N-banding, Q-banding and R-banding patterns[18] demonstrate genetic variability within the populations; we determined the chromosome frequencies across sites and studied the chromosome x site interaction.[12] Our hypothesis was: "chromosomal variants are not randomly distributed in populations of the species".

Single term tests for the chromosome-site data

"D of F" are degrees of freedom

Effect	D. of F.	Marginal χ^2	*p*-level
SITE (S)	4	294.74	< 0.000001
CHROMOSOME (C)	9	1500.00	< 0.000001
C × S Interaction	36	236.52	< 0.000001

The chromosome composition of *A. fraterculus* populations varied among localities.

		Chromosome Variant										
		X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Total
Site	BA	96	0	1	1	5	3	0	0	25	0	131
	MIS	124	78	5	6	15	6	0	0	32	15	281
	TUC	108	15	0	0	31	0	0	0	10	0	164
	BRAZ	148	58	0	0	33	9	8	9	10	0	275
	URUG	10	7	0	4	3	0	4	0	0	0	28
	Total	486	158	6	11	87	18	12	9	77	15	879

Figure 1 The chromosome composition of *A. fraterculus populations varied among localities*.

Taken from Basso et al.[10]

Previously, we studied the single term tests for the chromosome x site data and the estimates and hypothesis testing of model parameters[12] based on data from Argentina, Brasil and Uruguay.

But we have determined the karyotype of 879 individuals from Argentinian Brazilian and Uruguayan populations and studied the chromosome x site interaction.[10] All those natural populations showed chromosomal polymorphisms differing in their frequencies among localities.

Effect	Model Term	Percent Count	Effect (*u*)	St. Error	*z*-value
C × S Interaction	BA – X_1	10.67	+0.6975	0.2931	**+2.38**
	BA – X_2	0.06	-2.7776	1.0526	**-2.64**
	BA – Y_5	2.82	1.5072	0.4098	**+3.68**
	BRAZ – X_2	6.47	0.9154	0.3688	**+2.48**
	BRAZ – Y_3	0.94	1.1724	0.5620	**+2.09**
	BRAZ – Y_4	1.05	1.7009	0.6053	**+2.81**
	MIS – X_2	8.68	0.9378	0.3508	**+2.67**
	MIS – Y_1	1.71	-0.7175	0.3093	**-2.32**
	MIS – Y_6	1.71	1.8208	0.5785	**+3.15**
	TUC – X_1	12.00	0.7881	0.3153	**+2.50**
	TUC – Y_1	3.48	1.3059	0.3537	**+3.69**
	URUG – X_1	1.16	-1.1564	0.3651	**-3.17**
	URUG – X_4	0.50	1.8038	0.5928	**+3.04**
	URUG – Y_3	0.50	1.9699	0.6269	**+3.14**

Populations with the highest number of individuals analyzed (peaches from Montecarlo, guava from Posadas and arazá from Brasil) evidenced -through a hypothesis test- an association between native populations and karyotype frecuencies relative to chromosomal variants of X-chromosome (X_1, X_2, X_3, X_4) and Y-chromosome (Y_1 Y_2, Y_3, Y_4, Y_5, Y_6). Chi square test demostrated that all three populations differ very significatively in the frequencies of the Y (x2=40.015; p< 0.005**) and in those of the X-chromosome (x1= 1.710; p< 0.01**).[12]

Karyotype *"f Arg. 1"* was detected in 15 out of 19 Argentinian populations, in 3 out of 4 Brasilian hábitats and it was observed in a low proportion in Uruguay.

Reproductive compatibility tests IN *Anastrepha fraterculu*[1]

To measure the compatibility between germplasms of different origin, reciprocal crosses were made between individuals from stocks that differ karyotypically. In all cases, fertility was measured through the percentage of eggs that reach pupation. The cross with the highest yield was taken as 100%.

Crosses between stocks - from the same habitat - that differ karyotypically

M212 M25 M35

X1/Y1 X1/X2/Y1 X1Y3

Polyploid Mosaic B Chromosome II_BII_C-III_B-IV_BIV_C-V_AV_B

The objective of performing the reciprocal crosses between stocks that differ in their karyotypes (not shown) was to measure the reproductive compatibility between them. Results show that the reciprocal crosses produced fertile and viable progeny FI, F2 and backcrosses in all cases.[1]

Generations FI and F2

The comparative analysis of the F1 and F2 progenies from 14 crosses, indicated that in two of them there are no fertility differences

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between both progenies. However, in the remaining 12 there was evidence of an inverse relationship between the fertility of Fl and F2, so that when the former gave high values, they decreased in the respective F2 and vice versa.

F1 and backcrosses

The analysis of the paired data from different replicates and situations of the backcrosses was performed by non-parametric methods. This analysis showed that - in our conditions – the Hybrid females F1 were more efficient than hybrid males. It was found that in 16 out of 28 cases, the F1 females were more fertile than the paternal lines ("hybrid vigor" or heterozygous advantage) and in the remaining 12 cases they were found to be inferior. However, the data from Fl males showed that in 6 of the 28 cases they were superior to both parents and in the remaining 22 situations they were less fertile.[1]

The complete compatibility tests are available in the appendix??

Compatibility tests in *C. capitata* are available in Basso et al 2009.

The Sterile Insect Technique (SIT) consists of the massive rearing, sterilization and subsequent release to the environment of sterile specimens of Mediterranean flies thus, females althoug fertile do not leave offspring. The development of a unique genetic sexing strain to control fruit fly populations has repetedly failed.[7]

For the first time in Argentina as also in the world, pioneer studies succeded in developing a genetic sexing strain to control the *Medfly*.[8] A Population is a set of genotypes of the same species with the capacity to intercross and to produce millions of fertile offspring.

Populations of fruit flies compete with man for a common source of food in order ***to survive***. Man being trying different methods to "erradicate" them. The South American fruit fly *Anastrepha fraterculus* (Wied.) and the Mediterranean fruit fly are pests of economic importance. Our previous studies on populations -using cytogenetics along with compatibility tests- demonstrated that the autosexing mechanism to produce only males, must be developed on the germplasm of the population to be controled.[10] This was a major determination concerning behaviour of fruit flies contribuiting to improve the technique.

We emphasize that the purpose of control methods is to maintain the flies population number low enough so as not to damage fruits. It is not necessary neither positive to erradicate them. Is it possible *to erradicate them?* Well, it is not and, how can we be sure we have eliminated all the fruit flies? Only climate and /or ecological conditions could do it. We can define a population of fruit flies present in one tree, or within a park, or with reference to a particular location, or to a particular country. Different methods have been tryied and are applied at present ranging from application of chemical insecticides, introduction of parasitoids, entomopathogenic funghi. These methods are contaminant and/or modifiers of the environment. Genetic methods are originally based on sterilization of fruit flies in order ***to control*** fruit flies populations. These are the only method based on the use of the insect for its own control.

Fruit flies have a very long list of hosts, some of them being reservoirs of these flies. Many host fruits are not economically important at a particular country "during a period of years", but they serve as a reservoir for fruit flies. From this considerations we need to define a population of fruit flies as a group of genotypes within each population. Each population is characterized through its genotypic frequencies. We studied different geographic populations.

Since Genetic control is based on compatibility between the germplasm(s) of the genetic sexing strain(s) and the wild population of fruit flies to be controlled, compatibility studies are necessary.

A population is a set of genotypes, this means it is genetically heterogeneous. Thus, we need to study if those different genotypes are genetically compatible with the genetic sexing strain.

The present work integrates the results of previous works with unpublished data, analyzing the significance of chromosomal variability within and between different argentinian, brazilian and uruguayan populations of *C. capitata* and *A. fraterculus.*

The work demonstrates that

For the success of the Sterile Male Technique previous genetic samplings of the population to be controlled along with characterization of the karyotypes are needed. Unless population studies and while being applied based on the use of a unique genetic sexing strain, SIT will not be a useful technology.

Chromosome x site interaction studies are unavoidable and render rigurous data to ensure the reduction of females within the wild population to be controlled. Without knowledge on populations genetic variability, it is useless to apply SIT technology.[22–25]

F1 and backcrosses

The analysis of the paired data from different replicates and situations of the backcrosses is performed by non-parametric methods. This analysis showed that - in our conditions- the hybrid females Fl were more efficient than hybrid males. It was found that in 16 out of 28 cases, the F1 females were more fertile than the paternal lines ("hybrid vigor" or heterozygous advantage) and in 12 cases they were found to be inferior. However, the data from Fl males showed that in 6 of the 28 cases they were superior to both parents and in the remaining 22 situations they were less fertile.

The complete compatibility tests are available in Basso.[1]

Conclusions

When large samples from a population were available, eight out of ten variants were found.

Chromosomal variants are associated to different sites. There is variation in the frequencies of each karyotypic variant and there are strong interaction effects between these two factors. Karyotypes showed that variants do not recombine at random. Our evidence is consistent with the fact that *A. fraterculus* is a single polymorphic species within the populations studied. Present results point out that the study of genotype by environment interaction will allow to identify the right germplasms on which to develop the autosexing mechanism in order to control populations of fruit flies in a sustainable manner.

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Conflicts of interest

The author declares that there is no conflict of interest.

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