PROCEEDINGS OF THE
FINAL RESEARCH CO-ORDINATION MEETING
CLEARWATER, FLORIDA, 11–13 JUNE 1994
ORGANIZED BY THE
JOINT FAO/IAEA DIVISION OF
NUCLEAR TECHNIQUES
IN FOOD AND AGRICULTURE

Evaluation of Genetically Altered Medflies for Use in Sterile Insect Technique Programmes

EVALUATION OF GENETICALLY ALTERED MEDFLIES FOR USE IN STERILE INSECT TECHNIQUE PROGRAMMES

PANEL PROCEEDINGS SERIES

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FOREWORD

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), continues to be a major pest of fruit and vegetables throughout the world. It is mainly controlled through insecticidal applications in the form of bait sprays. However, there is increasing use of the sterile insect technique (SIT), to the point that this technology can now be considered in some regions of the world as a conventional approach to many medfly problems. Cheap and efficient mass rearing procedures combined with changing economic and trade practices have led to the establishment of many large scale SIT programmes. The invasion of the medfly into new areas has also strengthened interest in the concept of area-wide eradication as a feasible insect management approach.

In the early 1980s it was recognized that the release of sterile males only would have many advantages over releases of both sterile males and females in the application of the SIT; these have been well documented in many IAEA publications. This stimulated the development of genetic sexing strains for *C. capitata* mainly through FAO/IAEA co-ordinated research programmes (CRPs). This publication represents the completion of a second CRP on this subject and progress has been impressive.

The development of specialized strains has progressed from the establishment of the basic genetic tools through field testing of selected strains to the point of implementation in action programmes. To progress from a stage where only a few genetic markers were available to a situation where strains are demonstrating high efficiency in the field represents a tremendous achievement.

This publication summarizes the most recent developments in the field, which were presented at the final Research Co-ordination Meeting of the CRP at Clearwater, Florida, United States of America, in June 1994. This meeting was held in conjunction with the Fourth International Symposium on Fruit Flies of Economic Importance, which permitted the discussions to be expanded to include inputs from key people in related fields. Progress was such that the group was convinced that future activities should focus more on field applications and be broadened to include other fruit flies.

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CONTENTS

— Isolation of 11 Y–autosome translocations,
— Preparation and analysis of polytene chromosomes from trichogen and salivary gland cells,
— Development of sexing systems based on pupal colour and sensitivity to purine and ethanol,
— Field testing of a genetic sexing strain based on pupal colour.

A major difficulty encountered during the development of the genetic sexing strains was strain stability. This is a crucial factor and one which has to be solved if these sorts of strains are to be of any value to large scale SIT programmes for medfly control. This point was addressed in one of the recommendations of the meeting, where it was stated that the quality and stability of these strains under mass rearing conditions must be assessed. The meeting also recommended that evaluation of the strains in the field be considerably expanded to provide data on the effectiveness of all-male releases. Research on the use of the temperature sensitive lethal (*tsl*) approach for genetic sexing had not progressed very far when the CRP was concluded; in fact it was recommended that "research at Seibersdorf on EMS induced *tsl* mutants should be continued for at least one more year." This was certainly not a ringing endorsement of the approach and it is ironic that the major impact on genetic sexing technology in the past five years has been made in this area.

On the basis of the recommendations of the previous CRP, a new CRP entitled Laboratory and Field Evaluation of Genetically Altered Medflies for Use in Sterile Insect Technique Programmes was initiated and the results are published here. The final Research Co-ordination Meeting of the CRP was held in conjunction with the Fourth International Symposium on Fruit Flies of Economic Importance. It was clear from the results presented and discussed at the meeting that tremendous progress had been made in implementing the recommendations of the previous CRP. There are now available reliable field data on the differences between the release of males only and the release of males and females. Furthermore, many of the genetic sexing strains have now been reared under mass rearing conditions, so the situation is approaching where real comparisons can be made concerning the economic feasibility of using these strains in operational programmes. The most significant progress made can be seen in the understanding of the principles involved in the mass rearing of the *tsl* strain under small scale mass rearing conditions and the overwhelming evidence of the effectiveness of the release of males only for SIT application against the medfly. The ability to eliminate females completely from the flies to be released will now enable the SIT to be used for control as well as eradication, thus expanding the areas where this technique can be used.

The proceedings contain 11 papers, which range from an initial molecular analysis of the genome of the fly to a field experiment to assess the impact of an all-male release. It is clear, therefore, that there has been real progress during the course

of this CRP. This is probably a consequence of the composition of the group, which covered the field from the development of the genetic 'tool-box' to the practical application of engineered strains. The first four papers provide an overview of the current status of the genetics and cytology of the medfly and discuss the tools necessary for the development of genetic sexing strains. The next four papers describe the development of sexing strains using several different concepts and are restricted to small scale laboratory studies. More extensive data on large scale rearing and field testing of sexing strains are provided in the following two papers. The final paper of the proceedings presents an overview of the subject and indicates where future research should be focused.

The conclusions of the meeting centred on the need for immediate field testing of the available strains in order that their stability and mass rearing characteristics could be investigated. A major conclusion was that the CRP had been successful in achieving its aims of developing and field testing several genetic sexing strains. It is clear that without the stimulus of the two FAO/IAEA sponsored CRPs success in this area would not have been achieved, and the participants recognized the key role played by the Joint FAO/IAEA Division. However, the success already achieved was not the end of the road and the consensus of the meeting was that the work should be followed up by more extensive mass rearing observations coupled with large scale releases for field assessment. The participants felt that the progress made warrants the testing, for the first time, of the advantages of using these strains for field releases. Sufficient data are not yet available on several key aspects of the use of genetic sexing strains, including long term stability in mass rearing, dispersal, mating competitiveness and economic advantages. If a new CRP is initiated then the field application of the available strains should have a high priority.

Although the medfly is a key pest in many areas of the world, there are important fruit growing areas where other fruit flies are key pests. It was concluded that it would be of value for any future CRP on genetic sexing to include these species. For the development of genetic sexing systems in these species much can be gained from the experiences with the medfly. However, at present, a similar procedure will have to be followed which will entail the construction of genetic maps, the development of cytological techniques and the induction of male linked translocations. In future, the availability of molecular methods could enable sexing systems to be transferred between species.

The participants concluded that it was unwise to restrict the development of genetic sexing strains to the two marker genes currently being used, i.e. *white pupa* and *tsl*, both of which have some minor disadvantages. Within the now extensive gene bank of the medfly, the *tsl* mutation is the only one of its type. In *Drosophila,* where these mutations have been intensively studied, it is known that they can be induced with a high frequency following irradiation. The problem in the medfly is probably therefore not one of induction but of isolation. The necessary genetic tools

are simply not yet available to carry out effective screening for new *tsl* mutations. These genetic tools, i.e. balancers, as well as being used for mutation isolation, can also be used to stabilize current genetic sexing strains and their isolation is urgently needed.

At the initiation of the first CRP, many strategies were enlisted to achieve the goal of genetic sexing. However, it is clear that participants of both CRPs remained clearly focused as scientifically interesting but impractical approaches were quickly dropped. This restraint was instrumental in achieving success.

The multidisciplinary background of the participants, whose expertise ranged from fine grained cytogenetic analysis to medfly field behaviour studies, was also vital in contributing to the success of the programme. The common element linking the group was the insect being studied, coupled with a focus on practical application. The participants concluded that they and the CRP had all benefited from the multidisciplinary approach and thus they had been able to help identify solutions to problems in areas other than their own.

This publication clearly demonstrates the progress made in developing genetic sexing strains in the medfly to a point at which they can be incorporated into mass rearing facilities. This will enable considerable savings to be made in these large scale operational programmes within the near future. It is unlikely that these strains would have been developed without the initiative and support of the Joint FAO/IAEA Division in Vienna.

The initiative to develop genetic sexing strains in the medfly was started in 1980 with the organization of a Consultants Meeting by the Joint FAO/IAEA Division. At that time the genetics of the medfly was rudimentary and the calling of this meeting demonstrated a long term strategic vision by the staff of the Division and of the Insect and Pest Control Section. This vision has been expanded in the intervening years, with the result that at the completion of this CRP, the majority of the recommendations for future work now relate to the field implementation of these strains. To proceed from an idea to field testing of strains in 15 years is a tremendous achievement. Along the way, a highly motivated and focused group of researchers has been assembled that has constructed an extensive genetic and cytological tool-kit for the medfly and ensured that the practical objectives of the CRP have remained highly visible.

The majority of the present list of recommendations are field related and represent the start of the transfer of this technology to users in developing countries. The participants at the meeting recommended the following:

(1) That a new CRP be initiated with emphasis on the field application of genetic sexing strains for SIT control of the medfly. This new CRP should be expanded to include the development of genetic sexing strains in fruit flies other than the medfly.

(2) That the members of the new CRP include a nucleus from the current CRP but that every effort be made to identify new members working on other species and different approaches.

(3) That the necessary technical support be given to SIT programme managers who wish to incorporate a genetic sexing strain into an action programme.

(4) That a major effort be made to study the bionomics of mass rearing of the two sexing systems currently available, namely pupal colour and *tsl*.

(5) That alternative systems for genetic control be investigated, including the isolation of new *tsl* mutations and further studies on the mutations which increase egg and larval development time.

(6) That attempts be initiated to develop strain management procedures following the introduction of genetic sexing strains into mass rearing factories. Mass rearing of these strains at action programme levels will clearly expose them to new and unpredictable biological and genetic stresses.

(7) That the role of cytology, especially polytene chromosome analysis, in the development of genetic sexing strains continue to be stressed. The limited number of scientists with this expertise in fruit flies is a matter of some concern.

(8) That efforts be continued to induce and isolate medfly strains which reduce genetic recombination. The absence of these strains is a serious impediment to the construction of more stable genetic sexing strains. These strains have been readily induced and studied in other insects; their absence in the medfly, despite considerable efforts, is difficult to explain.

GENETICS OF THE MEDITERRANEAN FRUIT FLY IN THE STERILE INSECT TECHNIQUE

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Abstract

GENETICS OF THE MEDITERRANEAN FRUIT FLY IN THE STERILE INSECT TECHNIQUE.

Altogether 27 morphological mutations on the five autosomes of the Mediterranean fruit fly, *Ceratitis capitata* (Wied.), have been isolated and studied in the author's laboratory during 22 years of research on the genetics of this species. Of the 27 loci, 18 were located on chromosomes 4 and 5. No mutant loci were identified on the sex chromosome in the laboratory. Linkage relations, map distances and linear arrangements on the respective chromosomes were established for most of the 27 mutant traits. The *wp* and *dp* traits were utilized in the construction of genetic sexing lines in laboratories involved in studies of the sterile insect technique. The occurrence and consequences of male recombination are discussed.

1. INTRODUCTION

The study of the genetics of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wied.), was initiated in our laboratory in 1972, at Tel Aviv University. In 1975, the laboratory and all the mutant lines were moved to the Israel Cohen Institute for Biological Control (CMBI), where they are still located. The study was supported during the whole period through various research grants by the Insect and Pest Control Section of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

The medfly, a serious pest of over 260 fruit varieties throughout most subtropical and tropical countries, was targeted as a possible candidate for the implementation of the sterile insect technique (SIT). The technique was eventually used for the eradication of the medfly with great success in some Central American countries following the invasion of the pest from neighbouring countries. However, the SIT has not yet been adopted for the control of the medfly in regions or countries where the pest has been established for some time. A major hindrance to the utilization of this technique in countries which export fresh fruit, such as Israel and other Mediterranean countries, was the lack of an automated method for sexing during the rearing process. The release of large numbers of females, although sterilized, in these countries during an SIT programme could result in numerous 'sterile stings' by the

released females and make most of the fruit unsuitable for marketing. This would of course prohibit the use of the SIT. The construction of medfly lines amenable to automated sexing required a basic knowledge of the genetics of the target species. Chromosomes had to be multiply marked, so that they would be easy to follow and recognize during the steps carried out to develop such 'genetic sexing' (GS) lines. These lines had to be constructed with traits such as sex limited morphological or conditional lethal mutants, and had to be true-breeding for these traits.

During the current project we have isolated and studied various morphological mutants, mapped the chromosomes and developed some GS lines. A number of the mutants which were isolated in our laboratory were further used by other laboratories for genetic studies and the construction of the GS lines. Some of these lines have already been used in cage and field tests and proved the feasibility of using all-male releases with success.

2. MATERIALS AND METHODS

2.1. Rearing of medfly populations

Rearing of numerous medfly mutant lines in a limited laboratory space required the development and construction of small rearing units, which include an independent oviposition cage, a cup with larval media and a pupation chamber. A compact rearing unit containing all three elements was developed and has been used in our laboratory for the maintenance of the lines, and for the various crosses which were carried out with these lines in our laboratory [1]. The larval diet contained a sugar, brewer's yeast and wheat bran mixture, with sodium benzoate and a pH of 4.1 for fungal and bacterial control.

2.2. Isolation of mutant lines

Mutant flies were searched for by visual observation of adult flies and pupae. Most of the lines were detected in field collected material which was reared and inbred in the laboratory. A few mutants were observed and isolated from flies which were irradiated at low doses of 1–2 krad (10–20 Gy). Irradiation was mainly used for the construction of translocation lines for genetic sexing. No mutagenic agents were used in our laboratory during these studies.

Adult flies were anaesthetized with a small dose of CO_2, placed on a cold plate (4°C) and observed under a stereoscopic microscope. Flies deviating from the normal appearance in one or more characters were isolated and crossed to their kin; this was

followed by a sequence of inbreeding crosses, up to the third filial generation. Flies resembling the original deviant fly during these cross sequences were isolated and inbred until a true-breeding line was established. Such a 'cleaning' process could last in some cases for several generations and in a few cases the traits, which seemed to be inherited, could not be completely cleaned to produce true-breeding lines.

2.3. Determination of mode of inheritance and chromosome location

The mode of inheritance of the newly isolated and established mutant flies was determined during the process of establishing the mutant stock. The mutants were reciprocally crossed to wild type flies and the F_1 offspring were backcrossed to the mutant flies. Hence the dominance and the sex linkage of the trait could be ascertained.

Once the mutant stock was well established, the flies were crossed to existing mutants, representing the various chromosomes. (We did not have any sex linked mutant stocks, and could not ascertain directly the sex linkage of the isolated mutants.) The linkage relation between the two mutant traits was studied by inbreeding the F_1 hybrids of these crosses and scoring the phenotypes of the F_2 offspring.

2.4. Construction of multiple mutant lines

To determine map distances between mutants and to construct chromosomal maps of the existing mutants it was necessary to construct multiple mutant stocks with at least two linked traits. The method used was to carry the above crosses (between two linked mutant traits) to the F_3 generation and search for the double mutants among the offspring. This could be done as crossing-over occurred (although at a very low frequency) in males and a few multiple mutant flies could be detected among the large number of F_3 offspring. These flies were used to establish multiple mutant lines which were eventually used in further studies.

2.5. Chromosome mapping

The multiple mutant lines (with two or three markers on a single chromosome) were crossed to wild type flies and then test crossed, using classical genetic techniques. Phenotypes of the offspring of these test crosses were scored and the data enabled us in most cases to determine linkage distances and chromosome locations (using three point crosses). The data were not always clear-cut, and the linkage relations and chromosome map of some of these loci remained obscure.

3. RESULTS

3.1. Isolation and identification of morphological mutants

Throughout the operation of our laboratory we have isolated 27 mutant lines (Table I). During this period hundreds of deviant flies have been observed and studied. Most were found to be teratogenic in nature and others, which appeared promising, did not reproduce and were lost.

TABLE I. MEDFLY MORPHOLOGICAL MUTANTS ISOLATED IN CMBI LABORATORY

Symbol	Description	Mode of inheritance	Chromosome	Date found	Ref.
ey	Eyeless	?		22 Jun. 1989	–
hl	Hairless	Recessive		4 Jun. 1990	–
tg	Thoracic groove	Recessive		15 Jun. 1988	–
Pe	Popeye eye shape	Dominant		20 Jul. 1988	–
Oe	Oval eye	Dominant	2	29 Mar. 1989	–
ew	Eroded wings	Recessive	3	21 Sep. 1987	[2]
dp	Dark pupa	Recessive	3	1 Jan. 1974	[3]
sb	Stout bristles	Recessive	4	21 Oct. 1987	–
Sr	Sargeant abdomen	Dominant	4	19 Nov. 1986	[2]
vg	Vestigial wings	Recessive	4	28 Nov. 1990	–
db	Dark brown eye colour	Recessive	4	19 Sep. 1988	–
Sp	Spotty abdomen	Dominant	4	9 Jul. 1986	[2]
dc	Double SO bristle	Recessive	4	1 Jan. 1974	[3]
ap	Apricot eye colour	Recessive	4	1 Jan. 1974	[3]
B	Bar eye[a]	Dominant	4	1 Mar. 1979	[4]
rb	Ruby eye colour	Recessive	5	3 Mar. 1987	[5]
ro	Rough eye	Recessive	5	21 Nov. 1986	[5]
ye	Yellow body	Recessive	5	24 Oct. 1988	[5]
el	Elbowed setae	Recessive	5	9 Jun. 1991	–
we	White eye colour	Recessive	5	29 Jun. 1989	[5]
wp	White pupa	Recessive	5	1 Feb. 1977	[6]
Nw	Notched wings[a]	Dominant	5	29 Mar. 1990	–
or	Orange-red eye colour	Recessive	5	1 Oct. 1978	[7]
Cy	Curly wings[a]	Dominant	5	12 Jul. 1981	[5]
h	Harpoon SO bristle	Recessive	5	8 Jul. 1981	–
gr	Garnet eye colour	Recessive	6	1 Jan. 1984	[4]
bo	Brown-orange eye colour	Recessive	6	9 Aug. 1981	[4]

[a] Semilethal.

On chromosome 5 we identified and established ten morphological mutant loci, on chromosome 4 eight loci, and on chromosomes 2, 3 and 6 one or two loci. No mutant traits have yet been identified on the sex chromosome in our laboratory. These results are rather interesting as chromosomes 4 and 5 are not the largest in the complement. Chromosomes 2 and 3 are large chromosomes and yet showed the lowest yield of identified loci [8]. We have isolated 7 dominant traits and 20 recessive traits. Of the dominant traits, three were semilethal and could be maintained only in the heterozygous state. Three mutant lines, *Pe, tg* and *hl,* are still not true-breeding although they have been maintained for many generations now, and several attempts were made to clean them by various means, including numerous single-pair crosses within the line.

3.2. Recombination between morphological loci

We studied and recorded recombinations between 54 pairs of loci (Table II). These values were derived, for some of the pairs, from more than a single set or type of cross, and Table II lists the highest and lowest values (percentage crossing-over) obtained in these crosses. Recombination in males was observed in 35 pairs of loci, thus indicating that this phenomenon was rather common in the medfly. The recombination values in males were nevertheless much lower than in females. The recombination values associated with the *Sp* and *Sr* loci were quite bizarre. Recombination values for males and females were similar and close to 50% (*Sp & ap, Sp & dc, Sp & lt, Sr & db, Sr & lt, Sr & Sp*). These values (a seemingly independent assortment) could lead to the conclusion that *Sr* and *Sp* were not located on chromosome 4. Additional crosses (*Sr & ap, Sr & dc, Sp & ap* in some crosses) convinced us that both loci were actually on chromosome 4, and the odd data remained uninterpreted.

Recombination values in females were high, and only one pair of morphological loci showed no recombination (*sk & dc*). Ten pairs of loci had recombination values between 0% and 10%, 16 pairs between 10% and 30%, 6 pairs between 30% and 40%, and 21 pairs above 40% (Fig. 1). In males, 26 pairs of loci showed no recombination, 14 pairs between 0% and 2%, 8 pairs between 2% and 20%, and 6 pairs (associated with *Sr* and *Sp*) between 40% and 50% (Fig. 2).

3.3. Three point crosses — linear arrangement on chromosomes 4 and 5

As stated above, chromosomes 4 and 5 yielded the most morphological mutant loci. We found only two loci on chromosome 3 (*dp* and *ew*), two loci on chromosome 6 (*gr* and *bo*) and a single locus on chromosome 2 (*Oe*). The location of *Oe* on chromosome 2 was ascertained by crosses with the *Adh* translocation line by E. Riva in Spain. We constructed numerous mutant lines homozygous for three recessive loci

TABLE II. RECOMBINATION (%) BETWEEN MORPHOLOGICAL LOCI IN THE MEDFLY

Loci	Females	Males	Loci	Females	Males
	Chromosome 3			*Chromosome 5*	
dp & ew	21.57–	0.14	*ye & or*	23.48–	0.62–
			ye & ro	11.28–	0.57–
	Chromosome 4		*ye & rb*	40.95–45.37	0–0.57
ap & dc	7.64–19.17	0–	*ye & w*	39.87–	0.11–
B & ap	0.2–0.54	0–	*ye & we*	37.90–	0–
B & db	26.14–	0–	*Cy & ye*	44.41–	8.39–
B & dc	7.84–17.13	0–	*Cy & or*	22.50–27.85	7.72–15.79
B & lt	47.41–47.86	0.00–0.14	*Cy & ro*	43.48–	5.94–
B & Sr	0.18–	0.11–	*Cy & w*	42.77–46.80	5.94–8.73
db & sb	18.30–	2.45–	*ro & or*	28.56–29.22	0–0.12
dc & db	6.49–	0.68–	*ro & rb*	43.53–44.81	0–1.02
dc & lt	46.24–	0–	*ro & w*	33.36–	0–0.32
dc & sk	0.00–	0–	*rb & Cy*	12.25–13.71	8.39–10.59
lt & dc	42.22–43.88	0–0.14	*rb & or*	34.55–	0–
lt & Sp	53.00–	41.83–	*rb & w*	50.30–50.96	0.37–0.6
lt & Sr	49.75–50	47.7–49.25	*w & or*	44.53–51.87	0.24–1.51
sb & ap	9.02–	0.54–	*we & rb*	5.94–	0–
sb & B	4.50–	0–	*el & wp*	43.84–48.65	0–0.21
sb & dc	9.62–11.02	0.09–2.69	*el & we*	20.62–24.9	0–0.04
sb & lt	46.41–50.37	0.19–2.69	*el & ye*	48.55–	0–
sb & Sr	50.36–	49.95–	*Nw & wp*	44.62–51.9	1.78–
Sp & ap	46.35–48.27	14.37–51.27	*Nw & we*	2.72–	0–
Sp & dc	51.27–	48.43–	*we & wp*	37.67–48.7	0–
Sr & ap	36.09–	0.15–			
Sr & db	49.19–	49.08–		*Chromosome 6*	
Sr & dc	28.13–	0.15–	*bo & gr*	11.1–	3.8–
Sr & Sp	56.06–	47.51–			
vg & ap	27.80–	0.00–			
vg & dc	15.89–	0.00–			
vg & sb	22.09–	0.00–			
vg & B	26.05–	0.00–			
vg & lt	25.46–	0.00–			

(in tandem) each and utilized them for the three point crosses. They were crossed to wild type flies, or to the appropriate dominant mutant line, and the F_1 flies were then reciprocally backcrossed to the recessive multiple mutant flies.

We carried out a total of 17 such crosses (Table III) and were able to establish the linear arrangement in 16 of these crosses. The results were not always clear-cut

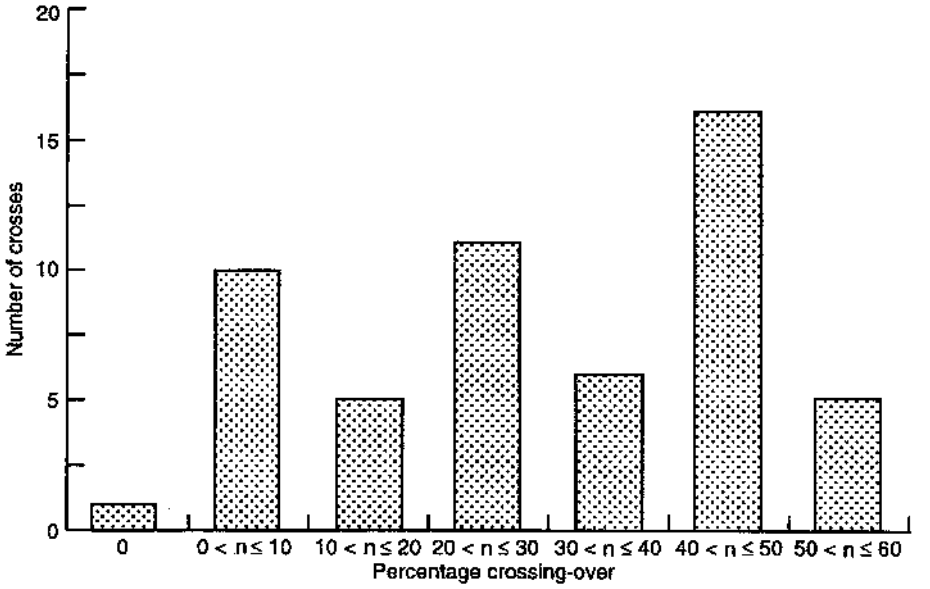

FIG. 1. Recombination in females.

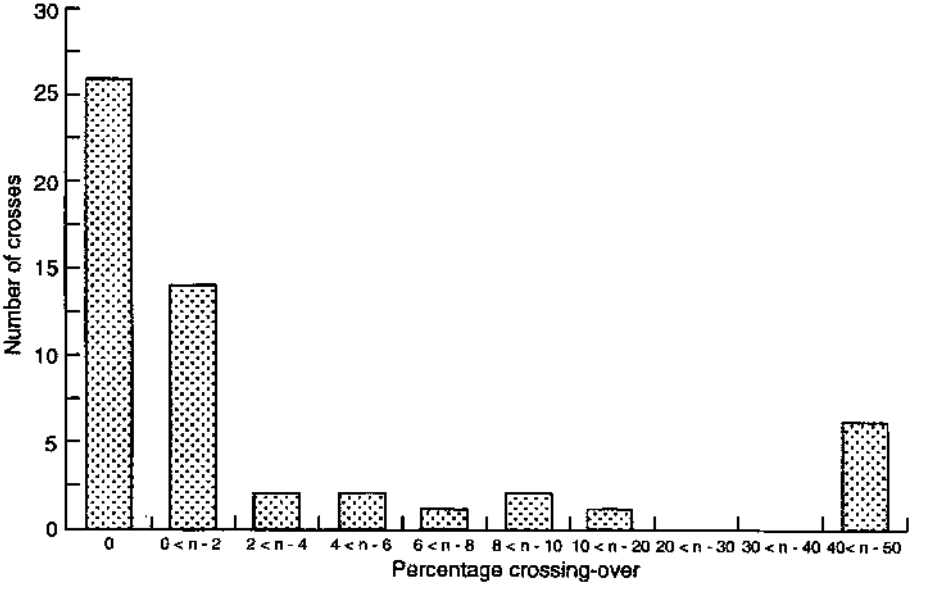

FIG. 2. Recombination in males.

TABLE III. THREE POINT CROSSES IN THE MEDFLY AND ASSUMED ORDER OF MORPHOLOGICAL LOCI

		Total flies	Observed[a] (%)				Expected (%)		Gene order
			n.c.o.	c.o.	c.o.	d.c.o.	d.c.o.	Interf.	
Male	*ye rb wp/+++*	1870	99.89	0	0.11	0	0.00		
Female	*ye rb wp/+++*	2340	33.68	26.45	23.85	16.03	6.31	2.541	*rb ye wp*
Male	*ye rb ro/+++*	881	98.86	0.57	0.57	0	0.00		
Female	*ye rb ro/+++*	1685	51.51	37.21	7.54	3.74	2.81	1.333	*rb ye ro*
Male	*Cy ++/+ ro wp*	539	94.06	5.94	0	0	0.00		
Female	*Cy ++/+ ro wp*	391	37.08	27.37	19.44	16.11	5.32	3.028	*Cy ro wp*
Male	*wp ro or/+++*	2465	99.76	0.12	0.12	0	0.00		
Female	*wp ro or/+++*	3645	46.45	24.33	20.19	9.03	4.91	1.838	*wp ro or*
Male	*wp ro rb/+++*	2186	99.63	0.05	0.32	0	0.00		
Female	*wp ro rb/+++*	2626	37.28	28.18	19.19	14.35	5.41	2.654	*rb ro wp*
Male	*ye we rb/+++*	1204	100	0	0	0	0.00		
Female	*ye we rb/+++*	657	57.08	36.99	5.02	0.91	1.86	0.49	*ye we rb*
Male	*Cy ++/+ wp or*	272	91.91	1.1	6.99	0	0.08		
Female	*Cy ++/+ wp or*	1239	40.44	32.61	10.73	16.22	3.50	4.636	*Cy or wp*
Male	*Cy ++/+ ye rb*	1573	91.61	0	8.39	0	0.00		
Female	*Cy ++/+ ye rb*	2290	48.25	38.03	6.38	7.34	2.43	3.025	*Cy rb ye*
Male	*Sp ++/+ ap dc*	1336	48.73	51.27	0	0	0.00		
Female	*Sp ++/+ ap dc*	1212	40.1	36.8	11.63	11.47	4.28	2.68	
Male	*Sr ++/+ ap dc*	1368	99.85	0.15	0	0	0.00		
Female	*Sr ++/+ ap dc*	1607	58.31	22.53	13.57	5.6	3.06	1.832	*Sr ap dc*
Male	*B ++/+ ap dc*	1422	100	0	0	0	0.00		
Female	*B ++/+ ap dc*	1480	92.16	7.64	0.2	0	0.02	0	*ap B dc*
Male	*B ++/+ lt dc*	1476	99.86	0.14	0	0	0.00		
Female	*B ++/+ lt dc*	1698	48.41	39.69	7.71	4.18	3.06	1.366	*B dc lt*
Male	*sb dc lt/+++*	1116	97.31	0	2.69	0	0.00		
Female	*sb dc lt/+++*	1888	46.19	42.8	7.57	3.44	3.24	1.062	*sb dc lt*
Male	*B ++/+ sb ap*	1598	99.87	0	0.13	0	0.00		
Female	*B ++/+ sb ap*	936	94.23	2.78	2.46	0.53	0.07	7.75	*ap B sb*

TABLE III. (cont.)

		Total flies	Observed[a] (%)				Expected (%)		Gene order
			n.c.o.	c.o.	c.o.	d.c.o.	d.c.o.	Interf.	
Male	*B ++/+ db dc*	1058	100	0	0	0	0.00		
Female	*B ++/+ db dc*	1457	86.48	7.41	4.87	1.24	0.36	3.436	*B dc db*
Male	*ye rb or/+++*	1590	100	0	0	0	0.00		
Female	*ye rb or/+++*	2201	58.88	23.44	15.17	2.5	3.56	0.703	*rb or ye*
Female	*Nw ++/+ wp we*	3521	54.16	43.11	1.51	1.22	0.65	1.874	*Nw we wp*

[a] n.c.o.: no crossover; d.c.o.: double crossover.

and the double crossover products could not always be clearly defined. Such was the case with the backcross of the *Cy ++/+ ye rb* females, which resulted in 38.03%, 6.38% and 7.34% crossing-over, and we used the data of the reciprocal cross of the *Cy ++/+ ye rb* males to ascertain the nature of the double crossover offspring.

The cross involving the *Sp ++/+ ap dc* males and females yielded inconclusive data and the order of the *Sp, ap, dc* loci remained undetermined.

On the basis of the data obtained from the three point crosses, and additional data on recombination between pairs of loci, we assumed the following linear arrangements on chromosomes 4 and 5:

— Chromosome 4: *ap, B, sb, dc, db, vg, lt.* The position of *vg* is still unclear. The positions of *Sr* and *Sp* are undetermined.
— Chromosome 5: *Cy, (Nw), rb, (Nw), we, or, ye, ro, wp.* The position of *Nw* is still unclear. The position of *el* on the chromosome is yet undetermined.

4. DISCUSSION

Most of the 27 mutant traits were assigned to linkage groups, which were initially described as linkage groups A–E [9]. The location of the *Oe* locus on the second chromosome was established using the linkage between that locus and the *Adh1* locus in a male Y–autosome translocation line [10]. Since 1987 the *ap* and *dc* loci have been assigned to the fourth chromosome [11] and the *dp* and *wp* loci to the third and fifth chromosome, respectively [12], with cytogenetic techniques and using chromosomal aberrations. The remaining linkage group (*gr, bo*) was then assigned to the sixth chromosome by elimination.

During 1991, all the morphological mutant traits in our laboratory were documented on diapositives, as part of a final thesis by a photography student (T. Shany). The photographic technique was standardized and documented. All the diapositives are maintained by us for future records.

Some of the mutant traits were used to construct GS lines for SIT projects. This was for the purpose of initiating genetic studies of the pest. The specific goal of these studies was to construct Y–autosome translocation lines involving morphological traits which could be used in automated sexing methods in the mass rearing of the pest. Obviously these traits involved mainly pupal colour (or shape) mutants. Several such lines were constructed using the *dp* locus [13–16] and the *wp* locus [17, 18]. The separation of males from females, by a photoelectric sorting machine, produced an almost clean male batch for release. The feasibility of using GS methods, based on the translocation of the *wp* locus to the Y chromosome, and the effectiveness of all-male releases were demonstrated in cage and field tests in Italy, Hawaii and Israel. There were, however, three types of problem which prevented the immediate adoption of the method for wide and commercial utilization.

(1) *Recombination in males is quite common, but low in the medfly.* This is a serious obstacle to the development and maintenance of GS lines based on Y–autosome translocations [7]. The first GS line involving the *wp* locus showed unexpected segregation ratios of the males and females [19]. That line, T:Y(wp^+)101, was later outcrossed to the double recessive *or wp/or wp* line to produce the T:Y(or^+ wp^+)*/or wp* GS line. Studies of this line showed that recombination in males occurred between the or^+ locus and the translocation breakpoint (0.07%) and between the wp^+ locus and the translocation breakpoint (0.24%) [20]. A more recent line, T:Y(wp^+)30C, was constructed at the laboratories of the International Atomic Energy Agency in Seibersdorf, Austria, and produced a few white-pupa males in every generation from the parental generation onwards, and 2.3% fertile brown-pupa females in ten generations of mass rearing [17]. Studies of Y–autosome translocation lines involving the *dp* or *ap* locus or both loci in Seibersdorf demonstrated also the instability of these lines, but in at least one of these lines, (T:Y;3)1-30, the instability was due to the survival of adjacent-1 segregation products to adulthood, rather than due to male recombination [13]. Early studies with Y–autosome translocation lines indicated that the introduction of a chromosomal aberration into the genome might enhance the occurrence of recombination in males [7]. Lines with Y–autosome translocations involving the *dp* locus were maintained in our laboratory for hundreds of generations. Sporadic checks showed that breakdown of such lines could reach high values (25.6% wild type brown-pupa females in 23 generations). A simple model demonstrated that given 0.1%

recombination in males we will obtain 10% wild type females (which are contaminants from the practical point of view) in 163 generations, and with 1% recombination in males we will obtain that level in 10 generations [21]. There are two ways to prevent or reduce the phenomenon and prolong the practical life span of such a GS mass reared line. We could develop lines with a very short distance between the 'sexing' locus and the translocation breakpoint, or introduce recombination suppressors, such as chromosomal inversions which include the region of the sexing locus and the translocation breakpoint or other chromosomal events which suppress crossing-over [11].

(2) *Separation of females and males is incomplete owing to technical problems.* In practical tests, when the available photoelectric devices were used for sex separation, the results were not always foolproof. Thus, during a field test of the T:Y(wp^+)30C line in Israel, flies of the line were reared in Seibersdorf, irradiated, sexed in the pupal stage and shipped to the test location in Israel. The arriving lots were checked, and we found 3.9–16.6% females (white pupa) among the male lots (brown pupa) arriving in the various shipments during 1989, and 0–4.92% females in the lots arriving in 1990 (unpublished data). The occurrence of females among the released male lots could have been reduced if pupae had been passed through the machine more than once. This would have affected the eclosion of the pupa. It should be noted that the release ratio (released to native flies) is set usually at 100:1. Thus, even 1% females among the released males will double the existing female population in the field and result in some of the fruit being made unsuitable for marketing through 'sterile stings'. The latter are the major reason for the adoption of an all-male release method. Another problem involved in the release of sterile females (even at a very low frequency) is the difficulty of assessing the decrease in the native female population through the adoption of the SIT, as the actual decrease of the native female population is an important parameter for assessing the success and progress of an SIT project. Thus, a reliable and successful GS method should completely eliminate the females from the released lot.

(3) *A genetic sexing system based on pupal colour discrimination is costly.* Flies of both sexes are reared to the pupal stage and only 50% (males) are used in the releases. Thus, there are no savings in the production cost. Additional expenses are incurred through the need to employ expensive photoelectric sorting machines. At best, the pupal colour separation method saves on release costs, as only males have to be released, and the lack of released females probably increases the efficacy of the released males, requiring less males per unit area than bisexual releases. This, of course, is speculative and awaits experimental proof.

During the last two decades, the various laboratories that have been involved in the genetic studies of the Mediterranean fruit fly have isolated and studied nearly one hundred morphological mutations. The co-ordination among these laboratories was only partial and they did not develop a unified nomenclature or test redundancy and duplication of mutant lines. It is highly probable that some of the lines that were isolated and described independently by the various scientists are actually identical. Only a central gene bank in a single, recognized location, assigned with the particular task of maintaining the hitherto accumulated mutants, will be able to bring some order to the present situation.

ACKNOWLEDGEMENT

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EFFORTS TO SYNTHESIZE 'BALANCER' STRAINS FOR CHROMOSOME 5 OF *Ceratitis capitata* AND CYTOLOGICAL MAPPING OF THE *Cy* MUTATION

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Abstract

EFFORTS TO SYNTHESIZE 'BALANCER' STRAINS FOR CHROMOSOME 5 OF *Ceratitis capitata* AND CYTOLOGICAL MAPPING OF THE *Cy* MUTATION.

The authors describe their first efforts to construct 'balancer' chromosomes for the Mediterranean fruit fly, *Ceratitis capitata*. The experiments were focused on chromosome 5, which is genetically the best characterized medfly chromosome. Following the synthesis of a multiple marker strain for chromosome 5, bearing the *Cy* mutation as a selectable marker, attempts were made to induce chromosome rearrangements by gamma irradiation and select those chromosomes showing crossing-over suppression. During this process the cytological mapping of the *Cy* mutation was achieved.

1. INTRODUCTION

'Balancer' chromosomes are special chromosome rearrangements which suppress exchange between homologous chromosomes owing to their complex structures. Such chromosomes, in addition to several chromosome rearrangements, usually bear at least one visible dominant mutation as well as one or more morphological, recessive mutations for identifying the heterozygotes and for easy chromosome manipulation. Balancer strains have proved very useful for genetic analysis in *Drosophila* and are expected to be important in the Mediterranean fruit fly, *Ceratitis capitata*, especially for the synthesis of new genetic sexing strains and/or to improve the existing one. So, having as a model the balancer *Drosophila* strains [1], we have undertaken the synthesis of analogous strains in *C. capitata*.

2. MATERIALS AND METHODS

2.1. Medfly strains

Benakeion is a mass reared strain which is considered as having the standard chromosome arrangement.

Cy we bb wp is a multiple marker strain having the dominant mutation *Cy* (curly, lethal as a homozygote) and the three recessives *we* (white eye), *bb* (blond body) and *wp* (white pupa) all mapped on chromosome 5 by Rössler and Rosenthal [2]. The *bb* mutation is homologous to *ye* (yellow body), characterized and mapped by Rössler and Rosenthal [2].

we bb wp is the multiple marker strain.

Both multiple strains were synthesized by crossing an initial *Cy wp* strain provided by Rössler to appropriate strains and selecting the respective genotypes and phenotypes.

2.2. Induction and selection of chromosome rearrangements

Cy we bb wp males, one to two days old, were gamma irradiated with a dose ranging from 30 to 40 Gy in five different experiments. The following crossing scheme was used for the isolation and maintenance of induced chromosome rearrangements:

P gen *Cy we bb wp*(*irrad* ♂) × Benakeion(♀) (mass culture)

F_1 gen *Cy*(♀) × *we bb wp*(♂) and *Cy*(♂) × *we bb wp*(♀) (single pairs)

↓ ↓

Genetic analysis in F_2 adults (screen for recombinants)

Cytological analysis in F_2 larvae

We maintained for detailed cytogenetic analysis those F_2 families either showing absence of recombination or bearing any chromosome rearrangement, by outcrossing *Cy* flies to the *we bb wp* strain. We considered the outcrossing necessary in order to improve the low fertility and viability associated with the irradiated chromosomes.

3. RESULTS AND DISCUSSION

3.1. Characterization of *Cy* mutant

Following a pilot experiment it became clear that *Cy* strains are always associated with a heterozygous translocation in which chromosomes 4 and 5 are involved (Fig. 1). At first it was considered a spontaneous chromosome mutation in a multiple marked *Cy* strain constructed for use in irradiation experiments. However, more detailed cytological analysis performed in all strains having the *Cy* phenotype revealed that the translocation is always present with *Cy*. It should be pointed out that *Cy* is an induced mutation isolated by Rössler (personal communication) in 1987 following irradiation. Since then, all the existing *Cy* strains have been derived from

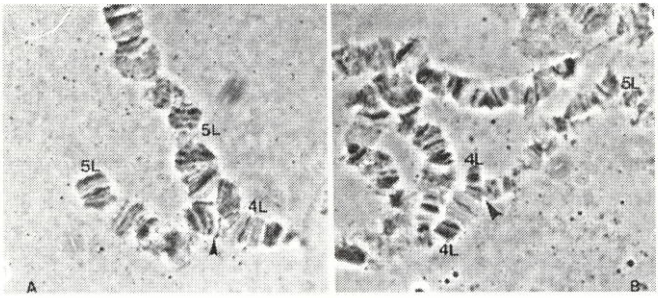

FIG. 1. Salivary gland polytene chromosome from Cy *strains. (A) The pairing cross of the heterozygous T(4;5)*Cy. *(B) The two homologous chromosomes 4, one normal and one translocated. The breakpoints are 41B and 63C for chromosomes 4 and 5, respectively.*

this induced mutation and maintained by crossing *Cy* flies in each generation. Thus, the mutation is not associated with the translocation but with one of the breaks. In view of these data the chromosome position of *Cy* had to be redetermined to find whether it was on chromosome 4 or 5.

Figure 2 shows the karyotype as well as the meiotic segregation of the heterozygous translocation T(4;5)*Cy*. According to this scheme we expect, in addition to the balanced gametes (1, 2) resulting from alternate segregation, two unbalanced gametes (3, 4) from adjacent-1 segregation. The contribution of these last gametes to the viable zygotes could give an answer regarding the position of the *Cy* mutation.

In trying to determine the chromosomal position of *Cy*, three types of single-pair crosses were set up (ten crosses in each case) and the genotypes of their F_1 progeny were examined during the larval stage. Figure 3 shows the crosses as well as the observed karyotypes following polytene chromosome analysis in F_1 larvae. Four classes of karyotypes were observed among the progeny in crosses of type A, where both mated flies had the *Cy* phenotype. Class 1 consists of the normal genotype while

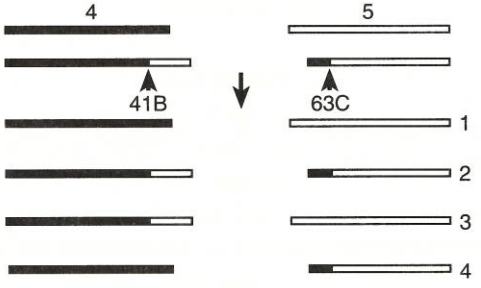

*FIG. 2. The karyotype and meiotic segregation of the translocation T(4;5)*Cy.

class 2 is heterozygous for the translocation T(4;5)*Cy*. The other two classes comprise individuals with an unbalanced chromosome constitution. Both are partially trisomic and monosomic for chromosomes 5 and 4, respectively. One of these has three complete chromosomes (two 5 and one 4) and one translocated, and the second has only one complete chromosome (chromosome 5) and three translocated (two 4 and one 5). Polytene chromosomes from those two aneuploids are shown in Fig. 4.

Three classes of genotypes were found in crosses of type B (Fig. 3), where only one of the mated flies had the *Cy* phenotype. A different situation was observed in

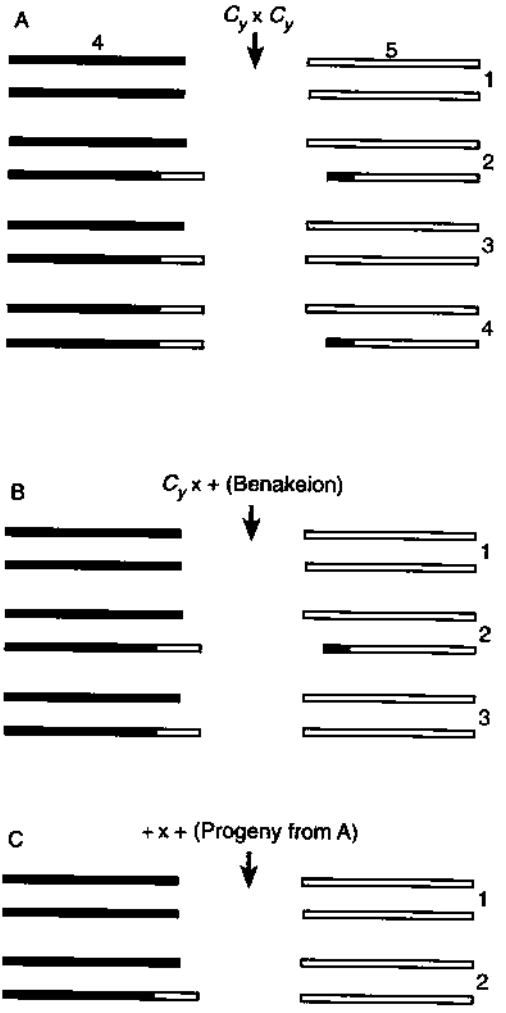

FIG. 3. Types of crosses and the karyotypes observed among their progeny (see text for a detailed description).

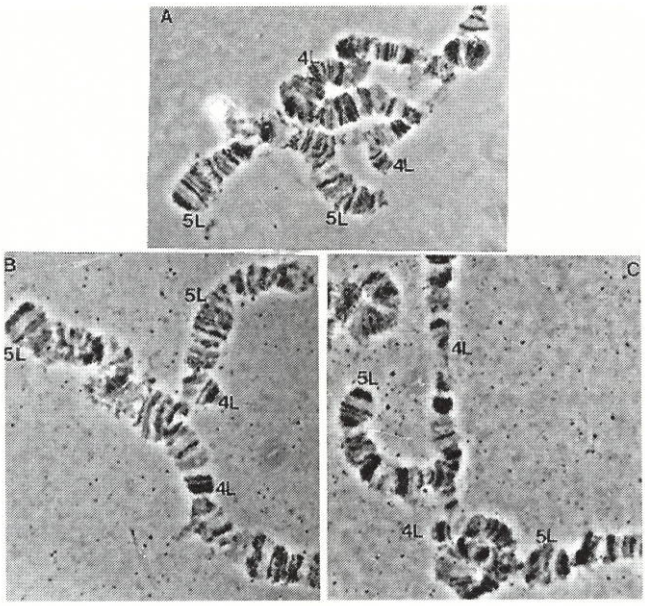

FIG. 4. Aneuploid larvae bearing a triplication for 5L tip and a deficiency for 4L tip. (A) One normal chromosome 4. (B, C) Both chromosomes 4 translocated.

crosses of type C (Fig. 3), where mated flies had the wild phenotype and both were progeny from crosses of type A. Some crosses produced only one normal genotype, while in others two classes of progeny were observed, one showing the normal karyotype and the second an aneuploid one.

These data clearly show that only one of the unbalanced gametes (class 3, Fig. 2) contributes to the viable zygotes at least up to the larval stage. The second (class 4, Fig. 2) may be due to the deleted part of chromosome 5, which does not contribute to the zygotes. In addition to the cytological analysis in the larval stage, F_1 progeny from all three crosses were also checked for the presence of the *Cy* phenotype. Flies showing the *Cy* phenotype were always observed in the first two crosses (A, B) but never in the third (C).

This observation, in combination with the genotypes found in F_1 larvae as well as with the recessive lethal condition of *Cy* mutation, clearly shows that *Cy* maps to chromosome 5 (supporting earlier genetic data [2]) and also to the translocation breakpoint. This position favours the stability of *Cy* strains. The *Cy* phenotype is always associated with the translocation T(4;5)*Cy*.

It is interesting to note that *Cy* in *Drosophila* is also mapped to an inversion breakpoint, although at the moment there is no evidence concerning homology between the two mutations.

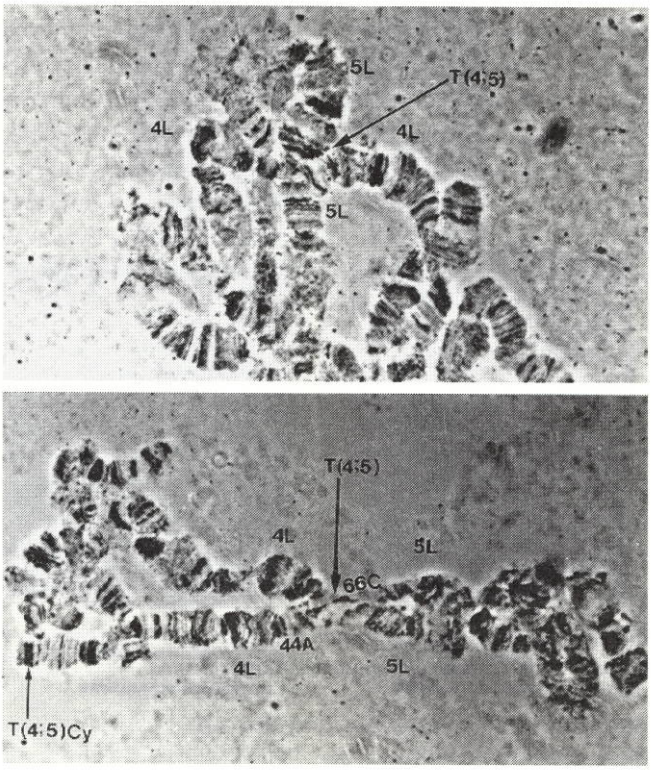

*FIG. 5. An induced translocation between chromosomes 4 and 5, in addition to the T(4;5)*Cy. *Arrows indicate the breakpoints 44A and 65C for chromosomes 4 and 5, respectively.*

3.2. Inversion induction

During two years of effort on the synthesis of a balancer strain for medfly chromosome 5, 450 single-pair crosses were set up in five irradiation experiments. However, most of these crosses exhibited low viability, and in others no progeny were produced, a fact that can be attributed to the induced mutations in the genome of the irradiated males. This of course is a problem in cytogenetic analysis, where a large number of progeny are necessary for a detailed analysis. To overcome these difficulties the *Cy we bb wp* flies were subsequently outcrossed to the *we bb wp* strain until the viability was improved. It should be emphasized here that cytological analysis in *C. capitata* is not as easy as in other Diptera, owing mainly to chromosome quality: chromosomes often break and usually show extensive ectopic pairing, so a large number of chromosome slides are needed for a complete analysis.

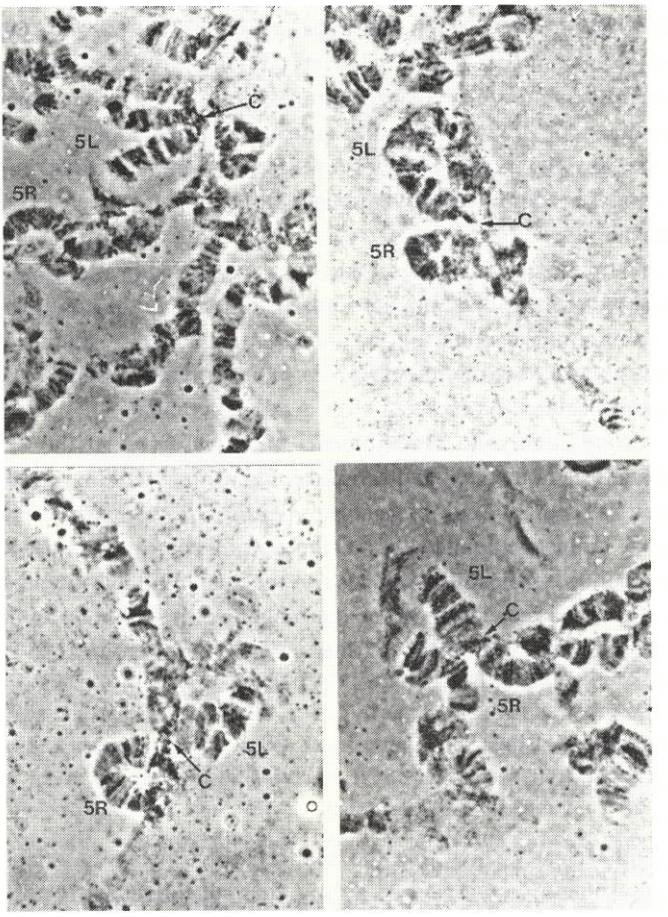

FIG. 6. Pairing configuration of the chromosome 5 centromeric region in the progeny of the F_2 family showed an absence of recombinants.

This condition, in combination with the low viability observed in most families, added to the difficulties. The genetic and cytological analysis could not be completed at the F_2 generation in most of the families.

The results of the first four experiments were disappointing, in that no crossover suppressor in chromosome 5 was found. Although a number of rearrangements, mostly translocations, were observed, none involved chromosome 5, a fact that is in very good agreement with the genetic analysis.

In the last experiment, we raised the irradiation dose to 40 Gy, hoping to increase the frequency of the induced chromosome mutations. A total of 150 F_1 *Cy* individuals (80 males and 70 females) were crossed to the *we bb wp* strain. However,

as a result of both low egg hatch and pupal eclosion, only 25% of them produced progeny, which in some cases were very few. Cytological analysis in these families, where it was possible, revealed a large number of chromosome rearrangements in all families examined, but owing to the difficulties mentioned above, it was not possible to identify the rearrangements. Therefore, we maintained most of the families by out-crossing to analyse them in subsequent generations.

In one family derived from F_1 *Cy* males during the fifth generation a new translocation between chromosomes 4 and 5 was identified in addition to the existing one. It is clear from Fig. 5 that both new breaks are in about the middle of the two left arms. The viability of the strain is good and it is maintained by mating only *Cy* flies. Genetic analysis to assess the effect of this induced rearrangement in combination with the *Cy* translocation on crossing-over is not yet complete.

In a second family no recombinants were observed in the F_2 generation. Cytological analysis in the next and subsequent generations did not show an obvious rearrangement, except a kind of unusual pairing in the centromeric region of chromosome 5. It must be pointed out that this pairing configuration (Fig. 6) is always present with the *Cy* translocation and also in the aneuploid karyotype (Fig. 3, cross A, class 4). If this is a real chromosome rearrangement it should be an inversion with the breaks close to the centromere. Further studies are in progress to confirm these observations.

4. CONCLUSIONS

The results clearly show that the synthesis of a balancer strain, at least for medfly chromosome 5, is not as easy as was first considered. It is possible that the strain used in these experiments is itself one of the reasons for this. However, other reasons related to the dose or the type of irradiator cannot be ruled out. Therefore, our next step will be, in addition to a complete cytogenetic analysis of all families maintained, to try a new irradiation experiment, using this time a different irradiator and possibly a different dose, in addition to a new multiple marker strain for chromosome 5.

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GENETIC STUDIES ON MEDFLY POPULATIONS AND RELATED SPECIES

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Abstract

GENETIC STUDIES ON MEDFLY POPULATIONS AND RELATED SPECIES.

Multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA were used to detect genetic markers in *Ceratitis capitata.* The authors employed both types of markers (1) to study the genome organization of the medfly, (2) to determine the level of intraspecific genetic diversity, and (3) to understand the evolution of the geographical populations. Sterility and high mutation rates in interstrain crosses were observed in *C. capitata,* reminiscent of hybrid dysgenesis in *Drosophila,* and may represent the activation of mobile elements, useful for medfly transformation. The biochemical, genetic and molecular characterization of the enzyme alcohol dehydrogenase clarified the peculiarity of this selectable system, compared with that of *Drosophila,* and revealed a surprisingly high sequence variability in medfly populations. The phylogenetic relationships between *C. capitata* and other Tephritidae species of economic importance were analysed by the MLEE approach.

1. INTRODUCTION

Genetic information on insect pest populations is of great importance to applied entomology, in particular to the long term success of sterile insect technique (SIT) programmes. Crosses between populations from different areas may reveal incompatibilities between geographical 'races'. The major fruit pest *Ceratitis capitata* (Mediterranean fruit fly) has not given rise to taxonomic problems, as there is an apparent morphological uniformity within this species. However, *C. capitata* is in reality a complex of several genetically differentiated populations [1].

Genetic analyses of *C. capitata* and related species may also offer a suitable approach to clarify the systematics and phylogeny of the family Tephritidae. Tephritid taxonomy is also of crucial importance to applied entomology [2]. Incorrect identification or the inability to recognize distinct populations can have drastic and costly consequences for pest control management.

Multilocus enzyme electrophoresis (MLEE), random amplified polymorphic DNA (RAPD), and polymorphism detected at single copy nuclear DNA (scnDNA) represent different methodological approaches applied to the study of *C. capitata* genetics. The knowledge of different aspects of variability extends and deepens our understanding of the biology of the medfly and of its populations, and thus improves the prospects of recognizing factors suitable for exploitation in planning new effective control methods.

2. GENOME ORGANIZATION

The genetic segregation of about ninety RAPD polymorphisms generated with six primers has been studied. These RAPD fragments segregate as expected for simple Mendelian inheritance of dominant markers [3]. Their linkage relations have been assessed, and they have been assigned to the five autosomal linkage groups of *C. capitata,* previously marked with morphological and biochemical markers [4]. The combining of morphological, biochemical and molecular markers has enriched the genetic maps of the medfly.

3. HYBRID DYSGENESIS

A syndrome of abnormal genetic effects, which resembles *Drosophila* hybrid dysgenesis, is present in *C. capitata* [5]. This syndrome includes high frequency of partial or complete female gonadal sterility, chromosomal rearrangements (bridges and fragments) at male meiosis and instabilities at the white-eye (*w*) locus. This syndrome was observed in hybrids of *C. capitata* when strains of different origin were mated. The morphology of the undeveloped ovaries recovered in the medfly is apparently very similar to the gonadal dystrophy (GD) which in *D. melanogaster* is associated with the P–M and *hobo* mediated dysgenic syndromes (Fig. 1). The amount of gonadal sterility that can be observed in medfly hybrids depends on the parental strains used, which exhibit specific differences in their inducing abilities. In the considered interstrain combinations there appears to be quantitative variation in the effect of temperature on GD sterility. The highest level of sterility occurs at 25°C. The pattern of abnormal traits observed in medfly hybrids appears to be the phenotypic expression of a rather complex interacting dysgenic system of inducer and

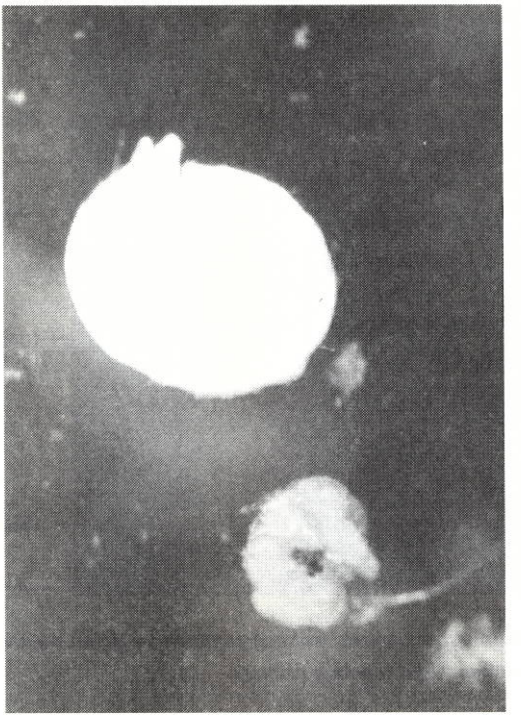

FIG. 1. Illustration showing a normal (top) and a defective ovary of C. capitata, *recovered in a dysgenic cross.*

suppressor effects; probably more than one system is activated in the considered crosses. Genetic instability at the *w* locus has also been observed. Genetic data seem to support the hypothesis that insertional mutations in the *w* locus cause the observed genetic instability [6].

4. GENETIC DIVERSITY AND EVOLUTION

Four African populations of *C. capitata* (Kenya, Libyan Arab Jamahiriya, Morocco, Réunion), five Mediterranean populations (Chios, Crete, Italy, Procida, Sardinia) and two from Guatemala and Hawaii, respectively, were examined by MLEE for genetic variability at 26 enzyme loci. Wright's F_{ST} and Slatkin's estimates, together with tree representations, were used to compare the African populations with the derived ones. Parameters using gene frequencies (F_{ST}, D, Nm) indicate the presence of substantial geographical heterogeneity correlated with the dispersal of the medfly from its source area (Sub-Saharan Africa) to the periphery. Significant

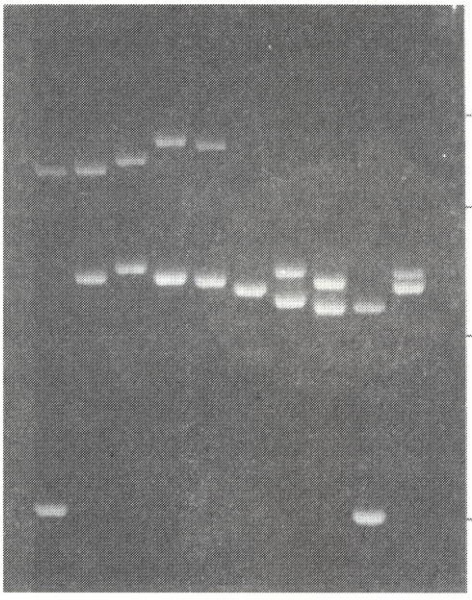

FIG. 2. Adh_1 *first intron size variants isolated from wild Kenyan populations of* C. capitata, *using specific polymerase chain reaction primers.*

estimates of gene flow between the African and derived populations support the hypothesis of recent colonization by *C. capitata* [1]. The analysis suggests that the genetic structure of medfly populations is correlated with the historical events of their colonization. In addition, seasonal and annual variation has been found in a Mediterranean population (Procida island), which chiefly involves the *Mpi* locus [7].

The results obtained by the RAPD markers are significantly correlated with those obtained with MLEE (Fig. 2). They are in agreement with the general trend of decreasing variability from African populations towards peripheral and laboratory ones. However, the RAPD technique reveals larger amounts of genetic variation than conventional MLEE, and can improve discrimination within and between populations. The extremely variable RAPD patterns in medflies from within the ancestral range can represent DNA fingerprints [3, 8].

5. GENETIC, BIOCHEMICAL AND MOLECULAR ANALYSIS OF ADH

The alcohol dehydrogenase (ADH) enzyme system of *C. capitata* has been analysed at the genetic, biochemical and molecular levels. Two ADH proteins exist in the medfly, differing in several features such as pI, tissue localization and

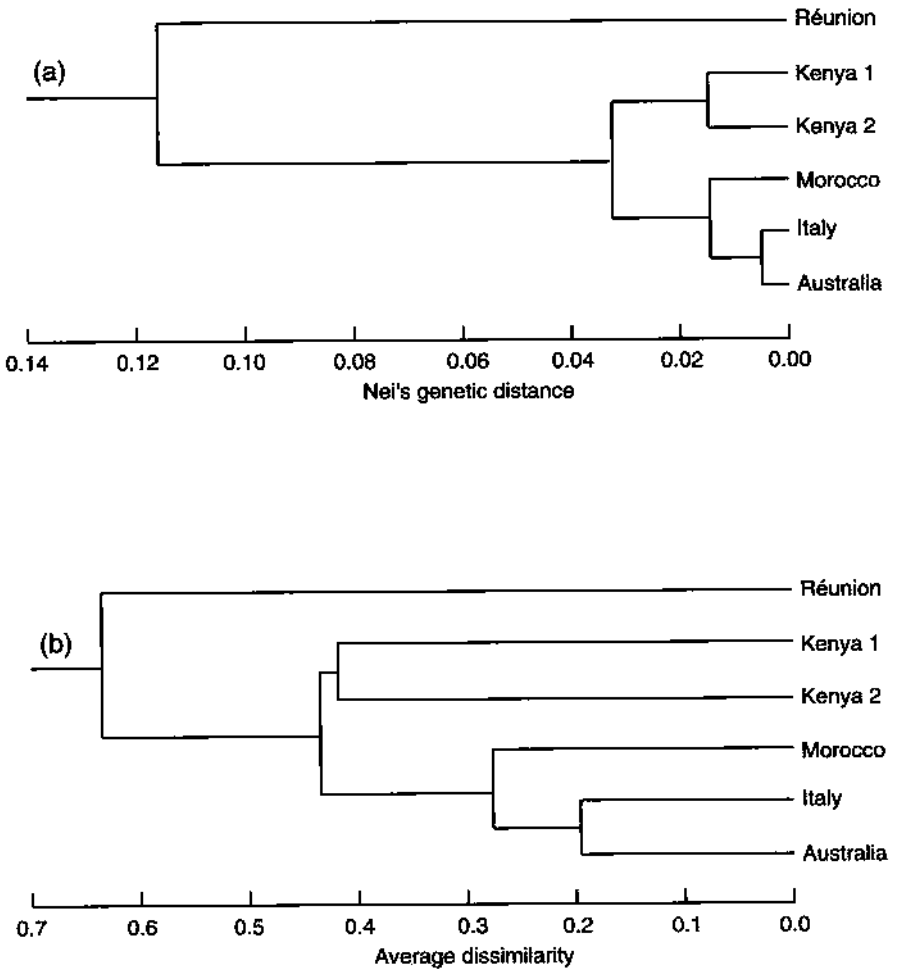

FIG. 3. Dendrograms of geographical populations of C. capitata, *derived from (a) genetic distances evaluated by MLEE and (b) dissimilarity values obtained by RAPD.*

developmental profile [9]. They are encoded by two tightly linked genes (Adh_1 and Adh_2) within 0.49 cmo on the left arm of the second chromosome [10]. These results suggest that the ADH isozymes have arisen by gene duplication. This hypothesis is supported by the immunochemical similarity of the two purified proteins [11].

The cloning of *Adh* genes has been achieved by generation of cDNA probes based on the amino acid sequence of ADH-1 [12]. The molecular characterization of the medfly *Adh* genomic region showed that the first intron of the Adh_1 gene is unexpectedly long, and contains a *mariner*-like element. Variation in the size of this

intron was detected in clones of a genomic library derived from a laboratory strain (Benakeion). Genetic crosses demonstrated codominant Mendelian inheritance for the variants present in this strain.

Further polymorphism at the first intron of Adh_1 has been detected using specific polymerase chain reaction (PCR) primers in a number of wild *C. capitata* populations. Populations from Kenya, within the supposed ancestral range of the species, contain an extremely high level of polymorphism for intron size (Fig. 3). Seventeen or more alleles have been found, each falling into one of three distinct size categories. The number of alleles in each population declines rapidly towards the periphery of the global distribution of *C. capitata*. In the majority of the populations analysed only two alleles, of 2060 and 2600 bp (corresponding to the 1.917 and 2.432 bp introns, respectively), are present. Analysis of the inheritance of the intron polymorphs by means of genetic crosses has confirmed their allelic status. Hybridization of the 1.4 kb intron PCR product to a Southern blot of PCR products from a number of populations indicates that there is a high level of homology between the fragments. Restriction maps of the alleles from the different populations are being constructed. Initial results confirm the presence of a high degree of homology between the fragments. Sequences of the most frequent alleles from the three size categories are nearing completion. It is noteworthy that, in some ancestral populations, intron variants have been found in which the *mariner*-like element is absent. If a functional element should be detected in medfly populations, it will represent the first active transposable element in *C. capitata*.

6. GENETIC AND EVOLUTIONARY RELATIONSHIPS AMONG RELATED SPECIES

There is no generally accepted classification of the Tephritidae, and the systems used are mainly based on morphological traits. Previous MLEE data were inconsistent with the taxonomic classification [13]. Twenty-four orthologous loci (212 alleles) were selected to elucidate the genetic divergence and the phylogenetic relationships among 11 taxa from six genera of the Tephritidae family *(Ceratitis, Anastrepha, Trirhithrum, Capparimyia, Rhagoletis, Bactrocera).* Nei's, Roger's and Cavalli-Sforza's genetic distance values were computed. Two methods of tree construction were employed. The first utilized the unweighted pair group method analysis (UPGMA), the other used the optimality criteria of Fitch and Margoliash [14]. Bootstrap resampling of loci from the original data set [15] was used to test the robustness of the tree topology derived from each method. All trees give the same topology (see Fig. 4). The two *Bactrocera* species, *B. oleae* and *B. dorsalis,* present the greatest range of distances. A common feature of the distance trees is the separation of *Ceratitis rosa* and the *Anastrepha* species *(A. suspensa, A. ludens,*

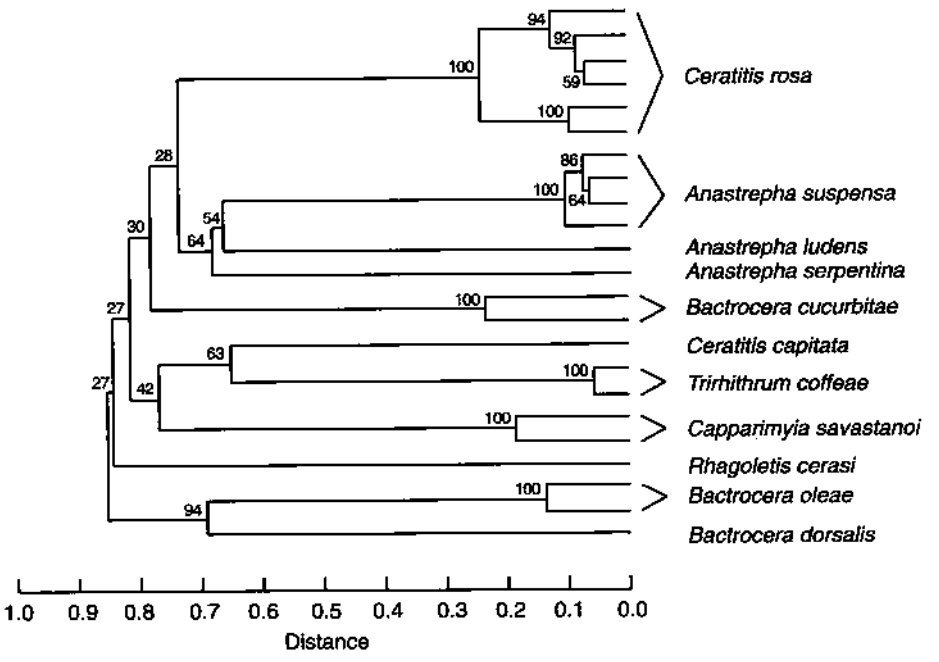

FIG. 4. Consensus tree showing relationships between several Tephritidae species, deduced from the Cavalli-Sforza and Edwards chord distances.

A. serpentina) into a single cluster, and the species *C. capitata*, *Trirhithrum coffeae* and *Capparimyia savastanoi* into another group. The origin of the lineages leading to these two groups of species from two different internodes indicates the possibility of two different ancestors. These findings indicate the need to reconsider the evolutionary relationships between these species.

7. CONCLUSIONS AND PROSPECTS

The biochemical and molecular approaches have provided information adequate to outline the amount and distribution of the genetic variability of the species *C. capitata*. Attention has mainly been centred on the well established multilocus enzyme electrophoretic method for mapping purposes, for assessing the population structures and for determining the phylogenetic relationships between tephritid species. However, the recent use of alternative and/or integrative molecular methods (RAPD and scnDNA) has revealed an additional ample reservoir of cryptic variability in geographical populations of the medfly. Fixed biochemical loci and

DNA fingerprints detected in certain genotypes can represent diagnostic characters for discrimination of medfly populations, and can help elucidate the route of the colonization processes.

The existence of hybrid dysgenic-like phenomena and genetic instability of the white-eye phenotype in *C. capitata* are highly indicative of transposable element activity, and may ultimately provide tools for the development of a germ line transformation system for this species along the lines of the model of *D. melanogaster* [16].

The genetic analysis of the phylogenetic relations of *C. capitata* with several other Tephritidae species can help clarify the controversies of the classification systems based on morphological traits.

ACKNOWLEDGEMENTS

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DEVELOPMENTS IN CYTOLOGICAL MAPPING OF *Ceratitis capitata* BY IN SITU HYBRIDIZATION

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Abstract

DEVELOPMENTS IN CYTOLOGICAL MAPPING OF *Ceratitis capitata* BY IN SITU HYBRIDIZATION.

Cloned DNA sequences were hybridized to salivary gland polytene chromosomes of the medfly, *Ceratitis capitata,* establishing molecular markers for 55 sites on all (ten) autosome arms. Fourteen of the markers correspond to characterized medfly transcription units, while the function of the remaining clones is unknown. Five additional markers were identified as repetitive elements that hybridized to a large number of autosomal sites and also to the granular network that represents the X chromosome. Some of the clones were also hybridized to polytene chromosomes from the orbital bristle cells of *C. capitata* to align the two types of polytene chromosomes, which differ significantly in their banding pattern.

1. INTRODUCTION

The correlation in the Mediterranean fruit fly, *Ceratitis capitata,* of polytene chromosomes [1, 2] to the mitotic chromosomes and to the genetic linkage groups [2] opened up the possibility of localizing genes by cytological analysis of chromosome rearrangements [3] and by in situ hybridization [4]. The latter technique provides a powerful tool for linking molecular and genetic studies, and supplements the classical approaches to establishing chromosome homologies among related species.

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Here, we summarize the current results of in situ hybridization studies in *C. capitata*. In addition to the salivary gland chromosomes, the male orbital bristle cells (trichogen cells) were used for the first time. This study permits a detailed comparison of the two chromosome maps in order to resolve the banding pattern differences between the polytene chromosomes from the two tissues [5].

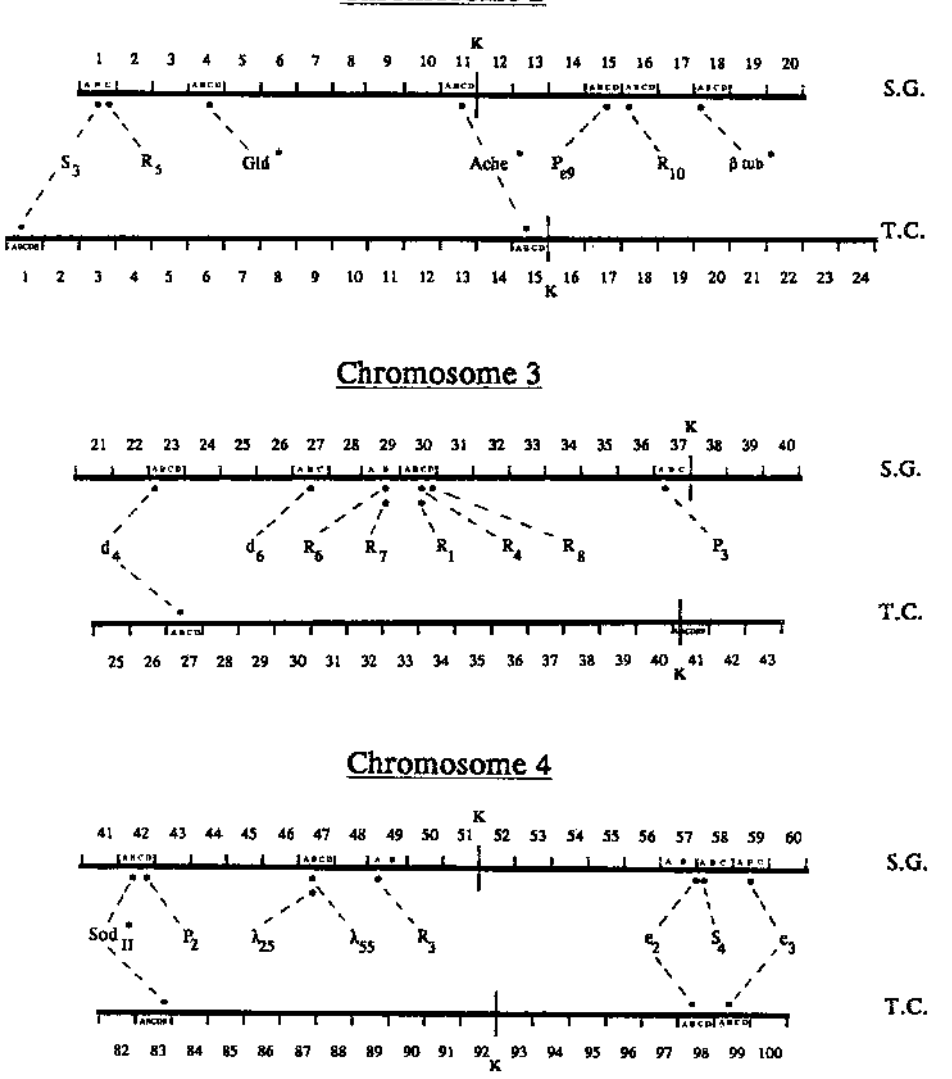

FIG. 1. The DNA cloned sequences and their cytological sites on salivary gland (S.G.) and trichogen cell (T.C.) polytene chromosomes. Asterisks indicate cloned medfly genes.

2. MATERIALS AND METHODS

Larvae and pupae of the Benakeion mass rearing strain were used for chromosome preparations. Squash preparations of salivary gland chromosomes were made from larvae five to six days old following the method of Zacharopoulou et al. [3]. In addition, squash preparations of trichogen cell chromosomes were obtained from male orbital cells according to the technique reported by Bedo [1], but omitting the staining step.

The cloned DNA sequences were isolated from genomic and/or cDNA libraries using in several cases *Drosophila* sequences as probes. Other clones represent random cDNA clones, clones obtained through amplification by the polymerase chain reaction, or clones originating from random amplified polymorphic DNA (RAPD) analysis. DNA labelled using biotin was used in a standard hybridization procedure [3]. The resulting hybridization signals were mapped on the basis of published maps for salivary gland chromosomes [2] and trichogen cell chromosomes [1].

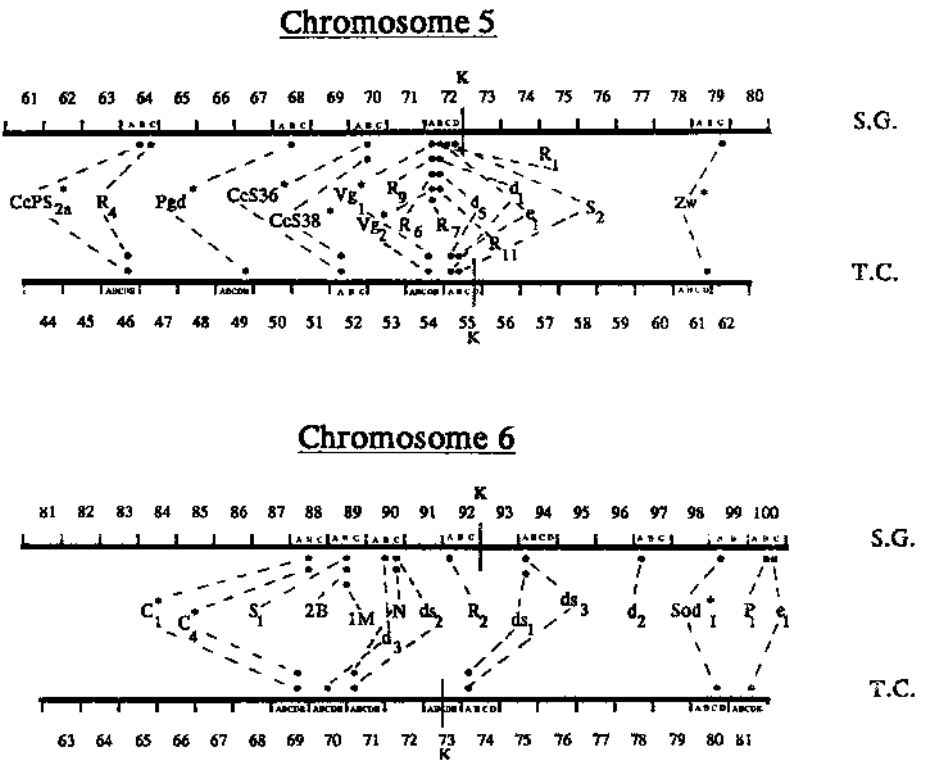

FIG. 1. (cont.)

3. RESULTS AND DISCUSSION

Polytene chromosomes in the medfly are difficult biological material. They often break and show extensive ectopic pairing, especially in salivary gland nuclei. Furthermore, there are only two orbital bristle cells in each male, a fact that adds to the above difficulties. In addition, resolution is lost during the in situ hybridization procedure. Nevertheless, our results clearly show that chromosomes from both tissues can be used for in situ hybridization. Figure 1 lists the clones used and the chromosomal sites to which they hybridize on both types of polytene chromosomes; in total this provides 55 molecular markers for all autosome arms.

Although we used almost all the clones for in situ hybridization in trichogen cells, only a small number could be mapped. This is, in addition to the difficulties mentioned above, due to possible differences in chromosome organization (e.g. degree of polytenization) between the two tissues. A comparison between the two sites in all different chromosomes favours the idea that differential activity of chromosome loci in different stages of development could be one reason for banding pattern differences [6, 7]. However, we cannot exclude other reasons, especially those concerning the structural organization of the chromosomes [8].

Among the molecular markers already mapped, 14 correspond to characterized genes of *C. capitata*. An interesting observation concerns the chromosomal localization of those genes that are homologous to sex linked *Drosophila* genes. All these genes not only mapped to medfly chromosome 5 but also showed a gene order similar to their homologues in *Drosophila* (Fig. 2). This is clear molecular evidence that the two chromosomes are homologous, and further supports the concept that distantly related Diptera species essentially maintain their chromosome identity [9, 10].

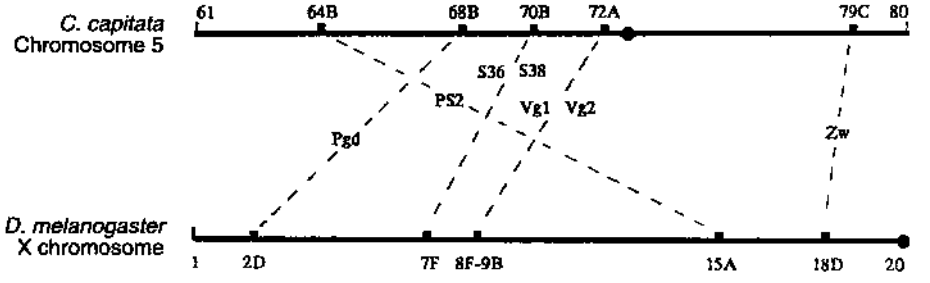

FIG. 2. Cytological sites of several genes mapped on chromosome 5 of C. capitata *compared with homologous sex linked genes of* D. melanogaster.

The function of all other markers is unknown, although some of them were isolated by using *Drosophila* genes as probes. Nevertheless, a number of them could be very useful in genetic mapping experiments to correlate genetic and cytological maps. Some of these clones, symbolized as 'R' in Fig. 1, are polymorphic in different medfly populations (restriction fragment length polymorphisms and RAPDs).

Finally, six of the DNA clones tested showed a hybridization pattern characteristic for repetitive sequences. They hybridized to a large number of chromosomal sites in all autosomes and to the heterochromatic network that represents the X chromosome [6, 7]. It would be interesting to examine whether such elements may be transposable in different medfly strains.

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GENES AND CHROMOSOME ARRANGEMENTS AFFECTING SEX RATIO IN THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata* (Wied.)

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Abstract

GENES AND CHROMOSOME ARRANGEMENTS AFFECTING SEX RATIO IN THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata* (Wied.).

The *MP* (male producing) factor, which shows temperature sensitive meiotic drive favouring the Y chromosome, proved to be highly variable in spermatozoal deficiency in different cysts within a single testis. However, the overall loss of sperm corresponded almost precisely with the loss of females. The minimum proportion of females consistently obtained in inbred lines was about 30–35%. On the basis of parallel studies with the mosquito *Aedes aegypti,* variability between cysts is open to interpretation in terms of different rates of senescence. The T:Y(wp^+)30C genetic sexing strain, which is designed to generate males with brown (wild type) puparia and females with white puparia, was contaminated artificially in a series of population experiments to investigate the pattern of breakdown. Wild type contamination with either sex caused an increase of brown pupae. The sex ratio became progressively distorted in favour of females after contamination with females, mated or unmated, but not after male contamination. The experiments revealed evidence of a low frequency of natural recombination between wp^+ and the translocation breakpoint on the Y chromosome, shown by the appearance of *wp* males. The frequency of male recombination (r) and the selection coefficient (s) against *wp/wp* were measured over 11 generations. The best fit to the observed data was obtained with $r = (0.14 \pm 0.04)\%$ and $s = (26.0 \pm 2.7)\%$. Using these estimates to predict the frequency of wp^+ females and *wp* males for up to 100 generations, it was concluded that white males would never exceed 0.5% whereas the frequency of brown females was expected to exceed 33% after 25 generations. Published data on the mass reared strain, maintained with a population size of 240 000 adult flies, were subjected to the same analysis. A

higher value of s (between (38.0 ± 3.2)% and (52.0 ± 0.3)%) was obtained under these conditions. Electrophoretic studies on esterases revealed a significantly higher activity in a recently colonized strain from Morocco than in two laboratory strains (H1 and TY4). No change in activity was observed in this strain during the first three generations of laboratory culture.

1. INTRODUCTION

The technical contract (5261/TC) of the International Atomic Energy Agency under which this work on genetic sexing of the Mediterranean fruit fly, *Ceratitis capitata* (Wied.), was carried out extended over the period 1989–1994. The aspect of the work relating to sex ratio distortion by meiotic drive at the *MP* (male producing) locus was already well ahead by then [1–4]. Our aim as we began the contract extension was to enhance the action of *MP*, with the hope of producing a greater reduction of females. To this end we engaged in a programme of selective inbreeding, and we also experimented with manipulating the parental rearing temperature, having demonstrated that the degree of distortion was temperature dependent. Temperature treatments were eventually abandoned as unproductive (see below). Selection experiments produced lines generating around 30–35% females in continuous culture for ten or so generations, without further selection [5, 6]. These strains were used for investigating the mechanism of distortion.

This evidence located *MP* (or a regulator of *MP*) on the Y chromosome, and indicated resistance to *MP* to be either X linked or autosomal. Cytological investigations revealed changes during spermiogenesis, to be reported below.

The second major component of our work arose out of a series of population experiments and computer simulations to investigate the process of breakdown of Y translocation based genetic sexing strains, carried out at the Agency's Laboratories in Seibersdorf [7–9]. Here we report the results of our joint studies on two aspects of work on strain T:Y(wp^+)30C: (1) the effect of adding wild type contaminants to the strain; and (2) measurements of recombination between *wp* and the Y chromosome. We have shown that these two effects have a different outcome in terms of the pattern of strain breakdown.

Recent work has centred upon investigating the impact of unconscious laboratory selection on sexing strains, with respect to which we shall report preliminary work on esterase activity.

2. MATERIALS AND METHODS

T:Y(wp^+)30C [= TY30C], isolated in 1985 by Busch-Peterson et al. [10], is a strain in which the wp^+ allele has been translocated on to the Y chromosome in a *wp*

background, so that males are T:Y-*wp*$^+$/*wp* (brown puparia) and females *wp/wp* (white puparia).

T:Y(*wp*$^+$)4 [= TY4] is a strain based on the same genotype as T:Y(*wp*$^+$)30C, supplied by G. Franz of the Agency's Laboratories, Seibersdorf.

MP comprises A425 and various selected substrains. A425 originated from a single X ray exposed male [1]. The substrain investigated for esterases was H1 [11]; the substrain investigated cytologically was H1-18-27/9 [6]. Both lines supported a significant excess of males.

CHIOS was recently collected on the Greek island of that name, and was obtained from G. Franz.

DOUBLE-CHAETA, obtained in 1979 from Y. Rössler in Israel, was originally homozygous for the mutant double chaeta (*dc*) although the mutant phenotype was no longer evident.

SEIBERSDORF was a long standing laboratory colony.

MOROCCO was obtained from P. Howse, of Southampton University, straight from the field, and was collected from Argon trees.

Most of the experimental methods have been published [12–14] or reported in University of Manchester postgraduate theses [4–6, 15]. Others are described briefly in the appropriate section.

3. DISCUSSION OF RESULTS

3.1. Strains with excess of males due to meiotic driving *MP* factor

The proportion of females in progenies of *MP* males was found to decrease significantly when such males were exposed as pupae to a reduction in temperature from (26 ± 2)°C to (18 ± 1.5)°C for 24 h from the end of day 3 of pupal development [1–4]. More recent studies have shown, however, that this only happens when the proportion of females is initially relatively high (around 45%). In genetically selected lines, yielding a lower proportion of females (30–35%), temperature treatments of 18°C were found not to reduce the proportion of females any further.

The holandric pattern of inheritance of *MP* [4] resembles that found in the better known *MD* (meiotic drive) system of *Aedes aegypti* [16]. The *MD* haplotype causes X chromosomes to fragment into two or more pieces. The result is a reduction in the number of spermatozoa and gross abnormalities in some of the remainder [16]. The extent of X chromosome fragmentation is more than enough to explain the deficiency of spermatozoa and almost sufficient to explain the loss of females (Table I). The reduction of sperm density in *MD* males is, however, insufficient to explain the depletion of females observed in the progeny of such males (Table I). The

proportion of abnormal sperm cannot be defined precisely in *Ae. aegypti*, which probably accounts for the disparity.

In *MP* males of *C. capitata* we have found a comparable depletion of sperm and similar types of abnormality: compound spermatozoa with multiple axial filaments and extra mitochondrial derivatives. However, the possibility of investigating the precise extent of spermatozoal loss and abnormality is more favourable in this species. This is because in the young testis of *C. capitata*, spermatozoa are arranged in defined cysts, those in the same cyst being the product of a single primordial germ cell [6]. The normal number per cyst in *C. capitata* is 256, the result of six mitoses plus meiosis. By counting the number of normal spermatozoa per cyst in *MP* males, it can readily be shown that the average depletion corresponds almost precisely with the loss to be expected from the sex ratio distortion observed in the progeny of such males (Table I). The correspondence confirms that it is mainly X bearing spermatozoa that are lost or abnormal.

One observation to be noted [6] is the wide variation between cysts within the same *MP* testis. Some show 50% spermatozoa missing or abnormal (100% expression of *MP*); others show the full complement of apparently normal spermatozoa (zero expression of *MP*, Fig. 1). Because the spermatozoa from different cysts become mixed together, it is the average content of spermatozoa per cyst which is reflected in the sex ratio (Table I).

TABLE I. PERCENTAGE OF FEMALES OBSERVED IN THE PROGENY OF *Ae. aegypti* MALES CARRYING THE Y LINKED *D* GENE AND *C. capitata* MALES CARRYING THE Y LINKED *MP* GENE, COMPARED WITH PERCENTAGE OF FEMALES EXPECTED ON THE BASIS OF (1) OBSERVED FRAGMENTATION OF X AND Y CHROMOSOMES AT SPERMATOGENESIS, (2) SPERM DEPLETION (DENSITY COMPARED WITH MALES NOT CARRYING THESE GENES), (3) SPERM DEPLETION PLUS SPERM ABNORMALITY

Species	Meiotic drive gene	Percentage female pupae or newly emerged adults				Ref.
		Predicted by X or Y breakage at spermatogenesis	Predicted by sperm density (including abnormal sperm)	Predicted by density of normal sperm	Observed	
Ae. aegypti	*D*	8.6	21	<21	3.8	[17]
C. capitata	*MP*	—	42.9	37.7	37.6	[6]

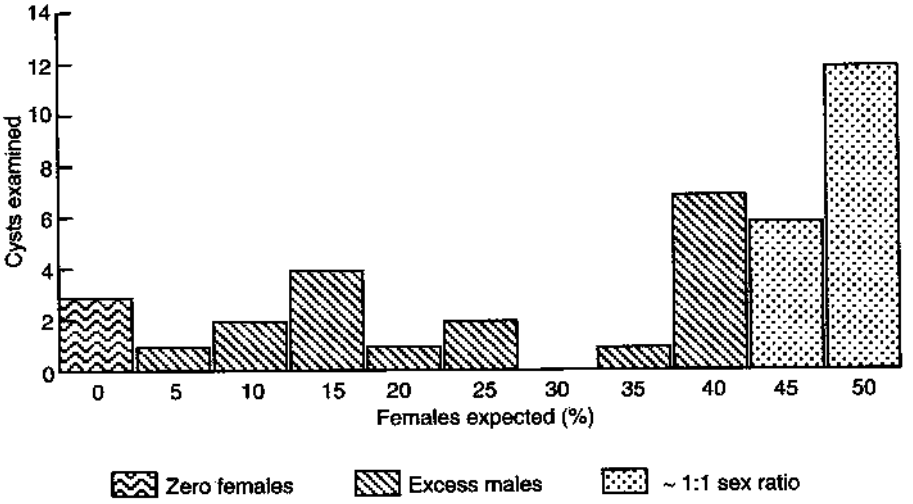

FIG. 1. Percentage females expected from the gametes present in 39 mature cysts taken from two testes of the MP *strain of* C. capitata.

To be of practical use as a genetic sexing technique, a meiotic drive system, such as *MD* or *MP*, needs to be conditional in its action, in a way that can be easily controlled. To breed such a strain in large numbers demands culture conditions in which the gene is not expressed. Then, with an appropriate stimulus applied, the strain can be transformed to provide only males. A possible approach to making *MP* conditional in its action could lie in exploiting the cause of the variation between cysts of the same genotype. The possibility of some definable stimulus which switches on the *MP* genes in some cysts but not others is intriguing and seems worth investigation. Evidence so far indicates that the variation is not defined by the age of the cyst. When spermatozoa of the first cysts produced were sampled by mating newly emerged males, the sex ratio in the progeny corresponded with the sex ratio predicted from the average degree of sperm loss/abnormality in the cysts examined from the testes of unmated sibs (Table I). Variation between cysts is a subject for our future attention.

Progress in enhancing the potential of meiotic drive genes must surely benefit from future developments at the molecular level. With respect to *MD*, we already have information about the map position of *D* in relation to *M* and to a closely linked band of guanine–cytosine enriched DNA, identified by Hoechst 33258 fluorescence [16]. With the *D* gene cloned, which must be the primary aim, it will be possible to examine its structure with the hope of gaining information on its mode of action. It may also be possible to use it as a probe for the *MP* factor.

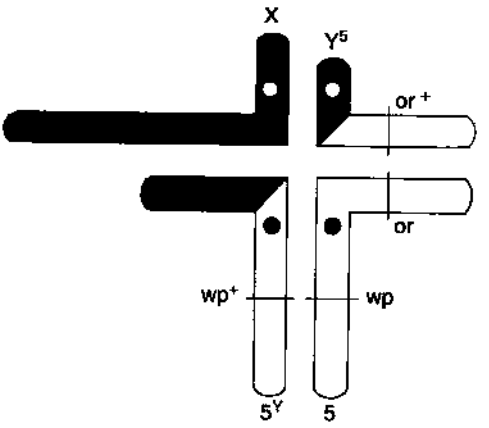

FIG. 2. Diagrammatic representation of the male karyotype of the T:Y(wp⁺)30C sexing strain of C. capitata (or: *orange eye;* wp: *white pupa).*

Many questions about *MP* remain unanswered: do X chromosomes fragment as in *Ae. aegypti*? If they do, what causes it and what protects the Y chromosome from fragmentation? In both species, questions arise which can only be answered by molecular study.

The *D* gene of the *Drosophila SD* system has recently been cloned and sequenced [18]. It may prove an effective probe for locating *D* in *Ae. aegypti* or *MP* in *C. capitata*. Also investigated in *Drosophila* has been the locus *R* which controls response to *D*. Wu and his colleagues have shown *R* to be a satellite DNA array with sensitivity related to copy number [18]. Clearly the potential for genetic manipulation of meiotic drive, as an approach to genetic sexing, is hardly yet exploited. While we may be sure that there is no 'quick-fix' solution to the problem, we feel bound to explore its possibilities.

3.2. Studies on stability of T:Y(*wp*⁺)30C sexing strain

Research on the sexing strain T:Y(*wp*⁺)30C (Fig. 2) by Kafu et al. [12, 13] was directed towards analysing the phenotypic consequences of strain breakdown arising from (a) recombination between *wp* and the Y–5 translocation junction, and (b) outside contamination with *wp*⁺ flies. Key changes in sexual pupal colour and sex ratio, which could be monitored in factory populations in order to identify the cause of breakdown and regulate strain replacement, were identified.

3.2.1. *Recombination*

Laboratory population experiments revealed a low frequency of natural recombination in males between wp^+ and the translocation junction on the Y chromosome, shown by the appearance of *wp* males and wp^+ females. The frequency of recombination (r) and the selection coefficient (s) against *wp/wp* were measured over 11 generations. The best fit to the observed data was found with $r = (0.14 \pm 0.04)\%$ and $s = (26.0 \pm 2.7)\%$. Using these estimates to predict the frequency of wp^+ females and *wp* males for up to 100 generations, it was concluded that *wp* males would never exceed 0.5% after 25 generations even in the absence of outside contamination. Published data on the strain, under conditions of mass rearing, maintained with a population size of 240 000 adult flies, were subjected to the same analysis. A higher value of s (between $(38.0 \pm 3.2)\%$ and $(52.0 \pm 0.3)\%$) was indicated under these conditions. The higher value of s implies greater selection against *wp/wp*, and therefore a more rapid increase in brown females under factory conditions [13].

3.2.2. *Outside contamination by wild type flies*

In laboratory population experiments [12], we showed that the effect of wild type contamination differed according to whether the contaminants were male or female, and if female, whether they had mated. Females became more in excess after female contamination, mated or unmated, but not after male contamination. The changes observed in brown and white frequencies under the three regimes are summarized in Table II. Outside contamination was clearly capable of producing a substantial increase in white male pupae, something that does not occur by recombination. This then is a sign to be looked for as an early warning that a factory strain of T:Y(wp^+)30C has been contaminated.

TABLE II. SUMMARY OF EFFECTS OF CONTAMINATING THE T:Y(wp^+)30C GENETIC SEXING STRAIN ON ONE OCCASION WITH WILD TYPE FLIES

Wild type flies added	Frequency of white male pupae	Frequency of brown female pupae	Percentage males	Frequency of brown pupae
Males	Increase	Increase	No change	(Increase)[a]
Virgin females	No change or slight increase	Increase	Decrease	Increase
Mated females	(Increase)[a]	Increase	Decrease	Increase

[a] Only at high levels of contamination.

3.3. Electrophoretic studies on esterases

Work on esterases in mosquitoes has shown that insect stocks remain highly polymorphic after many years of culture. The pattern of allele frequency distribution observed in *Ae. aegypti* [19] makes it unlikely that esterase variants are entirely neutral. Yet, different stocks are polymorphic for different combinations of alleles. If selection is to be invoked to account for this variability, the question naturally arises, what kind of selection could protect different sets of alleles in different stocks living under the same laboratory conditions in Manchester? Experiments to look for associations of esterase heterozygosity with fitness factors showed heterozygous larvae to be significantly larger and slower growing than homozygotes [20]. Recent work on *Culex quinquefasciatus* showed a comparable degree of variability in this species [21, 22]. It also revealed changes taking place upon laboratory colonization, including marked reductions in band density (i.e. esterase activity) during the first 30 generations of laboratory culture; these were associated with reduced resistance to organophosphorus insecticides.

Because these observations in mosquitoes have implications with respect to laboratory adaptation and quality control for mass sterilized release in general, we decided to investigate esterases in the medfly. The number and density of esterase isozymes active against α-naphthyl acetate were investigated in two laboratory adapted medfly strains, TY4 and H1, and compared with a wild caught strain from Morocco, tested over the first three generations of laboratory culture (Mor P, Mor F_1, Mor F_2). The aim was to answer two questions: (1) whether changes in esterase

TABLE III. DENSITY (ARBITRARY UNITS) OF ESTERASE ISOZYMES ON POLYACRYLAMIDE GELS OF THIRD INSTAR LARVAE OF THREE STRAINS OF *C. capitata*, ONE OF WHICH WAS INVESTIGATED DURING THE FIRST THREE GENERATIONS OF LABORATORY CULTURE

Strain/Generation	Est-2A			All isozymes		
	N	Mean	SD	*N*	Mean	SD
Mor P	24	1.03^{ac}	0.40	81	0.83^{a}	0.49
Mor F_1	21	0.93^{ac}	0.95	75	0.72^{ab}	0.69
Mor F_2	19	1.48^{a}	1.17	79	0.93^{a}	0.82
H1	32	0.62^{bc}	0.26	105	0.52^{bc}	0.27
TY4	18	0.56^{bc}	0.14	83	0.46^{c}	0.34

Notes: (1) Thirty-two larvae in four replicates were investigated in each strain/generation.
(2) Means followed by the same letter are not significantly different.

production can be used as an index of laboratory adaptation; and (2) whether inbreeding by sib mating (single family selection) for several generations, which had taken place extensively in the H1 strain, would affect esterase production.

The electrophoresis was carried out on vertical polyacrylamide gels, relative mobility being measured against bromophenol blue in 50% sucrose solution. The method is described by Khayrandish and Wood [21]. Band density was measured with an LKB 2202 Ultrascan Laser densitometer. Thirty-two 'jumping' (late third instar) larvae were investigated in each strain/generation on the basis of four replicates (gels) each. The replicates were derived from independent larval cultures. Nine isozymes were identified on the basis of relative mobility with bromophenol blue. The single most frequent isozyme present in a large population of larvae of all strains was the middle band of the nine, designated Est-2A (relative mobility RM = 0.26–0.27). The mean densities of this band and those of all bands are compared in Table III. The two laboratory strains show lower esterase activity than the MOROCCO strain. Statistical analysis using data on all nine isozymes revealed the greatest difference to be between MOROCCO and the two laboratory strains (Meddis test, $Z = 3.25$, $P < 0.001$). The variation between samples of MOROCCO revealed no progressive trend except an increase in variance. The data from Est-2A were broadly characteristic of the total picture.

Thus the data collected so far indicate the possibility that a reduction in esterase activity occurs upon laboratory colonization of *Ceratitis capitata*, similar to that observed in *Culex quinquefasciatus* [21, 22]. They also indicate that any reduction which does take place is unlikely to be apparent within the first three generations of laboratory culture. However, we need to look at more generations and more strains. Clearly, the variation between strains may have a different cause.

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STATUS OF MUTATION AND TRANSLOCATION STUDIES FOR MEDFLY GENETIC SEXING IN BRAZIL

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Abstract

STATUS OF MUTATION AND TRANSLOCATION STUDIES FOR MEDFLY GENETIC SEXING IN BRAZIL.

The induction and isolation of four morphological mutants and one temperature sensitive lethal strain of *Ceratitis capitata* are described. After ten generations, the *tsl* strain CENA-78 no longer showed temperature sensitivity during the egg stage, but the sensitivity was maintained in larvae. During tests involving the CENA-78 strain, many females laid few or no eggs. Dissection of the ovaries showed that females had either one or two abnormal ovaries with minor or severe atrophy.

1. INTRODUCTION

There are many advantages to be gained by releasing only males in sterile insect technique (SIT) programmes and these have been well documented. In essence, the elimination of the females from the fly production makes the SIT more efficient and less expensive [1–4]. Also, the monitoring procedures during control and eradication campaigns can be simpler and less expensive.

To achieve genetic sexing for the Mediterranean fruit fly, *Ceratitis capitata*, several different markers were considered. Among them were markers for sex separation based on pupal colour [5, 6]. Conditional lethal systems were also developed where the insects are sensitive either to purine [7, 8] or to allyl alcohol [9]. Cases of sex distortion were also studied [10, 11] but all of these systems presented some difficulties [12].

Recently, a genetic sexing strain based on a temperature sensitive lethal (*tsl*) mutation was constructed [13]. This strain is currently under evaluation in Guatemala (D.O. McInnis, personal communication, 1993). The *tsl* mutation has the advantage that the females can be eliminated during early stages of development with relatively inexpensive equipment that can be adapted to existing rearing technology. However, reliance on a single such mutation with some of its restrictions is unwise. Therefore, it is important to continue the induction and isolation of new *tsl* mutations.

2. RESULTS

2.1. Induction and isolation of morphological mutants

The isolation of *tsl* mutations can be achieved by following the traditional inversion scheme or by using schemes based on the male linked translocations [14]; both schemes could be used to isolate a *tsl* mutation in one specific autosome. An indirect method can be used in which any autosome that has received mutagenic treatment can be screened for new induced morphological mutants. These mutants are the indicators of the mutagenic action and subsequently there is a high probability that conditional lethal factors have also been induced.

Screening for the *tsl* mutation can be following Mendelian inheritance patterns of a sample of embryos after the temperature pulse. A second sample of embryos will serve as control. If the isolation of homozygous *tsl* is successful, the *tsl* linkage analysis can be done by a male linked translocation method [15].

In 1992, one hundred newly emerged wild type males from a *C. capitata* colony, maintained at the Centre for Nuclear Energy in Agriculture (CENA), were treated by feeding them with 1% ethylmethane sulphonate (EMS) in a 10% sugar solution as a drinking water supply for 24 h [16].

After the mutagenic treatment only four males survived. Each of them was crossed with five virgin females. In the resulting F_1 adults 60 single-pair families were set up and in the F_2 offspring three new families (one male with five females) were set up from each subfamily. The F_3 offspring (pupae and adults) were screened for morphological mutants.

From 219 treated sperm one family originated, 3-44-2, in which four morphological mutations were found. The family originated from F_1 family number 44 and carried white pupa (*wp*), black pupa (*bp*), grey pupa (*gp*) and orange-red eye (*or*). The eye colour mutation was classified using the universal colour chart [17] as orange-red No. 187. All mutant strains descended from this family were inbred in order to increase population size for subsequent linkage studies.

2.2. Isolation of temperature sensitive lethal factor

The *wp* strain was chosen for the isolation of a *tsl* factor. Thirty F_4 pairs were set up and the eggs were collected daily, counted and divided into two groups on strips of black moistened filter paper. The strips were placed into glass vials with a larval agar diet based on carrots. One group was incubated at 25°C and the other at 32°C for 48 h. After this period both groups were kept at 25°C until the third larval instar. The hatchability was determined four days after egg collection. Four strains were selected from these 30 initial pairs on the basis of the difference in egg hatch (>20%) between the 25°C and 32°C rearing conditions. Owing to microbiological

contamination of the larval medium, the egg–adult viability of all 29 families was very low. One family (No. 8) laid no eggs.

From the four potential *tsl* families (Nos 12, 21, 13, 24), only eight pupae were recovered from family No. 13; they were also homozygous for *or*. The emerged adults were inbred for two more generations. Adults from the F_6 generation were used for a second *tsl* test. Eighty single-pair families were set up and their offspring were treated as in the first *tsl* test. Five families showed low viability when treated at 32°C.

Family No. 78 was chosen for further analysis because of its low viability in the temperature test. A group of females from this family were crossed to translocation males, strain T(Y;5)(wp^+/wp^-)70, isolated at CENA [18]. Phenotypic analysis of the F_2 offspring (resulting from F_1 males backcrossed to family No. 78 females) showed that the newly isolated *wp* mutation is located on chromosome 5, because the males emerged only from brown puparia and females from white puparia. The potential *tsl* mutation is not located on chromosome 5 because the egg hatch at 32°C was the same for wp^+/wp and *wp/wp* genotypes.

2.3. Biological aspects of *tsl* strain CENA-78

After ten generations, 20 pairs were chosen from this strain and temperature sensitivity tests were run using 1–24-hour-old eggs incubated for 60 h at 33°C. The eggs were seeded on moistened filter paper inside glass vials with a special agar diet for the larvae. After the incubation period the filter paper was removed and the hatchability was determined. The diet vials were transferred to the control cabinet and kept at 25°C until pupation.

The CENA-78 strain did not maintain temperature sensitivity in the egg stage but lethality was observed during the larval stage. The diet vials were examined daily and larval mortality was checked by counting dead second or third instar larvae. The length of the larval development period was also checked. The mean larval period was 16.75 d for the high temperature treatment and 12.95 d for the control (25°C) temperature. In both cases the larvae developed significantly more slowly than larvae from a wild type strain, the difference being 6.67 d.

2.4. Linkage analysis of new eye colour mutation

The linkage of the newly induced *or* mutant was determined using different male linked translocations [15]. When homozygous *or* females were crossed to males heterozygous for an apricot eye (*ap*) sex linked translocation, T(Y;4)(ap^+/ap) [19], all F_1 descendants showed the *or* phenotype, indicating that *or* is a dominant allele of *ap* and located on chromosome 4 (Table I).

TABLE I. SEGREGATION OF CROSSES BETWEEN ORANGE-RED EYE (*or*) AND APRICOT EYE (*ap*) MEDFLY MUTANTS
(Piracicaba, 1993)

Crosses	F_1			F_2			Goodness of fit for 1:1 ratio
ap × *or*	Wild	*or*	*ap*	Wild	*or*	*ap*	χ^2
– × –	0	222	0	—	—	—	
– × –	0	243	0	—	—	—	
F_1 *or* × F_1 *or*	—	—	—	0	132	122	28.25***

*** Significant at 0.005 level.

2.5. Altered oogenesis and semisterility

In several experiments with the CENA-78 strain it was observed that some females laid either no eggs or only a few eggs. On the basis of this observation, an experiment with 20 pairs from this strain was set up and oviposition behaviour was observed. Eggs were collected for seven days and incubated at 25°C. The hatchability ranged from 40% to 80%. On the eighth day, the females were dissected and

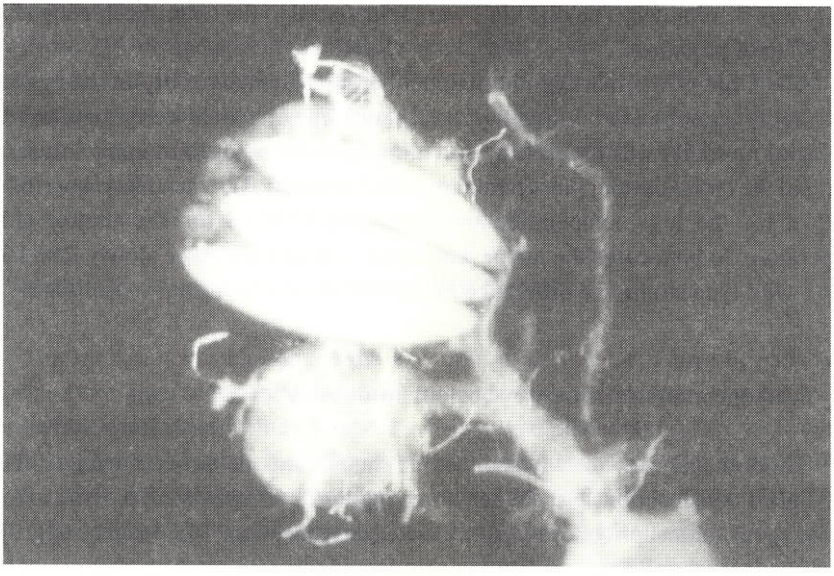

FIG. 1. Abnormal ovaries observed in an eight-day-old medfly female from strain CENA-78.

the ovaries examined. From a total of 20 females, 8 showed abnormal oviposition behaviour, i.e. these females laid very few eggs (0–100), and showed abnormalities in the morphology of the ovaries. Either one or both ovaries were rudimentary, and the observed structures suggested a reduced number of nurse cells in the egg chambers, tumorous follicles, and ovarioles with incomplete vitellogenesis (Fig. 1). The basis and genetic behaviour of this phenomenon are subjects for future studies.

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TRANSLOCATIONS AND MUTATIONS: TOOLS FOR MEDFLY CONTROL IN ARGENTINA

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Abstract

TRANSLOCATIONS AND MUTATIONS: TOOLS FOR MEDFLY CONTROL IN ARGENTINA.

The research objectives during the past five years involved a series of basic and applied studies for the genetic control of the Mediterranean fruit fly. Four new mutations were isolated, described and mapped. A set of three translocation strains was assembled as a tool to facilitate chromosome localization of these or any other new mutations. The stability of the pupal sexing strains T(Y;5)122 and T(Y;3;5)11 was studied under simulated mass rearing conditions. Two per cent of recombinant females were generated from brown pupae, in generations 17 and 15, respectively. Using wild type strains of different geographical origins, significant heterosis in F_1 and F_2 was detected for body weight, pupal survival, longevity and fecundity. A method is proposed to 'exploit' heterosis for genetic sexing. White-eye mutant males were unable to reach trimedlure traps in open areas; they were poor fliers and exhibited poor mating performance in field cages. The mutant behaviour augurs well for the future development of a 'genetically impaired female' technique for medfly control.

1. INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata,* became established in Argentina in the beginning of this century and has, since then, become a major pest of citrus and other fruits. It requires permanent chemical control, which is expensive. During 1992–1993, Argentina produced 49% of the grapefruit, 26% of the tangerine and 4% of the orange production in the Southern Hemisphere according to the FEDERCITRUS Association. These values indicate the magnitude of national citrus production and stress the importance of medfly control.

In the early 1970s, a large scale sterile insect technique (SIT) programme was initiated to suppress the medfly in Argentina [1, 2]. The project was abandoned for two decades but increasing limitations on fruit export combined with pressure to reduce insecticide applications resulted in the re-establishment of the 'National Programme' to eradicate the medfly in the Mendoza and Rio Negro provinces [3]. A

technical co-operation project (ARG/5/004) was established by the International Atomic Energy Agency (IAEA) to promote R&D in support of the national activities. The possibilities for applying the SIT to control the medfly in Argentina are described briefly by Zapater [4]. A large scale mass rearing facility started operation in 1992. A methyl bromide quarantine [5] was established for fruits and other susceptible products in the provinces mentioned above.

A genetic sexing system (GSS) would increase the efficiency of the SIT [6] considerably because releasing only males in the field favours matings between sterile males and wild females [7, 8]. Another advantage is that 'sterile stings' by released sterile females attempting to lay eggs are avoided. A GSS would also favour medfly suppression in economically productive areas of Argentina where eradication is not feasible, in the same way as it was applied successfully during 1989 and 1990 in Gvullot (Israel) on 22 ha of mango and citrus trees [9].

2. BASIC STUDIES

2.1. New mutations

Two experiments were performed to isolate new morphological mutations, following the protocol described in Fig. 1. In the first experiment, Castelar wild type virgin males and females were fed separately on a sucrose solution containing 3 mM ethylmethane sulphonate (EMS), and in the second experiment 3mM ethylnitrosourea (ENU) was used. In the third generation of inbreeding, 52 and 103 families were screened, resulting in the isolation of one and six mutations, respectively.

Experiment 1

Yellow body (*ye2*): The mutation is recessive and causes a decoloration of wings, body and bristles. The mutation shows consistent expression and high penetrance. Overall, *ye2* survival is significantly lower ($P < 0.001$) than in the wild type control. This is due to significantly lower hatch ($P < 0.001$) and pupal survival ($P < 0.001$) [10]. The gene is located on chromosome 2.

Experiment 2

Fragile setae (*fs*): All bristles of the body and the head (including male orbital bristles) break off gradually, starting with the emergence of the flies. The mutation shows consistent expression and high penetrance, is recessive and is located between 4 and 5 recombination units (r.u.) away from *bo* (brown-orange eye colour, allelic to *gr*, garnet eye [11]) on chromosome 6.

$$P \quad EMS \rightarrow\rightarrow\rightarrow \quad \frac{+}{+}\,\delta\delta \quad \times \quad \frac{+}{+}\,\varphi\varphi \quad \leftarrow\leftarrow\leftarrow EMS \qquad \text{(mass)}$$

$$F_1 \qquad \frac{*}{+}\,\delta \quad \times \quad \frac{+}{+}\,\varphi \qquad \text{(single)}$$

$$F_2 \quad \frac{*}{+}\,\delta\delta : \quad \frac{+}{+}\,\delta\delta \quad \times \quad \frac{*}{*}\,\varphi\varphi ; \frac{+}{+}\,\varphi\varphi \qquad \text{(mass)}$$

$$F_3 \quad \frac{*}{*}\,\delta\delta \text{ and } \varphi\varphi ; \frac{*}{+}\,\delta\delta \text{ and } \varphi\varphi ; \quad \frac{+}{+}\,\delta\delta \text{ and } \varphi\varphi$$

FIG. 1. Protocol used for isolating chromosome recessive mutations. Score for abnormal phenotypes in F_3. (mutant allele; $^+$ wild type allele.)*

Hazel pupae (*hl*): The mutation is recessive and causes a dark brown colour of the puparia. The expression and penetrance are fair.

Burgundy eye (*by*): The mutation causes a dull red-brown eye colour. Its penetrance is high and the expression is consistent. The mutation is recessive and is located on chromosome 5 between *wp* (white pupae [12]: 40.92 r.u.) and *ye* (yellow body [13]: 9.16 r.u.).

Dark pupae (*dp*): Identical expression and allelic to Rössler's *dp* [14].

Double chaetae (*dc2*): Identical expression and allelic to Rössler's *dc* [14]. It was confirmed that this mutation is present in the Castelar wild type stock at a low frequency.

Additional mutation

Curly wings (*cy2*): The wings are curved. The mutation has a variable penetrance and expression and is recessive. The mutation occurred spontaneously and was isolated during routine screening.

2.2. Method to assign new mutations to chromosomes

A quick method that employs three Y–autosome translocations was proposed to determine the chromosomal localization of any autosomal and recessive mutation. A

TABLE I. DETECTION SCHEME FOR CHROMOSOME LOCATION OF ANY NEW MUTATION

T(Y;A)	Pseudolinkage between sex and mutation, if located in autosome:				
	2	3	4	5	6
T(Y;3;5)11	No	Yes	No	Yes	No
T(Y;2;3)97	Yes	Yes	No	No	No
T(Y;4)104	No	No	Yes	No	No

series of crosses identifies pseudolinkage between sex and the mutation, indicating its chromosome location. The translocations used were T(Y;3;5), T(Y;4) and T(Y;2;3). The method requires the following steps. Males with the different T(Y;A) are mass crossed separately to females of the mutant strain under investigation. Their male offspring are backcrossed to the respective mutant females and the resulting F_2 is analysed according to the scheme in Table I.

2.3. Multiple marker strain

A multiple marker line for the five autosomes was established. This strain is homozygous for *ye2* (chromosome 2), *dp* (chromosome 3), *ap* (chromosome 4), *wp* (chromosome 5) and *fs* (chromosome 6). All mutations have a good expression, allowing adequate identification: *ap* and *ye2* interact, resulting in lighter colour than *ye2* alone; the presence of *wp* and *dp* produces puparia with grey colour. The strain has an overall viability of 68% in comparison with the wild type control. This reduced fitness does not limit the use of this line for most applications. The strain can be used to screen for translocations, e.g. as required for the construction of a GSS. It also allows individual chromosomes to be followed during basic genetic studies.

2.4. Ovipositor adaptations

Females can adapt to new oviposition substrates [15]. Our aim was to study the effects on aculeus anatomy after changing the oviposition substrates. Aculeus widths were measured in the 12th and 22nd generations after perforated plastic was replaced as oviposition substrate by a mesh with smaller mesh size. The results show that aculeus width was significantly smaller ($P < 0.001$) than in the parental line [16]. Understanding the underlying mechanisms could help to find the most suitable substrates to maximize egg production.

3. GENETIC SEXING

3.1. Stability

The stability of two medfly sexing strains, T(Y;5)122 and T(Y;3;5)11, based on the *wp* mutation as selectable marker, was studied for 21 generations under simulated mass rearing conditions. In these strains, isolated by Zapater [17], males emerge from brown puparia and females from white puparia. Larvae were reared in a carrot based medium. Samples of 750 pupae from each sex were screened every generation and aberrant individuals were separated to perform progeny tests. The *wp* phenotype and the presence of the translocation were determined.

Genetic recombination, which affects the stability of genetic sexing strains, stayed at a low level during 17 and 15 generations, respectively. During that stable period, the average percentage of aberrant females and the percentage of these confirmed to be recombinants (in brackets) were 0.27% (60%) in T(Y;5)122 and 0.85% (40%) in T(Y;3;5)11. The average frequencies of aberrant males were 0.69% in T(Y;5)122 and 1.17% in T(Y;3;5)11, but only 4.6% and 5.8% of these males were confirmed to be recombinants. After reaching a level of 2% (in G17 and G15), female recombinants accumulated rapidly in the population owing to lower viability of *wp* genotype. This resulted in a rapid breakdown of the strain. The relatively low recombination frequency observed in both strains indicates their potential, especially that of T(Y;5)122, for use in large scale SIT mass rearing programmes.

3.2. Fitness of *we* (white eye) mutation

The mating ability of flies homozygous for the white-eye (*we*) mutation was evaluated in standard field cages and their ability to reach trimedlure traps was measured. These experiments were carried out to explore the possibilities of using *we* as a conditional lethal in the field to develop a 'genetically impaired female' technique (GIFT) [18]. This technique was formerly called the 'field-female killing' (FK) system [19] and was employed successfully for sheep blowfly control.

Field cage mating experiments were carried out with 100 virgin flies from each sex and strain (*we* and a wild type strain symbolized by +). The flies were released into the cage on sunny mornings (8.00–9.30 a.m.). Mating couples were counted and removed every 30 min for 2 h. Results of the five experiments are presented in Table II. A total of 266 couples were scored during the five experiments: 136 ♂+ × ♀+ couples and 1 ♂*we* × ♀*we* couple were detected in the peach tree; on the wall and on the roof, 102 ♂+ × ♀+ couples were found; on the floor, 28 ♂+ × ♀+, 97 ♂*we* × ♀*we* and 43 ♂+ × ♀*we* couples were detected. No *we* flies were observed higher than 20 cm on the wall, tree or roof. No mating couples between ♂*we* and ♀+ were detected. The ratio of wild type couples to *we* couples was 2.7:1.

TABLE II. COUPLES ESTABLISHED IN A FIELD CAGE AFTER THE RELEASE OF 100 VIRGIN FLIES OF EACH SEX AND STRAIN (WILD TYPE AND WHITE EYE)

	♂ × ♀	30 min					Σ	60 min					Σ	
		Cage No.						Cage No.						
		I	II	III	IV	V		I	II	III	IV	V		
Floor	+ × +	—	4	—	11	1	16	1	2	—	3	3	9	
	we × *we*	7	7	—	1	4	19	5	6	3	6	18	38	
	+ × *we*	3	6	1	2	1	13	4	—	—	10	1	15	
Wall and roof	+ × +	9	12	12	4	4	41	7	5	4	10	4	30	
Peach tree	+ × +	—	4	25	—	24	53	2	13	11	1	17	44	
	we × *we*	—	—	—	—	—	—	—	—	—	—	1	1	
	♂ × ♀	90 min					Σ	120 min					Σ	ΣΣ
		Cage No.						Cage No.						
		I	II	III	IV	V		I	II	III	IV	V		
Floor	+ × +	—	1	—	2	—	3	—	—	—	—	—	—	28
	we × *we*	8	2	2	1	9	22	9	1	3	1	4	18	97
	+ × *we*	5	2	—	2	—	9	4	—	1	1	—	6	43
Wall and roof	+ × +	9	4	3	4	—	20	2	6	1	2	—	11	102
Peach tree	+ × +	—	10	4	—	8	22	1	4	4	—	8	17	136
	we × *we*	—	—	—	—	—	—	—	—	—	—	—	—	1

TABLE III. WILD TYPE ♂♂/♀♀ FOUND PER TRAP AFTER WILD TYPE AND WHITE-EYE RELEASES IN THE DAYS INDICATED AFTER RELEASE

Rep.	Trap	Days after release:							
		1	2	3	4	5	6	7	8
1	I			53/3			7/—		
	II			26/1			1/—		
2	I			12/—				4/—	
	II			6/—				2/—	
3	I				32/1				4/—
	II				12/2				5/—

The ability of the males to reach trimedlure traps was evaluated in the Botanic Garden of the Agriculture Faculty, where two such traps were located on external branches of a shrub 1.5 m high. They were located 5 and 8 m from the release point. A total of 300 flies of each sex and strain, three to five days old, were released simultaneously and three replicates were done. Sun, shadow and rain occurred during the first four days after the releases in all replicates. No we males were recovered in the traps in any of the replicates, whereas wild type males and a few females were detected (Table III).

Although more experiments in different environments are required, the results obtained so far indicate that *we* flies in Buenos Aires are unable to fly in field cages under sunny conditions, have a low mating ability and are, therefore, very poorly competitive in the field and will not survive. This conditional behaviour could be used to develop a GIFT strategy for the medfly.

3.3. Hybrids

Heterosis was studied in the medfly by measuring several different traits in the F_1, F_2 and F_4 generations of crosses between four different strains (including reciprocal crosses) and by comparing their average performance with that of the respective parental lines. The traits measured were adult dry weight, survival at different developmental stages, longevity and fecundity. The lines used originated from Argentina (A), Brazil (B), Costa Rica (C) and Israel (I) and have been cultured in the laboratory for one or two decades.

The adult dry weights for the parental lines and the F_1, F_2 and F_4 hybrids are shown in Tables IV and V. The mean F_1 heterosis for the four different crosses and both sexes was 7.9% as compared with the average of the parents; the value for the F_2 was 4.8%. The average weights in the F_1 were 81% higher than in the F_2 originating from the same crosses.

TABLE IV. AVERAGE MALE DRY WEIGHT FOR PARENTAL LINES AND F_s IN *Ceratitis capitata*

Line	G	*n*	Weight (mg) ($\bar{X} \pm$ SE) ♂♂	Scheffé reciprocal	Scheffé $\bar{X}P - F$	Het. (abs.)	Het. (%)	Scheffé $F_1 - F_2$ $F_1 - F_4$ $F_2 - F_4$
A	G_n	120	1.66 ± 0.13					
I	G_n	120	1.65 ± 0.11					
B	G_n	59	1.76 ± 0.13					
C	G_n	60	1.8 ± 0.10					
A × I	F_1	100	1.81 ± 0.15	n.s.	***	0.16	9.6	
I × A		80	1.79 ± 0.13		***	0.14	8.5	
B × C		60	1.93 ± 0.12	n.s.	***	0.15	8.4	
C × B		40	1.92 ± 0.1		***	0.14	7.9	
A × I	F_2	60	1.69 ± 0.13	n.s.	n.s.	0.045	2.4	***
I × A		40	1.74 ± 0.12		**	0.09	5.4	n.s.
B × C		60	1.88 ± 0.14	n.s.	***	0.1	5.6	n.s.
C × B		60	1.88 ± 0.15		***	0.1	5.6	n.s.
C × B	F_4	40	1.9 ± 0.13		***	0.12	6.7	n.s./n.s.

Note: n.s.: not significant; ** significant at 0.01 level; *** significant at 0.005 level.

The length of the developmental period from egg to pupa varied considerably between the different hybrids; emergence was always superior in F_s. The F_1 adult longevity varied from 30 to 47% but only one of the crosses gave significant differences. Hybrid fecundity exceeded the parental mean by 15–125%.

For most traits studied here, F_1 hybrids performed better than the parental lines and the F_2 or F_4 hybrids. It needs to be established whether the superiority of F_1 hybrids found in the laboratory can be reproduced in the field. If this is the case, one might consider mass rearing two separate pupal genetic sexing strains which are crossed together only to generate hybrids for the release material.

4. CONCLUSIONS

Our studies in the framework of the FAO/IAEA co-ordinated research programme led to the following results:

TABLE V. AVERAGE FEMALE DRY WEIGHT FOR PARENTAL LINES AND F_s IN *Ceratitis capitata*

Line	G	*n*	Weight (mg) ($\bar{X} \pm$ SE) ♀♀	Scheffé reciprocal	Scheffé $\bar{X}P - F$	Het. (abs.)	Het. (%)	Scheffé $F_1 - F_2$ $F_1 - F_4$ $F_2 - F_4$
A	G_n	100	1.81 ± 0.15					
I	G_n	90	1.80 ± 0.15					
B	G_n	60	1.87 ± 0.13					
C	G_n	39	1.94 ± 0.16					
A × I	F_1	100	1.89 ± 0.13	n.s.	***	0.09	5	
I × A		80	1.93 ± 0.16		***	0.125	7	
B × C		60	2.07 ± 0.11	n.s.	***	0.12	8.7	
C × B		60	2.07 ± 0.12		***	0.12	8.7	
A × I	F_2	60	1.83 ± 0.15	n.s.	n.s.	0.025	2	*
I × A		40	1.83 ± 0.12		n.s.	0.025	2	*
B × C		60	2.08 ± 0.17	n.s.	***	0.13	9.1	n.s.
C × B		60	2.04 ± 0.12		***	0.09	7	n.s.
C × B	F_4	39	2.01 ± 0.12		**	0.06	5.5	n.s./n.s.

Note: n.s.: not significant; * significant at 0.05 level; ** significant at 0.01 level; *** significant at 0.005 level.

(a) Four new mutations were isolated, described and mapped.
(b) The stability of our two pupal genetic sexing lines was determined.
(c) The flight ability and mating ability of flies homozygous for the mutation *we* were analysed. Their poor performance could become useful for genetic sexing strains or could be employed for developing GIFT strategies.
(d) The heterosis found for several traits in crosses between different strains could open the possibility to increase the fitness of mass reared strains by generating hybrids.

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GENETIC MARKERS, TRANSLOCATIONS AND SEXING GENES ON CHROMOSOME 2 OF *Ceratitis capitata*

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Abstract

GENETIC MARKERS, TRANSLOCATIONS AND SEXING GENES ON CHROMOSOME 2 OF *Ceratitis capitata.*

A review is presented of results obtained in a search for genetic markers, translocations and selectable genes obtained at the Instituto de Genética, Castelar, Argentina, with special reference to chromosome 2 linked mutations and genes useful for developing self-sexing strains in *Ceratitis capitata.*

1. INTRODUCTION

In any attempt to improve the sterile insect technique (SIT) by means of genetic sexing systems a fair amount of basic knowledge on genetic markers and chromosomal morphology (besides some selectable genes) is required. The linkage group associated with chromosome 2 of the Mediterranean fruit fly, *Ceratitis capitata,* is not as well studied as others. Rössler and Rosenthal [1] did not identify any morphological markers and Malacrida et al. [2] questioned the linkage of nine biochemical variants to any morphological marker of linkage group 2. A number of reports have been produced at the Instituto Nacional de Tecnología Agropecuaria (INTA) in Castelar but they remain unpublished (INTA Yearly Reports). The description of these markers and some information obtained on chromosome 2 will probably be useful in the general context of the genetics of the medfly and its application to the improvement of the SIT.

The purpose of this paper is also to clarify some lack of uniformity in the nomenclature of published and unpublished genetic variants and translocations in this linkage group and a first attempt is made to put them on the genetic map. Genes affecting the rate of development have been isolated within this linkage group. How they are now used for the construction of new genetic sexing strains is briefly described.

2. HISTORICAL PERSPECTIVE

Research on the biology and genetics of *C. capitata* started at Castelar in the mid-1970s, well before any institutional support was assured. At that time only a small number of papers were known on the existence of genetic variants in this species. Starting in November 1981, the author's laboratory entered a period of interaction with the International Atomic Energy Agency under Research Contract No. 2973/R3/RB. The title of the contract was "Induction and utilization of mutations to delineate the complexities of sex determination and the stability of Y–autosome translocations in *Ceratitis capitata*". This project was acknowledged by INTA as an institutional plan with the title "Determinación de métodos genéticos para el control de poblaciones de *Ceratitis capitata*" in December 1982. A good deal of knowledge has been gained in the last two decades, particularly with relevance to the development of genetic sexing strains.

3. MUTATIONS

The mutants used in this laboratory are listed in Table I. A condensed description of their phenotypes follows.

3.1. Niger

nig: black adult, black pupa and black larval spiracles. There are two alleles: nig^1, originally found at Castelar, and black fly (proposed symbol nig^{bf}), found at the Agency's Laboratories in Seibersdorf, near Vienna. Origins: nig^1: ethylmethane sulphonate (EMS); nig^{bf}: spontaneous [3, 4].

TABLE I. MUTATIONS OFTEN USED AT INTA, CASTELAR

Name	Symbol	Dominance	First cited	Refs
(a) On chromosome 2				
Niger	*nig*	Recessive	1979	[3, 4]
Pupal esterase	*Est-1*	Codominant	1981	[4, 5]
Long pupa	*lp-1*	Recessive	1985	[6]
Dark dorsal abdomen	*d*	Recessive	1985	[7]
Slow	*sw*	Recessive	1990	[8, 9]
(b) Not on chromosome 2				
Black pupa	See text	Semidominant	1985	[7]

3.2. Pupal esterase

Est-1: this gene is expressed only in pupae 24–48 h after pupariation. The preferred substrate for it is 2-naphthyl acetate. There are three alleles: $Est\text{-}1^c$ is found only in the field; the other two, $Est\text{-}1^a$ and $Est\text{-}1^b$, have been studied in the laboratory and found in the field. Origin: naturally occurring variation.

The same symbol has also been used for a gene active in adult flies. It would be possible to distinguish them simply by referring to pupal esterase 1 (*pEst-1*) and adult esterase 1 (*aEst-1*). Variation in pupal esterase does not show a correlation with any band observed in the adult [4, 5].

3.3. Long pupa

lp-1: pupal case 10–20% longer than in the wild type. The shape is not ovoid but rather club-like, half-way between the shapes of a larva and a pupa. There is only one allele.

lp-2: linked but not allelic; because of its variable and milder effect, not analysed in detail. Origin: X rays [6].

3.4. Dark dorsal abdomen

d: dark adult body with no effect on pupa. There are three alleles, identifiable by the growing intensities of their effects: $d^i > d^d > d^a$. The most commonly used allele is d^d. Origin: EMS [7].

3.5. Slow

sw: mutations at this locus cause a visible effect on eye iridescence and a more purple-reddish pigmentation than in the wild type. They also cause a slowing down of the rate of development. There are two alleles: slow-mazzard (sw^z) and slow-mulberry (sw^y), the latter having a stronger effect on development than the former. Origin: EMS [8, 9].

3.6. Black pupa

Although not linked to previous markers, black pupa is included here because, in the homozygous condition, it produces a phenotype indistinguishable from *nig* on the pupa. However, the heterozygous form is intermediate in colour. In addition, it causes very little, if any, effect on the adult. Three alleles have been isolated: *Bp*, *Bn* and *Bs*. Later they proved to be alleles of a gene previously described as *dp*. Therefore, it is proposed here to unify nomenclature as

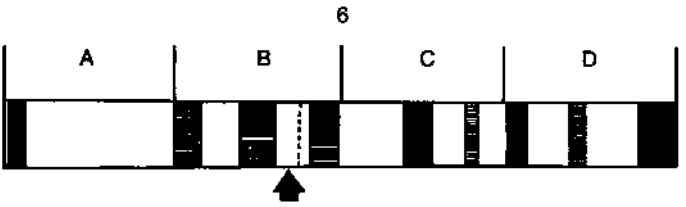

FIG. 1. Schematic representation of region 6 on the polytene banding pattern of chromosome 2, in translocation strain T5038, showing the site where the sex chromosome complex is associated to band 6B.

follows: dp^{1}, dp^{Bp}, dp^{Bn}, dp^{Bs}. Origins: dp^{1}: spontaneous; dp^{Bp}, dp^{Bn}, dp^{Bs}: EMS [7].

4. TRANSLOCATIONS

The first evidence associating the linkage group of *nig* to chromosome 2 came from a translocation between this chromosome and chromosome 4 induced by X rays, selected by its property to reduce hatchability, and identified just by cytological observations. The linkage of the inheritance of *nig* to the marker carmine eye indicated the association of *nig* and *ca* to chromosomes 2 and 4 (E. Lifschitz and F.C. Manso, unpublished observations). After a number of generations the strain carrying this translocation was lost.

Another translocation was isolated by its property to link *nig* to the sex: T(Y,nig^{+})5038. As it happened to be a translocation not visible in mitosis, the final proof for its association to chromosome 2 has only recently been obtained using

TABLE II. HOLANDRIC LINES DERIVED FROM T5038

Male		Female	
Phenotype	Genotype	Phenotype	Genotype
+	Y+/*nig*	*nig*	*nig/nig*
+	Y+/*lp*	*lp*	*lp/lp*
+	Y+/*d*	*d*	*d/d*
+	Y++/*lp d*	*lp d*	*lp d/lp d*
+	Y+++/*lp d sw*	*lp d sw*	*lp d sw/lp d sw*
sw	Y++*sw*/*lp d sw*	*lp d sw*	*lp d sw/lp d sw*
d sw	Y+*d sw*/*lp d sw*	*lp d sw*	*lp d sw/lp d sw*

polytene chromosome preparations (Fig. 1). The breakpoint has been localized at region 6B in the polytene chromosome map of orbital bristle trichogenic cells [10].

By means of T5038, a number of strains have been obtained, linking the inheritance of different genes to the sex (wild type allele linked to males and mutant alleles to females). Some of them are listed in Table II. The all wild type male strains were obtained simply by crossing T5038 males to the appropriate mutant female and backcrossing F_1 males to females of the same mutant strain. The two male marked strains (*sw* and *d sw*) were obtained by crossing two single individuals produced by male recombination events.

5. MALE RECOMBINATION

Early cytological observations of meiosis were not conclusive as to whether chiasmata were present or not in *C. capitata* males. Therefore, finding two linked genes, *Est-1* and *nig*, was very useful for performing a genetic test. The conclusion was that recombination does not occur in males to the same extent as in females [4]. Other observations followed, working with larger numbers and using morphological markers, the result of which was the demonstration of exceptions to that rule, usually occurring in the order of 10^{-2} [1].

A large scale experiment was then set up in the laboratory to find the magnitude of this phenomenon. Two morphological markers, *lp* and d^d, were chosen which are located far apart in the female linkage map. Overall 44 639 flies were scored, among which 49 progeny tested individuals proved to be true cases of male recombination (Table III). Therefore, the frequency of male crossing-over between two markers far apart on chromosome 2 was $(1.10 \pm 0.16) \times 10^{-3}$, remarkably low for two genes that distance apart [11].

6. CONDITIONAL LETHALS

A project directed towards the isolation of temperature sensitive lethal mutations in the medfly was initiated in Castelar around 1985. Information was obtained on the thermal tolerance of the embryo and the conditions required to perform a screening for such mutations [12]. Some strains were actually obtained [13] but this project had to be discontinued in 1988 for lack of support.

A more promising line of research then appeared: eye colour mutants with a conspicuously longer time of development. The first mutant, named slow-mazzard (sw^z) [9], requires about two days more than the wild type to reach the pupal stage at normal temperature (Fig. 2(a)). When a holandric line was built with T5038 (wild type male, mutant female), and temperature was reduced by 2°C, an automatic sexing

TABLE III. PROGENY TESTED CASES OF MALE RECOMBINATION AMONG 44 639 PROGENIES OF ++/*lp d* MALES

	(+) *d*	*lp* (+)	Total
Male	11	10	21
Female	14	14	28
Total	25	24	49

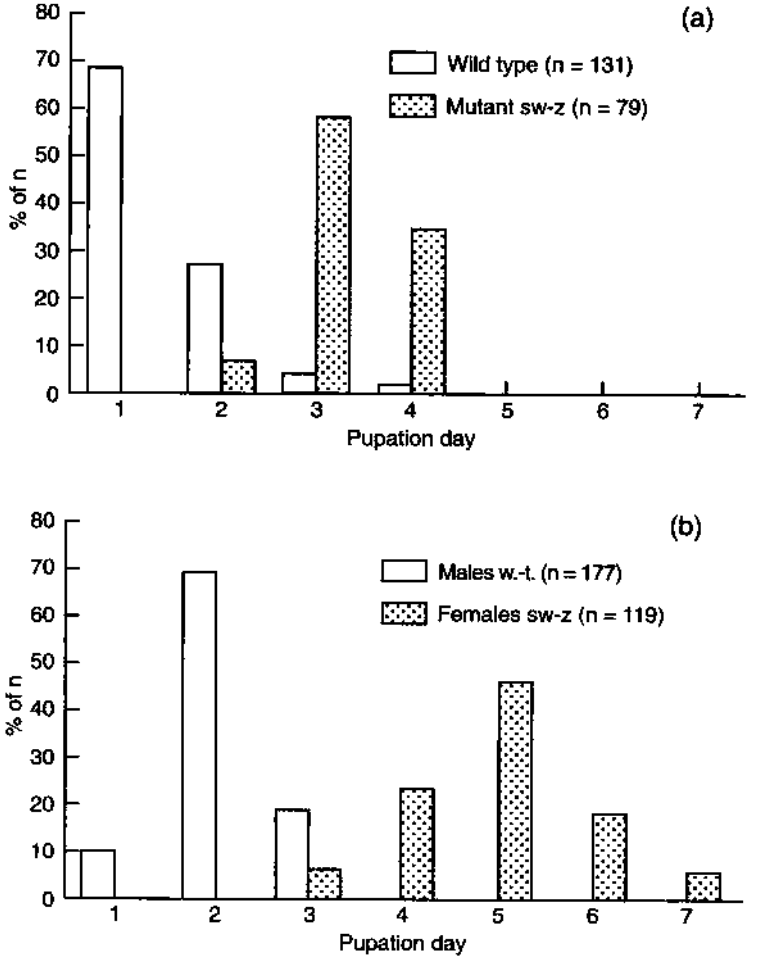

FIG. 2. Percentage of wild type and sw^2 *flies arising from successive days of pupation in (a) the two original strains (temperature 26°C) and (b) a strain with a Y–2(+/sw) translocation (temperature 24°C).*

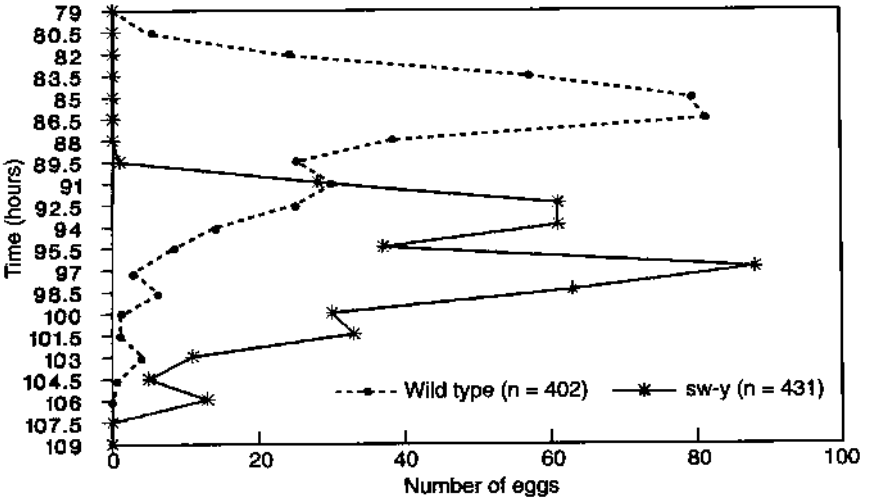

FIG. 3. Number of wild type and sw^y *flies arising from eggs hatching at different times after laying (temperature 22°C).*

strain was obtained in which the larvae jumping out of the food during the first days are the males and those jumping later are the females (Fig. 2(b)).

The embryonic development was also longer in this mutant than in the wild type, but not so much as to allow separation at that stage in the conditions of this experiment. A second mutant, named slow-mulberry (sw^y) [8], was also obtained; this was associated with a stronger reduction in the rate of development during both embryonic and larval development. Using this strain and lowering the temperature by another 2°C, a good separation of the two populations of embryos was obtained at the time of hatching (Fig. 3). It has been shown that both mutations are alleles of one

TABLE IV. LINKAGE MAP OF GENES LOCATED ON CHROMOSOME 2

Gene	T5038 *lp*	*nig*	*Est*	*d*	*sw*
Distance (c.o.)		15(18)	c. 50	20	33
Polytene	1A 6B		15D–16A		24D
			?		
Refs	[9, 10]	[6],[a]	[6, 9, 15]	[15]	[9],[a]

[a] Unpublished.

gene [14]. Research is in progress aimed at the use of this gene in a sexing strain at a mass rearing facility.

7. MAP OF CHROMOSOME 2

Most of the information available on the relative locations on the recombination map of the markers mentioned in this paper has been brought together in Table IV. Although some genetic distances have been included in the picture, they must be taken as approximate. The gene order, however, is reasonably certain.

What has been learned from working with the linkage group associated to chromosome 2 is interesting and contributes to the growing body of genetic knowledge that is accumulating on this species. It is hoped that this will be useful in the near future not only in helping to improve the SIT but also in developing newer and more sophisticated strategies for the control of this and other insect pests.

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DEVELOPMENT AND APPLICATION OF GENETIC SEXING SYSTEMS FOR THE MEDITERRANEAN FRUIT FLY BASED ON A TEMPERATURE SENSITIVE LETHAL MUTATION

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Abstract

DEVELOPMENT AND APPLICATION OF GENETIC SEXING SYSTEMS FOR THE MEDITERRANEAN FRUIT FLY BASED ON A TEMPERATURE SENSITIVE LETHAL MUTATION.

The present status in genetic sexing for the Mediterranean fruit fly is discussed. This includes the selection of the appropriate sexing gene (which determines the feasibility and practical applicability of the sexing system) as well as the selection of the appropriate Y–autosome translocation (which determines the stability of the sexing system). A temperature sensitive lethal mutation is used to eliminate females during the egg stage. This mutation in combination with new Y–autosome translocations allowed the construction of a genetic sexing strain, named VIENNA-42, that is stable enough for large scale mass rearing. Also described are the analysis of this strain under field cage and field conditions and, in preparation for large scale tests in Guatemala, the outcrossing of VIENNA-42 with genetic material from the target area.

1. INTRODUCTION

The sterile insect technique (SIT) is an environmentally friendly method for the control of pest insects. It has been applied successfully on numerous occasions and the number of ongoing or planned programmes increases constantly [1]. For the

medfly 12 mass rearing factories are in operation worldwide [2]. However, in all cases males and females are produced despite the fact that only the released sterile males contribute to the population suppression. The availability of a technique that allows the large scale elimination of females and, therefore, the release of only male flies can improve the SIT considerably in the following ways:

(a) *Reduction in programme costs*

— Reduced production costs: only the active agent is reared, transported and released.
— Simplified monitoring: trapped females can be used as direct indicators for the progress of the programme.

(b) *Increased efficiency*

— No assortative mating among the released flies and better dispersal of the flies in the target area.
— Improved male quality: in contrast to females, male pupae can be irradiated later, i.e. at an age where the radiation is less damaging to the overall viability of the fly.
— More flexibility in the timing of the release: in the absence of females, males can be released later, i.e. closer to their sexual maturity.

(c) *Increased application range*

— The SIT can be applied also in fruit exporting regions as the problems associated with sterile stings no longer exist (e.g. infection with pathogenic bacteria or fungi and transmission of these pathogens by females). As a consequence, application of the SIT is no longer restricted to eradication, but can also be used as a bioinsecticide to control the target population.

We describe the construction of sexing strains utilizing a temperature sensitive lethal mutation which allows elimination of the females at the egg stage. The genetic stability of these strains is primarily dependent on the structure of the Y–autosome translocation. Through genetic and cytogenetic analyses it was possible to develop strains with sufficient stability. Furthermore, some of these strains have been mass reared and evaluated under field cage and field conditions to assess their survival, dispersal and mating behaviour. Recently, one of these strains was outcrossed with genetic material from Guatemala in preparation for a larger scale mass rearing/field test in Guatemala. The aim is to produce 20 million males per week which will then be evaluated for their performance in the field.

2. RESULTS

2.1. Temperature sensitive lethal (*tsl*) mutation

By comparing and evaluating the existing selectable markers, it became apparent that none of them was suitable for a large scale mass production facility. In some cases, sexing would be too expensive (requiring expensive machines or chemicals), would not be accurate enough, would be allowed only at very late stages (no savings on larval diet) or would require the application of toxic chemicals. On the basis of these arguments it was suggested that the use of temperature inducible lethal mutations be considered instead. Within this group of lethal mutations it should be possible to identify those where lethality can be induced during early developmental stages, ideally during the egg stage. This stage is not only the earliest stage possible to eliminate the females, but it is also a very convenient stage to apply temperature as the eggs are maintained at high density in plastic containers.

To induce a temperature sensitive lethal (*tsl*) mutation, flies were treated with ethylmethane sulphonate (EMS), followed by the isolation of the treated chromosomes via single-pair mating steps. The treated chromosomes were made homozygous and each family was tested for temperature dependent lethality by incubating eggs at various temperatures and by recording the resulting egg hatch. In this large screen, one *tsl* mutation was recovered (G. Franz and E. Busch-Petersen, in preparation). In addition to the *tsl* mutation, this strain also carries a white pupa (*wp*) mutation. It turned out later that this is a very useful feature for the analysis as well as the practical application of the otherwise 'invisible' *tsl* mutation as it provides visual cues at the pre-release stage to whether the temperature treatment has been successful or not. This argument can be taken even further. Any new *tsl* based sexing system that is going to be isolated in the medfly, or any other fruit fly species, should contain a morphological mutation that is closely linked to the respective *tsl* mutation.

In a first series of tests we have shown that it is indeed possible to use the *tsl* mutation as a selectable marker when the mutation is combined with an appropriate Y–autosome translocation (G. Franz and E. Busch-Petersen, in preparation). In our tests, eggs were collected for 24 h, followed by a 24 h treatment with temperatures between 31 and 35°C. At 34°C all homozygous *tsl* genotypes are eliminated during very early stages, i.e. the lethality is visible primarily as reduced egg hatch (G. Franz, unpublished results).

Sublethal temperatures, e.g. 29°C, lead to a significant delay in development if the treatment period is extended to 48 h and longer. This effect leads to a separation of male (wild type) and female (*tsl*) pupation which can be used for sexing, though incomplete.

In addition to the initial tests mentioned above, the *tsl* mutation was analysed with respect to its genetic behaviour. If homozygous *tsl* females are crossed with wild

type males, the resulting heterozygous *tsl*/+ offspring are more temperature sensitive than the wild type strain, but less sensitive than the homozygous *tsl* strain. This finding, in combination with the result that the heterozygotes generated by the reciprocal cross are not sensitive, points towards an incomplete maternal effect of this *tsl* mutation (G. Franz, unpublished results). Furthermore, the sensitivity seems to be independent of the dose, i.e. *tsl*/+/+ genotypes are as sensitive as *tsl*/*tsl*/+ and *tsl*/+ genotypes [3].

Although the egg stage is the most temperature sensitive phase of the life cycle, the other stages, i.e. larvae, pupae and adults, also show varying degrees of sensitivity (G. Franz, unpublished results). On the one hand, this makes it necessary to avoid elevated temperatures during mass rearing, though only for that fraction of the production that is used to set up the next generation of the colony. Here the temperature is quite important, primarily because it affects indirectly the stability of the sexing system. Removal of the bacterial and fungal contamination of the diet by various means, and the use of liquid diets (P. Rendon, personal communication) in combination with the appropriate diet tray design (J. Hendrichs, personal communication) are possible alternatives for minimizing the temperature related effects. On the other hand, the temperature sensitivity of later stages, especially of *tsl* female adults, can be viewed as an additional safety feature, i.e. accidental release or escape from the factory will not be a problem as these flies will not survive.

2.2. Y–autosome translocations

The wild type allele of the respective selectable marker is linked to the male sex via a Y–autosome translocation [4]. In the medfly, the Y chromosome contains a dominant male determination factor [5]. The males in a sexing strain are heterozygous for the selectable marker, i.e. the free autosome carries the mutant allele. The following three primary deviations from pseudolinkage were observed during the analysis of various sexing strains.

2.2.1. Recombination in heterozygous males

During the mass rearing of genetic sexing strains it became apparent that sexing systems can break down over time. It was determined that genetic recombination in the males is the primary cause of this instability [6]. Genetic exchange in the chromosomal region between the translocation breakpoint and the location of the selectable marker will unlink the wild type allele from the male sex. This leads in the following generation to mutant males and wild type females. Especially the latter type of aberrant fly poses a severe problem because these females cannot be separated from the males and are therefore released. The recombination frequency in males is

very low [7]. However, as the resulting recombinant females are wild type, they possess a selective advantage over their mutant female siblings. This results in an accumulation rate that exceeds the underlying recombination rate. In the case of the *tsl* based sexing system, the recombinant females that have lost the *tsl* would accumulate faster if the rearing temperature were not maintained properly or if the next generation were set up exclusively with early pupae.

One strategy to overcome this stability problem is to generate translocations where the Y–autosome breakpoint is close to the selectable marker. This required two separate lines of experiments. First, the chromosomal location of the selectable marker had to be determined. Two mutations, *wp* and *tsl*, were mapped on the right arm of chromosome 5 [3, 8]. Secondly, a series of translocations, linking chromosome 5 to the Y chromosome, had to be induced and analysed with respect to the position of the Y–autosome breakpoint [8–10]. It was shown that sexing strains based on translocations with breakpoints close to *wp* and *tsl* are sufficiently stable that they can be reared for many generations without stability problems [9].

A second strategy to avoid the occurrence of recombinant adults is to incorporate pericentric inversions. In genotypes heterozygous for such an inversion, recombination in the inverted chromosome segment will lead to unbalanced gametes and, consequently, the resulting offspring will not be viable. We have initiated experiments to induce homozygous viable pericentric inversions in the *wp-tsl* chromosome. Applied to genetic sexing, such a strain would consist of females homozygous for the inversion (i.e. although female recombination frequency is high, this does not lead to semisterility) and males containing (a) a Y–autosome translocation with a breakpoint close to *wp* and *tsl*, and (b) a free autosome with the pericentric inversion. Male recombination between the breakpoint and the selectable markers is low and, consequently, the resulting sterility should be negligible, especially compared with the sterility caused by meiotic segregation of Y–autosome translocations.

Very little is known about recombination between the sex chromosomes in the medfly. Potentially, even unequal recombination between the two Y fragments of a Y–autosome translocation (or intrachromosomal recombination of the Y) is possible, especially considering the highly repetitive structure of the Y chromosome. The structure of the Y chromosome in certain aberrant flies, isolated from a mass rearing colony, might be evidence for this (U. Willhoeft, personal communication).

2.2.2. *Adjacent-1 segregation*

The segregation of Y–autosome translocation during the male meiosis is complex. Only alternate segregation produces balanced gametes, while adjacent-1 segregation generates genotypes with Y chromosome deletions and either autosome deletions or duplications. The frequency of such genotypes at any developmental stage depends on the following factors.

(a) *Relative ratio of alternate to adjacent-1 segregation*

It is very difficult to measure this ratio directly. However, by comparing egg hatch, pupation and emergence with a wild type strain one can deduce that some translocations, e.g. T(Y;5)30C, show primarily alternate segregation, while in the case of other translocations both types occur at equal frequencies. It is possible that the position of the breakpoint on the Y chromosome, relative to the Y centromere, is responsible for these differences.

(b) *Viability of these unbalanced karyotypes*

It is assumed that in most cases autosomal deletion leads to early, i.e. zygotic, lethality. A very short deletion, e.g. segment 61A to 62D (trichogen map), can potentially survive until the larval stage as judged by the egg hatch of more than 80% in the case of translocation T(Y;5)3-245 [10] (G. Franz, unpublished data). The viability of triplications appears to be inversely correlated to the length of the duplicated segment [10]. In many translocation strains, a certain fraction (up to 20% of all emerged adults) of the adjacent-1 segregation offspring (general structure: X/Y-A/A/A) reaches adulthood and can be made visible by the appropriate combination of mutations [3, 11]. The sex of these flies is dependent on the position of the Y-chromosomal breakpoint relative to the male determination factor (U. Willhoeft et al., in preparation). Generally, adjacent-1 flies are weak: their overall fitness and viability are reduced significantly. Furthermore, they are more temperature sensitive than balanced genotypes (G. Franz, unpublished results).

The sterility of Y–autosome translocation bearing males is directly proportional to the complexity of the translocation [8]. The more autosomes are involved, the more complex is segregation and the more unbalanced genotypes are generated. Therefore, for mass rearing only simple translocations are considered.

2.2.3. Non-disjunction

Non-disjunction of X and Y chromosomes has been reported [5]. Preliminary data from the Agency's Laboratories in Seibersdorf, near Vienna, show that certain Y–autosome translocations produce at relatively high frequencies unbalanced genotypes with two X chromosomes and one of the two translocation fragments, usually the one with the Y centromere. As this is not the case for all strains, one might speculate that the position of the Y-chromosomal breakpoint, and the resulting difference in size/quality of the two Y fragments, are responsible for this phenomenon. Whether these aberrant genotypes pose a threat to the sexing system depends on the position of the autosomal breakpoint (which determines the phenotype with respect to the selectable marker) in combination with the position of the Y-chromosomal

breakpoint (which determines whether the aberrant fly is male or female). A serious problem for mass rearing arises when wild type females are generated, while mutant males can be tolerated as long as their frequency does not severely reduce recovery.

2.3. Mass rearing of sexing strains based on *tsl* mutation

Several translocations have been used to construct sexing strains utilizing the temperature sensitive lethal as selectable marker, namely T(Y;3;5)1-56 (= GS-2), T(Y;2;5)2-82 (= GS-3), T(Y;5)1-61 (= GS-4), T(Y;5)2-22 (= GS-6) [3, 9] and T(Y;5)3-245 (= GS-8) [10]. These were selected on the basis that they have Y–autosome breakpoints on the right arm of chromosome 5 and show very good stability in laboratory tests. The strains GS-2 and GS-3 were used only for a relatively short period of time as they are difficult to rear owing to the complexity of the translocation and the resulting sterility of the males. The most promising results were obtained with GS-4, later named VIENNA-42.

The homozygous *tsl* strain and all translocation strains were outcrossed with our wild type strain EgII before they were combined to generate sexing strains and before they were transferred to our mass rearing facility. This was done (a) to remove other mutations present in the *tsl* strain after EMS mutagenesis and (b) to increase genetic variability. The outcrossing scheme was the same as the one proposed later for the outcrossing with genetic material from Guatemala. However, here no complications with sterility were encountered. This is most likely due to the fact that all our strains, including the translocations, are based on the EgII strain.

The mass rearing procedure had to be modified compared with the scheme used for rearing a sexing strain utilizing only *wp* as selectable marker:

- The adult colony was enlarged to obtain sufficient numbers of eggs.
- That fraction of the production that was put aside for setting up the next generation was reared separately.
- Especially for this fraction, temperature was controlled more carefully throughout the rearing process.
- Owing to the presence of the *tsl* mutation, the females pupate, on average, later than the males. This feature was used to set up the next generation primarily with material from pupal collections 3 to 5, thereby selecting against recombinant females that have lost the *tsl* and therefore pupate early, together with the males.
- To obtain only males, eggs were collected for 24 h and then treated with temperature during the 'bubbling' phase.

With these modifications it is possible to mass rear VIENNA-42 and to produce only males. On two occasions this strain was reared either for 21 or for 13 generations;

in both cases the level of recombinant females stayed below 1% (J. Hendrichs, unpublished results). However, increasing numbers of males emerging from white pupae were observed. Preliminary genetic tests show that these males have apparently lost the translocation (G. Franz, unpublished results). This was confirmed by cytological analysis on a few individuals (U. Willhoeft, personal communication). It is not clear yet whether these aberrant males are the consequence of intra-Y-chromosomal recombination or whether they were introduced into the strain during the outcrossing with EgII. As this phenomenon was not observed when GS-6 was mass reared for eight generations, we have started to investigate the potential of this strain.

2.4. Construction of VIENNA-42/Guatemala hybrid strain

In preparation for a large scale mass rearing and field test of the medfly sexing strain VIENNA-42 in Guatemala, it was suggested that the genetic background of this strain (derived from a wild type strain originating from Egypt) be replaced with genetic material from Guatemala. The following three arguments were considered:

— To increase the genetic variability
— To improve mating competitiveness relative to the target population
— To avoid introduction of non-native flies.

The two components of the sexing strain VIENNA-42, the temperature sensitive lethal (*tsl*; linked to the mutation *wp*) and the Y–autosome translocation T(Y;5)1-61, were outcrossed separately.

The original outcrossing scheme for the *tsl* consisted of three consecutive crosses with flies from a recently colonized mass rearing strain from Guatemala (Toliman strain). In the last two crosses, Toliman females were used, thereby maintaining the *wp-tsl* chromosome in the males to avoid recombination between these markers. In the next step, an attempt was made to reconstruct a strain homozygous for *wp* and *tsl*, i.e. the F_4 was inbred, followed by single-pair crosses with *wp* individuals. However, it became apparent that these females were sterile, i.e. they produced no or only very few eggs and all dissected females showed various forms of degenerated ovaries. This phenomenon gradually disappeared when the *wp-tsl* chromosome was maintained for three or more generations in a heterozygous condition. From these generations it was possible to recover 27 viable *wp* families and 9 of these contained the *tsl* mutation. By crossing females of 8 of these families with T(Y;5)1-61 males (also outcrossed with Guatemalan material), the VIENNA-42/Guatemala hybrid strain was constructed.

At the stage of outcrossing where all inbred females were sterile, alternative strategies were considered to overcome this problem. As the observed sterility was

reminiscent of a phenomenon called hybrid dysgenesis, we started a second set of crosses:

(a) The reciprocal outcrossing scheme: i.e. only the males were used from the Guatemala strain and the *wp-tsl* chromosome was maintained in the females.
(b) A second *tsl* subline was used.
(c) A second strain from Guatemala was used.

In all crosses severe sterility was observed. However, in general, using the males from either of the two Guatemala strains generated less severe effects. However, the recovery of *wp-tsl* families was reduced. This is due to the fact that the labelled chromosome had to be maintained in females in a heterozygous condition, resulting in an increased recombination between the two mutations.

2.5. Evaluation of VIENNA-42 under field cage and field conditions

So far, three extensive evaluations of the behaviour of VIENNA-42 have been performed:

(a) Tests on potted host and non-host trees in the greenhouse at Seibersdorf, assessing all aspects of the behaviour of these males in comparison with males of other laboratory strains used previously with success in the field;
(b) Tests involving the release of sterile VIENNA-42 males from a central point in a citrus area of Chios, Greece, to study their dispersal and survival under field conditions;
(c) Tests on field caged orange trees in Chios to assess the competitiveness of VIENNA-42 males relative to wild males for wild females.

Results of the first tests indicate that VIENNA-42 males exhibit all components of the normal sexual behaviour of this species, as well as a pattern of temporal and spatial distribution of activities similar to other strains [12]. The field dispersal and survival study showed mortality rates of sterile VIENNA-42 males comparable with results of survival studies of standard strains, with dispersal exceeding 100 m, the minimum distance required between 200 m flight lines during conventional sterile fly releases [12].

The field cage study indicated that sterile VIENNA-42 males attract a greater number of receptive females than wild males. However, wild males still had a moderate competitive advantage (confirmed by egg sterility) in terms of mating success, indicating some rejection of VIENNA-42 males by wild females during final courtship. It is concluded that VIENNA-42 is a viable genetic sexing strain, with a competitiveness comparable with that of other standard medfly laboratory strains.

3. CONCLUSIONS

Genetic sexing systems have reached a sufficient level of accuracy, stability and applicability that large scale mass rearing of these strains is possible. On the basis of experience that will be gained in this process, further refinements may be required in the future, e.g. development of optimized rearing with respect to temperature treatment and temperature control during the larval stages. Furthermore, additional measures to further improve stability could be included in the sexing strains. However, the existing systems have two principal drawbacks:

(a) As these strains are based on Y–autosome translocations, their inherent semisterility requires increased egg production.
(b) The transfer to other pest species is very difficult, i.e. for each species for which genetic sexing systems have to be developed, a rather extensive basic knowledge in genetics and cytology has to be established.

It is hoped that these limitations can be overcome by generating sexing systems with molecular techniques. Several laboratories are working on the identification of genetic elements that could be used as vectors in a transformation system for pest insects, and other groups have initiated research to clone genes that, after appropriate engineering and reintroduction into the insect, can be used as selectable markers in genetic sexing systems.

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FIELD EVALUATIONS OF A GENETIC SEXING STRAIN OF *Ceratitis capitata* IN HAWAII

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Abstract

FIELD EVALUATIONS OF A GENETIC SEXING STRAIN OF *Ceratitis capitata* IN HAWAII.

Large scale field studies of the Mediterranean fruit fly, *Ceratitis capitata,* were carried out in Hawaii using a genetic sexing strain based on pupal colour. In a ground release population suppression study, an all-male release was compared with a bisexual release; also included was a release of predominantly females. Following release, egg hatch measurements in the field indicated that the release of males was about four times more effective than the release of males and females. The release of females only, as expected, failed to show any effect on egg fertility. During the latter part of the releases it appeared that the wild medfly population was developing some form of behavioural resistance to the released sterile males.

1. INTRODUCTION

As reported in previous Research Co-ordination Meetings, organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, in Vienna (1985, 1987), Crete (1986, 1988), Guatemala (1990) and Italy (1992), the authors have been working with *Ceratitis capitata* (Wiedemann) (medfly) genetic sexing strains based upon sexual differences in pupal coloration [1]. One of these strains, Hawaiianized Robinson (RHW), was created through repeated backcrossing of males from *wp-23* [1] with standard Hawaii mass production females. This strain proved

itself superior to the original imported sexing strain (*wp-23*), received from A. Robinson, in a number of important fitness parameters, including: field cage mating competitiveness, small scale field dispersal and longevity, and rearing efficiency. The hybrid sexing strain also fared well against the standard bisexual laboratory strain (HI LAB) which has been in mass production for c. 38 years. On the basis of the encouraging preliminary test results with the RHW strain, in 1989 we began a programme of mass production and large scale field evaluation in order to evaluate the concept of releasing sterile males-only medflies in Hawaii.

2. PRELIMINARY LARGE SCALE FIELD STUDIES

Our initial field studies in coffee provided data comparing dispersal and longevity characteristics for a *C. capitata* pupal colour sexing strain and a standard bisexual strain. A third population (RHW-Mixed), differing from each of the other two populations by one parameter (either sex ratio or strain), served as a control. The inclusion of this third population proved to be important. Also of crucial value in evaluating the concept of releasing males-only strains is the need to maintain a highly pure stock and avoid genetic contamination and progressive breakdown. We endeavoured to provide as high a percentage of males as possible in the RHW-Males release population (98% or higher) and we established rearing and pupal sorting procedures to ensure the maintenance of this standard.

With respect to fly dispersal, the RHW-Males population dispersed significantly more than the HI LAB population, with c. 30% dispersing beyond 100 m compared with c. 10% for HI LAB. The ratio of the proportions of RHW-Males to HI LAB flies trapped increased with distance, exceeding a factor of 10 at the higher distances. The dispersal of the RHW-Mixed population could not be directly compared but, in general, was intermediate in magnitude between RHW-Males and HI LAB, on the basis of trap recaptures up to 400 m from the release point.

Regarding the longevity of the released populations, both the RHW-Males and RHW-Mixed flies had significantly lower mortality rates than HI LAB flies, and their estimated LT_{50} values were about twice that of HI LAB. This result indicates that the strain difference between RHW and HI LAB, rather than the males-only factor per se, was the significant factor in producing the observed difference.

3. GROUND RELEASE SUPPRESSION STUDIES

On the basis of the encouraging preliminary results of the ground releases discussed above, we proceeded with plans to attempt population suppression of medflies in coffee grown in Kauai [2]. For the first time in testing a medfly genetic

sexing strain, we collected sterility data to assess the competitiveness of released sterile flies. We compared two strains (standard HI LAB and RHW-sexing) and several sex ratios: (1) 50:50 (HI LAB and RHW), (2) 99:1 (RHW 'males-only') and (3) 5:95 (RHW 'high females'). The third treatment was a new one, made possible by the ability to save machine sorted white pupae (females). This treatment was included in order to better assess the effect of sterile females in the field.

The various strains involved were mass reared at the Tropical Fruit and Vegetable Research Laboratory in Honolulu, Hawaii. The RHW genetic sexing strain was reared at a level of c. 2 million adults per week, while the HI LAB standard strain was reared at a level of c. 5 million per week. Larval rearing followed the standard methods with an artificial wheat based diet [3]. Popping larvae were collected in water, then placed into fine vermiculite at the rate of 1 L of larvae to 4 L of vermiculite to allow pupation. At six days of age, c. 10 L of the RHW production was sifted, then rinsed briefly to remove any vermiculite, and allowed to dry overnight. At seven days of age, c. 8 L of clean RHW pupae were mechanically sorted by colour using a Sortex/Scancore Inc. Model 1121 bichromatic sorter at a speed of c. 300 000 pupae/h. Brown pupae were sorted twice to produce a c. 99.5% brown lot and white pupae were sorted three times to produce a 95–98% white lot.

After RHW pupae were machine sorted at seven days' pupal age, pupae of each treatment were dye marked with distinct Dayglo^{R} fluorescent dyes at a rate of 3 g per litre of pupae. Each week, 1 L (c. 60 000) of the bisexual treatments (HI LAB and RHW unsorted) and 0.5 L of the unisexual treatments (RHW males-only and RHW high females) were dyed and bagged for irradiation (at eight days). Pupae were gamma irradiated at 15 krad (150 Gy) under anoxia with a ^{60}Co pool type irradiator. While still bagged, irradiated pupae were shipped to Kauai and carried to field test sites.

A technique has been developed which can distinguish irradiated from wild sperm in mated female spermathecae [4]. The head lengths of irradiated sperm are significantly shorter than wild fly sperm head lengths (c. 26 μm compared with 30 μm average length), with less than 5% overlap in distributions. Measuring about 10 sperm per female with dark field microscopy at 1000× allowed one to make a correct determination as to the source of the sperm (sterile or wild). Presumptive cases of multiple mating can be recognized by the presence of bimodally distributed sperm head lengths. With the development of this procedure, for the first time the natural field mating behaviour of wild females can be monitored, and the resulting competitiveness of sterilized males deduced. Of course, the mating behaviour of sterile females in the field can also be determined, including the frequency of sterile–sterile matings. Two measures of sterility can be calculated, one based on hatchability of eggs and one based on the sperm identification technique described above.

Egg hatch measures were obtained from field collected coffee samples each week per test plot. Estimates were made each week as to the percentages of ripe and

infested berries at each trap site. Eggs dissected were scored first as to species type, *C. capitata* or *Bactrocera dorsalis,* the oriental fruit fly, then as either hatched or unhatched. During summer and fall months in Hawaii, *B. dorsalis* becomes the dominant tephritid species infesting coffee.

With only minor fluctuations, releases continued smoothly throughout the test. Pupal size, adult emergence and laboratory longevity remained high. Samples of pupae from the field buckets indicated, with a few exceptions, good adult emergence of 85–95%. Contamination of the RHW rearing stock remained low; nonetheless it did increase slightly as expected, resulting in a drop in purity of the brown pupae to c. 98% males and of the white pupae to c. 95% females.

Estimates of sterility induced by release of sterile flies were obtained by scoring egg hatch from coffee berry samples and correcting these by subtracting control field sterility. Egg sterility rose gradually over several weeks after the onset of pupal releases in all of the treatment fields except in Mahaulepu (wild population very low) and in Kapaa (RHW high females). Egg sterility remained low (<10%) in Kapaa throughout the test, indicating very little effect on sterility by sterile females. Indeed, the low sterility observed probably came from c. 5% sterile males emerging from ground released brown pupae in that field. In the two bisexual release fields, egg sterility rose to c. 45%, then dropped. This pattern differed sharply from that seen in the RHW males-only field. There, sterility rose to c. 40–50%, as happened with the two bisexual treatments, but instead of dropping off later, sterility rose quickly to 100% and remained there for several weeks until the end of the test.

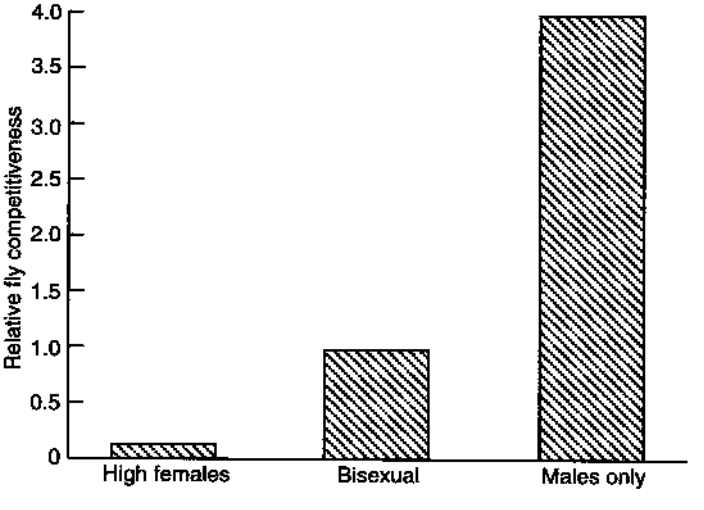

FIG. 1. Variation of sterile fly competitiveness with sex ratio.

In summary, the RHW males-only treatment caused significantly higher sterility in the wild population than bisexual populations. It was also trapped at significantly higher rates than bisexual populations in trimedlure (TML) traps. The high sterile female release did not reduce egg hatch or oviposition rates relative to control levels, and this resulted in a high trap-back of virgin flies coupled to a low trap-back of wild flies. In conclusion, males-only releases significantly increased the efficiency of the sterile insect technique (SIT) against wild medfly populations in coffee. The costs associated with each particular sexing system need to be accounted for in large scale SIT programmes.

Estimates of sterile fly competitiveness based on relative degrees of egg hatch suppression were calculated for each of the bisexual and unisexual treatments [2]. The competitiveness per fly for the high females treatment was estimated to be 0.04, where a value of 1.0 indicates that the sterile released flies are equally competitive with wild flies. The competitiveness of the HI LAB and RHW-Mixed treatments was estimated to be 0.14 and 0.21, respectively. The RHW-Males treatment, meanwhile, had competitiveness values of 0.62 and 1.33, depending on the formula used. These values indicate that the males-only treatment was 3–5 (average 4) times more efficient than the bisexual releases and c. 30 times more efficient than the c. 95% females treatment (Fig. 1).

In addition to relative field efficiency on the benefit side of the equation, one should consider the relative costs involved in rearing and releasing millions of sterile males. If the sexing operation is performed in the egg stage, one could save virtually 50% of the total costs by eliminating females that early, i.e. produce twice as many males for the same cost. The rearing efficiency in that case would be c. 2.0 (Table I). This maximum efficiency makes egg separation strains like the IAEA's current temperature sensitive lethal (*tsl*) strain very attractive, of course. Indeed, that strain is being field evaluated in Europe and Guatemala by several organizations. For the RHW pupal separation strain with which we worked, on the other hand, we have estimated a more modest rearing saving of c. 25%, leading to a relative efficiency of c. 1.25 (Table I). Combining both factors for rearing and field efficiencies, one can estimate the total increase in efficiency, considering only the benefits side of the cost–benefit equation. Clearly, our studies indicate that males-only releases, together

TABLE I. RELATIVE EFFICIENCY OF BISEXUAL AND MALES-ONLY STERILE FLY PROGRAMMES

Relative rearing saving/ million males sexed in:			Relative field efficiency (males-only/bisexual)		Total release efficiency
Pupal stage:	c. 1.25	×	c. 4	=	c. 5
Egg stage:	c. 2.0	×	c. 4	=	c. 8

with a rearing saving in the production of males, can result in very significant increases in efficiency — on the order of 8-fold for egg separation strains and 5-fold for pupal separation strains.

On the other side of the cost–benefit equation, one needs to consider the cost of creating a genetic sexing strain or system (if no new strain is involved), the cost of the sexing system in actually rearing all males and, finally, the cost of maintaining the sexing strain in a highly pure, stable condition. Depending on the strain (or system), the particular costs involved will be readily apparent. The ease of this exercise contrasts sharply with the difficulty in estimating the benefits side of the equation, as our lengthy field studies have shown. For the RHW pupal separation strain, for example, one has to consider the fixed, initial cost of one or more sorting machines, the labour costs to operate the machines, and the labour and materials costs to maintain the strain pure and the sorting machines operational. For egg separation sexing strains, the mass rearing and release costs will be much less, of course. However, the initial cost of creating such strains, e.g. the IAEA's *tsl*, can be high as rare genes are sought.

4. BEHAVIOURAL RESISTANCE TO THE SIT

Brimming with confidence after the exemplary performance of the RHW males-only releases in Kauai coffee, we initiated a programme to compare aerially dropped RHW males-only against the standard HI LAB strain already being air dropped in Kauai. Two plots each of 1 square mile (c. 2.6 km^2) were selected for the test. One of them, designated for the RHW males, had been kept as a control area free of flies until only several months prior to the onset of aerial releases in May 1992. The HI LAB test area was within the two-year-old aerial drop zone for standard HI LAB flies. Approximately 300 acres (c. 120 ha) of high yielding coffee were centred within each test area. Fly releases were made by a fixed wing aircraft twice weekly, yielding c. 1 million males dropped over each plot (i.e. c. 2 million HI LAB flies of both sexes). A series of standard male lure TML traps, and protein baited traps for both sexes, were emplaced in each area to monitor both sterile fly releases and wild fly populations. Fruit was routinely sampled weekly for dissection of eggs, as was done in the earlier ground release, small plot test. Sterility estimates were obtained from both egg dissections and sperm analysis, the latter from mated wild females caught in liquid protein traps.

Throughout the late spring and summer of 1992, the aerial releases proceeded smoothly, when good quality flies of both strains were dropped into their respective test areas. Sterility levels increased quickly in the males-only area, as expected, reaching 70% by the first week of September. Fly competitiveness was also good for the RHW strain since the sterile to wild ratio was relatively low — c. 20:1 during this

period of low wild fly population. However, in the HI LAB area, sterility remained at the relatively low level of 10–20% that it had been maintaining for about one year. Indeed, because the sterile to wild ratios were higher in this area (c. 50–100:1), the competitiveness per fly was even lower.

The appearance of a well controlled experiment, proceeding smoothly with clear-cut, consistent differences again showing a males-only advantage, was dramatically shattered by the advent of Hurricane Iniki on 11 September 1992. This storm, with winds in excess of 200 mph (320 km/h), devastated the island of Kauai, including the coffee growing area. It would be two months before any trap monitoring or coffee dissections could resume.

Unfortunately, the wild fly population recovered rapidly, no doubt owing to the protection offered pupae underground during the storm. Possibly as a result of some selective power of the storm, or simply through mere coincidence, the sterility levels in the males-only area never again reached 50%, and hovered around only 25% for most of the remainder of the aerial release period, which continued until May 1993.

During the whole period of aerial releases, we began investigating the mating behaviour of the RHW and HI LAB strains in outdoor mating cages. These tests were designed to measure the level of compatibility between laboratory reared flies and their wild counterparts from Kauai and other Hawaiian islands. Over the course of 1992 and into 1993, at the same time as problems developed in the field regarding low sterility, we began noticing problems with sterile males not mating with wild Kauai females in the outdoor cages. Interestingly, the reciprocal cross involving laboratory females and wild males remained at normal levels, indicating that the problem lay with a selective wild female. The corresponding declines in mating behaviour between sterile flies and wild flies from other non-SIT areas of Hawaii did not occur. These other sites included coffee growing areas on the islands of Maui and Hawaii, where, in the latter instance, even the same varieties of coffee, Red and Yellow Catuai, are grown.

In spite of these disappointing developments, yet another effort was made to compare RHW males-only with HI LAB releases in new 1 square mile plots within the previous HI LAB aerial release area. We hoped to increase greatly the number of flies actively participating in the field by ground releasing adults from roving vehicles thoroughly covering the entire test areas. This desired result was achieved as sterile to wild overflooding ratios reached 100:1 or higher for several weeks after releases began in June 1993. However, in both test areas the sterility again remained low (10–30%), though consistently somewhat higher for the males-only treatment. These results again strongly indicated a relatively poor mating performance by sterile released males with wild females. All lines of evidence — field sterility rates, wild population levels and cage mating studies — pointed to the dreaded monster, resistance to the SIT. It would appear as though a rapidly increasing level of resistance took hold about two years previously in Kauai in those areas which had received

continuous sterile fly pressure for several years. In other areas, such as the initial aerial release site for RHW, where no significant sterile fly releases had been made, sterility started out high, then dropped. Though such reported resistance is unprecedented in medflies, resistance to sterile insect releases was first reported for the melon fly, *Bactrocera cucurbitae,* during the eradication programme in Japan [5].

Faced with the reality of failure for even the vaunted males-only strain, we terminated fly releases in September 1993. Since that time we have focused our efforts on finding an effective way to counter the resistant population. We collected several wild populations from the resistant area and have been rearing and testing them in mating cages, in hopes of finding one which can successfully mate with resistant flies. Also, we have been trying to dissect the courtship behaviour of resistant and non-resistant flies through both visual observations in outdoor cages and analysis of video recordings of indoor and outdoor cage matings. This work is continuing at the present time. It is hoped that a solution will be found to the resistance problem in order to complete final testing on one or more males-only strains, and vindicate the initial highly encouraging results of males-only sterile medfly releases.

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FUTURE NEEDS IN RESEARCH ON GENETIC SEXING OF *Ceratitis capitata*

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Abstract

FUTURE NEEDS IN RESEARCH ON GENETIC SEXING OF *Ceratitis capitata.*

The author makes suggestions on the direction of research for genetic sexing over the next several years and prepared the paper as a guide for discussion. The literature of genetic and cytogenetic studies on insects as a whole is the basis for most of the approaches that the genetic control community has used, but only a tiny fraction of the literature is directed at genetic sexing and most of that is limited to small scale laboratory studies. The effort to use genetic sexing strains on the scale of mass rearing of medflies is unprecedented, and it is not surprising that a few problems have been encountered during implementation. Consideration of this fact leads to the conclusion that it is necessary to 'think big' and target the research.

1. BACKGROUND

Genetic sexing of the Mediterranean fruit fly, *Ceratitis capitata*, was set as a worthwhile goal about fourteen years ago by a Consultants Meeting convened by the Joint FAO/IAEA Division. The economic advantages of being able to kill females selectively during mass rearing of sterile flies were obvious. Large scale programmes for eradication of the medfly were anticipated, and a method was being sought to avoid having to rear the females and to eliminate oviposition stings by sterile females. At that time there was a general lack of information on the genetics of this species. The literature was so sparse that a complete literature survey could be accomplished in one afternoon at the library. On the basis of the information available for the medfly and other insects (for which genetic sexing was documented), priorities were outlined and a plan was formulated for a balanced programme of research and development aimed at both the accumulation of basic information and studies for applied objectives.

In 1980 most of the existing genetic sexing systems were based on genetic markers such as dominant insecticide resistance or recessive colour variants that had been rendered male linked by the use of translocations and inversions. There were a

few innovative systems that employed recessive, temperature sensitive lethals or other recessive balanced lethal combinations. At that time, eukaryotic molecular biology was also underdeveloped. For example, there were no methods for germ line transformation for any insect. Cloning and characterization of genes were still fairly difficult, and this whole area, although potentially of great promise, was not well advanced enough to offer any obvious solutions to genetic sexing of the medfly. Therefore, the original blueprint for R&D aimed at genetic sexing of the medfly was a combination of classical genetic and cytogenetic techniques based on the successful application of such efforts with other insects. Although some early work was directed towards insecticide resistance, this was discouraged to some extent because of the possibility of the accidental release of resistance into field populations.

Over the course of these past fourteen years, a great deal of progress has been achieved. An extensive list of mutants (and genetic map) and detailed maps of polytene chromosomes from trichogen cells and salivary glands are available. Significant advances have been made in radiation genetics and population genetics of this fruit fly. Our current understanding of the medfly is continuing to accumulate at an advanced level. This total effort should provide a sound basis for future genetic manipulation of the medfly and also provides a useful plan of action and expected results for other tephritid flies. The overall prospects for future breeding programmes are certainly enhanced by the intensive efforts of those scientists involved in basic studies over the past several years.

Genetic sexing strains have been assembled with a white-pupa mutant and a temperature sensitive lethal (*tsl*) as the means for the automated separation (with seed sorting machinery) of males and females and the selective killing of females, respectively. Other efforts, e.g. based on alcohol sensitivity, were promising but complicated by mitigating factors of the alcohol dehydrogenase system in the medfly that led to interesting results but no sexing system. A dieldrin resistance factor was abandoned because of cross-resistance and difficulty in establishing a clear-cut inheritance. A system based on purine sensitivity was also discarded.

Two major problems were encountered with the system assembled for the pupal mutant. First, the machinery used to separate the wild type from the mutant is not completely effective, which means that a minor fraction of the released insects are females that will impart oviposition stings, with accompanying blemishes, on the fruit. This problem is not too important if eradication, without subsequent reinvasion, of the medfly is achieved, because under those circumstances the damage caused by the females occurs in only one growing season. However, where the sterile insect technique is used as a control measure or for quarantine purposes, the damage to fruit will be an ongoing process that will invariably be accompanied by complaints from growers.

The other problem was simply breeding a strain that did not deteriorate under conditions of mass rearing. Because the males of these strains are heterozygous for a

holandric translocation, any contamination of the strain with a normal male, either through an accidental introduction from a wild type strain or via genetic recombination, both meiotic and mitotic, will destroy the ability to maintain the genetic sexing strain in a usable state. The release of a small fraction of females has not been overcome, but the complication of recombination has been circumvented by the isolation of translocations that are more stable. Large scale field tests with the white-pupa strain were a resounding success, with the very promising result that a males-only release led to a much better performance of the sterile males. Elimination of females from the releases increased the effectiveness of the sterile males in mating with wild females. The original idea of saving half of the rearing costs is further enhanced by this development, and there is now no doubt of the importance of devising a female-killing system that will produce only males for sterilization and release.

Two problems also surfaced with the effort to make sexing systems by using temperature sensitivity. The early embryonic stage and neonate larva are normally sensitive to and also adversely affected by temperature changes, and this situation has led to a sexing strain in which some of the males are also killed by a discriminating temperature that kills the females. It might be possible to isolate a more effective lethal gene. The second problem is related to genetic stability under the conditions of mass rearing, but by the time the *tsl* was being implemented, the experience with translocation and recombination in the pupal mutant strains was used to good advantage to construct a stable strain. This strain is currently being tested for its suitability for mass production.

After germ line transformation methods were developed for *Drosophila melanogaster,* several groups of scientists tried to adapt P element to the medfly and other insects. These efforts were not successful, because the transposon vector seems to be useful for only closely related species. Investigations on the molecular biology of the medfly and other fruit flies are continuing, with important data being collected. Current efforts on germ line transformation are aimed mostly at the other *Drosophila* transposons, viz. *mariner* and *hobo.* Recently, a possible hybrid dysgenesis system was described, and developments such as this might expose a useful transposon.

2. THE FUTURE

At this point in time, the recommendation for future work on development of genetic sexing obviously involves a heavy emphasis on molecular biology; however, there is no way to predict when useful technology will be available. There are perhaps a few immediate improvements that can be made with classical genetic and cytogenetic methods. One important fact must be emphasized, and that is that the goal in research on the medfly is genetic sexing and sterilization for use in eradication

programmes. Restraint should be exercised to avoid unnecessary research; in other words, avoid the 'drosophilazation' of the medfly as a laboratory insect for basic studies that are not critical to getting the job done.

2.1. Classical genetics

To begin with, potential improvements can be envisaged for strains based on white pupae. After overcoming the restraint of genetic recombination, which caused strain deterioration, via the synthesis of stable translocations, the main problem now is to eliminate females that slip through the machine used for separation. Probably, it will be impossible to obtain perfect separation, but we could include mutants in the genetic sexing strains that would render the females harmless. Scientists with good skills in mutagenesis and radiation genetics could set about to develop strains in which the females are homozygous for white pupae and some other trait, e.g. an eye colour mutant that causes blindness or a flightless mutant, both of which already exist in the medfly. New translocations would probably be necessary, but the techniques for inducing and screening translocations are available. Perhaps a better idea would be to induce a mutant condition (more than likely affecting the muscles) whereby the females could not insert their ovipositor into a fruit, or a modification of the sensory organs so that the flies would not be able to identify fruit. These latter mutants may already exist in the large factory strains, since those strains do not have to pierce fruit to reproduce under the conditions of the factory or laboratory.

For strains based on temperature sensitivity, there should be a new effort to induce and study additional *tsl* mutants, with the hope that a better system can be assembled. The linkage and cytogenetic maps for the medfly and the accompanying knowledge on translocations and inversions are much more extensive since the initial work on *tsl* mutants. With the proper tools in hand, the work of a breeder is much less complicated. Schemes for inducing, detecting and evaluating *tsls* should be much easier and more sensitive than in the original work, where only one stable mutant was isolated. The use of temperature as a discriminating condition is still a very attractive approach, and there may be some gems of wisdom gained that will be useful later when genetically engineered systems based on heat shock promoters are assembled.

Genetic leakage is a problem that will continue to plague the use of genetic sexing systems, and this will probably be true for genetically engineered strains as well as the current strains. A better knowledge of genetic and cytogenetic maps will be invaluable as a means for tracking what is happening when a strain begins to break down. For that reason, traditional genetic and cytogenetic studies should continue for the near future. This work should include the development of deletions and inversion balancers and can be integrated with molecular markers, so that mutants, microsatellites and restriction fragment length polymorphisms are used to map the genome and study genomic organization.

2.2. Population genetics

The most important aspect of population analysis that should be pursued is within the area of searching for hybrid dysgenesis, because presumably this phenomenon would signal the existence of usable transposons. For surveillance purposes, the identification of DNA sequences that differ from one major population to another would be very useful in tracking the source of reinfestation after the medfly is eradicated from an area.

2.3. Molecular genetics

The field of molecular genetics is wide open, but until a good technique for germ line transformation is a reality; there will be no substantial advance in genetic engineering of genetic sexing systems. P element, which is so effective in *D. melanogaster,* has been tested and abandoned, and current efforts are aimed at *mariner* and *hobo.* If *mariner* and *hobo* are not useful, then the prudent approach would be to pursue transposons in the medfly. In a recent report, a condition that looks like hybrid dysgenesis was described for the medfly, and the strains reported there would be a good starting point for screening for an active transposon. There are other ways to look for transposons, e.g. screening highly mutable strains, cloning and sequencing mutants, screening repetitive elements, and cloning the DNA around naturally occurring chromosomal aberrations.

Recently, there was a report on a simple approach of using restriction enzymes to transform a slime mould. Whether this method would work on insects is a big question. Perhaps a cleverly executed combination of first irradiating embryos with a low dosage, to cause minor damage to the chromosomes and DNA, followed by injecting a restriction enzyme and a plasmid containing a suitable reporter gene should be tried. This strategy would take advantage of the natural repair mechanisms and the ability of restriction enzymes to cut DNA.

The cloning of reporter genes and construction of plasmids that could be used whenever germ line transformation is finally worked out would be useful. Cloning and sequencing of the genes could be a part of the screen for transposons. Easily identifiable phenotypes, e.g. body or eye colour, are probably the best target genes.

Whenever transformation is possible, then decisions have to be made on what genes to engineer. From the standpoint of genetic sexing, the gene should be a conditional female-killing trait. The condition could be any physical variable (e.g. temperature) or any toxic substance. For the near future, work should be done on characterizing the genome in terms of sex determination (Y chromosome) and promoters for genes that are expressed in only one sex. Any information developed will be useful, but the key element in deciding what to work on should be whether the data will be useful for genetic sexing and sterilization.

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