Chapter 9

Reference Karyotypes and Chromosomal Variability: A Journey with Fruit Flies and the Key to Survival

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69835

Abstract

My goal is to present the analysis of concepts on the "normal" karyotype and chromosomal abnormalities through comprehension of chromosomal variation within fruit flies populations, advantages of these insects as models to study genetic polymorphisms and the methodology from field to strains. Cytological preparations were obtained from cerebral ganglion. Staining methods include routine and fluorescent bandings as well as *in situ* hybridization using DNA probes. We define a more frequent karyotype of each one species and take them as the reference karyotype. The reference as well as the chromosomal variants studied within each species were isolated in different strains. The techniques applied revealed differences among individuals belonging to different strains, thus documenting the mutations into the DNAr cluster, variation in the patterns of heterochromatin, mosaic specimens carrying nuclei with different chromosomal numbers. Hoecht revealed double-minute chromosomes and CG- rich banding marked somatic crossing over between sister chromatids. The most frequent karyotype is the reference karyotype, namely, the normal karyotype. Chromosomal mutations produce variability. In man, a number of these mutants are considered chromosomal abnormalities. We learnt that variation is the key to survival and that many individuals could be in the right place in the wrong moment.

Keywords: *Anastrepha fraterculus*, chromosomal abnormalities, somatic crossing over, sister chromatids interchange, chromosomal rearrangements, evolutive advantage, hybridization, rDNA cluster, genetic disorders, chromosomal mutations, aneuploidies, mosaic specimens, double-minute chromosomes

1. Introduction

When we talk about chromosomal abnormalities, we are referring to a particular group of karyotypes which differ from the most frequent one known as the "species karyotype." The "species karyotype" is known as "normal" karyotype.

The denomination "chromosomal abnormalities" refers to human mutations involving entire chromosomes or large segments of them which are missing, duplicated, and rearranged, showing that the relevant cellular processes are prone to a high level of error.

The consequences of such changes are physiogenetic disorders which are more evident in diploid species such as human beings and flies. The reason why fruit flies are a good model to study the inheritance of chromosomal mutations is because of their short life cycle, the possibility of their artificial rearing, and their large progenies.

Most genetic abnormalities appear spontaneously. Physical or chemical agents in the environment are capable of causing mutations in genes, and these mutations will be passed from parents to offsprings.

The purpose of this chapter is to clarify the concepts of normal karyotype or chromosomal composition throughout my prolonged research on populations of fruit flies, the materials and methods I used in my research, the lessons I learnt on this subject, and the conclusions I drew concerning chromosomal abnormalities on human beings.

I organized the main subject into different sections: (i) the South American fruit fly and the laboratory rearing technique for genetic studies; (ii) some concepts when studying karyotypes in men and flies; (iii) working with fruit flies; and (iv) original research findings in fruit flies.

1.1. The South American fruit fly *Anastrepha fraterculus* (Wied.)

A. fraterculus and *C. capitata* belong to the Tephritidae family which groups the true fruit flies. Both are agricultural pests with complete metamorphosis. The complete life cycle begins when females oviposit their eggs inside fruits where eclosion takes place and the larvae feed and develop passing through three instars. Immobilization of larva III marks the beginning of metamorphosis along with chitinization of the larval cuticle. Inside the puparium, histolysis of many larval tissues is followed by a *de novo* synthesis to reconstruct the adult fly. Ecdysis takes place at approximately 45 days from egg eclosion.

Their life cycles last between 35 and 45 days, while human beings produce one generation each in 20–25 years.

1.1.1. A laboratory rearing technique adjusted for genetic studies

An artificial rearing technique of the species for genetic studies begins with a representative sampling of a particular population and ensures abundant offspring. A genetic study looks for understanding chromosomal variation; thus, it is based in the study of families [1] founded by one male and one female. Reference works for the laboratory rearing techniques of *C. capitata* are Refs. [2–4] and for *A. fraterculus* is Ref. [5].

http://dx.doi.org/10.5772/intechopen.69835

These techniques allow us to establish colonies and to study families of flies in order to isolate chromosomal variants and to understand their behavior and significance within a population [6].

1.2. Some concepts when studying karyotypes in man and flies

The karyotype is the full set of chromosomes in a person's cells or in a fly's cell.

A very particular feature of flies is somatic pairing, an unusual event that makes the recognition of chromosomal pairs and their changes during mitosis easier.

A chromosome contains hundreds to thousands of genes. A gene is a segment of DNA containing the code used to synthesize a protein, an enzyme, an RNAt, or RNAr, so a gene can code for different polypeptides at different moments of life and in different organs.

Sexual reproduction of these organisms occurs in cycles of alternated phases and delineate times between two generations, and it is represented as:

Female 2n---MEIOSIS----n

FERTILIZATION produces a new zygote 2n

Male 2n------MEIOSIS-----n

where "n" is a gamete and "2n" is a zygote. We use a slightly different nomenclature to clearly distinguish between the zygote and the number of chromosome sets or ploidy level.

Almost every human cell is diploid (2x), since it contains two sets of 23 chromosomes inherited or received from each parent, for a total of 46 chromosomes/cell.

The human karyotype is composed of 23 chromosomal pairs, so almost every cell carries 46 chromosomes. Sperm cells and egg cells are gametes (n) which are haploid (x) since they carry only one set of 23 chromosomes (n = x = 23), and during fertilization (n + n), the new fertilized egg called zygote (2n) will be diploid 2n = 2x = 46 chromosomes (two sets of chromosomes). So almost all of the persons' cells are diploid 2n = 2x = 46 except for their gametes, either sperm or oocytes, which are haploid n = x = 23.

The fruit flies *C. capitata* and *A. fraterculus* are also diploid species with six pairs of chromosomes for a total of 12 chromosomes (2n = 2x = 12). Sperm cells and egg cells carry six chromosomes, so gametes are n = x = 6.

Karyotypes are obtained from good mitotic metaphases by cutting each chromosome and its homolog and ordering pairs from the largest to the shortest: a normal human karyotype will show 46 chromosomes, and a normal *Anastrepha's* or *Ceratitis'* karyotype will show 12 chromosomes.

We define a more frequent karyotype of each one species and take it as the reference karyotype, ordinarily known as the "normal karyotype." The reference karyotype as well as the chromosomal variants studied within each species were isolated in different laboratory strains and maintained throughout the generations in order to understand their significance. Chromosomal variants arise by mutations which are changes affecting chromosomal structure and/or chromosomal number. The rearing methodology allowed to associate each chromosomal mutation to particular physiological or morphological mutations or types of behaviors.

When chromosomal variants are found to be associated to physiological or morphological disorders, they are called chromosomal abnormalities. In human beings, they produce disorders known as syndromes and cancers. They are chromosomal mutations detected through cytological techniques. Chromosomal mutations are changes in chromosome structure which involve at least one chromosome breakage. Changes in chromosome number mostly arise as a consequence of failures during cell division, although they can also be produced by breakage of a chromosome segment. Sometimes, a change in chromosome structure causes a change in chromosome number.

1.3. Chromosomal mutations affecting chromosome number

1.3.1. Polyploids

Duplication of complete sets of chromosomes will modify ploidy levels and consequently chromosome number. For instance, we could use triploid flies carrying 2n = 3x = 18 chromosomes in their somatic cells to study sex determination in *Ceratitis capitata*. Triploidy is caused because of nondisjunction of chromosomes during meiosis I of one of the parents. Although this phenomenon could be an extremely rare event in living babies, a triploid bearing 2n = 3x = 69 was reported to live 9 months (Conference: La Española Hospital, 2013).

1.3.2. Aneuploids: nondisjunction of homologous chromosomes

Trisomy 21 in humans 2n = 2x + 1 = 47 is known as Down syndrome. Most affected persons have an extra copy of chromosome 21 due to nondisjunction of chromosome 21 in a parent with normal karyotype. This syndrome can eventually be produced by a translocation which occurs when the long arm of chromosome 21 breaks off and attaches to another chromosome at the centromere.

Monosomy: One chromosome of a pair is missing (2n = 2x − 1). A nulisomic is 2n = 2x − 2 because a complete pair is absent, which could be detected in triploid individuals or others with higher levels of ploidy.

1.4. Chromosomal mutations altering chromosome morphology

One chromosome breakage causes deletion of a chromosome segment in one chromosome of a pair.

Two chromosome breakages involving two chromosomes of a pair "o" from different pairs, cause translocations, inversions, and duplications of chromosomal segments

1.5. Original research findings in fruit flies

The main topics of this subsection are the comprehension of chromosomal variation within populations of fruit flies and the advantages of these insects as models to study genetic polymorphisms. We define a more frequent karyotype of the species and considered it as the reference karyotype, ordinarily known as the "normal karyotype." The reference as well as the chromosomal variants studied in the species were isolated in different laboratory strains and maintained throughout the generations in order to understand their significance.

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2. Methodologies

A very particular feature of flies is somatic pairing, an unusual event that makes the recognition of chromosomal changes easier.

A genetic study is based on the transmission of traits from one generation to the next. A long time between generations as well as a reduced progeny, greatly delays the comprehension on how mutations transmission is carried out.

An artificial rearing technique of the species for genetic studies begins with a representative sampling of a particular population and ensures abundant offspring (**Image 1**). A genetic study looks for understanding chromosomal variation; thus, it is based in the study of families [1] founded by one male and one female (**Image 1**). A good rearing technique ensures a good oviposition rate.

Reference works for the laboratory rearing techniques of *C. capitata* are Refs. [2–4] and for *A. fraterculus* see Ref. [5].

Cytological preparations were obtained from cerebral ganglion of third instars. The preparation of ganglia was as described in Ref. [7].

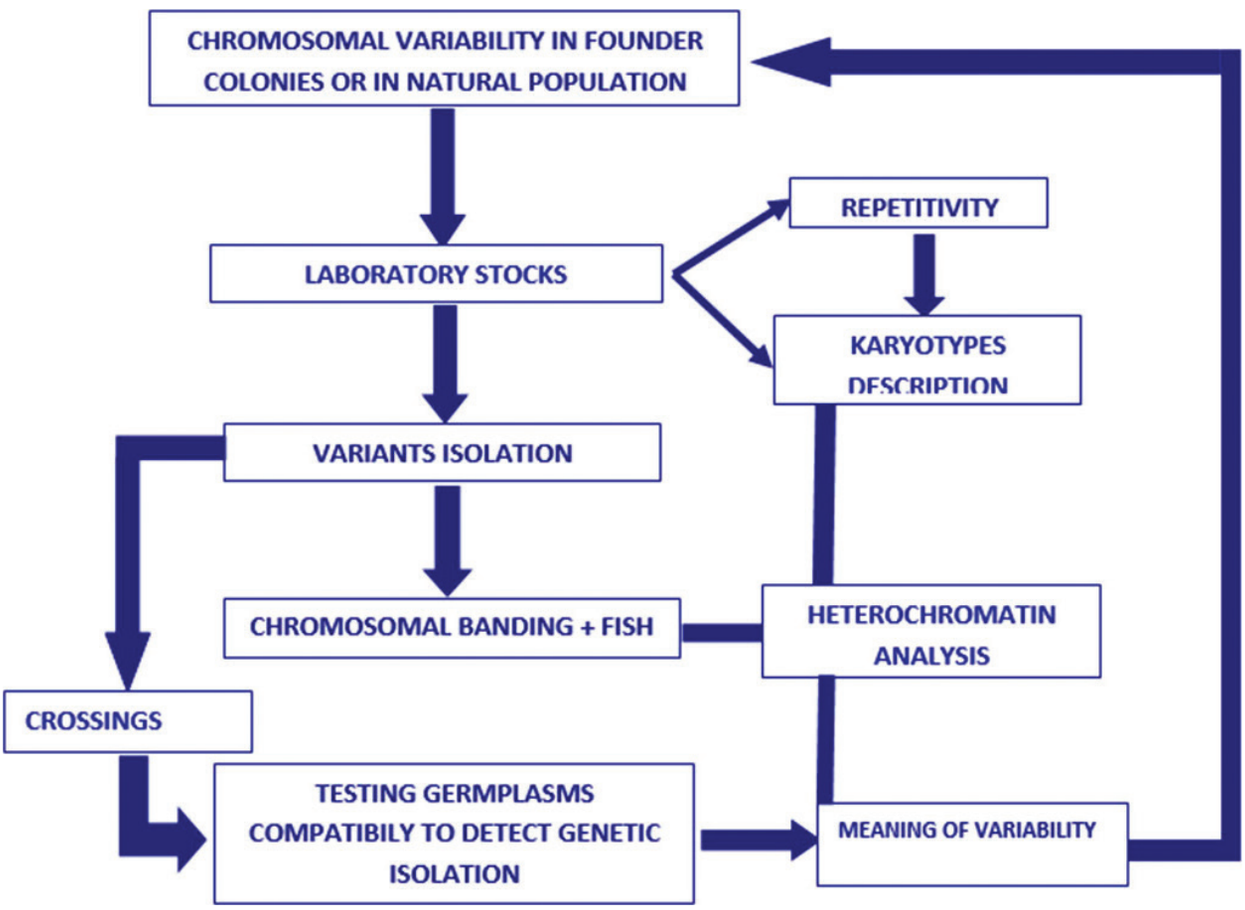

Image 1. Genetic methodology: work design scheme.

Chromosome banding and ideograms were from mitotic chromosome spreads from cerebral ganglia. C-banded preparations were obtained using the technique of Ref. [8]. H-banding was as described in Ref. [9]. GC-rich banding using $CMA_3$was as described in Ref. [10]. Mounting was performed in McIlvaine buffer with pH = 7 (0.16 M dibasic sodium phosphate, 0.04 M sodium citrate). Preparations were kept in the dark during 24 hours before examination under a Zeiss Axioplan fluorescence microscope. N-banding was obtained as described in Ref. [11]. At least 10 metaphase plates per chromosome spread were analyzed. Approximately 5000 larvae were dissected to obtain 1654 cytological preparations of *A. fraterculus* with good quality metaphases.

The fluorescent *in situ* hybridization (*FISH*) technique described by Willhoeft and Franz [7] was carried out using three different probes. (1) The *Drosophila hydei* probe pDh2-H8 corresponds to a cloned genomic fragment of 310 bp containing a 28S rRNA coding region interrupted by an intron [12]. (2) The *C. capitata* probe pCc-18S corresponds to an AT-rich cloned fragment of 720 bp derived from the 18S gene [13, 14]. (3) The *A. fraterculus* probe pK18 corresponds to a genomic fragment of 300 bp originated from a differential sex band [13]. All three probes were labeled by random priming with Digoxigenin-11-dUTP and revealed with anti-Digoxigenin-Fluorescein using propidium iodide as counter staining. The preparations were kept in the dark during 24 hours before examination under a Zeiss Axioplan fluorescence microscope. Images were recorded with an Olympus DP72 digital camera, time exposure being manually adjusted.

3. Results

Key results change two paradigms: *C. capitata* is a species considered to have karyotypic uniformity, and *A. fraterculus* was considered a complex of cryptic species. The possibility of rearing these species under laboratory conditions made possible the isolation of strains with complexities previously detected in natural populations of these flies (**Image 1**). Throughout 30 years, we could study polyploids, sexual aneuploids, and chromosomal rearrangements like translocations, inversions, deletions, duplications, ring chromosomes, jumping elements, cell mosaic specimens, B-chromosomes, and double-minute chromosomes. The rearing technique gave as the possibility of repeating a technique and/or applying different chromosomal markers on the same genetic material as well as to perform compatibility tests to understand the significance of chromosomal variation (**Image 1**).

Comprehension of populations' structures is an unavoidable task for geneticists.

3.1. *Ceratitis capitata*

The analysis of genetic variation within and between natural populations of *C. capitata* [15] explains the history of our reference laboratory strain *Arg 17* as well as all the morphological, chromosomal, and physiological variants along with the study of Mendoza polymorphisms colonies used in control strategies [15, 16]. We now summarize the chromosomal mutations found in the species throughout the years: reciprocal translocations, multiple translocations,

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and translocations between a sex chromosome and an autosome. The X-chromosome polymorphism due to attachment of a B-chromosome producing the X_L [16] could be understood through the analysis of 64 families involving reciprocal crosses of one male and one female. The transmission of the Y + B = Y_L was analyzed in Ref. [15]. The deletions affecting the long arm of the sexual Y chromosome produces a mutant Y named Y_B [15]. The polymorphism Ya-Yb is analyzed in Ref. [15]. Other chromosomal mutations were also isolated in different families such as inversions involving the autosomes, sexual aneuploidies: sexual trisomics 2n = 2x + 1 = 13 XXX, XXY, and sexual tetrasomics 2n = 2x + 2 = 14 XXYY. Finally, we studied triploids: 2n = 3x = 18 XXY and tetraploids: 2n = 4x = 24.

3.2. *Anastrepha fraterculus*

The taxonomic status of *A. fraterculus* has been a controversial subject, mainly because of misinterpretation of the observed chromosomal variation. In an 11 years work, the different karyotypes and DNA polymorphism of geographically defined populations from Argentina were studied, using derived stocks maintained in the laboratory during 25 generations.

This fruit fly is the main native tephritid pest and only second to the invading Mediterranean fruit fly *C. capitata*. Previous to this work, almost 38 species have been written after or are synonymies of *A. fraterculus*. Our studies have been performed utilizing wild flies as well as laboratory stocks. This was the first time that *A. fraterculus* stocks were successfully isolated and maintained. The emphasis of this work was in the analysis of chromosomal characteristics since misinterpretation of genetic variation has been the origin of the current taxonomic confusion. More than 2500 specimens from 24 habitats (host-fruit/locality) were cytologically analyzed using specific cytological techniques. The different approaches (cytological, biochemical, and molecular) including *in situ hybridization*, on the same genetic material (stocks) made it possible the rigorous karyotypic and molecular analysis of the stocks and population samples [13].

The main results obtained are:

That—contrary to what many specialists have postulated—the chromosomal polymorphisms in *A. fraterculus* described and analyzed throughout this work are not a barrier for intercrossings (in the wild and in laboratory conditions) and represent a single species.

The basic knowledge of the species' chromosomal variability was widened for different populations of South America: Argentina, Brazil, and Uruguay.

This was the first time the rDNA cluster is localized and the autosomes of the species are described and identified.

This was the first time that cytological ploidy mosaicism in natural populations of *A. fraterculus* is described, assigning a role in the regulation of differential gene expression during insect development.

For the first time, double-minute chromosomes are described in natural populations of an invertebrate, a physiological adaptive role is proposed.

It was determined that the different chromosomal variants can be associated to particular host fruits or particular geographic localities.

It was demonstrated that habitat heterogeneity maintains the coexistence of different karyotypes and rearrangements present as polymorphisms whose frequencies vary from one population to the other.

We demonstrated that no correlation exists between data from traps and those obtained from samples of infested fruits, strengthening that *A. fraterculus* mating system is not based in larval feeding resources. This is highly significant since host registering must be unavoidably done on the base of effectively infested fruits. Pest status must depend on registration of hosts.

The reference karyotype *fraterculus Arg 1*, from now on *fArg1*, carries a 2n = 2x = 12 chromosome complement composed—as revealed by C-banding by an acrocentric X-chromosome, a quasi-metacentric Y-chromosome and 4 autosomal pairs not easy to distinguish except for chromosomal pair II which is the largest of the complement [13, 17, 18].

We found variants for all the chromosomal pairs in comparing them with *fArg1*. We studied and documented 1654 specimens of good cytological quality, applying different techniques on the same material. We maintained stocks of flies and founded 85 families as described previously. We had to confine our study to the sexual chromosome variants.

C-banding of the most frequent karyotype named *fArg 1* shows two telomeric bands on the X-chromosomes and one on the Y-chromosome [13, 17]. N-banding was a valuable marker of chromosome 3 which otherwise is difficult to distinguish from chromosome 4: a negative N-band resulted as a strong marker of pair 3 (**Figure 1A**). In the same family, we could detect the presence of triploid individuals (**Figure 1B**).

We described four variants of the X-chromosome and six variants of the Y-chromosome [13].

C-banding of the X_1, X_2, X_3, X_4, Y_1, Y_2, Y_3, Y_4, and Y_5 variants can be found in Refs. [13, 17, 18].

H-banding along with somatic pairing revealed the heterozygous autosomal rearrangements which are clearly seen and indicated by arrows (**Figure 2**).

The combination of banding techniques provided profiles to characterize the 10 sex chromosomal variants isolated in laboratory stocks (**Images 2–5**).

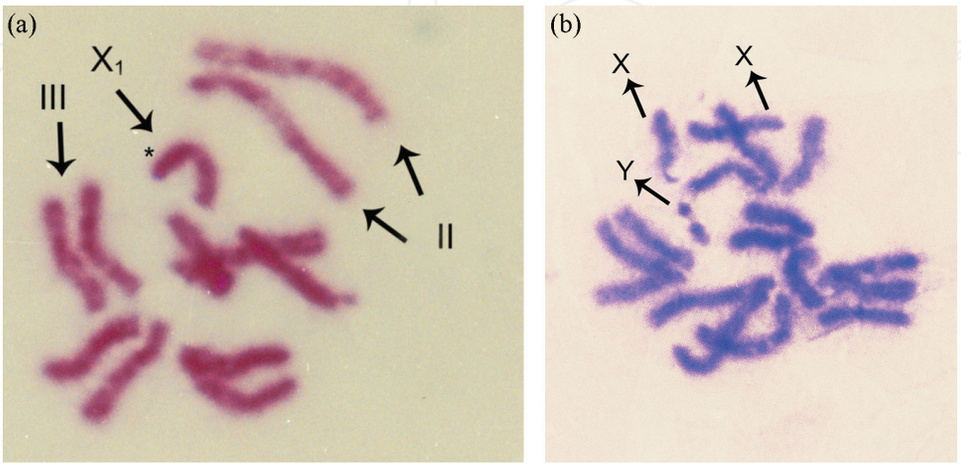

Figure 1. *N-banding* of neuroblast metaphases in *fArg1*. (A) Diploide X_1 Y_1 where asterisk indicates differential staining between positive banding and negative banding. 2800×. (B) Metaphase plate from a triploid male XXY. 2200×

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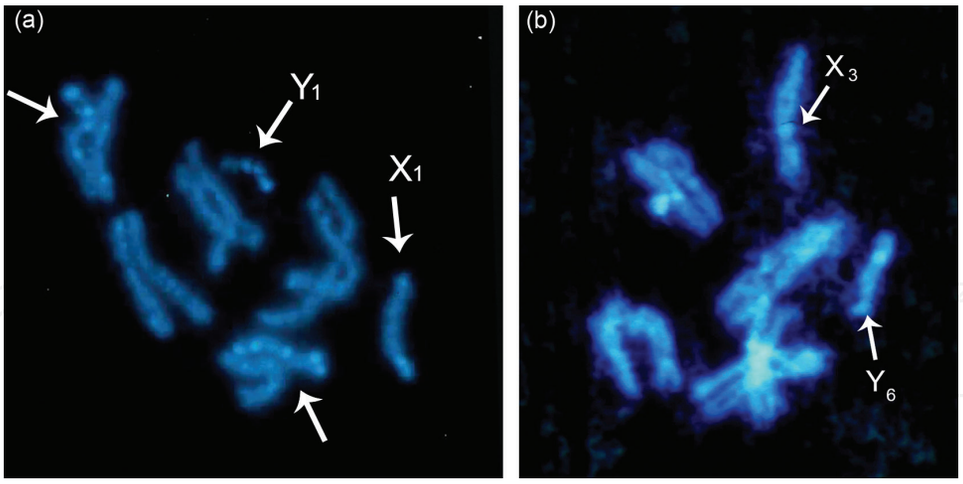

Figure 2. H-banding of metaphase plates. (A) X_1Y_1 specimen, arrows show heterozygous rearrangements. 2800×. (B) X_3Y_6 specimen. 2600×.

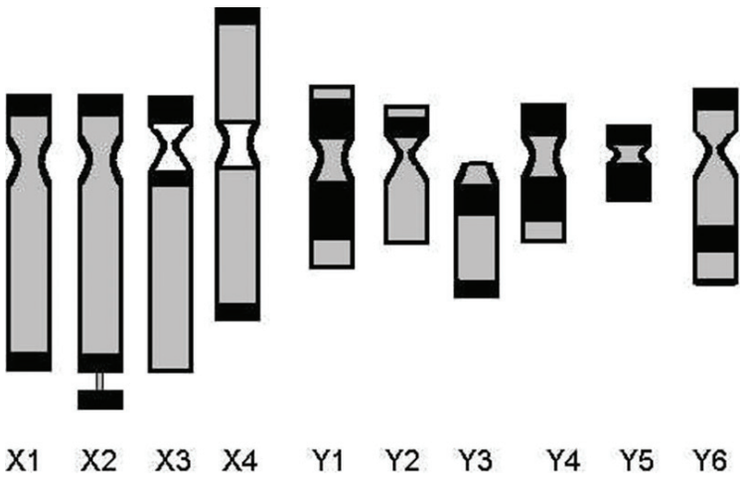

Image 2. Idiograms of the main variants of sex chromosomes for *Anastrepha fraterculus:* C-banding.

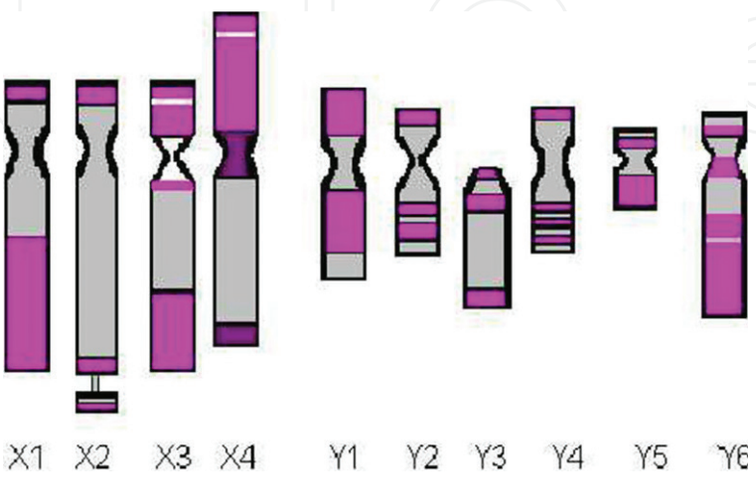

Image 3. Idiograms of the main variants of sex chromosomes for *Anastrepha fraterculus:* N-banding.

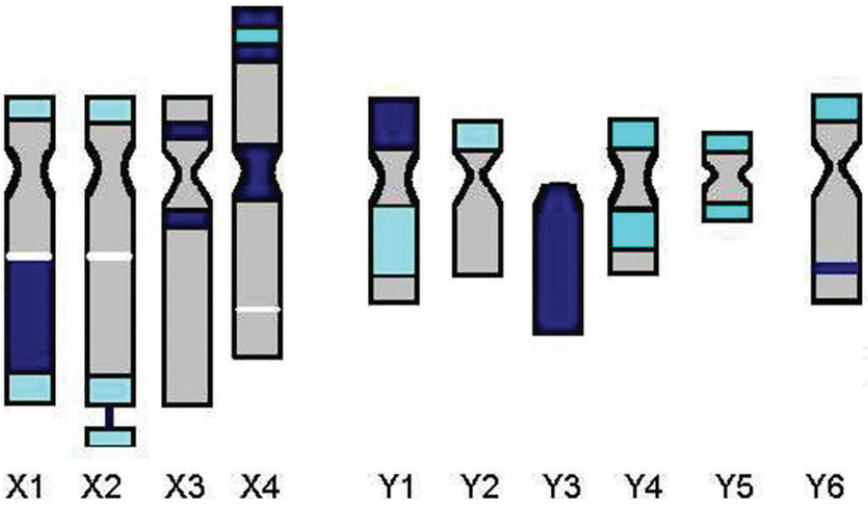

Image 4. Idiograms of the main variants of sex chromosomes for *Anastrepha fraterculus:* H-banding.

Ring chromosomes such as the X in (**Figure 3**) and the X-w (**Figure 4**) are evidence for duplication events. The X-w is shown in a prometaphase plate, as well as in an anaphase plate with bridge. This chromosomal rearrangement is a noticeable system during the evolution of *A. fraterculus*.

Ploidy mosaics (**Figures 5–7**) and double-minute chromosomes are also evidences for duplication events (**Figure 8**).

Mosaic individuals carrying diploid nuclei along with sexual tetrasomic nuclei X1X1Y5Y5 were found within some families of flies (**Figure 7**).

We also detected aneuploids such as monosomics (**Figure 9**) and sexual trisomics (**Figure 11**). Chromomicin A3 evidenced chromosomes with unequal sister chromatids (**Figure 10**) as a result of the somatic crossing over with interchange between sister chromatids (**Figure 11**) [19, 20].

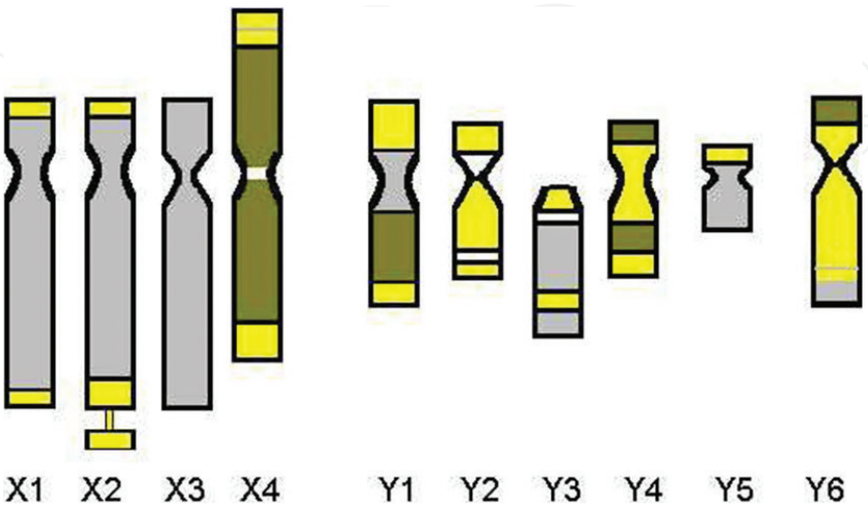

Image 5. Idiograms of the main variants of sex chromosomes for *Anastrepha fraterculus:* CMA_3-banding.

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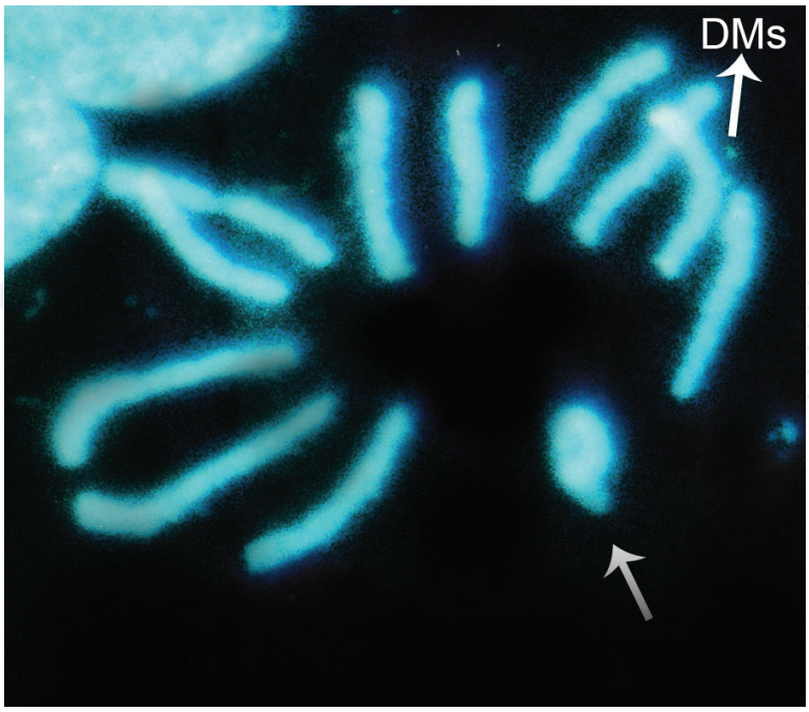

Figure 3. Metaphase carrying an X_2 ring chromosome. See arrow. 3200×.

The ribosomal DNA cluster was first located in *A. fraterculus* and in *C. capitata* using the 720 bp probe of the 18S gene of *C. capitata* [13]. This probe tested on the reference stock of *A. fraterculus* hybridized the short-arm telomere of X1 chromosome and the centromere and pericentromeric region of the short arm of Y1 chromosome in *fArg1* (**Figure 12**). In different strains of *A. fraterculus,* the localization of the ribosomal cluster was observed on the variants of sexual chromosomes such as Y_2 and Y_5 (**Figures 13–14**).

Except for the sexual karyotypes X_3X_3 and X_3X_4, we found all the combinations among the X chromosomes and among the X and Y chromosomes (**Image 6**).

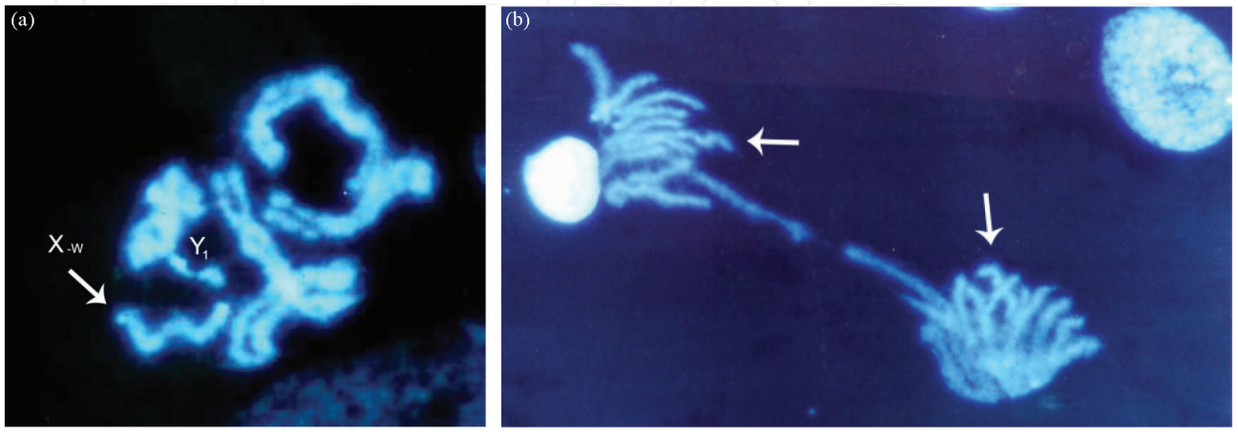

Figure 4. The X-w chromosome. (A) Prometaphase carrying X-w Y_1 and translocations. 2600×. (B) Anaphase with bridge, arrows show the X-w chromosome in each pole. 2600×.

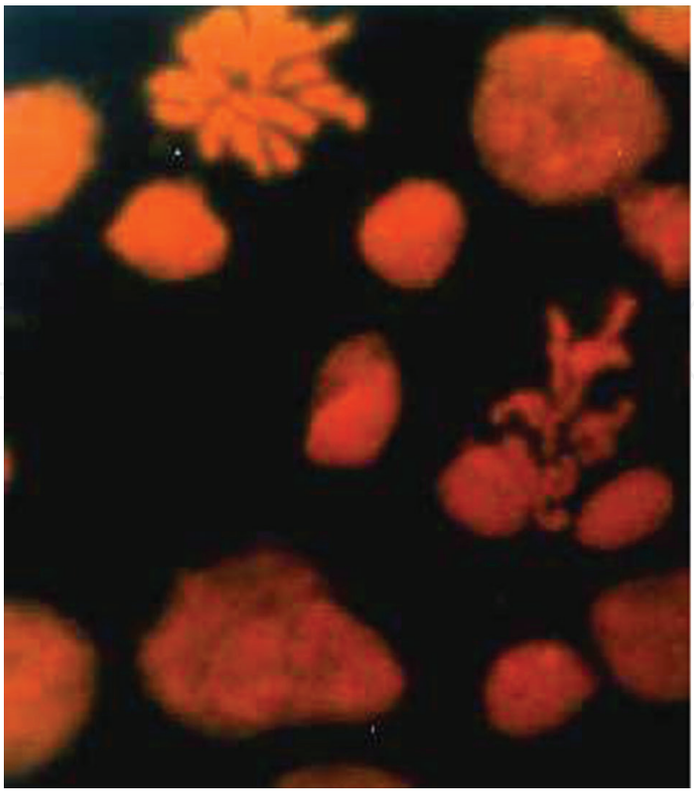

Figure 5. Propidium iodide staining of a mosaic specimen 2n = 2× – 3×. Metaphase plate showing two nuclei: diploid-triploid. 2600×.

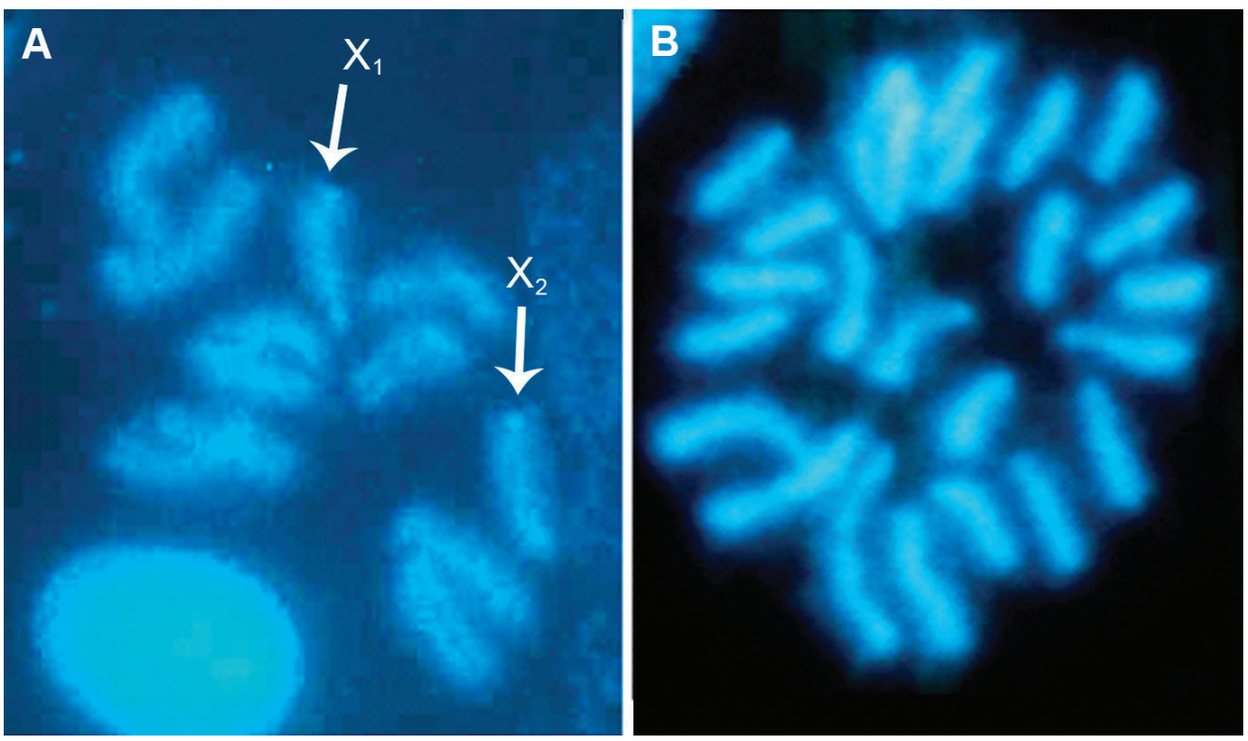

Figure 6. Hoechst staining on a mosaic specimen X_1X_2 from Brazilian stock 1220x. (A) Diploid metaphase. (B) Tetraploid metaphase. 2800×.

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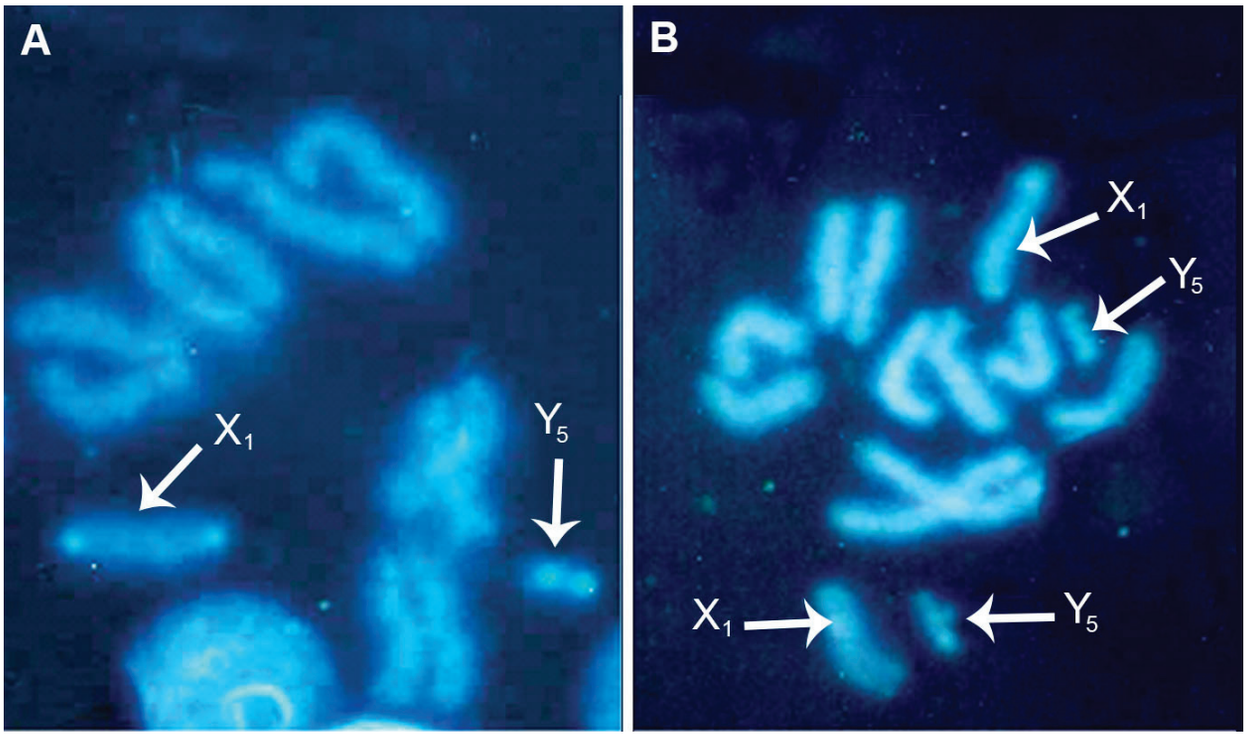

Figure 7. Hoechst staining on a X_1Y_5 mosaic individual from stock 244C. Two metaphases of the same specimen. (A) Diploid metaphase X_1Y_5. (B) Aneuploid metaphase $X_1X_1Y_5Y_5$ (sexual tetrasomic). 2600×.

Figure 8. Metaphase showing chromosomes with unequal sister chromatids (see arrows) and double-minute chromosomes. 2800×.

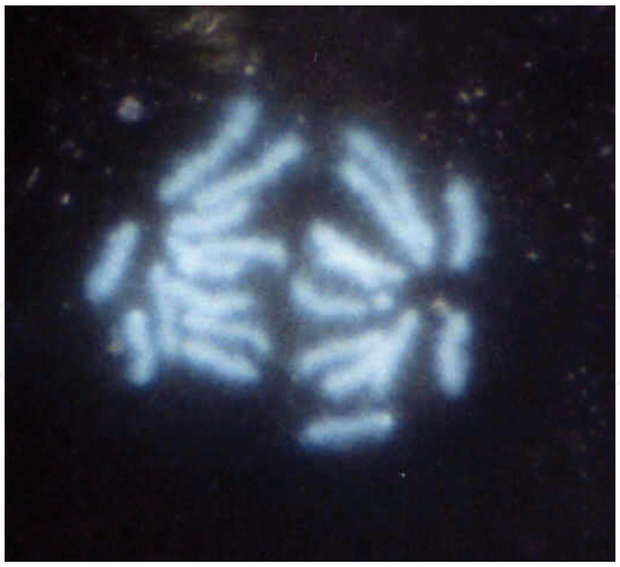

Figure 9. H-staining of a monosomic specimen 2n = 2× − 1. 2500×.

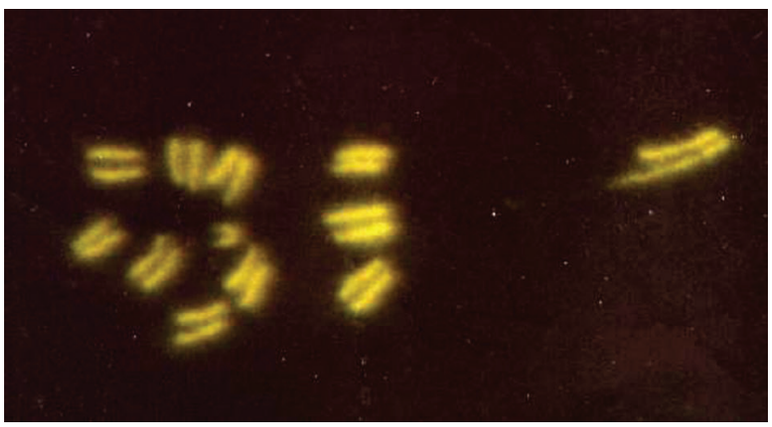

Figure 10. CMA 3 staining. Metaphase showing chromosomes with unequal chromatids.

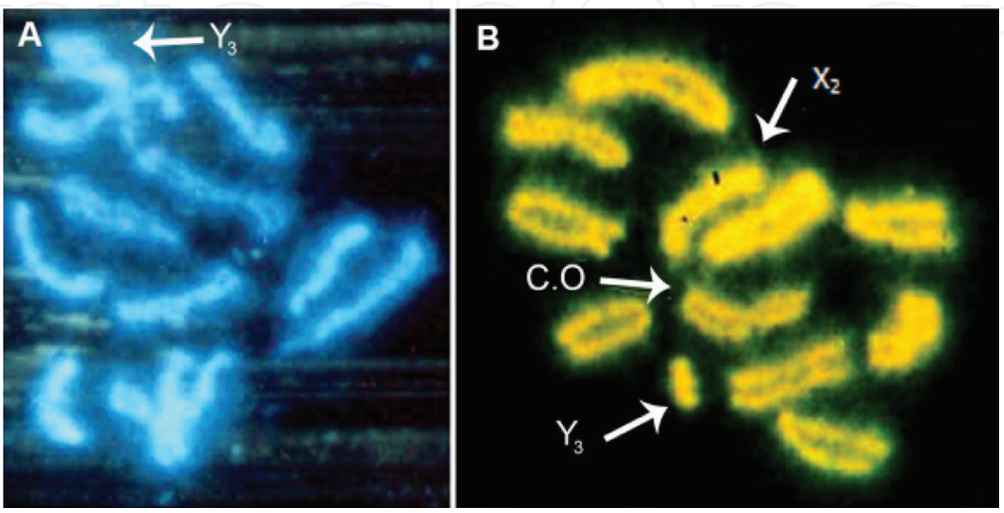

Figure 11. Stock 286. (A) Sexual aneuploid specimen 2n = 2× + 1=13, trisomic $X_1X_2Y_3$. (B) Specimen X_2Y_3 showing somatic C.O., interchange between sister chromatids.

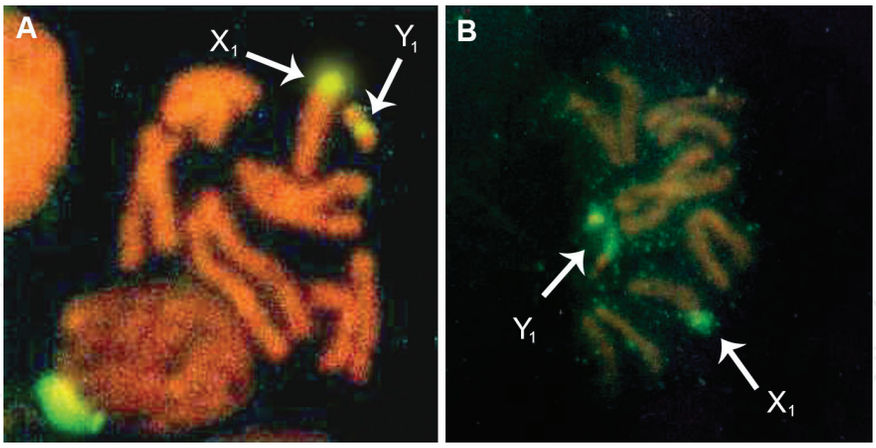

Figure 12. FISH with *C. capitata p18S* on "*f. Arg. 1.*" (A and B) Different specimens from stock 215M. 2800×.

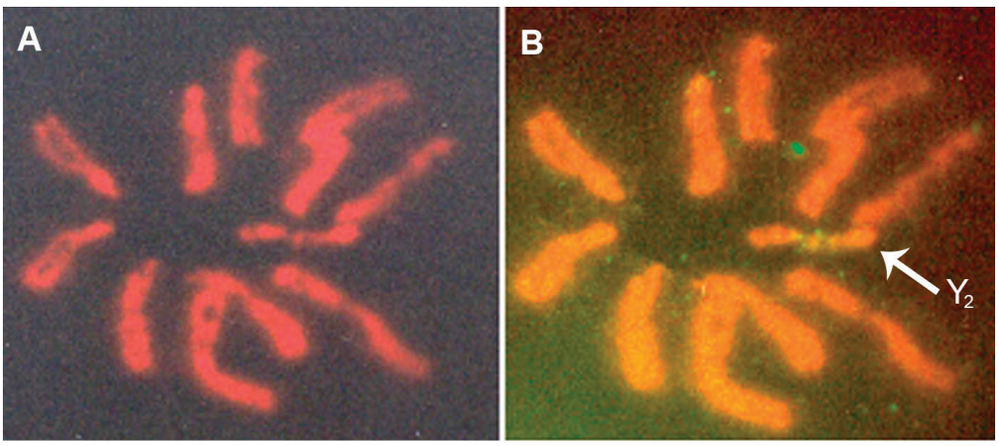

Figure 13. FISH with *C. capitata p 18S* on mitotic metaphase from stock 23M. (A) Counterstaining with propidium iodide. (B) Probe hybridizes Y_2 centromere. 2600×.

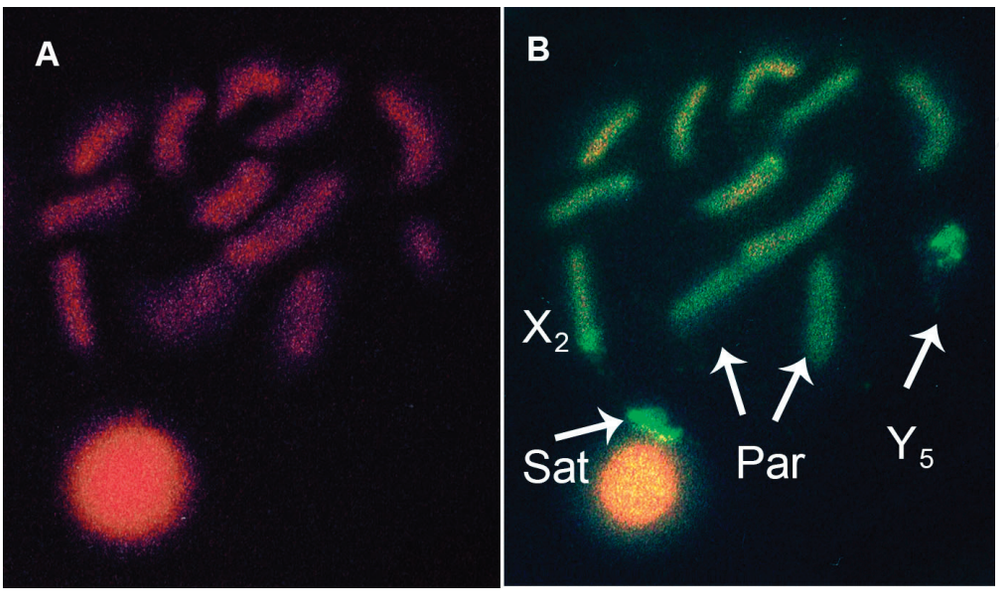

Figure 14. FISH with *C. capitata* p18S on mitotic chromosomes from a specimen X_2Y_5 of stock 1222. (A) Counterstaining with propidium iodide. (B) The probe hybridized the telomere and satellite of X_2 chromosome the whole Y_5 and an autosomal pair. 2500×.

MALES		FEMALES	
X1Y1	X1Y1+Ah	X1X1	X1X1+Ah
X2Y4	X1Y2+Ah	X1X2	X1X2+Ah
X1Y3	X1Y3+Ah	X2X2	X2X2+Ah
X2Y2	X2Y4+Ah	X2X3	X2X3+Ah
X2Y3	X2Y3+Ah	X1X3	
X2Y1	X1Y5	X1X4	X1X4+Ah
X2Y5	X2Y5+Ah	X2X4	X2X4+Ah
X1Y6	X3Y1	X4X4	X4X4+Ah
X2Y6	X3Y6		
X4Y1	X4Y5		
X4Y2	X4Y6+Ah		
X4Y4	X1Y4		
X4Y3	X3Y2		

Image 6. Karyotypes biologically compatible which were found in the studied natural populations and tested in laboratory stocks.

4. Conclusions

The normal karyotype is the most frequent karyotype known as the reference karyotype: *f Arg 1* in *A. fraterculus* and *Arg 17* in *C. capitata.*

Population cytology studies using large numbers of specimens allowed us to detect all possible combinations across generations.

The fruit fly is oviparous, its life cycle lasts around 45 days, it oviposits large numbers of eggs, has complete metamorphosis of egg and larva lives inside the fruit, pupae in the ground, and adults in the leaves of trees. Chromosomal rearrangements maintain within populations, some of them as polymorphisms similar to those of the chromosomal variants described for

Anastrepha and for *Ceratitis.* The study of the families carrying different variants proved their transmission from parents to offsprings through successive generations.

The techniques applied in our studies revealed many changes in heterochromatin and assisted in recognizing variants: N-bands are the best marker for autosome III; H-banding revealed autosomal mutations and sexual chromosomal variants such as the Y6 and double-minute chromosomes; Chromomicin A3 assisted in revealing the somatic crossing over, and FISH in recognizing rearrangements of the ribosomal cluster.

The long stretches of DNA in heterochromatin contain important sequences in health and disease that, for the most part, need to be silenced for cells to work properly.

In humans, one banding technique is applied to diagnose illnesses. It would be useful to apply different banding techniques in order to recognize new chromosomal rearrangements associated with physiological disorders. The use of several techniques on the same material should help to determine if the same mutation produces different phenotypes or behaviours when comparing different geographical populations.

Think about balanced polymorphisms such as the malaria—anemia in Eurasia, where different genotypes persist through heterozygote superiority. Could a genetic mutation that puts populations at risk for illnesses in one environmental setting expresses itself in positive ways in a different setting?

Chromosomal mutations produce variability. Variation is the key to survival and many individuals could be in the right place but in the wrong moment.

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