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RESEARCH ARTICLE

Evaluation of *Drosophila* chromosomal segments proposed by means of simulations of possessing hybrid sterility genes from reproductive isolation

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Abstract. In heterozygote state, we interogressed three chromosomal segments of *Drosophila koepferae* in *D. buzzatii*. The effect of each introgression was evaluated in the fertility of the segmental males, quantifying the amount of offspring produced. Through specific crosses method, we generated *Drosophila* segmental isolines carrying specific chromosomal introgression segments. The introgressions were monitored cytogenetically by the method of molecular markers of chromosomal asynapsis. The statistical analysis showed that none of the three segments evaluated, introgressed individually or in pairs, as well as *cis* or *trans*, do not produce sterility in the segmental males, as determined by the normal productions of offspring. Additional introgressions using other larger segments show that when the introgressions reach a minimum size of 31.15%, they produce sterility. It is concluded that the hybrid sterility genes present in the three segments evaluated did not act in strong epistasis, but show a pattern of gradual additive behaviour by requiring a minimum threshold size to produce sterility. Finally, we also isolated the smallest introgressing segment that has been reported for these species (2.19%), and for the first time we have managed to place it in homozygous state (data not shown), so we are now in the process of evaluating the ability to these segments in homozygous state.

Keywords. reproductive isolation; hybrid sterility; hybrid inviability; hybridization; *Drosophila*; chromosome introgression.

Introduction

The hybrid sterility represents a postzygotic reproductive isolation mechanism, generated by the interaction between genes of hybrid incompatibility, due to the impossibility of mating between individuals of different populations, consequently leads to the absence of genetic exchange and reproductive isolation. This is observed in many pairs of sibling species in insipient states of speciation. All this is a prevailing mechanism in the evolutionary process that gives rise to the emergence of new species (Mallet 1995; Gavrilets 2003; Wu and Ting 2004; Dzur-Gejdosová *et al.* 2012; Xie *et al.* 2017).

Sibling *Drosophila* species have been widely used to identify hybrid incompatibility genes and the manner by which they control reproductive isolation. *Drosophila* species perform an important role, as their complete genome sequence is known for 11 species of this genus, and many pairs of fruit fly sibling species have been used intensively to study the architecture of hybrid incompatibility or potzigotic copulation (Coyne and Orr 1998; Civetta and Gaudreau 2015; Gomez and Civetta 2015; Brill *et al.* 2016; Manzano-Winkler *et al.* 2017). Based on the genetic hybrid sterility producing elements, three types of genetic architecture have been postulated (Templeton 1981): type I, many segregating factors, each one with small effects. Type II, one or a few factors with greater epistatic effects. Type III, duplicated or complementary loci. To discern among genetic architecture types, the availability of a large number of markers is indispensable (Vos *et al.* 1995; Lynch and Walsh 2001; Mueller and LaReesa 1999; Laayouni *et al.* 2000; Wu and Ting 2004).

Since the pioneering suggestion of Dobzhansky, that at least two complementary loci with epistatic effect are needed for hybrid sterility to evolve, much effort has been dedicated to explaining the genetic architecture of reproductive isolation those and then the Dobzhansky–Muller two loci model of epistatic genetic incompatibility is considered best suited for this purpose (Dobzhansky 1936). Through various approaches and many others researchers have tried to identify individual genes (Perez and Wu 1995; Coyne and Orr 1998; Gavrilets 2003; Michalak and Noor 2004; Cooper and Phadnis 2016).

In several studies of hybrid sterility between *D. simulans* and *D. mauritiana*, it was estimated that in 10.5% of the echromatic genome studied, the number of genes involved in hybrid sterility was 15 genes, i.e., more than one gene (1.5) in 1% of the genome. Because the X chromosome represents 20% of the genome in *Drosophila*, then ∼40 genes of the chromosome X + 80 of the autosomes, indicates that ∼120 genes are involved (Wu and Palopoli 1994; Coyne and Kreitman 1986).

Now, with the methods of saturating, the chromosomal map with genetic markers, the estimate of 120 genes turns out to be very low, since extending the studies throughout the *Drosophila* genome, including the Y chromosome, its distribution is approximately proportional to the relative length of each chromosome, and its total number is estimated to be around 500 (Lindsley and Tokuyasu 1980).

The fitness reduction can range from ecological maladaptation or behavioural aberration to inviability or sterility. The loci that underlie such reductions in fitness might be considered 'speciation genes' so the identities of speciation genes, and their normal functions, must be known, as are the case of los genes Xmrk-2, OdsH, Hmr, Nup96, desat-2 (Wu and Ting 2004).

However, many results show that an abnormal chromosome organization, with a strict dependence on the size of the chromosome segments involved is the responsible of hybrid sterility. Genes responsible for intraspecific gene sterility show an easily recognizable, clear-cut segregation, and are generally recessive (Wu and Ting 2004). The way these genes perform is still a subject of much controversy, but experiments on genomic fragment introgression from one species into another suggest two possible types of action: (i) additive with a threshold effect (Naveira and Fontdevila 1986); and (ii) epistatic (Palopoli and Wu 1994). Although, both architectures are polygenic, they do not exclude the presence of genes with greater effects and can be cloned (Ting *et al*. 1998). Further, several studies using chromosomal introgression between sibling species of *Drosophila* concluded that a large number of genes act epistatically, affecting the fertility of hybrid males (Dobzhansky 1941; Naveira and Fontdevila 1986; Wu and Palopoli 1994; Sawamura *et al.* 2000; Sawamura *et al*. 2004). However, in a theoretical work it has been suggested that a small number of sterility factors, three to six per autosome, acting in pairs to produce sterility between *D. buzzatti* and *D. koepferae* (Marín 1996).

D. koepferae (*Dk*) and *D. buzzatii* (*Db*) are sibling species that coexist in various arid and semi-arid zones in Bolivia and the northwest of Argentina (Fontdevila *et al*. 1982, 1988; Naveira *et al*. 1984, 1989). They are closely related species belonging to the *buzzatii* complex (*repleta* group). This group has several species with various degrees of evolutionary divergence (Rodriguez-Trelles *et al*. 2000; Celeste *et al.* 2000) which generates a broad spectrum of reproductive interactions among them. Apart from its value as a colonizing species, *D. buzzatii* occupies an ecological niche restricted to cactus (*Cactaceae*), thus permitting many studies on population structure and coexistence with other related species. Among those species, *D. koepferae* females can be crossed with *D. buzzatii* males under laboratory conditions producing sterile hybrid males and fertile hybrid females, although reciprocal crosses never produce offspring (Naveira and Fontdevila 1986).

This hybridization facilitates performing advanced studies on ecology and genetics of speciation, generating testable hypotheses on species evolution. Many studies on the hybrid incompatibility between sibling species of *Drosophila* using chromosome introgression have focussed on the 'type 2 architecture' explanation; some have proposed the action of a single sterility gene (Ting *et al*. 1998; Barbash *et al*. 2000; Presgraves *et al*. 2003; Orr *et al*. 2004; Barbash *et al*. 2004).

Research based on the sterility between hybrid males of *D. buzzatii* and *D. koepferae* (Naveira and Fontdevila 1986, 1991a, 1991b) has contributed to the study of 'type 1 architecture'. These studies, in which the polytene chromosome asynapsis was used as a marker for hybrid regions (Naveira *et al*. 1986) concluded that hybrid male sterility could be affected by multiple factors of cumulative action with a threshold effect (threshold additive model). Further, they revealed that these factors are located throughout the chromosomes. Chromosome X introgressions produce

sterility regardless of their size and therefore the number of factors seems to be much greater in this chromosome.

Another theoretical study (Marín 1996) supports the epistatic model, interpreting the data of the previous work (Naveira and Fontdevila 1986, 1991a, 1991b) to infer that a maximum three to six factors per autosome is responsible for sterility. Moreover, the study predicts that four specific *D. koepferae* chromosome segments contain these sterility factors and when at least two are introgressed together—heterozygotically—into the *D. buzzatti* genetic background, sterility is induced in carrying males.

By means of careful introgressions of *D. buzzatii* segments, and testing the fertility in male carriers, our research evaluated three *D. koepferae* segments in chromosome number 4 that had been identified previously as carriers of strong sterility factors (Marín 1996). The main purpose of our research has thus been to increase our knowledge of the genetic architecture of reproductive isolation in two sibling species, *D. koepferae* and *D. buzzatii*.

Materials and methods

Standard cytological map of chromosome number 4 for species of the D. repleta group, and segments of interest

The cytological maps for both *D. koepferae* and *D. buzzati* have the same polytene karyotype as species of the *D. repleta* group (Wharton 1942), consisting of six chromosomes. Each chromosome is divided into cytological intervals, identified by capital letters (*A* to *H*). Each interval contains a specific amount of subintervals identified by numbers (1 to 5). Each subinterval is divided by a series of bands, identified by lowercase letters (*a* to *h*), in alphabetical order from telomere to centromere (Schaeffer *et al.* 2008) see figure 1. The symbols of letters and numbers describe the localization and length of each chromosomal segment according to the cytological map (figure 1).

Method for molecular genetic markers (asynapsis)

For each respective genotype identified, the offspring were analysed from a sample of six to 10 third instar larvae, identifying introgressed chromosomal segments by the presence of genetic markers known as chromosomal asynapsis (Naveira *et al*. 1986). Each asynapsis is formed by an incomplete pairing of chromatin fibres in the junction zone between a pair of homologous chromosomes, exclusively at the sites where introgression has been successful (figure 2).

The length and location of the introgressant segment in the cytological map indicates each specific asynapsis. Each asynapsis segregates according to Mendel's laws, representing the genotypes of male segmental hybrids and their offspring, further allowing inference on the parent's genotype. The absence of chromosomal asynapsis in all cells observed was taken to mean that the studied segment was homozygous and therefore had no chromosomal introgression in the specific larvae analysed. The procedure to identify chromosomal asynapsis of polytene chromosomes is performed by the standard squashing method: the salivary glands from a third instar *Drosophila* larva are extracted in 45% acetic acid and placed on a slide with a drop of standard dye solution lacto–aceto–orcein (Henderson 2004). This preparation is sandwiched between a cover glass, placed between a paper towel and crushed with the thumb tip to release the gland polytene chromosomes and allow them to extend over the surface of the cover glass. Finally, the preparation is observed under the microscope and its image captured with an adapted digital camera.

General conditions for crosses

All *Db* specimens were seven days old, whereas segmental specimens were between six to 10 days old. For all crosses, parental flies were transferred every five days to a new flask with fresh feeding medium and maintained at 25°C on a 12:12 light: dark regime. During specimen manipulation,

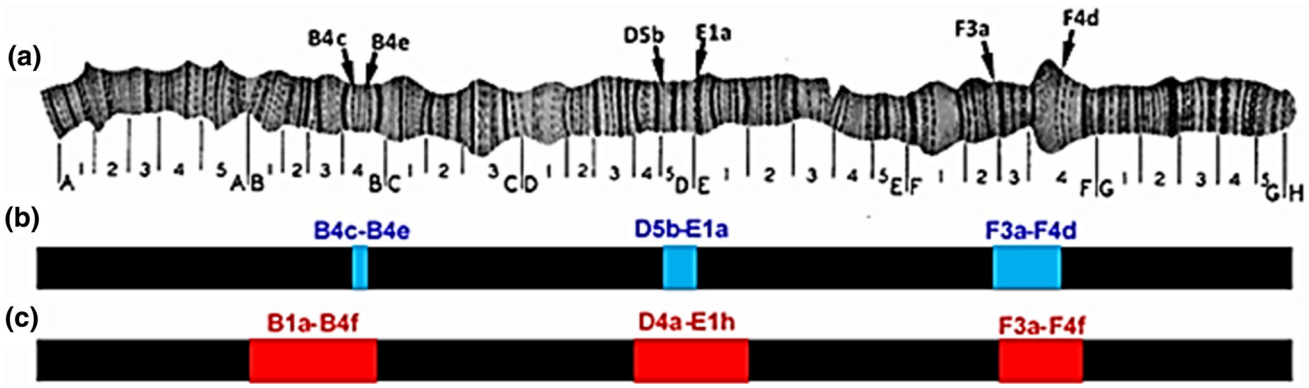

Figure 1. Simplified image of chromosome 4. (a) Cytological map of chromosome 4 of *D. Repleta* group species. (b) Small blue bars represent the location of introgressed chromosomal segments previously proposed to carry strong sterility factors (Marín 1996). (c) The small red bars define the location of experimental chromosomal segments (introgressions) obtained and evaluated in our research.

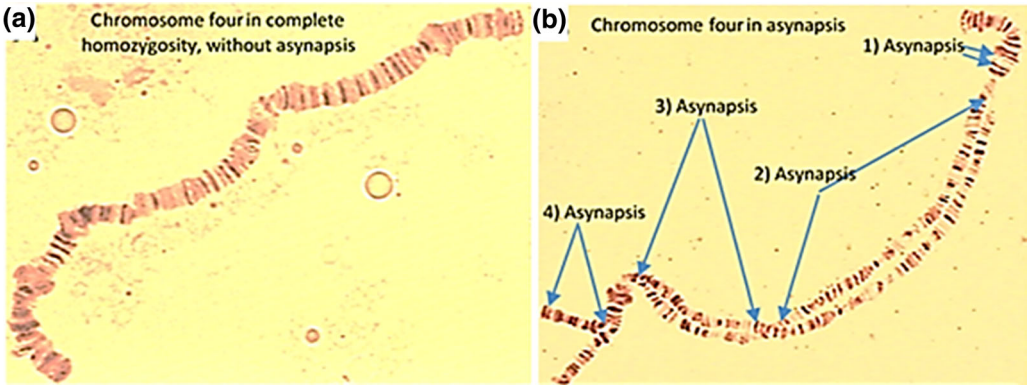

Figure 2. Chromosome squash from salivary glands of *D. buzzatii* larvae. Image taken with optical microscope to 40×. (a) Pair of homologous chromosomes four are complete aligned without formation of asynaptic regions. (b) Pair of chromosomes four show several regions in asynapsis action.

temperature never exceeded 25°C. We performed cytogenetic analysis of at least nine larvae from the offspring of each cross to look for introgressions and infer the parental genotype. For all individual crosses and backcrosses made to obtain the *Drosophila* segmental lines, as well as to test introgressant male fertility, we used randomly selected males from each of the chosen single or double-segmental lines with *Db* females. We also performed every corresponding reciprocal cross between *Db* males and introgressant females. Further, we performed control crosses, using males from single-segmental control lines, double-segmental and triple introgressant strains. For internal cross control, we used males with wild-type genotype (*bu/bu*) originating from each one of the corresponding single segmental line selected and the double-segmental strains, crossed with wild-type females (*bu/bu*).

Original fly strains

The *D. buzzatii* Bu-28 strain—designated *Db* with genotype *bu/bu*—originated in a sample collected from a natural population at Los Negros, Bolivia in 1982; the strain *D. koepferae* KO-2, designated *Dk* with genotype *ko/ko*, was collected in December 1979 at the Sierra de San Luis, Argentina. The F_1 progeny from each strain was placed in a population cage, later maintained in mass cultures at 25°C at the Universidad Autónoma de Barcelona *Drosophila* fly strain collection.

Obtaining single-segmental lines

A strict mating strategy was enforced to obtain single segmental lines with as described below, consisting of the massal type crosses (50 males with 50 females) and individual crosses (one male with one female) see figure 3.

Whereas each new offspring has a specific chromosomal arrangement, due to the chromosomal recombination during meiosis process, exclusively in drosophila females but not in males, which through the random exchange of different segments between homologous chromosomes, result in new and diverse combinations of chromosomal segments in each offspring. Thus, each new offspring was analysed cytogenetically, using at least six larvae from each culture, thus allowing identifying the presence of asynapsis, each of which represents a segment of an introgressed chromosome. Sequentially, we selected all the types of offspring carrying introgressions of interest located on chromosome number 4 as outlined in figure 3.

Obtaining double and triple segmental lines

To produce the segmental lines with double and triple introgressions, both in the *trans* and *cis* positions, it was necessary to carry out a strict crossing scheme (shown in figure 4), initially using flies from the simple segmental lines (B1a-B4f/bu, D4a-E1h/bu and F3a-4f/bu) see table 1.

To identify the genotype label, the new segmental lines according to the presence of their double or triple introgressions (as shown in table 1), at least six larvae of each offspring were analysed, using the polytene chromosome squash method (figure 2).

Test and additional crosses

To establish overall success of our experimental crosses—used to evaluate the fertility of simple, double or triple introgression of segmental males—we performed at least 15 individual test crosses (one male with one female) for each segmental line, using *Db* females and their corresponding males, randomly selected from each segmental strain. In the same way, we made all respective crosses by additional lines

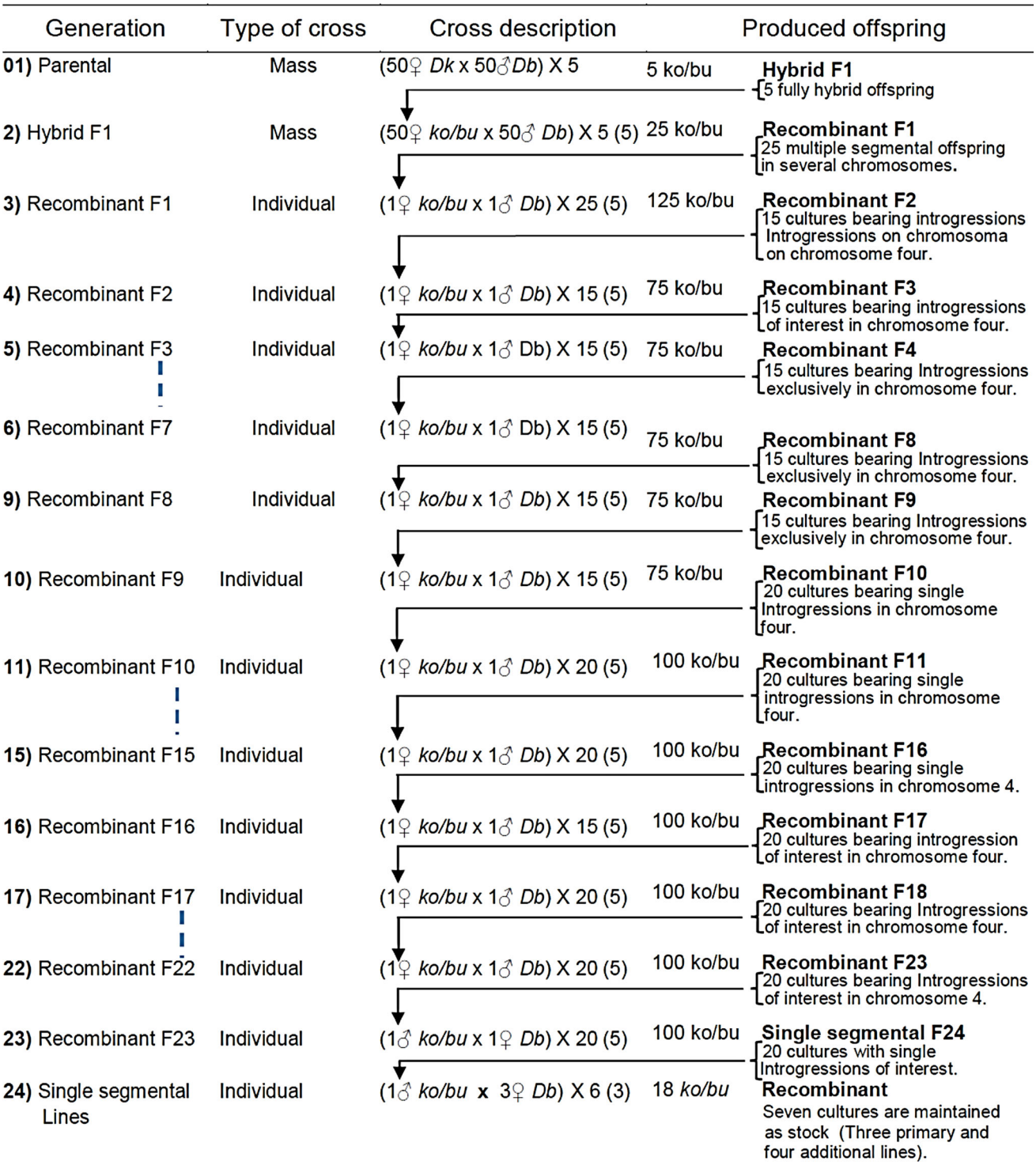

Figure 3. Crossbreeding protocol to obtain single-segmental lines. Genotype: *D. koepferae* (ko/ko); *D. buzzatii* (bu/bu). Parental strains: *D. koepferae* (Dk); *D. buzzatii* (Db).

(tables 2 and 3). Similarly, we performed all corresponding reciprocal crosses between females extracted from each segmental line with *Db* males (tables 2 and 3). To identify all the expected parental genotype in the offspring and deduce from it the parent's genotype, at least six third instar larvae from each offspring were cytogenetically analysed.

Male fertility tests

***Amount of offspring produced*:** The number of adults produced from each cross was counted during 20 days from the first day of larvy emergence. In crosses that failed to produce offspring, we added two new virgin females to confirm male sterility.

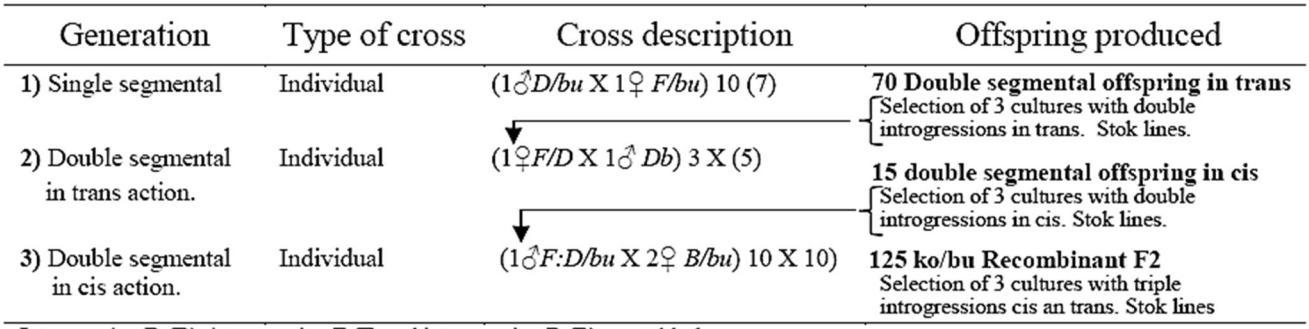

Figure 4. Protocol of crosses to obtain *trans*-acting and *cis*-acting double and triple segmental lines.

Table 1. Segmental lines obtained according to the genotype of their introgressions.

	Segmental lines (genotype)	Name of segments	Abbreviated genotype	% Size with respect to chromosome 4
Experimental segmental lines				
Single segmental lines				
1	*B1a-B4f/bu*	*B*	*F/bu*	10.59
2	*D4a-E1h/bu*	*D*	*D/bu*	9.06
3	*F3a-F4f/bu*	*F*	*B/bu*	7.87
Double-segmental *trans* lines				
4	*D4a-E1h/F3a-F4f*	*D* + *F*	*D/F*	16.93
5	*B1a-B4f/F3a-F4f*	*B* + *F*	*B/F*	18.46
6	*B1a-B4f/D4a-E1h*	*B* + *D*	*B/D*	19.64
Double-segmental *cis* lines				
7	*D4a-E1h:F3a-F4f/bu*	*D* + *F*	*D:F/bu*	16.93
8	*B1a-B4e:F3a-F4f/bu*	*B* + *F*	*B:F/bu*	18.46
9	*B1a-B4e:D4a-E1h/bu*	*B* + *D*	*B:D/bu*	19.64
Additional segmental lines				
Single segmental lines				
1	*D4a-E4h/bu*	*D4a*	*D4a/bu*	18.81
2	*D4a-E5d/bu*	*D4b*	*D4b/bu*	20.56
3	*F3a-G5d*	*F3*	*F3/bu*	22.97
4	*D3a-F3d/bu*	*D3*	*D3/bu*	34.78
Double-segmental *trans* lines				
5	*D4a-E5d/F3a-F4f*	*D4a* + *F*	*D4a/F*	28.43
6	*B1a-B4f/D4a-E5d*	*B* + *D4b*	*B/D4b*	31.15
7	*B1a-B4f/F3a-G5d*	*B* + *F3*	*B/F3*	33.55
Double-segmental *cis* lines				
8	*D4a-E5d:F3a-F4f/bu*	*D4a* + *F*	*D4a:F/bu*	28.43
Triple-segmental *cis* lines				
9	*D4a-E1h:F3a-F4f/B1aB4f*	*D* + *F* + *B*	*D:F/B*	27.52

Male progenitors are considered fertile when their corresponding crosses produce a number of offspring similar to that of control crosses. They are considered semi-sterile when their offspring represent only a low percentage of the control's progeny. Finally, they male were considered sterile when no offspring are produced.

Statistical analysis

A factorial ANOVA-univariate analysis was performed to estimate differences of the number of descendants between offspring by genotype, and a Friedman ANOVA to estimate total offspring by sex. The analyses were performed using the 'STATISTICA' statistical package, v. 6. StatSoft 2003.

Table 2. Test crosses, amount of offspring and their respective statistical ANOVA.

Number of crosses	Crosses	Number of offspring per genotype				Total	*P* one-way ANOVA between genotypes	*P* factorial ANOVA between test and reciprocal
D/F trans		*bu/bu*	*F/bu*	*D/bu*	*D/F*			0.8053
17 Test	♂*D/F* × ♀*Db*	1108	530	864	484	2986	0.7423	
19 Reciprocal	♀D/*F* × ♂*Db*	1231	819	642	538	3230	0.2989	
D:F cis		*bu/bu*	*F/bu*	*D/bu*	*D:F*			0.1750
22 Test	♂*D:F/bu* × ♀*Db*	993	983	1309	514	3799	0.6705	
23 Reciprocal	♀*D:F/bu* × ♂*Db*	878	1236	1144	644	3902	0.2498	
B/F trans		*bu/bu*	*B/bu*	*F/bu*	*B/F*	Total		|0.9515
29 Test	♂*B/F* × ♀*Db*	1392	227	526	791	2936	0.2498	
16 Reciprocal	♀*B/F* × ♂*Db*	460	209	398	700	1767	0.7327	
B:F cis		*bu/bu*	*B/bu*	*F/bu*	*B:F*			0.5563
34 Test	♂ *B:F/bu* × ♀*Db*	795	833	799	758	3185	0.2158	
31 Reciprocal	♀ *B:F/bu* × ♂*Db*	1033	1124	764	840	3761	0.9308	
B/D trans		*bu/bu*	*B/bu*	*D/bu*	*B/D*			0.5505
18 Test	♀ *B/D* × ♂ *Db*	958	732	486	613	2789	0.7511	
17 Reciprocal	♂ *B/D* × ♀*Db*	589	491	973	952	3005	0.4205	
B:D cis		*bu/bu*	*B/bu*	*D/bu*	*B:D*			0.3245
23 Test	♀ *B:D/bu* × ♂*Db*	411	1242	1248	441	3342	0.4287	
24 Reciprocal	♂*B:D/bu* × ♀*Db*	899	901	1515	573	3888	0.5182	

Table 3. Additional crosses, number of offspring by type of parent.

Fly crosses	Num. crosses	Individual crosses	Minimum number of offspring produced by the parents of each genotype					
D4a/F-trans			*bu/bu*	*F/bu*	*D4a/bu*	*D4a/F 4a/F*	–	–
Experimental	29	♂*D4a/F* × ♀Db	> 80 (6)	> 80 (8)	> 80 (7)	< 15 (8)	–	–
Reciprocal	20	♀*D4a/F* × ♂Db	> 80 (7)	> 80 (4) (19)	> 80 (4)	> 80 (5)	–	–
D4a:F-cis			*bu/bu*	*F/bu*	*D4a/bu*	*D4a:F/bu*	–	–
Experimental	*18*	♀*D4a:F* × ♂Db	> 80 (4)	> 80 (4)	> 80 (5)	< 13 (5)	–	–
Reciprocal	21	♂*D4a:F* × ♀Db	> 80 (7)	> 80 (4)	> 80 (6)	> 80 (4)	–	–
B/D4b-*trans*			*bu/bu*	*B/bu*	*D4b/bu*	*D4b/B*	–	–
Experimental	28	♂B/D4b × ♀Db	> 80 (5)	> 80 (5)	< 22 (8)	0 (10)	–	–
Reciprocal	18	♀B/D4b × ♂Db	> 80 (8)	> 80 (4)	> 80 (4)	> 80 (2)	–	–
B/F3-trans			*bu/bu*	*B/bu*	*F3/bu*	*B/F3*	–	–
Experimental	27	♂*B/F3* × ♀Db	> 80 (11)	> 80 (4)	< 20 (8)	0 (4)	–	–
Reciprocal	21	♀*B/F3* × ♂Db	> 80 (7)	> 80 (3)	> 80 (6)	> 80 (5)	–	–
D3/bu			*bu/bu*	*D3/bu*	–	–	–	–
Experimental	*38*	♂*D3/bu* × ♀Db	> 80 (19)	0 (19)	–	–	–	–
Reciprocal	15	♀*D3/bu* × ♂ Db	> 80 (7)	> 80 (8)	–	–	–	–
D:*F/B*			*bu/bu*	*B/bu*	*F/bu*	*D/bu*	*D:F/bu*	*D:F/B*
Experimental	39	♀*B:D/F* × ♂Db	> 80 (9)	> 80 (7)	> 80 (3)	> 80 (5)	> 80 (6)	< 12 (9)
Reciprocal	28	♂*B:D/F* × ♀Db	> 80 (4)	> 80 (9)	> 80 (3)	> 80 (4)	> 80 (5)	> 80 (3)

Results

Segmental lines obtained

From two original strains of respective sister species of drosophila, we obtained nine particular segmental hybrids lines, constitute by three single segmental lines, three double segmental lines in *trans* and three double segmental lines in *cis* (table 1). In addition, we obtained nine additional segmental lines, constitute by four single additional segmental lines, three additional double-segmental lines in *trans*, one additional double-segmental line in *cis* and one triple segmental line (table 1).

Test crosses

We performed individual test crosses (one male with one female), using males obtained from each double segmental line, both *trans* and *cis* action (*D/F*, *D:F*, *B/F B:F*, *B/D* and *B:D*) with *Db* females. Parallel to each cross, they made their respective reciprocal cross between corresponding females

from the respective segmental line with *Db* males (table 2). We performed statistical analyses, with an ANOVA, for total offspring of test crosses lines.

Additional crosses

We performed individual supplementary crosses (one male with one female), using males obtained from each additional segmental line, both *cis* and *trans* action (*D4a/F*, *D4a:F*, *B/D4b*, *B/D4b*, *B/F3*, *B/D4b*, *B/F3*, *D4a*, *D:F/B* with *Db* females. Parallel to each cross, they made their respective reciprocal cross between corresponding females from the respective segmental line with Db males (table 3).

Semiesterile males

The evaluation of introgressed segments is indirect. The introgressed segments are located in the carrier males, while the females used to cross with these segmental males come from a wild-type genotype strain, female were randomly selected.

With five types of segmental male, in 38 crosses (D4a-*E5d/bu*, *F3a-G5d/bu*, *D4a-E1h:F3a*, *D4a-E5d/F3a-F4f*, *D4a-E5d:F3a-F4f/bu*; with percentage records of specific segments 0.56, 22.97, 27.52, 28.43, 28.43 respectively) classified as semi-sterile (table 3). Those males always produced less than 20 individuals. While with the control males of wild genotypes, crossed with the females of the same wild-type strain they always produced at least 80 and up to hundreds of individuals, so the cut was too evident in less than 20 and more than 80 emerged adults.

Discussion

This study shows the effect in male sterility from three specific chromosomal segments previously proposed in a theoretical way of carry strong sterility factors, Marín (1996), suggest which operate with a small number of sterility factors, three to six per autosome, acting in pairs to produce sterility in hybrid segmental males.

There is a clear threshold pattern with very narrow size limits between each level of fertility, show difference between maximum size for fertility (19.64%) and minimum size for semisterility (20.56%) is 0.92% (table 4 and figure 5). While the difference between the maximum sizes for semi-sterility (28.43%) and minimum size to sterility (31.15%) is 2.72%. Allowing a margin of 7.87% between the smallest segment (20.56%) that produces semi-sterility and the largest segment (28.43%). This leads us to the

Table 4. Level of fertility for each type of segmental male.

Number of parental males	Segmental line	Genotype	Size of segment length (%)	Offspring average	Fertility level
Test crosses					
35	Bu	Bu-(IC)	–	139.45	Fertile
20	F/bu	F3a-F4f/bu	7.87	126.00	Fertile
23	D/bu	D4a-E1h/bu	9.06	166.75	Fertile
27	B/bu	B1a-B4f/bu	10.59	144.40	Fertile
3	D/F	D4a-E1h/F3a-F4f	16.93	161.30	Fertile
7	B/F	B1a-B4f/F3a-F4f	18.46	113.00	Fertile
4	B/D	B1a-B4f/D4a-E1h	19.64	153.25	Fertile
3	D:F/bu	D4a-E1h:F3a-F4f/bu	16.93	171.30	Fertile
9	B: F/bu	B1a-B4f:F3a-F4f/bu	18.46	84.22	Fertile
3	B:D/bu	B1a-B4f:D4a-E1h/bu	19.64	147.00	Fertile
Additional crosses					
30	Bu	bu/bu-(IC)	0	> 80	Fertile
15	F/bu	F3a-F4f/bu-(IC)	7.87	> 80	Fertile
5	D/bu	D4a-E1h/bu-(IC)	9.06	> 80	Fertile
16	B/bu	B1a-B4f/bu-(IC)	10.56	> 80	Fertile
6	D:F/bu	D4a-E1h:F3a-F4f/bu-(IC)	16.93	> 80	Fertile
12	D4a/bu	D4a-E4h/bu	18.81	> 80	Fertile
8	D4b/bu	D4a-E5d/bu	20.56	< 22	Semi-sterile
8	F3/bu	F3a-G5d/bu	22.97	< 20	Semi-sterile
9	D:F/B	D4a-E1h:F3a-F4f/ B1a-B4f F4f/B1aB4f	27.52	< 12	Semi-sterile
8	D4a/F	D4a-E5d/F3a-F4f	28.43	< 15	Semi-sterile
5	D4a:F	D4a-E5d:F3a-F4f/bu	28.43	< 13	Semi-sterile
10	B/D4b	B1a-B4f/D4a-E5d	31.15	0	Sterile
4	B/F3	B1a-B4f/F3a-G5d	33.55	0	Sterile
19	D3/bu	D3a-F3d/bu	34.78	0	Sterile

IC, internal control.

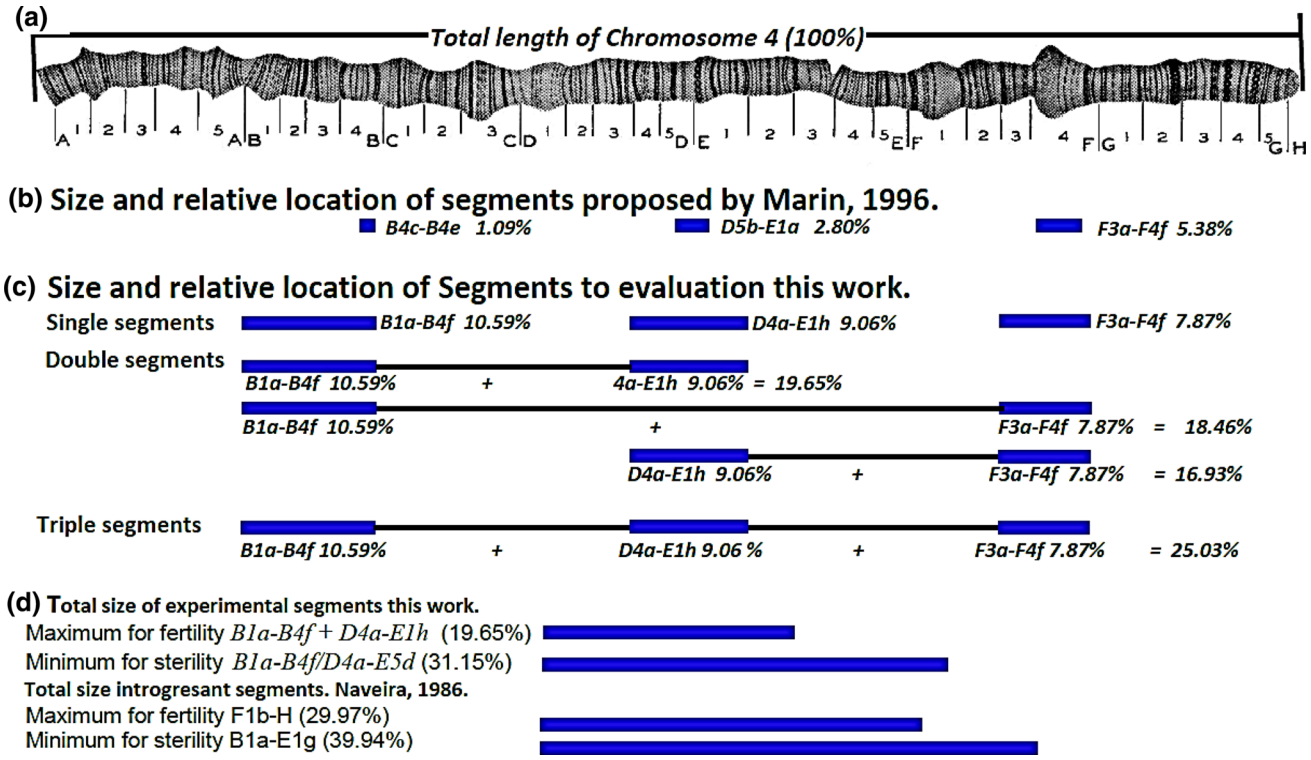

Figure 5. (a) Total length of chromosome 4. (b) Location and length of segments proposed to possess hybrid sterility factors by Marín (1996). (c) Length and location of the segments evaluated in this work. (d) Maximum length of intriguing segments, capable of producing fertility and minimum size of segments with capacity to produce sterility, for the segments evaluated in this work and for the segments previously identified by Naveira *et al*. (1986).

conclusion that, apart from obtaining smaller sterility segments than those previously reported, and, with more than 80 larvae (table 3). Therefore, we consider that the parent males of these crosses are sterile in the first class, semi-sterile in the second class and fertile in the third class. In all reciprocal crosses, segmental progenitor female falls in the third class; having more than 80 larvae (table 3).

All expected genotypes were identified in these offspring, except for the sterile male parents (table 3).

All corresponding reciprocal crosses produced—individually—many descendants and all expected genotypes were identified, including *D3/bu*, *D4b/B* and *B/F3*, corresponding to the genotypes of sterile segmental males (table 3), implying that all segmental female are fertile. The length and location of the introgressant segments (with respect to the total size of chromosome 4), proposed by Marín 1996, to have the capacity to produce sterility in segmental males (figure 5b) were evaluated as they were included in the intriguing segments evaluated in this work (figure 5c). While the introgressant segment of greater length, with the capacity to produce fertility was 16.95%, being smaller than the segment published in previous works (29.97%) by Naveira *et al.* (1986), figure 5d (table 4; figure 5). Also, the smaller segment with the capacity to produce sterility, evaluated of this work, is also smaller than the previously reported Naveira *et al*. 1986.

Also the probability that an introgressed segment confers sterility depends on both the number of factors and the magnitude of their effects, have suggested a larger density of chromosome factors (Coyne and Orr 1998; Naveira *et al.* 1989; Wu and Palopoli 1994), involved comparison of hemizygous with heterozygous autosomal segments.

All previous studies only compared heterozygous autosomal segments, finally, we also, isolate the smallest introgressing segment that has been reported for these species (2.19%), and for the first time we have managed to place it in homozygous state (data not shown), so we are now in the process of evaluating the ability to these segments in homozygous state.

In conclusion, the segments evaluated (*B1a-B4f*, *D4a-E1h* and *F3a-F4d*), do not contain strong sterility factor segments under any scenario: singly or jointly introgressing, in *trans*-acting or *cis*-action.

For all the above, we conclude that none of the three chromosomal segments proposed by Marín 1996 have strong sterility factors of hybrid epistasis, confirming the threshold size model. Where the chromosomal segments are carriers of weak sterility factors that act summation way until reaching a threshold to produce hybrid sterility in the segmental males, supporting the additive model with a threshold effect (Naveira *et al*. 1986). And because the chromosomal segments identified here are on the order of 10% smaller, the

chromosomal segments of introgression may contain less than six factors as previously proposed.

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