Method to obtain homozygous introgressing segments in *Drosophila* to identify the presence of hybrid sterility genes of the reproductive isolation

Francisco García-Franco (✉ f franco68@hotmail.com)

Universidad Autónoma Metropolitana <https://orcid.org/0000-0002-3424-5271>

Lilian Barandica-Cañón

Universidad de Cartagena

Ezel Galindo-Pérez

Universidad Autónoma Metropolitana

Martha Martínez García

Universidad Nacional Autónoma de México

Blanca Chávez-Sandoval

Universidad Autónoma Metropolitana

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Method to obtain homozygous introgressing segments in *Drosophila* to identify the presence of hybrid sterility genes of the reproductive isolation

Corresponding authors: *ffranco68@hotmail.com and *blanchavez29@gmail.com

ABSTRACT

Here, we present for the first time, a method to generate homozygous segmental introgressions, by means of crosses between a pair of symorphic species. The introgressions were monitored by the cytogenetic method of polygenic chromosome asynapses. Later the introgressions were evaluated in their capacity to produce sterility in segmental males. Also, the smallest segment with the capacity to produce sterility in segmental males was mapped by in situ hybridization of polytene chromosomes, using 8 sequences of BACs clones as probes. Finally, a bioinformatic analysis was carried out to identify the presence of particular genes. From 2 parental strains, *D. buzzatii* and *D. koepferae*, 6 simple segmental hybrid lines were generated, whose introgressing segments are distributed along chromosome 4 of these species. From the 6 simple segmental lines and by means of a new crossing strategy, the 6 respective homozygous segmental hybrid offspring were obtained, each of them carrying a specific homozygous introgression. None of the 6 heterozygous introgressions was capable of producing sterility in segmental males, while 4 of the same homozygous introgressions produced total sterility in segmental males, including in this group the two smallest introgressive segments, one of 5.03 % and the other 7.87% with respect to the total length of chromosome 4, which are located in the region F2 to F4 of the standard cytological map based on polytene chromosomes of the *Drosophila* Repleta group. In situ hybridization, using 8 clones from contig 1065 located along the F2 to F4 region of the physical map of *D. buzzatii* constructed in BACs, confirmed the precise location of the 6 clones in the chromosomal region F2 to F4 of chromosome 4 of the polygenic chromosomes of both *D. buzzatii* and *D. mojavensis*. The bioinformatic analysis of the F2 to F4 region, using the complete genetic sequence of the contig 1065 of *D. buzzatii* shows the presence of two predicted genes in the genomic map of *D. buzzatii* (g.1313.t1 and g.1314.t1), and the orthologous association of these 2 genes both with the *D. moj_GI22766* gene of *D. mojavensis* and with the *Trivet* gene of *D. melanogaster*.

1 INTRODUCTION

One long-standing issue of debate in speciation theory concerns the number of genes that are necessary for speciation to occur.

In *Drosophila*, it is suggested that the density of sterility factors may be of the order of one per 1 percent of the genome and, by extrapolation, a minimum of 40 of these loci has been estimated on the X chromosome, which gives an estimate of the sterility genes between *D. simulans* and *D. mauritiana* of the order of 100 (Wu, 1994) (Palopoli, 1994). Under the classic Bateson–Dobzhansky–Muller (BDM) model, speciation requires changes in at least two genes (Nei and Zhang, 1998) (Orr, 2005) (Rieseberg and Blackman, 2010) (Nosil and Schluter, 2011) (Blackman, genes 2016) (Cooper, 2016) but there are several examples of empirical evidence for the determination of mating traits by a single locus, Allen Orr (1991), in his model demonstrates the possibility of single-gene speciation is possible if the character causing reproductive isolation is maternally inherited. While Gregorius (1992), does it with a model where he involves three alleles and the speciation by the reinforcement. For their part, Yamamichi and Sasaki (2013), propose that the pleiotropy of a single gene on delayed inheritance and dominance is capable of allowing speciation. In the same way, as Phadnis and Orr (2009), describe the effect of a single gene, both on hybrid sterility and on the distortion of segregation.

Genes responsible for intraspecific gene sterility (Rieseberg and Blackman, 2010) show an easily recognizable, clear-cut segregation, and are generally recessive. Experiments on genomic fragment introgression from one species into another suggest two possible

types of action: 1) additive with a threshold effect (Naveira, et al., 1984) (Naveira & Fontdevila, 1998) and 2) epistatic effects between conspecific genes in a heterospecific background (Palopoli, 1994); (Presgraves, 2007). Both architectures are polygenic, they do not exclude the presence of genes with greater effects with strong or additive effects, and that when identified can be cloned (Ting, *et al.*, 1998), supporting the view that alleles of large effect that distinguish species may sometimes reflect the accumulation of multiple mutations of small effect at select genes (McGregor, *et al.*, 2007). And Feller *et al.*, 2020, found that several of the characters selected for reproductive isolation are assigned to a region of significant QTLs, supporting the theory of the presence of major effect loci.

Many researchers seek to do identify individual genes from reproductive isolation scattered throughout the *Drosophila* genome, and they do so by examining F2 backcross hybrids and high resolution analysis between closely related species (Perez and Wu, 1995) (Coyne and Orr, 1998) (Palopoli and Wu, 1994). In addition to genomic studies compared between the sequenced species of the genus *melanogaster* (Song et al., 2011) (*Drosopgila* 12 genome consortium, 207).

The *Drosophila repleta* group is an array of about 100 cactophilic species endemic to the New World, many of them cactophilic species living in the deserts and arid zones of the American continent and has become a model group for studies of ecological adaptation and speciation for over 70 years (Ruiz *et al.*, 1982) (Fontdevila, *et al.*, 1982) (Naveira, et al., 1984) (Fontdevila, *et al.*, 1988) (Santos, *et al.*, 1989) (Naveira & Fontdevila, 1991) (Ruiz and Wasserman 1993) (Oliveira *et al.*, 2012).

The *buzzatii* cluster consists of the seven cactophilic species, *D. antonietae*, *D. borborema*, *D. buzzatii*, *D. gouveai*, *D. koepferae*, *D. serido* y *D. seriema*.

They are closely related species belonging to the *buzzatii* complex (*repleta* group). This species with various degrees of evolutionary divergence (Rodríguez-Trelles, *et al.*, 2000); (Durando, *et al.*, 2000) (Manfrin *et al.*, 2001) which generates a broad spectrum of reproductive interactions among them.

They are closely related species belonging to the *buzzatii* complex (*repleta* group). This species has various degrees of evolutionary divergence (Rodríguez-Trelles, *et al.*, 2000); (Durando, *et al.*, 2000) which generates a broad spectrum of reproductive interactions among them. Recently Hurtado *et al.*, 2019, analyzed the phylogeny of this group using a large transcriptomic dataset, *D. buzzatii* split from the ancestor of the *D. serido* sibling set approximately 1.5 Myr ago, the same divergence time between *D. buzzatti* and *D. koepferae*, forming two different clades. The radiation of the remaining six species seems to be extremely recent, < 0.5 – 1 Myr ago.

Considering the replacement rates of 6.85 3 1029 per site per year for mtDNA, the estimated age for the separation of the clades comprised by *D. koepferae* and *D. buzzatti* from the remaining *D. buzzatti* cluster species is approximately 6-12 Myr, whereas separation of the clades composed of *D. sp.D* and *D. seriema*, *D. borborema*, *D. serido*, and *D. sp. B*, is estimated at 3-6 Myr (Manfrin *et al.*, 2001).

The divergence time estimates revealed that the *buzzatii* cluster is very young, with the first split that separates *D. buzzatii* occurring about 1.5 Myr ago and the remaining species diverging within the last ~ 0.5 Myr (Hurtado *et al.*, 2019).

Among those species, *D. koepferae* females can be crossed with *D. buzzatii* males under laboratory conditions (Naveira & Fontdevila, 1986) (Machado, 2006) producing sterile hybrid males and fertile hybrid females, although reciprocal crosses never produce offspring.

2 MATERIAL AND METHODS

2.1 EVALUATION OF CHROMOSOMAL SEGMENTS IN HOMOZYGOSITY

2.1.1 Standard cytological map of chromosome 4 for *Drosophila repleta* group

The cytological physic map for both *D. koepferae* and *D. buzzati* have the same polytene karyotype as species of the *Drosophila repleta* group 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 (Wharton, 1942), (Ruiz et al., 1982) (Naveira et al., 1986) (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.

Figure 1. A) Cytological map of polytene chromosome 4 of *Drosophila Repleta* group species. The vertical lines divide each cytogenetic interval (**A** to **H**) into subintervals identified by Arabic numerals 1 to 5. The arrows show one example of the classification of bands identified by lowercase letters (**a** to **h**) to subinterval 4 into the cytological interval **B**.

2.1.2 Method for genetic markers by chromosomal asynapsis

Each *Drosophila* culture produced from cross and backcrosses, was analyzed from a sample of six to ten third instar larvae. Each offspring were genotyped based on the presence of genetic markers known as chromosomal asynapsis, each chromosomal segments introgressed produce one asynapsis is formed by an incomplete pairing of chromatin fibers in the area of union between a pair of homologous chromosomes, exclusively at sites where introgression has been successful. 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 percent acetic acid and placed on a slide with a drop of standard dye solution lacto-aceto-orcin. This preparation is sandwiched between a cover glass, placed between a paper-towel and crushed with the thumb tip to release the gland.

The length and location of the introgressing segment in the cytological map indicate 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 asynapsis in the chromosomes of all the cells present in a squash preparation indicates that there was no integration of any chromosomal segment. When this happens in the squash preparations of at least 6 different larvae of a culture, then we consider that this offspring is of the genotype of the parental type without introgressions 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 (Naveira et al., 1986) (Henderson, 2004).

2.1.3 Initial 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 F1 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.

Table 1. Initial strain

Initial strain	Specie	Genotype	Origin	Obtained
Bu-28	<i>Drosophila buzzatii</i>	<i>bu/bu</i>	Los Negros, Bolivia (1982)	Universidad Autónoma de Barcelona <i>Drosophila</i> fly strain collection.
KO-2	<i>Drosophila koepferae</i>	<i>ko/ko</i>	Sierra de San Luis, Argentina. (1979).	

Each strains were derived from a single wild inseminated female (isoline), taken from a respective population cage founded by the combination of F1 progenies of approximately 60 wild-inseminated females (isolines) collected. Both strains were kept by mass culturing thereafter.

2.1.4 General conditions for crosses

All *Db* specimens were 7 days old, whereas segmental specimens were between 5 to 8 days old. For all crosses, parental flies were transferred every 5 days to a new flask with fresh feeding medium and maintained at 25° C on a 12:12 light: dark regime. During specimen manipulation, temperature never exceeded 25° C. We performed cytogenetic analysis of at least 9 larvae from the offspring of each cross to look for introgressions and infer the parental genotype.

For all type crosses (individual, massal or backcrosses) to obtain the *Drosophila* segmental lines as well as to test and reciprocal crosses, we used randomly selected males and females.

2.1.5 Method to obtaining single segmental lines in heterozygous state

To obtain segmental hybrid lines of *Drosophila buzzatii* and *Drosophila koepferae*, it was necessary to follow a strict crossing scheme (Naveira *et al.*, 1986, García-Franco 2020) that is summarized below in Figure 2. It is also necessary to consider, 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. And also, the genetic recombination only happens during meiosis in *Drosophila* females, allowing the appearance of new chromosomal arrangements (Naveira & Fontdevila, 1986) (Machado, 2006).

On the other hand, it is also important to bear in mind that each new offspring is analyzed for the presence of cromosomal asynapsis to identify the type of introgressions present and thus be able to characterize and select them according to their segmental genotype.

1) Parental cross: 5 mass type crosses of 50 male flies of the Bu-28 parental strain of *D. buzzatii* with 50 female flies of the other parental strain KO-2 of *D. koepferae*, which will produce 5 fully hybrid offspring type F1.

2) Backcross 1 (BC₁): five mass type crosses of each of five F2 offspring, between 50 females of the hybrid offspring F1 with 50 parental males *Db*, which will give rise to the twenty five recombinant cultures of multi-segmental offspring designates as F2.

3) Backcross 2 (BC₂): five individual-type crosses of each of twenty five offspring F2, using 1 female of the F2 segmental offspring with 1 male *Db*. Thanks to genetic recombination, these 125 new cultures produce more chromosomal arrangements and therefore new types of segments, therefore it is called multi-segmental F3 offspring.

4) Backcross 3 (BC₃): represents the first selection of 20 introgression-bearing offspring on chromosomal 4. Perform five individual-type crosses of each of twenty F3 cultures selected for the presence of introgressions on chromosome 4. Using 1 female of the selected F3 segmental offspring with 1 male *Db*. This offspring is designated as F4.

5-14) Backcrosses 4 to 12 (BC₄-BC₁₃): They represent a series of 10 backcrosses in sequence to continue producing new chromosomal arrangements in each new offspring, until obtaining the introgressions of interest and being able to select them. Each one similar to BC₃, where 20 cultures of each new offspring are selected for having introgressions on chromosome 4.

This new offspring (F5 to F14) are characterized by being multi-segmental and introgressive on chromosome 4.

15-24) Backcross 14 to 20 (BC₁₄-BC₂₀): They represent a series of 10 backcrosses in sequence to continue producing new chromosomal arrangements in each new offspring, until obtaining specific introgressions of interest and being able to select them. Each one similar to BC₁₄, where 20 cultures of each new offspring are selected for having specific and single introgressions of interest on chromosome 4.

These new offspring (F15 to F24) are characterized by carrying specific single introgressions of interest exclusively on chromosome 4.

25) Backcross 24 (BC₂₄): To keep the introgressions stable, the following backcrosses are now performed using the segmental male, since in *Drosophila* the males do not recombine during meiosis.

Selection of offspring with introgressions of interest on chromosome 4. Perform 3 individual-type crosses of each of twenty F25 cultures selected for the presence of specific introgressions on chromosome 4. Using one male of the selected F14 segmental offspring with 1 female *Db*. This new offspring F25 are characterized by being specific single segmental on chromosome 4. From now on, the backcrosses will be carried out in this direction, the male of each new segmental offspring specific x female *Db*. Each of these selected offspring for their specific chromosomal segment of introgression represent stock lines in maintenance.

No.	Parents' generation	Type of cross	Cross description	Offspring
1	Parental	Mass	(50♀ <i>Dko</i> x 50♂ <i>Db</i>) X 5	F1
2	BC ₁	Mass	(50♀ F1 x 50♂ <i>Db</i>) X 5 [5]	F2
3	BC ₂	Individual	(1♀ F2 x 1♂ <i>Db</i>) X 25 [5]	F3
4	BC ₃	Individual	(1♀ F3 x 1♂ <i>Db</i>) X 20 [5]	F4
5	BC ₄	Individual	(1♀ F4 x 1♂ <i>Db</i>) X 20 [5]	F5
14	BC ₁₃	Individual	(1♀ F13 x 3♂ <i>Db</i>) X 20 [5]	F14
15	BC ₁₄	Individual	(1♀ F14 x 3♂ <i>Db</i>) X 20 [5]	F15
24	BC ₁₅	Individual	(1♀ F23 x 3♂ <i>Db</i>) X 20 [5]	F24
25	BC ₁₆	Individual	(1♂ F4 x 3♀ <i>Db</i>) X 20 [5]	F25

BC: Backcross.
 HC: Hybrid cross.
 F: Filial offspring.
Db: Parental strain of *Drosophila buzzatii*.
Dko: Parental strain of *D. koepferae*.
 [] Number of replicates of each series.

Figure 2. Crossbreeding protocol to obtain single-segmental lines: 1) obtaining hybrid offspring; 2) recombinant offspring; 3) multi-segmental offspring in all chromosomes; 4) multiple segmental offspring just in the four chromosome; 5 to 14) selection of single segmental lines exclusively in the four chromosome; 15 to 24) Just single segmental lines with segments of interest; 25) lines in stock.

2.1.6 Method to obtaining segmental offspring in homozygous state

The series of crosses to produce homozygous segmental offspring are summarized in figure 3 and described below in a scheme comprising 7 general stages.

According to this crossing scheme, the first obtaining of homozygous segmental offspring corresponding to stage 4 is obtained from the crossing between heterozygous segmental males and females, which for this pair of sibling species *D. buzzatii*-D and *Koepferae* are fertile (Naveira and Fontdevila 1986) (García-Franco et al, 2020).

1) Backcross 1 (BC₁) and Reciprocal 1 (BC_{1R}): as an example, for obtain new offspring with homozygous (B1/B1) and heterocigous (B1/bu) genotypes, we perform ten individual crosses, using a male of the single segmental line (B1) in maintenance (table 1) with a female from parental strain *D. buzzatii* Bu-28 (*Db*) (table 1). In parallel, 10 individual reciprocal crosses were carried out, using in each crossing 1 female of the B1 single segmental line with a male of the parental strain *Db*. These new offspring were designated as type F2 (bu/bu, B1/bu) and F2_R, (bu/bu, B1/bu) respectively.

2) Isolation of B1 parents of BC₁ and BC_{1R}: for to deplete parent females BC_{1R} from egg laying, four days after the start of the crosses, the 10 male flies of the segmental line B1 used as progenitors in the BC₁, as well as the 10 female segmental flies B1 used as progenitors in the BC_{1R}, were removed from each of their respective cultures, and each

of those 20 flies was isolated in a new culture vial, and kept isolated independently until these females stopped laying eggs, 15 to 20 days.

3) Identification genotype parents BC_1 and BC_{R1} : To infer the genotype of each of the 10 male B1 parents of the backcrosses, and of the 10 female B1 parents of the reciprocal cultures, kept in isolation. At least 6 larvae from each offspring F2 and F2_R were analyzed cytogenetically for asynapses on polytene chromosomes.

4) Hybrid crosses type 1 (HC_1). To obtain homozygous hybrid segmental offspring (B1/B1), we made individual crosses using the same parent male of the BC_1 cross, previously isolated and identification of its genotype (B/bu), with the same parent female of the BC_{R1} cross, previously isolated until complete depletion of the laying of eggs (15 to 20 days) and identification of its genotype (B/bu). These new descendants were designated as segmental homozygous type F3 offspring (bu/bu, B1/bu and B1/B1).

5) Backcross 2 (BC_2) and Reciprocal 2 (BC_{R2}): we made individual crosses (1 male X 1 female) between the males of the F3 offspring with females of the parental strain Db, and in parallel, we made reciprocal crosses using the females of the F3 offspring with males of the parental strain Db.

These backcrosses represent a dual purpose, 1) they produce, among others, heterozygous segmental cultures with 100% of the B1/bu flies, which can be used to produce new homozygous offspring, avoiding the isolation processes of the progenitors and their crossing without being virgins, as done in stage 6.

2) function as test crosses that allow evaluating the fertility of segmental flies (heterozygous and homozygous). These new offspring were designated as type F4 and F4_R respectively.

6) Hibryd segmental cross HC_1 : we made individual type crosses (1 male X 1 female), using male and female siblings only of the F4_R heterozygous segmental offspring, from the reciprocal cross (BC_{R2}), but we only used flies from F4_RC cultures with 100% of flies of genotype B1/bu.

We use only the offspring of the reciprocal crosses (BC_{R2}) because the segmental parents of homozygous genotype (B1/B1) of these BCR2 crosses are fully fertile while the male parents of homozygous segmental genotype (B1/B1) of the backcrosses (BC_2) are affected in your fertility, and therefore do not produce offspring. These new descendants were designated as type F5.

7) Backcross 3 (BC_3): we carried crosses of individual type (1 male X 1 female), using males of homozygous segmental offspring F5 with parental females Db. In parallel, individual reciprocal crosses were carried out, using in each crossing 1 female of the F5 single segmental line with a male of the parental strain Db.

This crossing will allow the second evaluation of the fertility of segmental flies, both heterozygous (B1/bu) and homozygous (B1/B1), according to the number of larvae produced in each of their respective cultures. These new descendants were designated as type F6.

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Figure 3. General scheme to obtaining segmental offspring in *homozygosity*.

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2.1.7 Test crosses

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We made individual test crosses, one male with two females, using males obtained from each homozygous simple segmental offspring (table 3) with parental *D. buzzatii* Bu-28 strain (*Db*) females (table 1).

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Parallel to each test cross, we made their respective reciprocal cross, between corresponding females from each homozygous simple segmental offspring (table 3) with Bu-28 strain (*Db*) males (table 1).

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

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Male progenitors are considered fertile when their corresponding crosses produce a number of offspring similar to that of control crosses (>60), they are considered semi-sterile when their offspring represent only a low percentage of the control's progeny (at least 1 less than 15), finally, they are considered sterile when no offspring are produced.

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3.2.1 Mapping the smallest homozygous sterility segments

1.1.1 In situ hybridization

1.1.1.1 Obtaining BAC clones of Genomics library of *Drosophila buzzatii*

The mapping and obtention the genetic sequences, of the small chromosomal fragment of interest (F2-F4), it has been made using the genomics CHORI-BAC 265 (Bacterial artificial chromosome) library of *D. buzzatii* (fig. 6), obtained from the BACPAC Resource Center (BPRC) attached to the Oakland Children's Hospital Research Institute (CHORI).

This genomics BAC library was built by The Research Group on Genomics, Bioinformatics and Evolution of the Autonomous University of Barcelona, based in a physical map of the six chromosomes that make the *Drosophila buzzatii* genome, for this purpose it assembled the all fragments of the 6 chromosomes in BACs (Gonzalez *et al*, 2005). The following figure shows the physical map of chromosome 4, where the positions of the respective BAC clones containing each identified chromosomal fragment (figure 4).

Figure 4. Integrated BAC-based physical map of the *Drosophila buzzatii* genome. Vertical lines indicate the relative position of the each of the 65 BAC clones label. The 31 contigs to which the hybridized clones belong are represented by short horizontal lines below the chromosomes along with the respective contig number. Into the red rectangle, the position of contig 1065 is indicated where the chromosomal fragments of interest F2, F3, and F4 are located. (Modified image from Gonzalez *et al*, 2005).

Of the 31 contigs of chromosome four (fig. 4), the contig selected for our research is number 1065, which is made up of 63 BAC clones, each registered with a consecutive number that goes from number 28 to number 90 (table 1).

The chromosomal region encompassing contig 1065 includes the chromosomal segment of interest F2 (F2a-F3d) (Table 3 and Fig. 4). Therefore, in order to map said segment with genetic tags, we decided to acquire 8 clones (41.Z006D14, 47. Z025K20, 53.Z021N13, 59. Z015H23, 64. Z005G15, 76. Z005012, 82. Z002K06 and 86. Z002K22) of the contig 1065 that are distributed along said chromosomal region see table X. Said clones were acquired from the company BACPAC Resource Center (BPRC) attached to the Oakland Children's Hospital Research Institute (CHORI).

Table 2. Key assigned to each of the 63 BAC clones that make up contig 1065, labeled with consecutive numbers ranging from number 28 to number 90.

28. Z006E21	36. Z019M16	44. Z012N02	52. Z001C10	60. Z003M07	68. Z024F14	76. Z005012	84. Z018E16
29. Z010D13	37. Z025C20	45. Z021A12	53. Z021N13	61. Z017K07	69. Z001M11	77. Z005M13	85. Z000G17
30. Z010C14	38. Z022E22	46. Z009D02	54. Z006L04	62. Z026H09	70. Z016A09	78. Z024A05	86. Z002K22
31. Z006P02	39. Z009K38	47. Z025K20	55. Z008M20	63. Z002023	71. Z026L2D	79. Z012H20	87. Z018H13
32. Z017D13	40. Z006C14	48. Z005120	56. Z010A09	64. Z005G15	72. Z026P16	80. Z004M16	88. Z005023
33. Z017H02	41. Z006D14	49. Z025K11	57. Z0118A20	65. Z006M04	73. Z003A15	81. Z007N14	89. Z017P09
34. Z007C02	42. Z020E10	50. Z006D03	58. Z019E05	66. Z010A10	74. Z018L10	82. Z002K06	90. Z008H18
35. Z009C17	43. Z009D01	51. Z024C01	59. Z015H23	67. Z012K04	75. Z022B21	83. Z008020	

1.2.1.2 In situ hybridization using BACs clones as probes

The DNA of BAC clones was used as an in situ hybridization probe, to confirm the hybridization of these clones in the F2 and F3 (fig. 6) banding region of chromosome 4 of *D. Buzzatii*, as well as in the species *D. Mojavensis*, a symorphic species of the group packed.

The BAC clones were labeled with biotin by the "Nick Translation" method, using the protocol of the commercial kit (BioNick kit, Gibco-BRL).

After labeling the probe, the next steps of the in situ hybridization process consist of preparing the polytechnic chromosomes, prehybridization, hybridization, post-hybridization, development and visualization, as described in Carbajal-Rodriguez, 1999.

The labeled probes hybridized homologously to the polytechnic chromosomes of *D. buzzatti*, and heterologously to the polytechnic chromosomes of *D. mojavensis*.

Each of the in situ hybridization preparations were observed with an optical microscope and photographs were taken at 40 and 100X.

The in situ hybridization signals have been located and numbered based on the band pattern determined by chromosome 4 of *D. repleta*.

3. Results

3.1 Evaluation of chromosomal segments in homozygosity

3.1.1 Obtaining simple segmental lines in heterozygosity

The original fly strains, *D. koepferae* KO-2 (Dko) and *D. buzzatii* Bu-28 (Db), provided the material to obtain single segmental lines (Table 1) following a strict mating strategy (fig. 2).

According to the scheme of crosses, proposed in figure 2, six simple segmental lines in heterozygous state were obtained, which are described below in table 3.

3.1.2 Obtaining single segmental lines in homozygosity

By strictly following the crossing protocol, described in figure 3, it was possible to obtain six segmental lines in the homozygous state (Table 3).

Figure 3 describes the crossing scheme that includes up to 27 crosses, which depends on the selection of segments of interest obtained in each new offspring.

Table 3. Segmental lines obtaining

No	Heterozogous single segmental lines		Homozygous segmental offsprings		Segment size relative of chromosome 4 (%)
1	Name	Genotype	Name	Genotype	
2	F2	F2/bu	FF2	F2/ F2	6.25 (F2a-F3d)
3	F3	F3/bu	FF3	F3/ F3	7.87 (F3a-F4h)
4	B1	B1/bu	BB1	B1/ B1	10.59 (B1a-B4f)
5	FG	FG/bu	FFGG	FG/ FG	21.87 (F3a-G5)
6	D3	D4/bu	DD4	D4/ D4	9.06 (D4a-E1h)
7	D1	D1/bu	DD1	D1/ D1	15.62(D1a-D5d)

3.1.3 Test crosses of segmental offspring in homozygosity

We made individual test crosses, one male with one female, using males obtained from each homozygous simple segmental offspring (table 3) with parental type Db females (table 1). Parallel to each test cross, we made their respective reciprocal cross, between corresponding females from each homozygous simple segmental offspring (table 3) with Db males (table 1).

According to the results of the test crosses recorded in table 4, we used three fertility criteria (table 5), based on the total number of larvae present in all the culture flasks that make up each of the different offspring, including the total number of larvae from each offspring of parent females isolated during depletion of egg-laying:

Fertile: the presence of at least 60 larvae.

Semi-sterile: at least one larva and less than 60.

Sterile: number zero of larvae.

Table 4. Result of the number of descendants of each individual test crosses, according to the type of genotype of each parent.

Genotype of each type of parent, and the number of descendants for each type of									
Offspring by genotypes					Inference of parents genotypes				
Number	Description	Number of larvae by genotype in each culture			Parents by sex	Number of parent by genotype			Total
		bu/bu	F2/bu	F2/F2		bu/bu	F2/bu	F2/F2	
35	1♂ FF2 X 1♀ Db	>60 Fertile	>60 Fertile	0 Sterile	Male Percent	13 37.14	14 40.00	8 22.86	35 100
38	1♀ FF2 X 1♂ Db	>60 Fertile	>60 Fertile	>60 Fertile	Female Percent	16 42.11	18 47.37	4 10.53	38 100
		bu/bu	F3/bu	F3/F3		bu/bu	F3/bu	F3/F3	
52	1♂ FF3 X 1♀ Db	>60 Fertile	>60 Fertile	0 sterile	Male Percent	16 30.77	26 50.00	10 19.23	52 100
73	1♀ FF3 X 1♂	>60 Fertile	>60 Fertile	>60 Fertile	Female Percent	23 31.51	34 46.58	16 21.92	73 100
		bu/bu	B1/bu	B1/B1		bu/bu	B1/bu	B1/B1	
103	1♂ BB1 X 1♀Db	>60 Fertile	>60 Fertile	<15 Semi-sterile	Male Percent	39 37.86	40 38.86	24 23.30	103 100
87	1♀ BB1 X 1♂ Db	>60 Fertile	>60 Fertile	>60 Fertile	Female Percent	33 37.93	37 42.53	17 19.54	87 100
		bu/bu	FG/bu	FG/FG		bu/bu	FG/bu	FG/FG	
26	1♂ FFGG X 1♀ Db	>60 Fertile	>60 Fertile	0 Sterile	Male Percent	9 34.62	12 46.15	5 19.23	26 100
16	1♀ FFGG X 1♂ Db	>60 Fertile	>60 Fertile	0 Sterile	Female Percent	5 31.25	7 43.75	4 25.00	16 100
		bu/bu	D3/bu	D3/D3		bu/bu	D3/bu	D3/D3	
80	1♂ DD3 X 1♀Db	>60 Fertile	>60 Fertile	0 Sterile	Male Percent	29 36.25	31 38.75	20 25.00	80 100
77	1♀ DD3 X 3♂Db	>60 Fertile	>60 Fertile	>60 Fertile	Female Percent	28 36.36	38 49.35	11 14.29	77 100
		bu/bu	D1/bu	D1/D1		bu/bu	D1/bu	D1/D1	
17	1♂ DD1 X 1♀ Db	>60 Fertile	>60 Fertile	0 Sterile	Male Percent	5 29.41	8 47.06	4 23.53	17 100
33	1♀ DD1 X 1♂ Db	>60 Fertile	>60 Fertile	0 Sterile	Female Perce	18 54.55	11 33.33	4 12.12	33 100
Green color: parental homozygous genotype Yellow color: heterozygous segmental genotype Red color homozygous segmental genotype Db: <i>Drosophila buzzatii</i>									

The figure 5 shows offspring according to their introgressive segments (B1, D3, D1, F2, F3, and FG), differentiating the numbers of males (m) and females (f) organized in their respective genotypes (parental, segmental heterozygous, segmental homozygous).

While in figure 6, the data of the total numbers of parents of each offspring are grouped, differentiating the trend of the presence of females and males by the types of genotypes of each offspring.

Fig. 5 **Fig. 6**

Figure 5. Offsprings grouped according to their introgressive segment (B1, D3, D1, F2, F3 and FG), and their sex (m = male, f = female).

Figure 6. Comparison of the number of male and female parents of each type of offspring (B1, D3, D1, F2, F3, and FG) according to their three respective genotypes, parental, segmental heterozygous, and segmental homozygous.

3.1.4 Fertility heterozygous and homozygous segments

From the data obtained in the results of the test crosses (Table 4) it was possible to observe the effect that each segment produces on the fertility of males and segmental females.

The effect on fertility is shown in three levels: 1) total sterility; 2) semi-sterility and 3) fertility, and it turns out to be complex as it depends on several factors: segment size, position on the chromosome, sex and genotype, as shown in Table 5.

Table 5. The fertility of parents according to their segmental genotype and sex

Segment size relative of chromosome 4 (%)	Heterozygous segmental parents		Homozygous segmental parents	
	Name	Fertility	Name	Fertility
6.25 (F2a-F3d)	♂ F2	Fertile	♂ FF2	Sterile
	♀ F2	Fertile	♀ FF2	Fertile
7.87 (F3a-F4j)	♂ F3	Fertile	♂ FF3	Sterile
	♀ F3	Fertile	♀ FF3	Fertile
12.25 (B1a-B4f)	♂ B1	Fertile	♂ BB1	Semiesterile
	♀ B1	Fertile	♀ BB1	Fertile
21.87 (F3a-G5c)	♂ FG	Fertile	♂ FGFG	Sterile
	♀ FG	Fertile	♀ FGFG	Sterile
12.25 (D3a-E1h)	♂ D3	Fertile	♂ DD3	Sterile
	♀ D3	Fertile	♀ DD3	Fertile
15.62(D1a-D5d)	♂ D1	Fertile	♂ DD1	Sterile
	♀ D1	Fertile	♀ DD1	Sterile

In Figure 7 the twelve segments (Table 3) typical of this study (6 analyzed in heterozygosity and 6 analyzed in homozygosity) are represented in a schematized way, the segments of the studies by Garcia-Franco et al 2020 are

also found, the de Naveira and Fontdevila 1986, 1991a, 1991b, as well as the segments proposed by I. Marin, 1996.

All those chromosomal segments mentioned above, with the exception of the 6 segments analyzed in this work, were evaluated in their respective publications, only in the heterozygous state for their ability to possess hybrid sterility genes.

In the following diagram, each of these segments are located in their corresponding position within chromosome 4 of the full group and according to their relative size in percentage in relation to the total size of the chromosome 4.

Figure 7. (A) Total length of chromosome 4 of the Repleta group. (B) Representation of the 6 segments evaluated in heterozygosity and the 6 segments evaluated in homozygosity in this work. (C) representation of segments evaluated in heterozygosity in García-Franco et al, 2020. (D) representation of segments evaluated in heterozygosity Marin, 1996. (E) representation of segments evaluated in heterozygosity in Naveira and Fontdevila 1986, 1991a, 1991b.

Each bar of a certain color represents each chromosomal segment, and each color indicates a level of sterility or fertility that each segment produces:

Green: fertile Blue: semi-sterile Red: sterile.

The name and size of each segment can be seen at the top of its respective segment.

3.2 Mapping the smallest homozygous sterility segments

3.2.1 BAC clones of *D. buzzatii*

Eight BACS clones out of a total of 63 that make up contig 1065 (Tables 2 and 6), was acquired from the Oakland Children's Hospital Research Institute (CHORI).

The Contig 1065 encompasses almost the entire F2 to F4 region (Fig. 4) of chromosome 4 of the physical map of *D. buzzatii*.

The eight clones were selected to demonstrate, by in situ hybridization, the correspondence of Contig 1065 in the F2 to F4 region.

Table 6. Selected clones of Contigo 1065

Clones de Conti 1065	
Número	Clave
41	Z006D14
47	Z025K20
53	Z021N13
59	Z015H23
64	Z005G15
76	Z005O12
82	Z002K06
86	Z002K22

3.2.2 In situ hybridization

According to the proposed cytogenetic relationships between *D. buzzatii* and *D. mojavensis*, the two species are close enough to maintain a high homosequence ratio, especially for chromosome 4 because to the high colinearity (Guillen et al, 2018) so we performed in situ hybridizations with the same probes for both species.

In situ hybridizations were carried out using each of the 8 BAC clones as probes, marking them with biotin. The probes were hybridized in polythene chromosomes of *D. buzzatii* and *D. mojavensis*, respectively. The eight probes performed by in situ hybridizations were positive. In figure 7, 4 of them are shown.

Figure 8. Positive signals from in situ hybridizations. A) and B) positive signals from probes 25K20 and 2K06 in *D. buzzatii*. C) GD142 probe in *D. buzzatii*. D) GD142 probe in *D. mojavensis*.

The eight probes prepared from the BACs clones hybridized in the precise position expected for both *D. buzzatii* and *D. mojavensis*, in both species within

the F2 to F4 region, according to the physical map of chromosome 4, as outlined in figure 98.

Figure 9. In situ hybridizations of 8 BACs clones in *D. buzzatii* and *D. mojavensis*. The arrows in the upper part corresponding to the hybridizations in polygenic chromosomes of *D. buzzatii*, and the arrows in the lower part those of *D. mojavensis*. Each arrow indicates the position of the hybridization signal corresponding to the respective probe according to the indicated key. The black line under the chromosome image represents the full length of contig 1065. The 3 regions F2, F3 and F4 of chromosome 4 are indicated.

3.3 Bioinformatic analysis

3.3.1 BLAST Procedure

Using the gene browser of the *D. buzzatii* from the *Drosophila buzzatii* Genome Project (<http://dbuz.uab.cat>) we obtained the nucleotide sequence of contig 1065 and access to the genomic map of *D. buzzatii* to know the genes and genetic accessories annotated that are associated with contig 1065.

The complete sequence of contig 1065 has 4233 nucleotides, it is found within the scaffold2 between positions 308944 to 313176 of the genomic map of *Drosophila buzzatii* (Fig. 10A).

BLAST 1: We performed a Standard Nucleotide BLAST (Basic Local Alignment Search) process with the BLAST-suite software from the National Center for Biotechnology Information (NCBI), hosted on the GenBank website, using the complete nucleotide sequence of the Contig 1065 of *D. buzzatii* comparing it with the sequences annotated for the species of the family Drosophilidae (taxid: 7214) housed in GenBank (Thurmond et al., 2020).

The BLAST procedure with the megablast algorithm for highly similar sequences, identified the region with the highest homology:

Is a zone of 3428 nucleotides in the R arm of chromosomal 3 of *Drosophila melanogaster*, with 11 different segments homologous, which is located within the trivet

gene in to contig 1065, between the nucleotide region Dmel-chr3R 28074717-28065867 (fig10B and 11C).

We performed a second BLAST procedure, between the nucleotide sequence of contig 1065 and the nucleotide sequences of the *Drosophila* genus species housed in the FlyBase Gene Bank. We identified an area of 3704 nucleotides with 20 different homologous segments, which is located within the splicing factor (*D. moj_GI22766*) in to contig 1065 belonging to *Drosophila mojavensis* in the genomic region 19163410-19167114 of scaffold 6540 (Fig 10C and 11B).

A) *Drosophila buzzatii* Genome Project (DBGP)

D. buzzatii-chr_4. Scaffold2, contig 01065, length= 4233nt (308944 a 313176)

B) GenBANk. BLAST. Job title: Contig 01065 length= 4233

Optimize for Highly similar sequences (megablast)

D. melanogaster-chr3R (28074717-28065867)

C) FlyBase. BLAST. Contig01065 length=4233 bp

BLAST 3 Dmoj scaffold 6540 (19163410-19167114)

Figure 10. Schematic representation of the homologous sequences identified by the Blast procedure, between Contig 1065 and the nucleotide sequences of the species of the genus *Drosophila*. A) Contig 1065 of 4233 nucleotides in total length. B) Eleven homologous segments between the *D. melanogaster* sequences and the 1065 contig. C) Twenty homologous segments between the *D. mojavensis* sequences and the 1065 contig.

Each rectangle indicates a homologous segment between the respective *Drosophila* species with the 1065 contig of *D. buzzatii*.

3.3.2 Genes and genetic accessories associated with contig 1065

According to the genomic map of *D. buzzatii* (*Drosophila buzzatii* genome project <http://dbuz.uab.cat>) the nucleotide sequence of contig 1065 is part of the predicted gene Dbuzlg1313.t1, which is located at position 294049-325520 and showing the presence of an orthologous gene (fig 11A).

The orthologous gene is called DmojG122766 and is described as the splicing factor, proline- and glutamine-rich (splicing factor, proline- and glutamine-rich) whose genomic position includes nucleotides 19150747-19219807 in the mojavensis genomic map (GenBank_LOC6573952) (fig. 11B).

Dmoj_GI22766 gene is also orthologous with Trivet gene of *D. melanogaster*, identified as regulation of alternative mRNA splicing via spliceosome, being its position within the genomic map of *D. melanogaster* 28026615-28090740 (Genbank_Trivet) and in that genomic region it is found locally associated with a natural mobile element (Dmel\I {1441}), and 4 other mobile element insertion sequences (1) Mi {ET1} trvMB02668, 2) PBac {WH} trvf00299, 3) Mi {ET1} trvMB04653 and Mi {ET1} MB00028) (fig.11C).

On the other hand, and interestingly, in the genomic map of *D. mojavensis* the DmojG122766 gene in position 19150747-19219807 is located adjacent to the Dmoj_GI22767 gene, which is a gene characterized in GenBank as nucleosin TIA-1 (nucleolysin TIA-1) located at position 18977154-19072104 (LOC6573951), and is orthologous to the D. mel_CG34354 gene of *D. melanogaster*, and is characterized as an RNA-binding domain. This CG34354 gene, described as Trivet's paralogue, in the genomic map of *D. melanogaster* *D. melanogaster* is associated with a natural mobile element (Dmel\mdg1 {1442}), and 6 insertion sequences of mobile elements numbered from 1 to 6; (1 Dmel\Mi {ET1} CG34354MB01203, 2) Dmel\Mi {ET1} CG34354MB01203,

3) Dmel \ Mi {MIC} CG34354MI03256, 4) Dmel\Mi {ET1} CG34354MB07567, 5) Dmel \ Mi {MIC} CG34354MI13643, 6) Dmel \ P {GSV2} GS51766 (Fig. 11C).

Figure 11. Genetic regions of the genomic maps. A) *D. buzzatii*; B) *D. Mojavensis* and C) *D. melanogaster*, which are associated with the nucleotide sequence of contig 1065 of *D. buzzatii*. The numerical scale in kilobases at the top indicates only the relative distance of each genetic element on its respective genomic map.

The long black lines indicate the genomic region represented, respective of each species. Black rectangles with yellow margins represent the position of each of the indicated genes.

The small rectangles in red color indicate the positions of the homologous sequences between the 1065 contig with the genome of *D. buzzatii*, *D. mojavensis* and *D. melanogaster* respectively.

The smaller black rectangles at the bottom locate the natural transposable elements of each gene. The numbers 1 to 6 indicate the presence of the mobile element insertion sequences and the lower black lines represent the regions of the cytogenetic bands 98B6 to 98C2 of the cytogenetic map of *D. melanogaster*.

4. DISCUSSION

Previous studies on reproductive isolation between *Drosophila buzzatti* and *Drosophila koepferae* were always carried out with introgressions of heterozygous chromosomal segments (Naveira and Fontdevila 1986) (García Franco et al., 2020) and it was never possible to identify individual genes with strong effect, although it was able to demonstrate the effect of sterility in segmental males due to a threshold size when it was possible to introgress a segment with a minimum total size of 31.15% with respect to the total size of chromosome 4, either individually, in pairs or trios, or, both in cis action and in trans action, but always in heterozygosity, as shown in Figure 7. Therefore, with this methodology for obtaining homozygous introgressing segments, we now have the ability to produce an enormous range of homozygous introgressive segments and be able to evaluate them, not only for these species but for any group of species that present e fertile segmental males in heterozygosis, and importantly, being able to isolate groups of small individual introgressing segments that could be associated with one or a few hybrid sterility factors and be able to differentiate them from those that act epistatically between genes of different loci.

Obtaining 6 introgressive segments in a heterozygous state (fig. 2) with sizes between 6.25% the smallest and up to 21.87% the largest (table 3), did not produce any effect of sterility in males or segmental females, such as can be seen in table 5.

But when they were obtained in a homozygous state (figure 3), they now showed that the 6 segments were capable of producing some effect on sterility (table 5), and not only in males but also in segmental females, depending not only on size of the segment, but of its position within chromosome 4.

In such a way that, the segments of size 6.25% (F2a-F3d), 7.87% (F3a-F4j) and 12.25% (B1a-B4f) located in the F region and the first two and the third in the D region respectively, they were able to produce sterility in segmental males, although in females, its effect was null. While an intermediate size segment (12.25%) located in region B produced semi-sterility in males and no effect in segmental females. Unlike the larger segments, 15.62% (D1a-D5d) and 21.87% (F3a-G5c), located in the D and F regions respectively, produced sterility in both females and segmental males.

The above indicates the potential presence of a single strong effect factor with the capacity to produce sterility in segmental males carrying the smallest segments F2a-F3d (6.25%) and F3a-F4j (7.87%), although we cannot rule out the possibility of epistasis with other factors.

In addition, three of the segments (B4c-B4e, D5b-E1a and F2a-F3d) (fig 7D), proposed by Marin, 1996 as having epistatic sterility factors, coincide in their position with segments of this work (B1a-B4f, D3a-E1h, F2a-F3d) (Fig. 7B), especially the region that overlaps between segments F2a-F3d and F3a-F4j, and that indicates an even smaller region (F3a-F3e), with the possibility of the presence of a single strong effect factor, which could act independently, the same happens with the overlapping segment between the segments D1a-D5d and D3a-E1h, the small region D1a-D2e.

Also in relation to size, it is very revealing that the segments when they are small only affect segmental males but when a larger region is considered (F3a-G5) they also affect segmental females, which suggests additive or epistatic action. Including the different effect observed between two segments of the same size (12.25%), B1a-B4f and D3a-E1h, the first produce semi-sterility and the second sterility, in males (table 5 and figure 7).

On the other hand, the effect observed in the 6 segments regardless of their position within chromosome 4 indicates the presence of factors distributed throughout the entire chromosomes, which supports Naveira's original proposal (Naveira and Fontdevila, 1986) (Naveira et al., 1986) Naveira and Fontdevila, 1991a) (Naveira and Fontdevila 1991b) in the extensive study that he has carried out with this pair of species, where he has demonstrated the effect of segment size throughout the genome.

Although each of the 6 homozygous segments is of comprehensive interest, we decided to focus on the smaller F2a-F3d and F3a-F4j segments (Table 5) that produce sterility in segmental males. Thanks to the physical map of *Drosophila buzzatii* based on BACs (Gonzalez et al, 2005), we managed to obtain (from the BACPAC Resource Center CHORI) the 8 clones, which are located along the small region F2 to F4 of the chromosom map of *D. buzzatii* (Figure 4 and Table 2, and successfully hybridize each one of them when using them as probes, in the polygenic chromosomes of *D. buzzatii* and *D. mojavensis* (Figures 8 and 9). With which we show that we are within region F2 to F4.

The bioinformatic study, using BLAST processes and identification of genes and annotated genetic structures of the genomes of *D. buzzatii* (*Drosophila buzzatii* Genome Projet), *D. mojavensis* and *D. melanogaster* (GenBank and Flybase) allowed us to focus the genetic region of *D. buzzatii* where the contig 1065 sequence is located within the genome of *D. buzzatii*, turning out to be an interesting region, which although in *D. buzzatii* only shows the presence of two predicted genes, g1313.t1 and g1314.t1, (figure 11A). An orthologous gene of the g1313.t1 gene is also shown, which is located in the genome of *D. mojavensis* and is known as the Dmoj_Gl22766 gene (Fig. B), which has a biological function of participating in the regulation of splicing processes, in turn. Once this gene Gl22766 is orthologous with its counterpart in *D. melanogaster* known as the Trivet gene (Fig. 11 C) and which has the same biological function. And these regions, both of *D. mojavensis* and *D. melanogaster*, represent regions of high genetic mobility, since at least one mobile element and several insertion sequences of mobile elements are associated with these genes (Fig. 11C), and Interestingly, this entire region is located in a space of only 5 cytogenetic bands of the *D. melanogaster* genome (Fig. 11 C). And although the diagram in figure 11 only shows a small region of 280 kilobases, when comparing the regions of adjacent to this region it is possible to identify that the groups

of syntenic genes are maintained with great consistency between *D. buzzatii* and *D. melanogaster*, and they are also maintained, although to a lesser degree, between *D. mojavensis* and *D. Buzzatii*.

With all of the above, we conclude that the method to obtain homozygous segmental offspring allows for the first time homozygous introgressions with the ability to independently evaluate introgressive segments, or it is possible to extend the scheme for joint-segment analysis.

None of the 6 introgressing segments in heterozygosis produce effects on sterility. In contrast, in the homozygous state, the 6 segments produce different effects on sterility, when they are small segments (5 to 7%) they produce effects on the sterility of segmental males, and when they are larger (12.25% to 21.87%) they produce semi-sterility. or sterility in segmental males or sterility in both females and segmental males. That the sterility factors extend throughout the entire chromosome 4 considering that they have evaluated in percentage 57% with the 6 homozygous segments. And that the genes *g.1314.t1* and *g1313.t1* of *D. buzzatii*, both still in a state of prediction, represent being candidate genes involved in the reproductive isolation of these symmorphic species.

Although under the classical Bateson-Dobzhansky-Muller model, we are observing changes in at least two genes, due to the differential interaction and in size and position of large segments or in different positions of the genome, now we also observe the possibility of the action of a single allele with strong effects on non-heterozygous sterility and without epistasis to other genes, even considering the decreased aptitude of that allele that makes it difficult to fix this allele in the population, therefore, it would then be necessary to consider both possibilities, appealing to both models and using new strategies to understand the relationship between both possibilities, especially in small populations and in high-mobility genes. On the other hand, the dominance models show us an additional possibility to address the situations revealed in this work, although it is necessary to know the type of dominance of the allele in question under the current models in relation to the genotypic and phenotypic values and their position within the metabolic flow, and additionally it is relevant to consider the type of inheritance especially if it turns out to be of maternal origin. Finally, It is now feasible to study allele fixation processes as one of the fundamental processes of the hybrid sterility effect.

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Figures

Figure 1

A) Cytological map of polytene chromosome 4 of *Drosophila Repleta* group species. The vertical lines divide each cytogenetic interval (A to H) into subintervals identified by Arabic numerals 1 to 5. The arrows show one example of the classification of bands identified by lowercase letters (a to h) to subinterval 4 into the cytological interval B.

No.	Parents' generation	Type of croos	Cross description	Offspring
1	Parental	Mass	(50♀ <i>Dko</i> x 50♂ <i>Db</i>) X 5	F1
2	BC ₁	Mass	(50♀ F1 x 50♂ <i>Db</i>) X 5 [5]	F2
3	BC ₂	Individual	(1♀ F2 x 1♂ <i>Db</i>) X 25 [5]	F3
4	BC ₃	Individual	(1♀ F3 x 1♂ <i>Db</i>) X 20 [5]	F4
5	BC ₄	Individual	(1♀ F4 x 1♂ <i>Db</i>) X 20 [5]	F5
14	BC ₁₃	Individual	(1♀ F13 x 3♂ <i>Db</i>) X 20 [5]	F14
15	BC ₁₄	Individual	(1♀ F14 x 3♂ <i>Db</i>) X 20 [5]	F15
24	BC ₁₅	Individual	(1♀ F23 x 3♂ <i>Db</i>) X 20 [5]	F24
25	BC ₁₆	Individual	(1♂ F4 x 3♀ <i>Db</i>) X 20 [5]	F25

BC: Backcross.
 HC: Hybrid cross.
 F: Filial offspring.
Db: Parental strain of *Drosophila buzzatii*.
Dko: Parental strain of *D. koepferae*.
 [] Number of replicates of each series.

Figure 2

Crossbreeding protocol to obtain single-segmental lines: 1) obtaining hybrid offspring; 2) recombinant offspring; 3) multi-segmental offspring in all chromosomes; 4) multiple segmental offspring just in the four chromosome; 5 to 14) selection of single segmental lines exclusively in the four chromosome; 15 to 24) Just single segmental lines with segments of interest; 25) lines in stock.

Figure 3

General scheme to obtaining segmental offspring in homozygosity.

Figure 4

Integrated BAC-based physical map of the *Drosophila buzzatii* genome. Vertical lines indicate the relative position of the each of the 65 BAC clones label. The 31 contigs to which the hybridized clones belong are represented by short horizontal lines below the chromosomes along with the respective contig number. Into the red rectangle, the position of contig 1065 is indicated where the chromosomal fragments of interest F2, F3, and F4 are located. (Modified image from Gonzalez et al, 2005).

Figure 5

Offsprings grouped according to their introgressive segment (B1, D3, D1, F2, F3 and FG), and their sex (m = male, f = female).

Figure 6

Comparison of the number of male and female parents of each type of offspring (B1, D3, D1, F2, F3, and FG) according to their three respective genotypes, parental, segmental heterozygous, and segmental homozygous.

Figure 7

(A) Total length of chromosome 4 of the Repleta group. (B) Representation of the 6 segments evaluated in heterozygosity and the 6 segments evaluated in homozygosity in this work. (C) representation of segments evaluated in heterozygosity in García-Franco et al, 2020. (D) representation of segments evaluated in heterozygosity Marin, 1996. (E) representation of segments evaluated in heterozygosity in Naveira and Fontdevila 1986, 1991a, 1991b. Each bar of a certain color represents each chromosomal segment, and each color indicates a level of sterility or fertility that each segment produces: Green fertile

Blue: semi-sterile Red: sterile. The name and size of each segment can be seen at the top of its respective segment.

Figure 8

Positive signals from in situ hybridizations. A) and B) positive signals from probes 25K20 and 2K06 in *D. buzzatii*. C) GD142 probe in *D. buzzatii*. D) GD142 probe in *D. mojavensis*.

Figure 9

In situ hybridizations of 8 BACs clones in *D. buzzatii* and *D. mojavensis*. The arrows in the upper part corresponding to the hybridizations in polygenic chromosomes of *D. buzzatii*, and the arrows in the lower part those of *D. mojavensis*. Each arrow indicates the position of the hybridization signal corresponding to the respective probe according to the indicated key. The black line under the chromosome image represents the full length of contig 1065. The 3 regions F2, F3 and F2 of chromosome 4 are indicated.

Figure 10

Schematic representation of the homologous sequences identified by the Blast procedure, between Contig 1065 and the nucleotide sequences of the species of the genus *Drosophila*. A) Contig 1065 of 4233 nucleotides in total length. B) Eleven homologous segments between the *D. melanogaster* sequences and the 1065 contig. C) Twenty homologous segments between the *D. mojavensis* sequences and the 1065 contig. Each rectangle indicates a homologous segment between the respective *Drosophila* species with the 1065 contig of *D. buzzatii*.

Figure 11

Genetic regions of the genomic maps. A) *D. buzzatii*; B) *D. Mojavensis* and C) *D. melanogaster*, which are associated with the nucleotide sequence of contig 1065 of *D. buzzatii*. The numerical scale in kilobases at the top indicates only the relative distance of each genetic element on its respective genomic map. The long black lines indicate the genomic region represented, respective of each species. Black rectangles with yellow margins represent the position of each of the indicated genes. The small rectangles in red color indicate the positions of the homologous sequences between the 1065 contig with the genome of *D. buzzatii*, *D. mojavensis* and *D. melanogaster* respectively. The smaller black rectangles at the bottom locate the natural transposable elements of each gene. The numbers 1 to 6 indicate the presence of the mobile element insertion sequences and the lower black lines represent the regions of the cytogenetic bands 98B6 to 98C2 of the cytogenetic map of *D. melanogaster*.